Optimizing the Separation of 20 Nitro-aromatics Using Consecutively, a Phenyl-Hexyl Column with π-π Interaction and a C-18 Column on the Agilent 1290 Infinity Quaternary Method Development Solution

Application Note

Environmental

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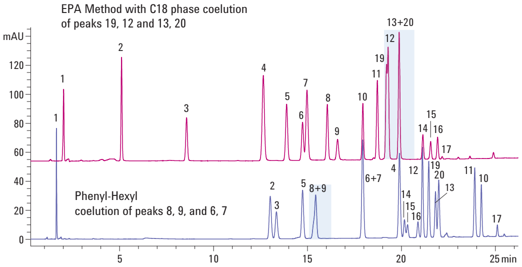
Abstract

The π-π interaction of Phenyl-Hexyl stationary phases with aromatic compounds has been proven by Kirkland and other authors[1,2]. This Application Note explores the influence of methanol, acetonitrile, and a mixture of both for the separation of nitro-aromatics on an Agilent Phenyl-Hexyl phase. In addition, the EPA method 8330A/B based on a C18 phase was used for further separation techniques. Solvent and gradient scouting for the two columns was done using the Agilent 1290 Infinity Quaternary Method Development Solution. Applying the EPA method and the method developed using the Phenyl-Hexyl column, all nitro-aromatics could be separated. A UV spectral library was used to identify the compounds in the different chromatograms. The 1290 Infinity Quaternary Method Development Solution offered the possibility to run both chromatographic methods on a single system, using different columns, mobile phases, and gradients, without the need for manual interaction.



Introduction

The separation of nitro-aromatics is a challenge due to the fact that some compounds are structural isomers and tend to coelute such as 2,6 and 2,4 di-nitrotoluene. Especially for aromatic compounds, the use of Phenyl-Hexyl columns is advantageous due to an additional separation mechanism. Among others, Kirkland stated that:

"Phenyl-Hexyl can retain analytes via several different mechanisms, including π–π interactions between the overlap of the delocalized electrons on the analyte and the stationary phase phenyl group, and via partitioning between the mobile phase. Acetonitrile tends to decrease the π–π interactions between aromatic and polarizable analytes and a phenyl stationary phases, but methanol enhances those same interactions, giving both increased retention and changes in selectivity. This does not mean that acetonitrile should not be used with a phenyl bonded phase or that it might not provide an acceptable separation, but methanol is more likely to deliver the additional selectivity that is desired from a phenyl phase"[1].

The influence of methanol, acetonitrile, and mixtures of both was evaluated on the separation of 20 nitro-aromatics, using a Pheny-Hexyl column. To evaluate these data, the 1290 Infinity Method Development System, in combination with the Method Scouting Wizard[3,4] was used for solvent and gradient scouting. Identification of compounds was done through a spectral library. The results were compared to results using the EPA method, which uses a C18 phase and also methanol as organic phase.

The Agilent 1290 Series Method Development Solution allows applying methods with different columns, gradients, and solvents without the need for manual interaction. Solvents and columns are method parameters and are automatically changed while uploading a new method. This is especially advantageous if two or more different sets of chromatographic conditions are needed to separate all compounds in complex mixtures.

Experimental

Instrumentation

Table 1. Instrumentation used.

	Agilent 1290 Infinity LC Method Development Solution
Quaternary Pump	G4204A
Auto sampler	G4226A
ALS cooler	G1330B
Column compartment 1 equipped with high pressure column switching valve	G1316C +Option #058
Column compartment 2 equipped with low pressure column switching valve	G1316C +Option #058
Diode array detector	G4212A
Method development kit +capillary kit	G4230B +Option #003

Chromatographic conditions

Compounds	20 nitro-aromatics were purchased from Dr. Ehrenstorfer, Nitroaromatic-Explosive Mix. 3 (08330300) 10 mg/each
Column 1	Agilent Poroshell 120 Phenyl Hexyl, 4.6 × 100 mm, 2.7 µm, p/n 695975-912
Column 2	Agilent Poroshell 120 C-18, 4.6 × 100 mm, 2.7 µm, p/n 685975-902
Mobile phases	(A) Water, (B) Acetonitrile, (C) Methanol
Gradient	see Figure 1
Flow rate	0.8 mL/min for C18 phase and 1 mL/min for Phenyl-Hexyl phase
Injection volume	3 µL
Column temperature	45 °C
Detection	214,235,254/10 nm, Reference 400/80 nm, 20 Hz, 10-mm cell, every second spectra was taken

