

Sensitive Analyses of Genotoxic Analytes by HPLC-ECD and HPLC-FLD



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ABSTRACT

Genotoxins are compounds that have the potential to chemically modify DNA and are considered to be mutagenic and carcinogenic. If these genetic modifications occur within an organism's gametes, descendants who were never exposed to the initial genotoxin may be adversely affected. Many genotoxins can be found in the environment, both natural and man-made (e.g., polycyclic aromatic hydrocarbons [PAHs], mitomycin C, aflatoxins, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, and formaldehyde). Exposure to genotoxic impurities/residues is also of great concern to the pharmaceutical industry and is regulated by the FDA, as active pharmaceutical ingredients (APIs) can be contaminated with genotoxins resulting from their manufacture (e.g., intermediates, catalysts, and solvents). Due to their potential toxicity, these compounds are being limited to a daily exposure of less than 1.5 μg in APIs.¹ Consequently, highly sensitive analytical methods are required. Two approaches were evaluated—HPLC with electrochemical detection (ECD) and HPLC-fluorescence (FLD)—for the analysis of genotoxins.

Most genotoxins are not electrochemically active and cannot be measured with traditional HPLC-ECD techniques. However, this can be overcome through the use of a novel boron-doped diamond (BDD) electrode at high potentials. Here, the production of hydroxyl free radicals are used to electrotag the inert analyte, thereby rendering it electrochemically active. As discussed in detail, this approach was used to measure alkyl tosylates, alkyl besylates, aminopyridines, and PAHs, with mid-pg limits of detection. Sensitivity for PAHs was further improved by the use of FLD. Using a novel, programmable FLD with rapid changes in wavelengths, 17 PAHs were determined in 6 min with limits of detection of <10 fg on column.

INTRODUCTION

During the synthesis of many APIs, impurities may either be present or generated that may have genotoxic properties associated with them. For example, some of these impurities—which include aminopyridines, alkyl tosylates, and alkyl besylates—result from their use as protecting groups. Other genotoxic compounds that are ubiquitous in the general environment include PAHs, which are caused by the manufacture of fossil fuels and as a waste product of general combustion.

To measure these compounds in various matrices, including APIs and environmental samples, sensitive separation and detection techniques are required. Both liquid and gas chromatographic (GC) methods are used. The use of GC methods for API analysis requires the injection of relatively large amounts of API, which can cause problems with injector linings and deterioration of capillary columns. HPLC, on the other hand, offers greater instrument stability. The two means of detection detailed here—ECD with the BDD electrode and fluorescence—enable the use of low-level sensitivity detection required for these determinations, without the degradation of system performance.

A unique working electrode material, BDD can operate at higher applied potentials than other working electrode materials such as glassy carbon, gold, or platinum, which tend to form oxide layers when elevated potentials are employed. When operating at higher electrode potentials, certain organic compounds that typically do not react at lower potentials can be forced to react at the BDD electrode surface. The oxidation of organic compounds at BDD can be divided into two mechanism types,^{2,3} depending on the applied potential:

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1. Direct electrochemical oxidation occurs in the region of water stability before oxygen evolution, where only reactions involving simple electron transfer can occur. One of the more important aspects of the BDD electrode is that higher potentials can be used to force electrochemically nonreactive analytes to oxidize at potentials that are impractical with conventional carbon electrodes, due to the adverse effects of mobile phase (water) oxidation.
2. Electrotagging describes an indirect electrochemical oxidation caused by a sequence of reactions that are initiated by HO• radicals formed at the BDD surface in the potential region of oxygen evolution (water electrolysis). Due to the high reactivity of HO• radicals, these reactions are confined to an adsorbed thin film adjacent to the electrode surface. The resulting hydroxylated adducts of these inactive analytes become electrochemically active, and it is the oxidation of these hydroxylated products that is being measured.

Both the HO• consumption and the applied measurement occur on the same BDD electrode. Although some aromatic compounds are inherently electrochemically active, due to heteroatoms present in aromatic rings (e.g., indole) or by the presence of substituents on the ring (e.g., phenol, aniline, etc.), PAHs are highly stable. Sixteen PAHs are shown in Figure 1. Like many other aromatic compounds and impurities, these PAHs do not respond to electrochemical detection using typical carbon-based electrodes, but they do react using the BDD electrode chemistry.

Several methods are presented here using the ECD-BDD approach for the determination of a few classes of genotoxic analytes with low-level sensitivity. A rapid separation LC (RSLC) method using a rapid wavelength changing fluorescence detector is also described using a short, 6 min analysis of 17 PAH analytes at very low levels.

