analytical chemistry

Article

pubs.acs.org/ac

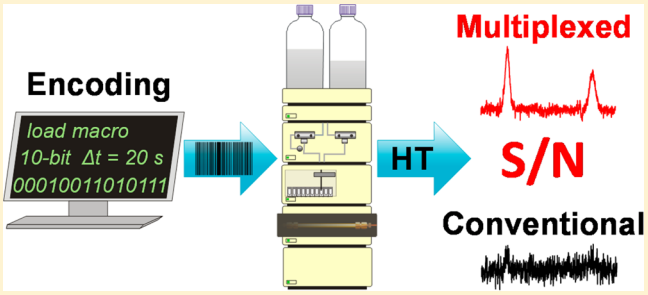
Development of a Straightforward and Robust Technique to Implement Hadamard Encoded Multiplexing to High-Performance Liquid Chromatography

Alexander F. Siegle and Oliver Trapp*

Organisch-Chemisches Institut, Ruprecht-Karls-Universität Heidelberg, Im Neuenheimer Feld 270, 69120 Heidelberg, Germany

Supporting Information

ABSTRACT: High-performance liquid chromatography (HPLC) is an indispensable technique to separate, quantify, and identify a broad range of compounds. Recent advances in HPLC technology led to the development of ultrahigh-performance instruments that allow rapid sample analysis with high efficiency. Nevertheless, there is still the opportunity to increase the sample throughput and to improve the signal-to-noise ratio by the application of multiplexing, where the injected samples are encoded with defined sequences. The obtained signal is then deconvoluted to give conventional chromatograms. In this work we present a method and technique which can be easily implemented in commercially available HPLC instruments to perform multiplexing analysis. Using our approach, multiplexing can be performed on standard laboratory equipment by software control and offers an inherent advantage in sensitivity and minimization of analysis time, demonstrated for the analysis of highly diluted polynuclear aromatic hydrocarbon (PAH) samples in water.



Separation techniques are commonly employed in trace analysis for instance to detect impurities in chemical and pharmaceutical processes and products, to monitor critical limits of hazardous compounds in the environment, and to expose drug abuse. Thus, great effort has been put into research and development of sensitive detection methods. Combination of sensitive detection methods with mathematical techniques such as multiplexing where information is combined, overlapped, and subsequently deconvoluted offers an inherent advantage in trace analysis and helps to overcome limitations of the detection principle. Multiplexing techniques are usually based on Fourier and Hadamard transforms and utilize injection patterns which are derived from pseudorandom binary sequences.[1,2] Their use in spectroscopy[3] and mass spectrometry (MS)[4−7] is well-known. The application of pseudorandom binary sequences in chromatography was first described by Izawa in 1967 who used a pseudorandom signal generator in process gas chromatography (GC) for the continuous analysis of oxygen/nitrogen mixtures.[8] Shortly thereafter, Smit modulated sample injections according to a pseudorandom binary sequence in correlation gas chromatography.[9] Application of a 6-bit pseudorandom binary sequence ($2^6 - 1 = 63$ elements) and subsequent cross-correlation with the measured signal led to decreased background noise. This technique uses cross-correlation functions that enable alignments between samples and recorded signals,[10−14] in the simplest form if there is no peak overlapping every single peak can be assigned to an analyte in a certain sample. Later the scope of cross-correlation techniques was extended beyond GC[15−20] to liquid chromatography[21−26] and capillary electrophoresis.[27,28] Coupling of separation techniques with multiplexing based on the Hadamard transform (HT) was first introduced in capillary electrophoresis.[29−34] Herein the signal-to-noise ratio (S/N) of highly diluted samples could be significantly improved after inverse Hadamard transformation of the encoded electropherogram. Lin and co-workers reported the construction of devices which allowed the application of HT to GC/MS[35−40] and LC/MS.[35] In HT GC/MS the S/N could be improved up to ∼20-fold by applying 11-bit sequences (2047 matrix elements). We reported on the development of high-throughput multiplexing gas chromatography (htMPGC) which utilizes structured modulation sequences and HT to achieve very high sample throughputs (up to 453 samples/h).[41−44]