Gradient 1

Minutes	A water	B ACN
0	80	20
10	70	30
30	10	90
31	10	90

Gradient 2

Minutes	A water	B ACN
0	80	20
10	70	30
30	10	90
31	10	90

Gradient 3

Minutes	A water	B ACN	C MeOH
0	75	5	20
10	70	5	25
30	15	5	80
31	5	5	90

Gradient 4

Minutes	A water	B ACN	C MeOH
0	75	5	20
10	85	5	25
30	15	5	80
31	5	5	90

Gradient 5

Minutes	A water	B ACN	C MeOH
0	75	5	15
10	70	5	25
30	5	5	90
31	5	5	90

Gradient 6

Minutes	A water	B ACN	C MeOH
0	75	5	20
10	70	5	25
30	5	5	90
31	5	5	90

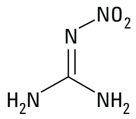
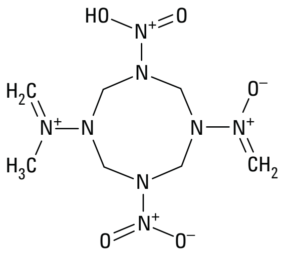
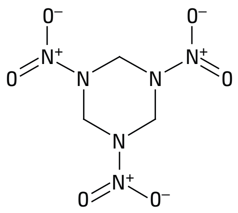
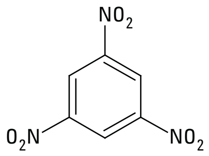
Gradient 7

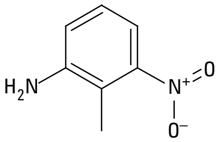
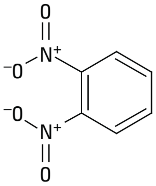
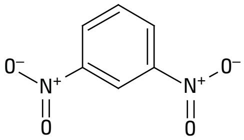
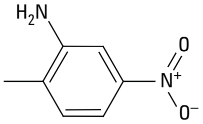
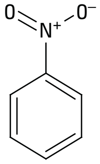
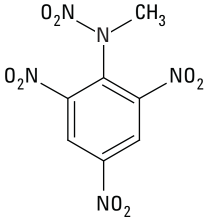
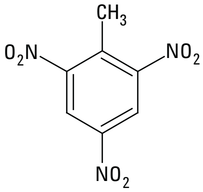
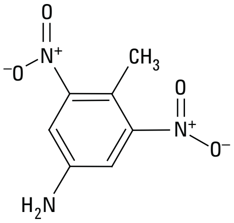
Minutes	A water	B ACN	C MeOH
0	80		20
10	70		30
17.99	50		50
18.00	45	5	50
30	20	5	75
31	5	5	90

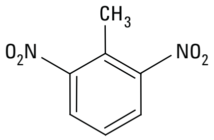
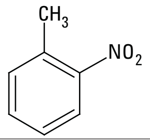
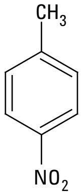
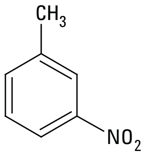
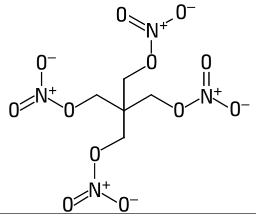
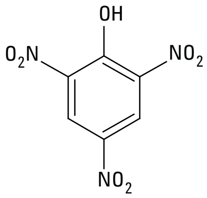
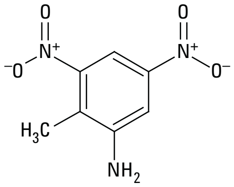
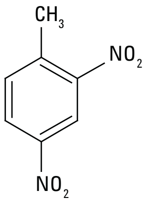
Figure 1. Binary and ternary gradients applied.

Analyzed compounds

Table 2. Compounds analyzed, the peak ID number is used in the chromatograms.