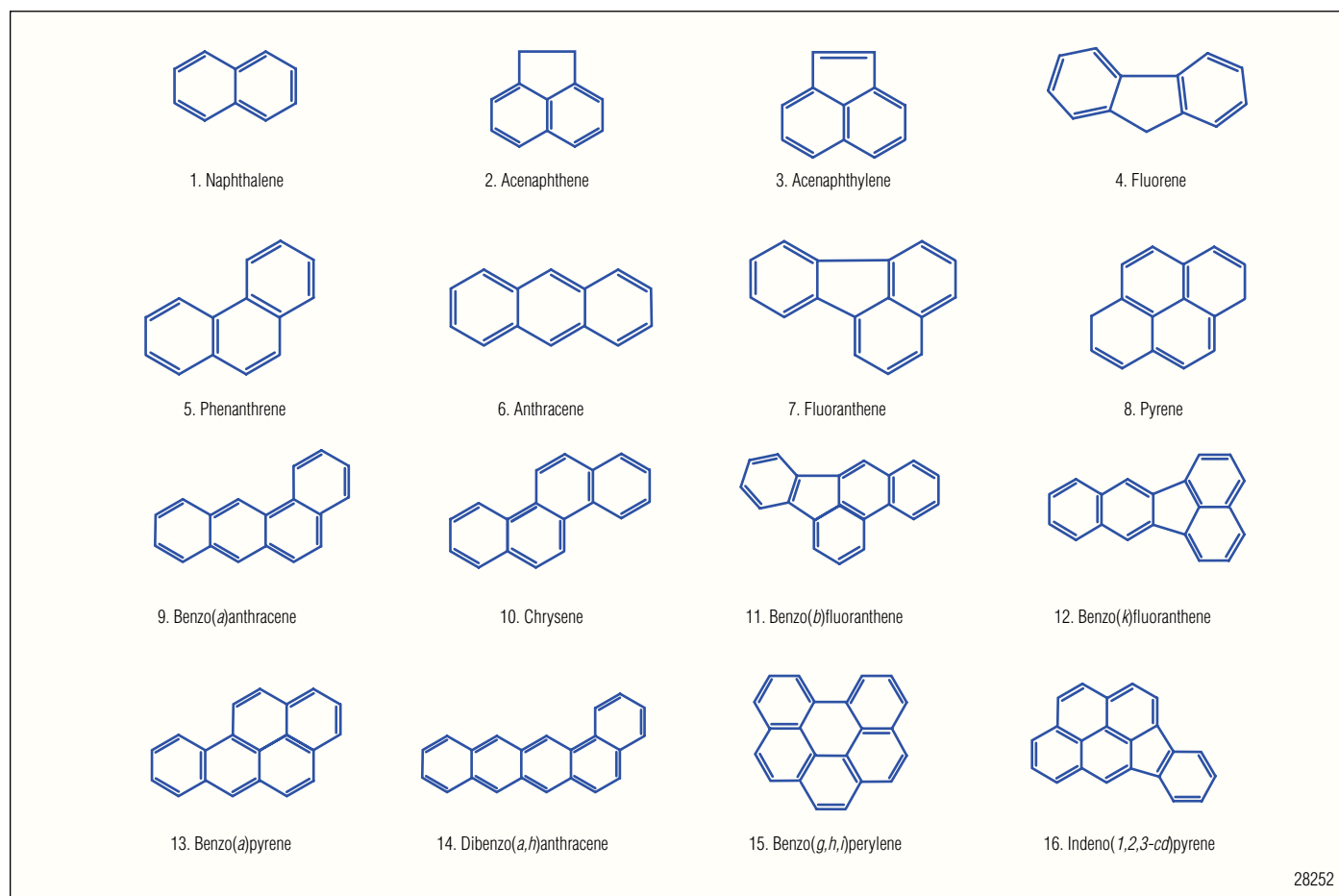


Figure 1. Structures of 16 PAH compounds analyzed by HPLC-ECD and HPLC-FLD.