All known methods to couple separation techniques and multiplexing have in common that they rely on an external device like a custom-made injector which modulates the injection or the flow of the analyte according to a pseudorandom binary sequence. Encoding sequences are applied to the injector by pulsing the sample or opening an injection valve; a sequence element "1" injects a sample, while a sequence element "0" means no injection. Using an external injection device leads to higher costs for multiplexing techniques and causes further issues such as stability of sample

Received: August 6, 2014
Accepted: October 8, 2014
Published: October 8, 2014

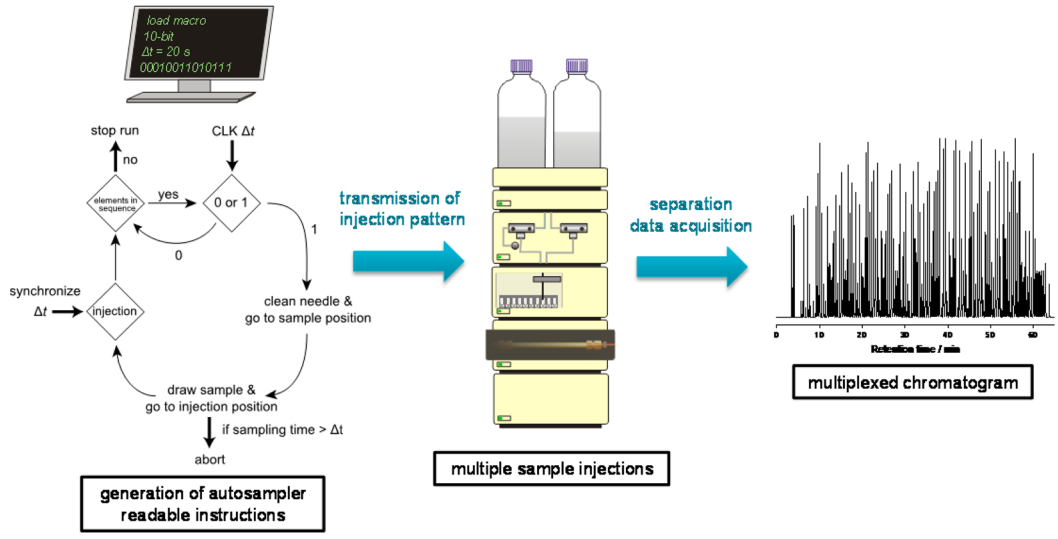


Figure 1. Schematic depiction of the experimental setup.