Peak ID number	Name of compound	Structure
Peak 1	Nitroguanidin	
Peak 2	Octogen (HMX)	
Peak 3	Hexogen (RDX)	
Peak 4	1,3,5 Trinitrobenzene	

Peak ID number	Name of compound	Structure
Peak 5	2-Amino-6-nitrotoluene	
Peak 6	1.2-Dinitrobenzene	
Peak 7	1,3-Dinitrobenzene	
Peak 8	2-Amino-4-Nitrotoluene	
Peak 9	Nitrobenzene	
Peak 10	Tetryl	
Peak 11	2,4,6-Trinitrotoluene (TNT)	
Peak 12	4-Amino-2,6-dinitrotoluene	

Peak ID number	Name of compound	Structure
Peak 13	2,6-Dinitrotoluene	
Peak 14	2-Nitrotoluene	
Peak 15	4-Nitrotoluene	
Peak 16	3-Nitrotoluene	
Peak 17	Nitropenta	
Peak 18	2,4,6-Trinitrophenol (not found)	
Peak 19	2-Amino-4,6-dinitrotoluene	
Peak 20	2,4-Dinitrotoluene	

Acquisition and evaluation software

OpenLAB CDS Chemstation version C.01.05 and Method Scouting Wizard version A.02.02

Results and Discussion

The following experiments were performed:

- Analyzing the 20 nitro-aromatics using a binary gradient and either acetonitrile or methanol as organic phase and the Phenyl-Hexyl column
- Analyzing the compounds using a ternary gradient and a mixture of acetonitrile and methanol as organic phase and the Phenyl-Hexyl phase
- Analyzing the compounds using the EPA method using a binary gradient, a C18 column, and methanol as mobile phase
- Comparison of both separation methods

Influence of methanol or acetonitrile as mobile phase

The compounds were analyzed using either methanol (gradient 2) or acetonitrile (gradient 1) as organic mobile phase. In Figure 2, the resulting chromatograms are overlaid. There were significant differences in selectivity. The numbers above the peaks reflect the peak numbers in Table 2. Peaks 5, 8, 9, 19, and 12 eluted significantly later using methanol as organic mobile phase. This confirms that methanol strengthens the π-π interaction of the nitro-aromatics with the Phenyl-Hexyl phases. The number of nitro-groups determined the elution order due to the ability to interact with the Phenyl-Hexyl phase. For example, the mono-nitro-toluenes (peaks 14, 15, and 16) eluted before the dinitrotoluenes (peaks 20 and 13) followed by the trinitrotoluene (peak 11). The first peak, nitroguanidin, eluted below 2 minutes and is not shown in Figure 2. This compound showed no selectivity change regardless which gradient or mobile phase or column was applied and is, therefore, not shown in the chromatograms in Figure 2.

Analysis using a mixture of acetonitrile and methanol as organic phase

The 1290 Infinity Series Method Development Solution in combination with the Method Scouting Wizard allows not only column scouting but also solvent, gradient, and temperature scouting. A set of different binary and ternary gradients were set up by just a few mouse clicks. The resulting sequence was automatically created including all equilibration, flushing, and column storage methods.

A mixture of methanol and acetonitrile was tested for its influence on the separation. In Figure 3, the chromatogram obtained using methanol only (gradient 2) and the chromatogram obtained by adding 5 % acetonitrile (gradient 3) are overlaid.

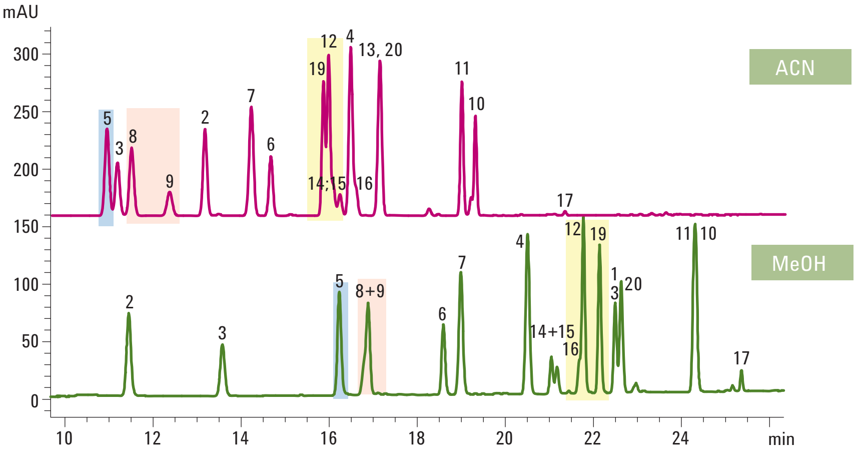


Figure 2. Analysis of nitro-aromatics using acetonitrile or methanol as mobile phase.