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METHODS

HPLC Method Conditions 1

Isocratic ECD of Aminopyrindines⁴

Column: Obelisc™ R, 5 µm, 4.6 × 150 mm
Mobile Phase: 40 mM Ammonium acetate, pH 4.4/acetonitrile (1:1)
Column Temperature: 35 °C
Sample Temperature: Ambient
Inj. Volume: 40 µL
Flow Rate: 1.00 mL/min
Detector: CoulArray® Coulometric Array Detector
BDD Cell: +1400 mV (vs Pd reference)

HPLC Method Conditions 2

Isocratic ECD of Alkylsulfonic Esters^{5,6}

Column: Fused-Core® C18, 2.7 µm, 3 × 75 mm
Mobile Phase: 40 mM Sodium perchlorate, 20 mM perchloric acid, 25 µL/L hydrogen peroxide in polished water*/acetonitrile (65:35)
Column Temperature: 35 °C
Sample Temperature: 10 °C
Inj. Volume: 10 µL
Flow Rate: 0.45 mL/min
Detector: Coulochem® III Electrochemical Detector
Guard Cell: +1000 mV (vs Pd reference)
BDD Cell: +2000 mV (vs Pd reference)
Filter: 5 s

HPLC Method Conditions 3

Gradient ECD of PAHs⁷

Column: Pinnacle® II, 4 µm, 2.1 × 100 mm
Mobile Phase A: Buffer/water/acetonitrile (225:675:100), 25 µL/L hydrogen peroxide
Mobile Phase B: Buffer/acetonitrile (200:800), 25 µL/L hydrogen peroxide
Buffer: 200 mM Sodium perchlorate, 100 mM perchloric acid in polished water*

Column Temperature: 35 °C
Sample Temperature: 10 °C
Inj. Volume: 10 µL
Flow Rate: 0.60 mL/min
Guard Cell: +500 mV (vs Pd reference)
BDD Cell: +1750 mV (vs Pd reference)
Filter: 5 s

Gradient:

Time (min)	%B
0.0	30
7.0	58
16.0	100
25.0	100
25.1	30
30.0	30

HPLC Method Conditions 4

Gradient Fluorescence Detection of PAHs

Column: Nucleodur® C18 PAH, 3 µm, 100 × 3.0 mm
Mobile Phase A: Deionized water
Mobile Phase B: Acetonitrile
Column Temp: 30 °C
Inj. Volume: 1 2.5 µL
Flow Rate: 2.0 mL/min
Fluorescent Wavelength Program: See program in Figure 6

Gradient:

Time (min)	%B
0.000	45
0.500	45
3.762	90
4.275	90
5.288	95
5.500	45
6.250	45

*Polished water is deionized water that has been pulled through a C-18 Sep-pak under vacuum: http://www.esainc.com/docs/spool/70-1668P_Water_Polishing.pdf

RESULTS AND DISCUSSION

Analysis of Aminopyridines by ECD-BDD

Aminopyridines were analyzed using the BDD electrode at +1400 mV. The three isomers, 2-, 3-, and 4-aminopyridines, were readily resolved using Method Conditions 1. This method had an LOQ value of <700 pg on column (o.c.) (17 ng/mL) for all three analytes. Calibration curves were linear from 400–4000 pg o.c., with a high-precision, peak area RSD of <0.9% at 160 pg o.c., and recovery values of 95–114%. For comparison, a GC method⁷ using a nitrogen-phosphorus detector had LOQ values of 1000–2800 ng/mL for these three analytes.

Analysis of Alkylsulfonic Esters by ECD-BDD

Analysis of alkylsulfonic esters required the use of higher oxidation potentials, possibly due to the presence of the electron withdrawing $-\text{SO}_3-\text{R}$ on the aromatic system. It was found that the addition of oxidative mobile phase modifier, perchlorate, lowered the analytical oxidative potential and improved response. Using the parameters outlined in Method Conditions 2, methyl tosylate and alkyl besylates were analyzed and resolved in under 4 min, as shown in Figure 3.

The calibration curves for the besylates and tosylate (only methyl tosylate shown here) were linear from their LOD to 12,700 pg on column. The besylates had an LOD value of 400 pg o.c., whereas that of the tosylates was <70 pg o.c. Precision was high with peak area RSD values <10% for all amounts >700 pg o.c. Interestingly, during development of this method, it was found that dissolved oxygen was necessary for the alkyl besylate analytes to respond: before a degasser was put in-line, both the besylate and tosylate compounds responded to the BDD potential at +2000 mV. When the degasser was installed to stabilize the baseline, the besylate responses vanished, leaving only the tosylate response. The response for the besylates could be re-established if a small amount of hydrogen peroxide was added to the mobile phases. The addition of hydrogen peroxide was found to also be necessary to obtain responses for the PAH compounds.