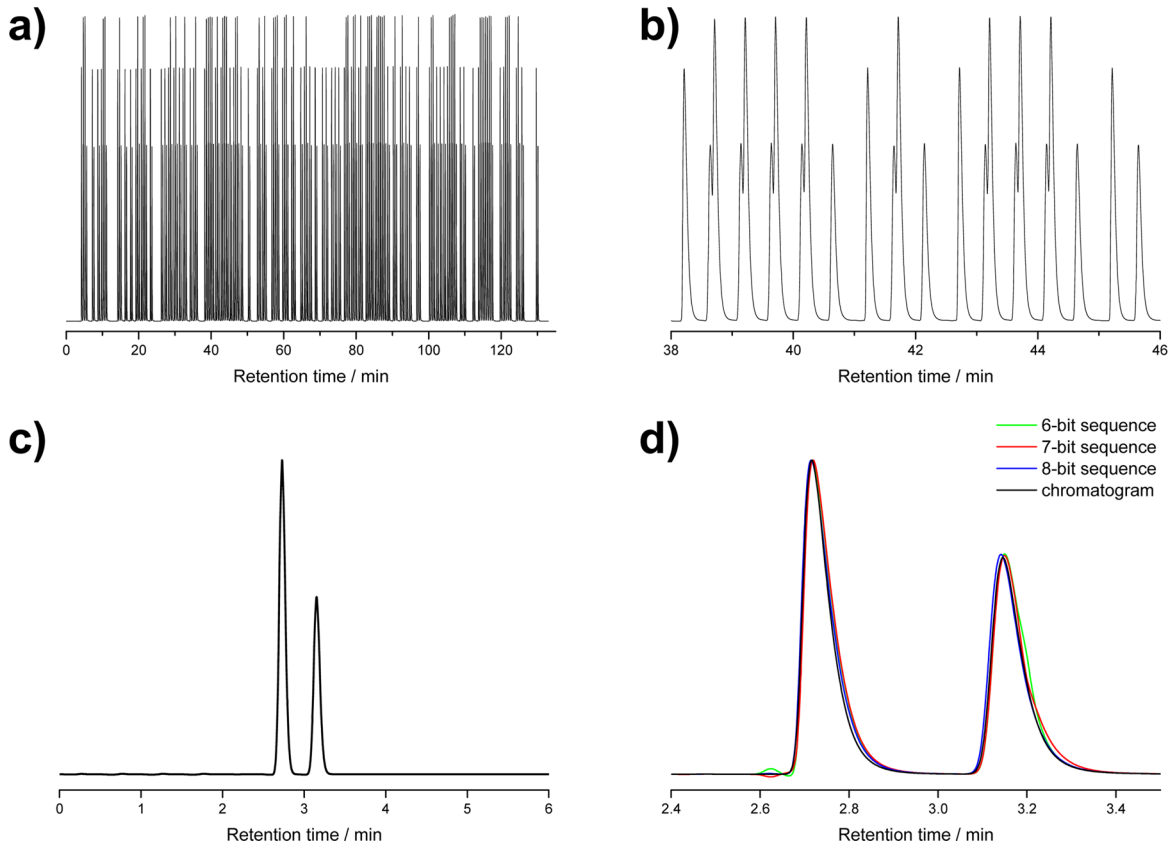


Figure 2. Utilization of a regular HPLC for Hadamard transform multiplexing leads to excellent injection stability. (a) Multiplexed chromatogram of an acetophenone/biphenyl mixture obtained by injection according to an 8-bit Hadamard sequence with a modulation time interval of Δt = 30 s; (b) zoomed area of the chromatogram; (c) corresponding Hadamard transformed chromatogram; (d) comparison of peak profiles obtained by using conventional liquid chromatography and varying modulation sequences in multiplexing HPLC.

injection, more complicated handling, and generally lower availability. In particular in liquid chromatography the construction of a device which allows hundreds to thousands of sample injections in a single multiplexing experiment poses high technical and engineering requirements regarding injection stability, solvent flow, and pressure. Here we present a method and technique which can be applied to a commercially available HPLC instrument using standard analytical columns and equipment without any instrumental modifications to perform multiplexing analysis and considerably improve detection sensitivity.

EXPERIMENTAL SECTION

Chemicals. Polynuclear aromatic hydrocarbons (PAHs) were supplied as a kit by Sigma-Aldrich (Steinheim, Germany). All other solvents and chemicals of analytical grade were

dx.doi.org/10.1021/ac502933f | Anal. Chem. 2014, 86, 10828−10833

purchased from Sigma-Aldrich (Steinheim, Germany) and used as received.

Apparatus. A commercially available Agilent 1260 Infinity system (Agilent Technologies, Palo Alto, U.S.A.), equipped with a high-performance autosampler model HiP-ALS SL+ and a DAD detector, was used for the multiplexing experiments. The HPLC was controlled by Agilent ChemStation software. We applied binary modulation sequences to the autosampler by creating a macro which can be read and executed by the Agilent ChemStation software. The macro contains a conditional loop, which creates a list of instructions for the autosampler of the HPLC setup based on the chosen Hadamard sequence (Figure 1). The amount of sample injected for multiplexing measurements was typically in the range of 1−10 μL although it is possible to use the full range of injection volume provided by the autosampler. Separation of the acetophenone/biphenyl mixture was conducted on a Daicel Chiralpak IB-3 column (3 μm, 4.6 mm × 150 mm, *n*-hexane/isopropyl alcohol 90:10, 1 mL/min). Toluene and biphenyl were separated on an Agilent Eclipse Plus C18 column (3.5 μm, 4.6 mm × 100 mm, acetonitrile/water 75:25, 1 mL/min). Reversed-phase separation of the PAH sample was performed on an Agilent Eclipse PAH column (1.8 μm, 4.6 mm × 30 mm, acetonitrile/water 95:5, 2.5 mL/min and 3.5 μm, 4.6 mm × 100 mm, acetonitrile/water 95:5, 2.5 mL/min, respectively). All samples were dissolved in the eluent.