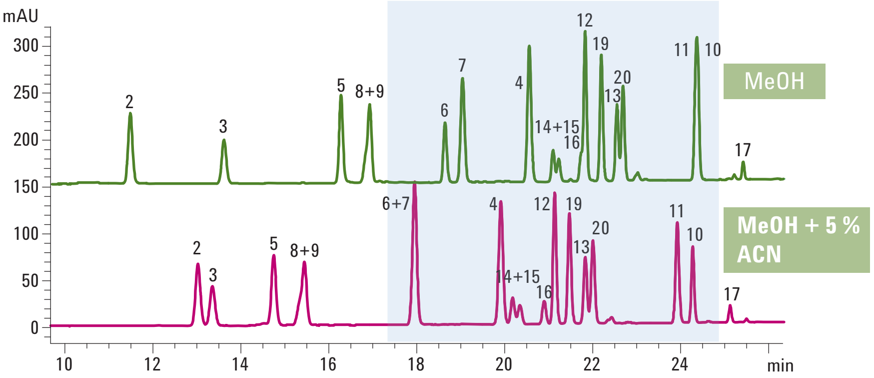


Figure 3. Analysis of nitro-aromatics using methanol and a mixture of 5 % acetonitrile in methanol as organic phase.

The addition of 5 % acetonitrile improved the separation of peaks 14,15,16 (2-, 3-, and 4-nitrotoluene). Also peak 11 (TNT) and 10 (tetryl) are separated whereas peak 6 (1,2-dinitro toluene) and peak 7(1,3-dinitrotoluene) coeluted using gradient 3.

Small gradient changes created significant differences in selectivity, (Figure 4).

Identification using a UV library

Identification of compounds was done using a UV spectral library (Figures 5 and 6). A spectral library was set up for explosives and was used as the identification tool. Every second spectra was taken during the analysis. This also allowed the identification of coeluting peaks.