Data Acquisition and Deconvolution. Data was typically collected with an acquisition rate of 40 Hz using the Agilent Chemstation software and exported as ASCII file for further data processing. Construction of pseudorandom binary sequences and inverse Hadamard transformation of the convoluted chromatograms was performed by a program written in Delphi (Embarcadero Technologies, South San Francisco, U.S.A.). The *n*-bit pseudorandom binary sequences used in all experiments contain $m = 2^n - 1$ elements ($2^n/2$ elements "1" and $2^n/2 - 1$ elements "0") and are derived from $(m + 1) \times (m + 1)$ Hadamard matrices. These were constructed by the use of a virtual shift register in silico. The full algorithm used therefor is described elsewhere.[45]

RESULTS AND DISCUSSION

In the here presented multiplexing approach commercially available HPLC systems without further hardware modifications can be employed. Thus, decades of development which led to advanced HPLC technology can be utilized advantageously. The design and development of a switching valve that is fast enough and suitable to be operated at high solvent pressures is highly challenging. Furthermore, switching of valves can add artifacts to the recorded signal that complicate the deconvolution of overlapping signals obtained by multiplexing. Therefore, we developed an algorithm and approach to apply binary modulation sequences to commercially available autosamplers by creating a macro which can be read and executed by the instrument's software, here an Agilent ChemStation. It has to be pointed out that the here presented macro is based on a very general algorithm (Figure 1 of the Supporting Information depicts a flowchart of the algorithm), which can be applied and transferred to any other instrument able to process commands and continuous sequence of injections with reliable temporal precision. The macro contains a conditional loop, which creates a list of instructions for the autosampler of the HPLC setup based on the chosen Hadamard sequence (Figure 1). The amount of sample injected can be adjusted in the macro, which was typically set to the range of 1−10 μL. Figure 2a shows a multiplexed HPLC trace of an acetophenone/biphenyl mixture obtained by execution of the constructed macro under normal phase conditions. The sample was injected according to an 8-bit Hadamard sequence comprising $2^8 - 1 = 255$ elements with a time interval of $\Delta t = 30$ s between two elements of the sequence. For 128 of those elements (number of elements "1" in the sequence) a sample injection was performed. The high reliability and stability of the injected sample volume manifests in three highly stable and reproducible peaks (two peaks corresponding to the analytes and the convoluted signal of both) as can be seen in Figure 2, parts a and b. Statistical evaluation of the peak areas yields a variation of 0.45% in the residual standard deviation (RSD) and confirms the observed signal stability. By inverse Hadamard transformation of the multiplexed chromatogram the transformed chromatogram of the sample can be obtained (Figure 2c). The accuracy of the transformation is demonstrated in Figure 2d. The comparison of chromatograms obtained by conventional HPLC with Hadamard transformed chromatograms corresponding to various modulation sequence lengths shows that peak profiles as well as peak areas are accurately conserved by the Hadamard encoding.

Precise determination of peak areas is required for the quantitative analysis of trace compounds. Hadamard transform multiplexing techniques are suitable for trace analysis since theory predicts an increase in S/N by a factor of $(m + 1)/2\sqrt{m}$ for sequences derived from Hadamard matrices of the order $m + 1$. In Figure 3 the analysis of an extremely diluted biphenyl/anthracene mixture (injected amounts of 50 and 10 pg,

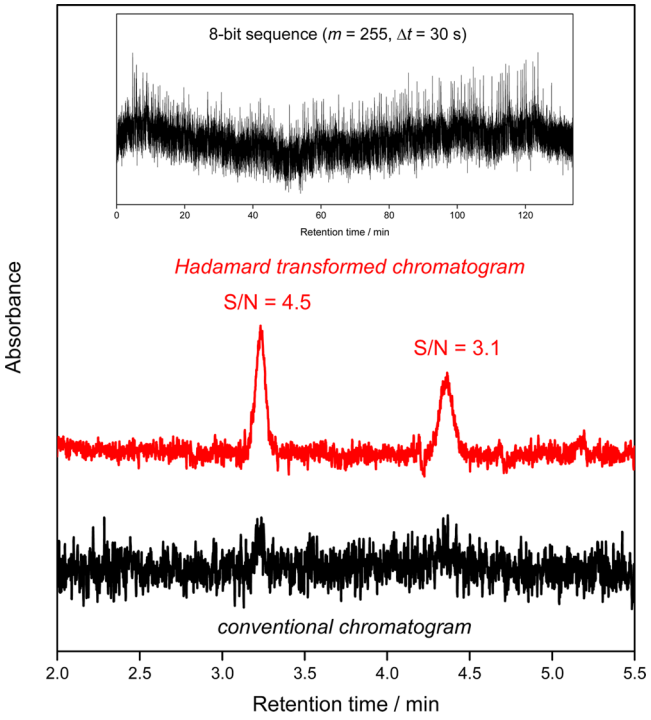


Figure 3. Conventional chromatogram (bottom) of an extremely diluted sample of biphenyl and anthracene and Hadamard transformed chromatogram (red line) with significantly enhanced analyte peaks and reduced noise. The inset (top) shows the multiplexed chromatogram obtained by applying an 8-bit Hadamard sequence with a modulation time interval of $\Delta t = 30$ s.

dx.doi.org/10.1021/ac502933f | *Anal. Chem.* 2014, 86, 10828−10833

respectively) under reversed-phase conditions is shown. Single injection of the sample yields a chromatogram wherein the analyte peaks are practically undetectable (S/N < 1). The inset of Figure 3 shows the multiplexed chromatogram which results from applying an 8-bit (m = 255) modulation of the injection with a time interval of Δt = 30 s. Inverse Hadamard transformation of the multiplexed chromatogram leads to a chromatogram in which the analyte signals are significantly enhanced. With an S/N of 4.5 and 3.1, respectively, the signals are now above the limit of detection (LOD). This corresponds to a 7.5-fold increase in S/N for the biphenyl peak and a 5.5-fold increase for the anthracene peak, which makes previously undetectable peaks now reliably detectable. It has to be noted that because of the extremely low signal intensity in the conventional chromatographic run the S/N of the analyte signals is hard to determine precisely.

An area of interest for trace analysis by liquid chromatography is the detection of polynuclear aromatic hydrocarbons (PAHs) in drinking water. PAHs are carcinogenic byproducts of the combustion of hydrocarbons, and their concentration in drinking water is strictly regulated. A sample containing trace amounts of six PAHs which are regulated in the European Drinking Water Directive[46] was analyzed using multiplexing and a fast separation on an ultraperformance separation column (Agilent Eclipse PAH, 1.8 μm, 4.6 mm × 30 mm) in reversed-phase mode (Figure 4). In the chromatogram resulting from a standard HPLC measurement only two peaks are visible, and all signals are below the LOD. The multiplexed chromatogram shown in the inset was obtained by injecting the same sample according to a 9-bit (m = 511) modulation sequence with a time interval of Δt = 18 s. In order to increase speed of analysis the time interval was decreased to 18 s, which is currently the time limit to equally address all vials on the sample rack and to complete the injection cycle. Inverse Hadamard transformation yields a chromatogram where all peaks are visible and four of the analyte signals are above the LOD. This demonstrates that the presented technique can be applied to complex analyte mixtures without sacrificing resolution and accuracy.

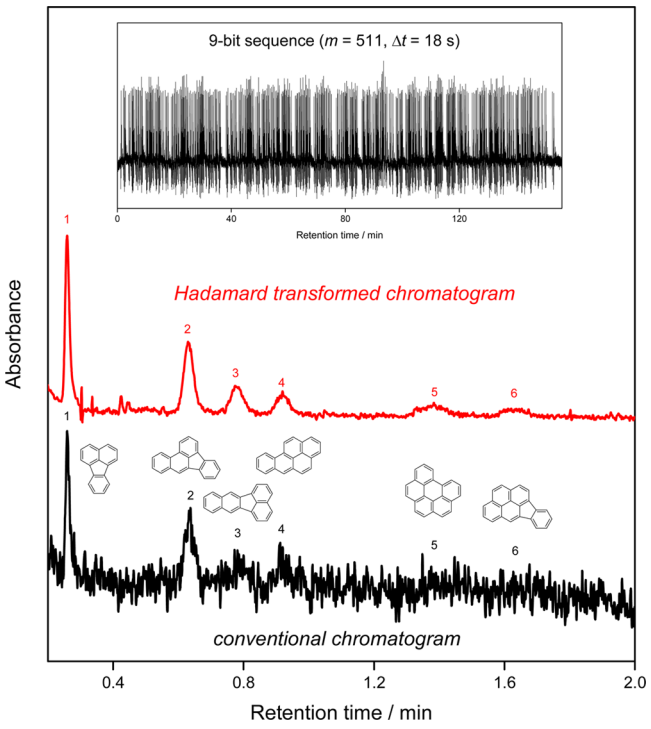


Figure 4. Conventional chromatogram (black line) and Hadamard transformed chromatogram (red line) of a sample containing trace amounts of six PAHs which are regulated by the European Drinking Water Directive. The inset (top) shows the multiplexed chromatogram obtained by injection according to a 9-bit Hadamard sequence with a modulation time interval of Δt = 18 s.