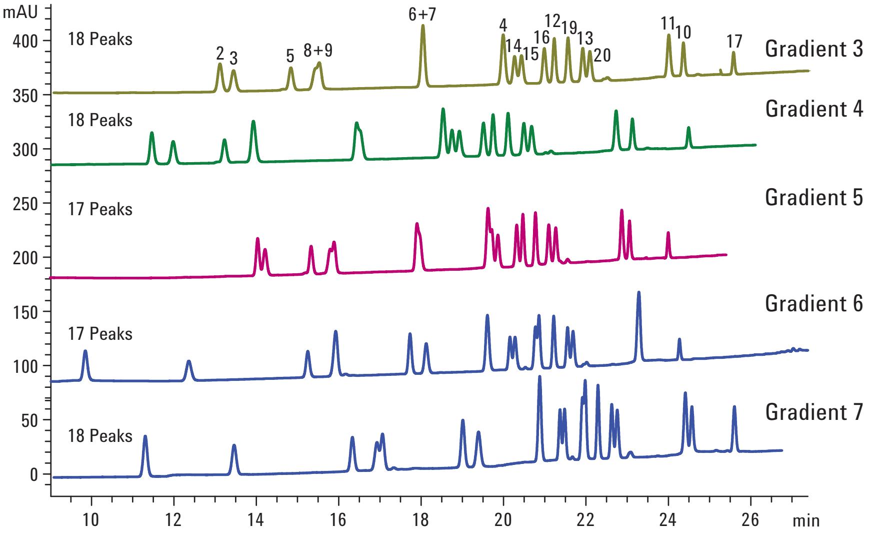
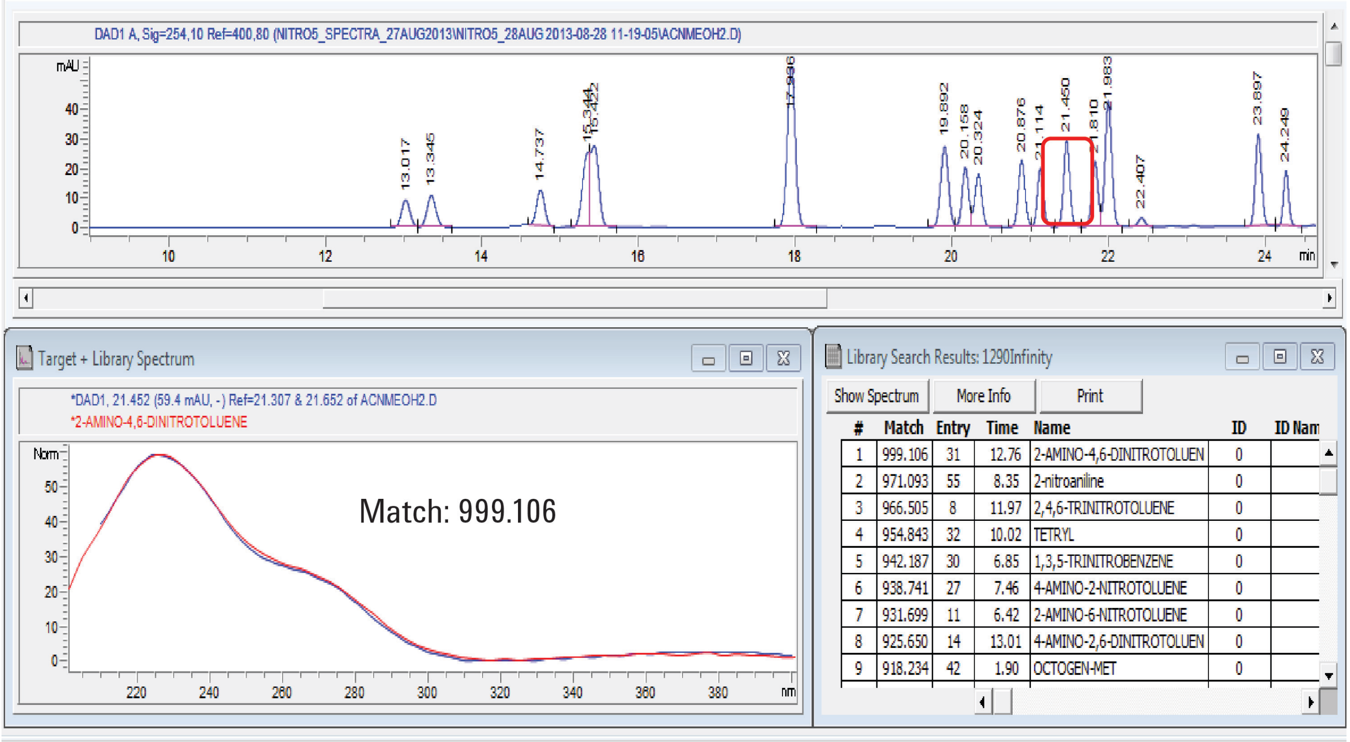


Figure 4. Influence of different gradients on selectivity.



DAD1 A, Sig=254,10 Ref=400,80 (NITRO5_SPECTRA_27AUG2013\NITRO5_28AUG 2013-08-28 11-19-05\ACNMEOH2.D)