In the next step we investigated the influence of the modulation sequence length on the S/N of the PAH sample (Figure 5). All separations were performed on a standard column (Agilent Eclipse PAH, 3.5 μm, 4.6 mm × 100 mm) under the same experimental separation conditions as reported in Figure 4. To obtain precise values for the S/N enhancement a less diluted sample was used. Modulation sequence lengths of 8-bit (m = 255), 9-bit (m = 511), and 10-bit (m = 1023) have been applied while the time interval was Δt = 18 s for all measurements. Figure 5 shows a comparison of the chromatograms obtained by Hadamard transformation with the conventional chromatogram of the same sample. Again all peaks and their respective intensity relations are accurately preserved while the S/N is significantly enhanced. In comparison, in the single injection chromatogram (Figure 5) only the first two peaks can be reliably detected (S/N > LOD), whereas the Hadamard transformed chromatograms allow reliable detection of all peaks.

This demonstrates that column length and speed of the separation method do not influence the quality of the Hadamard transformed chromatograms.

A summary of evaluated values for the S/N enhancement is given in Table 1. It can be seen that the S/N enhancement increases for longer sequences (higher matrix order m) as predicted by theory. However, longer sequences are more prone to small temporal fluctuations in the injection interval, which becomes apparent by small equidistant wiggles in the Hadamard transformed chromatogram (cf. Figure 5, 10-bit sequence). The S/N enhancement is best for peak 1 (corresponding to fluoranthene) which shows good agreement with theoretically predicted values and gets progressively smaller for the broader peaks. A more detailed analysis of the S/N enhancement (cf. Figure 2 in the Supporting Information) depending on the peak width at half-height w_h, which in turn depends on the separation efficiency and the retention time, shows that the experimental S/N converges toward the theoretical value only for maximum separation efficiencies.

In summary these experiments show that the S/N can be considerably improved and the overall analysis time is shortened compared to summing up single measurements.

■ CONCLUSION

In the present study we developed a technique which allows performing multiplexing on a commercially available HPLC system by software modification. No additional sophisticated instrumentation is necessary, making this technique interesting for broader application. Our technique allows the reliable detection and quantification of substances below the detection limit of standard HPLC while using an identical setup and seems ideally suited for trace analysis. The simplicity and robustness of the approach makes multiplexing feasible for the ordinary user in a standard laboratory setup. Limitations currently include the speed of the autosampler, which increases the overall analysis time, and the prerequisite of reproducible