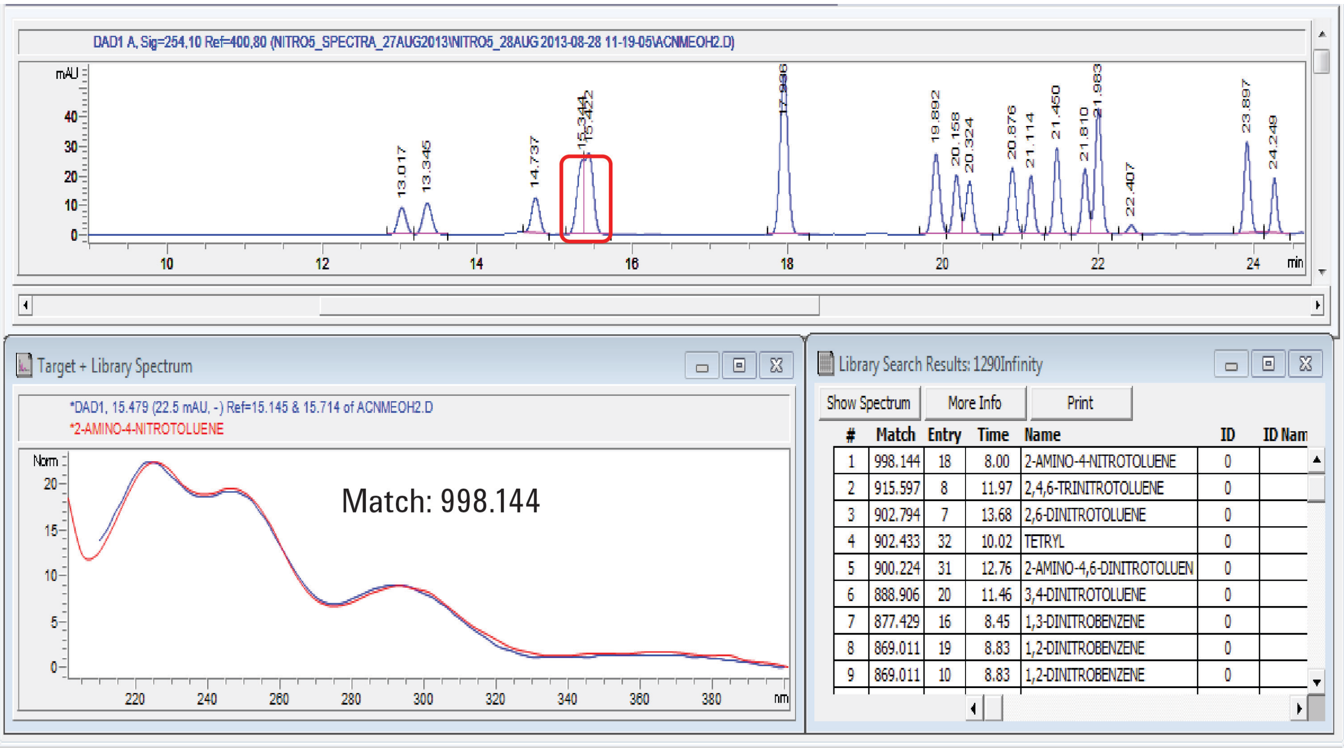
*DAD1, 21.452 (59.4 mAU, -) Ref=21.307 & 21.652 of ACNMEOH2.D
*2-AMINO-4,6-DINITROTOLUENE

Match: 999.106

#	Match	Entry	Time	Name	ID	ID Nam
1	999.106	31	12.76	2-AMINO-4,6-DINITROTOLUEN	0	
2	971.093	55	8.35	2-nitroaniline	0	
3	966.505	8	11.97	2,4,6-TRINITROTOLUENE	0	
4	954.843	32	10.02	TETRYL	0	
5	942.187	30	6.85	1,3,5-TRINITROBENZENE	0	
6	938.741	27	7.46	4-AMINO-2-NITROTOLUENE	0	
7	931.699	11	6.42	2-AMINO-6-NITROTOLUENE	0	
8	925.650	14	13.01	4-AMINO-2,6-DINITROTOLUEN	0	
9	918.234	42	1.90	OCTOGEN-MET	0	

Figure 5. Identification of 2-Amino-4,6-dinitrotoluene, fully resolved peak.

Identification of nonresolved peaks was also possible with high reliability by taking spectra at the upslope and at the downslope of the peak (Figure 6).


DAD1 A, Sig=254,10 Ref=400,80 (NITRO5_SPECTRA_27AUG2013\NITRO5_28AUG 2013-08-28 11-19-05\ACNMEOH2.D)

*DAD1, 15.479 (22.5 mAU, -) Ref=15.145 & 15.714 of ACNMEOH2.D
*2-AMINO-4-NITROTOLUENE

Match: 998.144

#	Match	Entry	Time	Name	ID	ID Nam
1	998.144	18	8.00	2-AMINO-4-NITROTOLUENE	0	
2	915.597	8	11.97	2,4,6-TRINITROTOLUENE	0	
3	902.794	7	13.68	2,6-DINITROTOLUENE	0	
4	902.433	32	10.02	TETRYL	0	
5	900.224	31	12.76	2-AMINO-4,6-DINITROTOLUEN	0	
6	888.906	20	11.46	3,4-DINITROTOLUENE	0	
7	877.429	16	8.45	1,3-DINITROBENZENE	0	
8	869.011	19	8.83	1,2-DINITROBENZENE	0	
9	869.011	10	8.83	1,2-DINITROBENZENE	0	

Figure 6. Identification of 2-amino-4-nitrotoluene, partially resolved peak.