dx.doi.org/10.1021/ac502933f | Anal. Chem. 2014, 86, 10828−10833

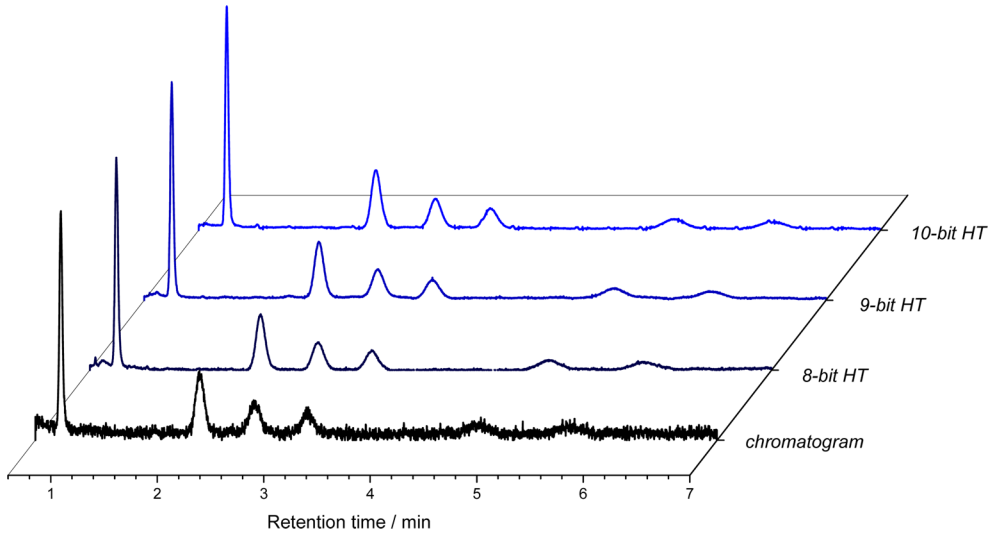


Figure 5. Comparison of Hadamard transformed chromatograms of different modulation sequence lengths (255, 511, 1023 matrix elements, respectively) with a conventionally obtained chromatogram.

Table 1. Influence of Varying Modulation Sequence Lengths on S/N Enhancement of Six Analytes in a Diluted PAH Sample

	S/N enhancement						
sequence length	theor	1	2	3	4	5	6
8-bit	8.02	7.36	6.83	6.37	5.87	4.40	4.04
9-bit	11.32	10.35	9.33	8.79	8.33	5.80	5.00
10-bit	16.01	15.42	14.14	13.37	11.53	8.50	8.40

and stable analysis conditions during sample modulation, which currently requires isocratic separation conditions.

Coupling with other detection techniques such as mass spectrometry, refractive index, or optical rotation measurements opens the avenue for a number of potential applications in the future and makes this technique broadly applicable.

Furthermore, it can be envisaged that the combination of the presented technique with algorithms developed for high-throughput multiplexing GC will maximize sample throughput of common HPLC systems. The high reliability of the injected sample volume and the possibility to run long continuous sequences is of great interest in process analysis, continuous reaction, and quality monitoring.

■ ASSOCIATED CONTENT

Supporting Information

Macro and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: trapp@oci.uni-heidelberg.de. Fax: +49-6221-544904.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the European Research Council under Grant Agreement Nos. StG 258740 and PoC 310196 and the Agilent Foundation, Santa Clara, California, for financial support. We thank Matthias Wetzel, Agilent Technologies Waldbronn, Germany, for helpful discussions.

■ REFERENCES

(1) Griffiths, P. R. *Transform Techniques in Chemistry*; Plenum Press: New York, 1978.
(2) Marshall, A. G. *Fourier, Hadamard, and Hilbert Transforms in Chemistry*; Plenum Press: New York, 1982.
(3) Ernst, R. R.; Anderson, W. A. *Rev. Sci. Instrum.* **1966**, *37*, 93.
(4) Comisarow, M. B.; Marshall, A. G. *Chem. Phys. Lett.* **1974**, *25*, 282.
(5) Brock, A.; Rodriguez, N.; Zare, R. N. *Anal. Chem.* **1998**, *70*, 3735.
(6) Fernández, F. M.; Vadillo, J. M.; Kimmel, J. R.; Wetterhall, M.; Markides, K.; Rodriguez, N.; Zare, R. N. *Anal. Chem.* **2002**, *74*, 1611.
(7) Trapp, O.; Kimmel, J. R.; Yoon, O. K.; Zuleta, I. A.; Fernandez, F. M.; Zare, R. N. *Angew. Chem., Int. Ed.* **2004**, *43*, 6541.
(8) Izawa, K. *Ind. Chim. Belge* **1967**, *32*, 223.
(9) Smit, H. C. *Chromatographia* **1970**, *3*, 515.
(10) Annino, R.; Grushka, E. *J. Chromatogr. Sci.* **1976**, *14*, 265.
(11) Phillips, J. B.; Burke, M. F. *J. Chromatogr. Sci.* **1976**, *14*, 495.
(12) Laeven, J. M.; Smit, H. C.; Kraak, J. C. *Anal. Chim. Acta* **1987**, *194*, 11.
(13) Fister, J. C.; Jacobson, S. C.; Ramsey, J. M. *Anal. Chem.* **1999**, *71*, 4460.
(14) Wong, J. W. H.; Durante, C.; Cartwright, H. M. *Anal. Chem.* **2005**, *77*, 5655.
(15) Annino, R.; Bullock, L. E. *Anal. Chem.* **1973**, *45*, 1221.
(16) Kaljurand, M.; Külllik, E. *J. Chromatogr.* **1979**, *171*, 243.
(17) Annino, R.; Gonnord, M.-F.; Guiochon, G. *Anal. Chem.* **1979**, *51*, 379.
(18) Villalanti, D. C.; Burke, M. F.; Phillips, J. B. *Anal. Chem.* **1979**, *51*, 2222.
(19) Valentin, J. R.; Carle, G. C.; Phillips, J. B. *Anal. Chem.* **1985**, *57*, 1035.
(20) Kaljurand, M.; Koel, M.; Küllik, E. *Anal. Chim. Acta* **1990**, *239*, 317.
(21) Lub, T. T.; Smith, H. C.; Poppe, H. *J. Chromatogr.* **1978**, *149*, 721.
(22) Smit, H. C.; Lub, T. T.; Vloon, W. J. *Anal. Chim. Acta* **1980**, *122*, 267.
(23) Laeven, J. M.; Smit, H. C.; Kraak, J. C. *Anal. Chim. Acta* **1983**, *150*, 253.
(24) Mars, C.; Smit, H. C. *Anal. Chim. Acta* **1990**, *228*, 193.
(25) Engelsma, M.; Kok, W. T.; Smit, H. C. *J. Chromatogr. A* **1990**, *506*, 201.

(26) Kaljurand, M.; Urbas, E.; Haldna, U. *Chromatographia* **1992**, *34*, 417.
(27) van der Moolen, J. N.; Louwerse, D. J.; Poppe, H.; Smit, H. C. *Chromatographia* **1995**, *40*, 368.
(28) van der Moolen, J. N.; Poppe, H.; Smit, H. C. *Anal. Chem.* **1997**, *69*, 4220.
(29) Kaneta, T.; Yamaguchi, Y.; Imasaka, T. *Anal. Chem.* **1999**, *71*, 5444.
(30) Kaneta, T.; Kosai, K.; Imasaka, T. *Anal. Chem.* **2002**, *74*, 2257.
(31) Hata, K.; Kichise, Y.; Kaneta, T.; Imasaka, T. *Anal. Chem.* **2003**, *75*, 1765.
(32) Hata, K.; Kaneta, T.; Imasaka, T. *Anal. Chem.* **2004**, *76*, 4421.
(33) Braun, K. L.; Hapuarachchi, S.; Fernandez, F. M.; Aspinwall, C. A. *Anal. Chem.* **2006**, *78*, 1628.
(34) Braun, K. L.; Hapuarachchi, S.; Fernandez, F. M.; Aspinwall, C. A. *Electrophoresis* **2007**, *28*, 3115.
(35) Lin, C.-H.; Kaneta, T.; Chen, H.-M.; Chen, W.-X.; Chang, H.-W.; Liu, J.-T. *Anal. Chem.* **2008**, *80*, 5755.
(36) Fan, Z.; Lin, C.-H.; Chang, H.-W.; Kaneta, T.; Lin, C.-H. *J. Chromatogr. A* **2010**, *1217*, 755.
(37) Cheng, C.-C.; Chang, H.-W.; Uchimura, T.; Imasaka, T.; Kaneta, T.; Lin, C.-H. *J. Sep. Sci.* **2010**, *33*, 626.
(38) Cheng, Y.-K.; Lin, C.-H.; Kaneta, T.; Imasaka, T. *J. Chromatogr. A* **2010**, *1217*, 5274.
(39) Cheng, Y.-K.; Lin, C.-H.; Kuo, S.; Yang, J.; Hsiung, S.-Y.; Wang, J.-L. *J. Chromatogr. A* **2012**, *1220*, 143.
(40) Fan, G.-T.; Yang, C.-L.; Lin, C.-H.; Chen, C.-C.; Shih, C.-H. *Talanta* **2014**, *120*, 386.
(41) Trapp, O. *Angew. Chem., Int. Ed.* **2007**, *46*, 5609.
(42) Trapp, O. *J. Chromatogr. A* **2010**, *1217*, 6640.
(43) Trapp, O. *LC·GC Eur.* **2011**, *24*, 172.
(44) Siegle, A. F.; Trapp, O. *Chem. Ing. Tech.* **2014**, *86*, 1044.
(45) Harwit, M.; Sloane, N. J. A. *Hadamard Transform Optics*; Academic Press: New York, 1979.
(46) Council Directive 98/83/EC.