Comparison with EPA Method 8330A/B

Following the EPA method[5], a C18 phase was used and compared with the results obtained on the Phenyl-Hexyl phase. The selectivity and, consequently, the peak elution order was completely different (Figure 7). Interesting was that the mono-nitrotoluenes (peaks 14, 15, and 16) eluted after the di- (peaks 6 and 7) and trinitrotoluene (peak 11). This supports the assumption that the C18 phase had, in this case, a different retention mechanism than the Phenyl-Hexyl phase.

Both columns showed coelution of some peaks but not for the same peaks. Peaks which are separated with C18 and methanol as mobile phase (gradient 2) are not separated using the Phenyl-Hexyl phase and methanol with 5 % acetonitrile (gradient 3) as mobile phase and *vice versa*. This offered the possibility to separate all compounds using two injections with two different chromatographic parameters.

Conclusion

Mixtures of nitro-aromatics often contain structural isomers which coelute using the EPA method (8330A/B). In this case, it was advantageous to apply a further method with different selectivity to separate peaks which were not separated. An alternative was using a Phenyl-Hexyl column, which provides an additional separation mechanism through π-π interaction of electrons of the aromatic compounds and the stationary phase phenyl groups of the column. By combining these two methods, a complete characterization of 19 nitro-aromatics was possible. A UV spectral library was used for identification. The Agilent 1290 Infinity Quaternary Method Development Solution offers the possibility to set up a workflow where first the EPA method and afterwards the Phenyl-Hexyl method is applied. Conditions like columns, mobile phases, and gradients are changed automatically while uploading the new method.

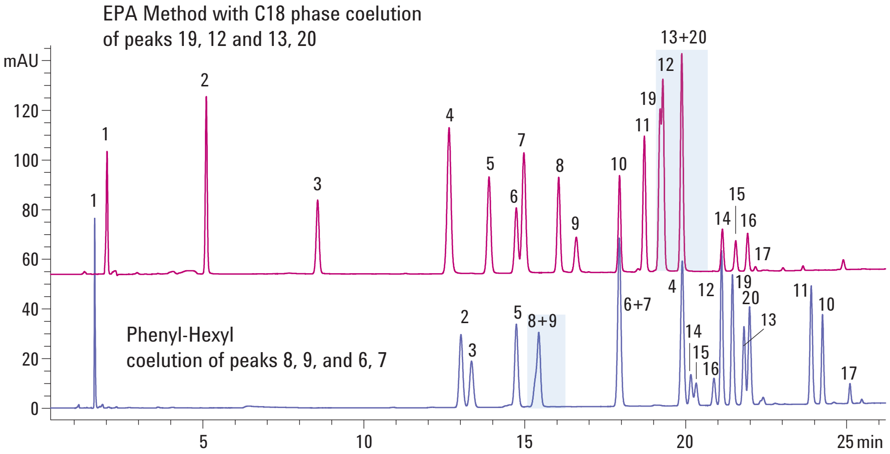


Figure 7. Comparison of EPA conditions using a C18 phase and conditions using a Phenyl-Hexyl column.

References

1. http://www.hplc.eu/halo.htm
2. M. Yang, S. Fazio, D. Munch, P. Drumm "Impact of methanol and acetonitrile on separations based on pi-pi interactions with a reversed-phase phenyl column" *J. Chromatogr. A* **2005** Dec 2; 1097 (1-2):124-9. Epub 2005 Aug 30.
3. A.G. Huesgen "Agilent 1260 Infinity Method Development Solution - Automatic Scaling of Gradient Times and Flow Rates for Different Column Lengths and Diameters Using the Agilent ChemStation Method Scouting Wizard" Agilent Application Note, Publication number 5990-6863EN, December **2010**.
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5. A.G. Huesgen "Seamless instrument-to-instrument method transfer of the EPA method 8330A/B for nitro-aromatics from an Agilent 1200 Series LC to the Agilent 1290 Infinity Binary LC using ISET" Agilent Application Note, Publication number 5991-1194EN, Oct **2012**.

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