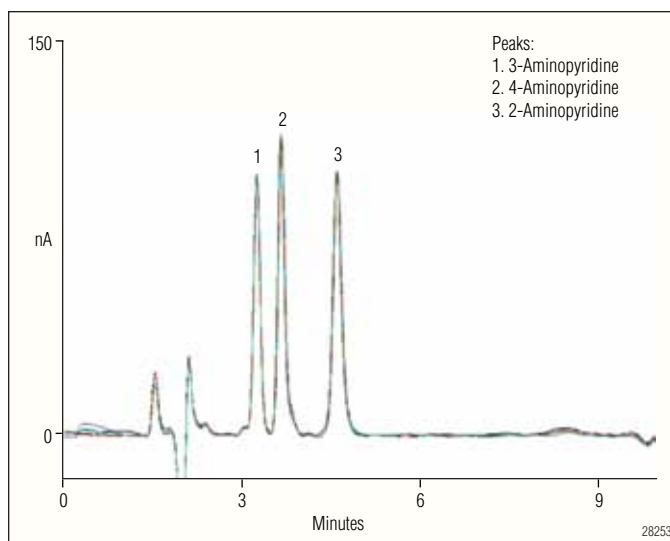


Figure 2. Overlaid chromatograms of three aminopyridines at 2000 pg o.c. ($n = 5$).

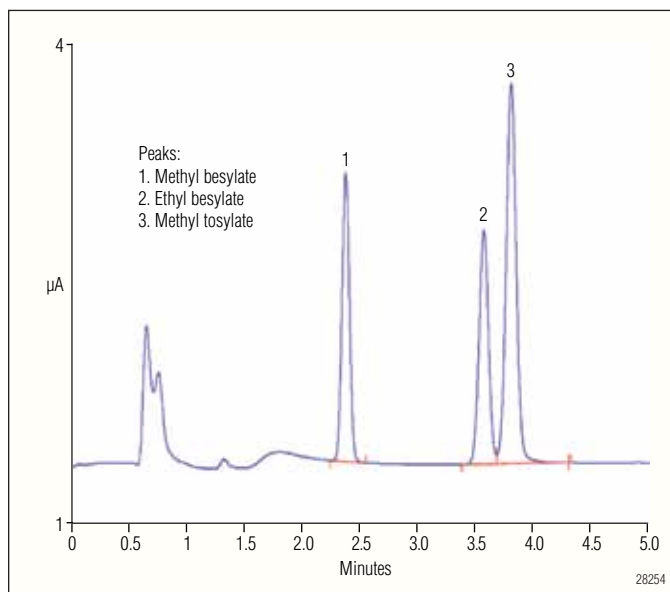


Figure 3. HPLC-ECD chromatogram of methyl and ethyl besylate and methyl tosylate.

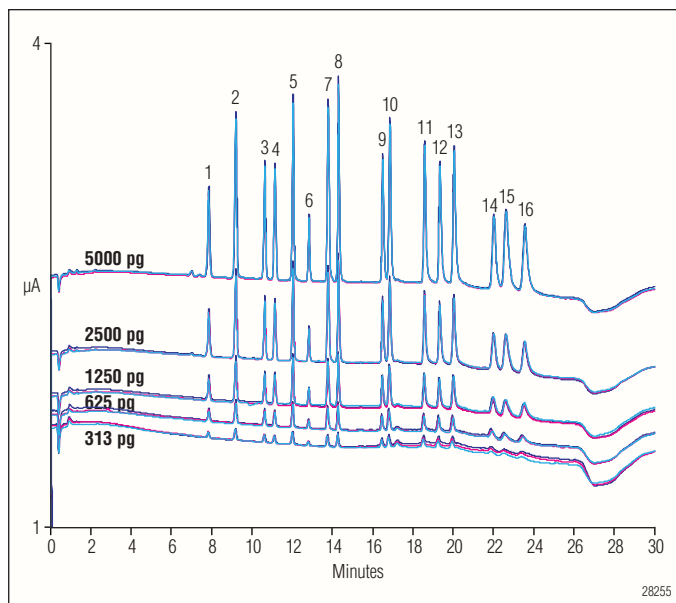


Figure 4. Overlaid HPLC-ECD chromatograms of 16 PAHs, from 313 to 5000 pg on column, using the BDD analytical electrode.

Analysis of PAHs by Gradient ECD-BDD

All 16 PAHs were detected by ECD using the peroxide-modified mobile phases and were well resolved in under 30 min using Method Conditions 3. Overlaid chromatograms, from 313–5000 pg o.c., are presented in Figure 4. Calibration curves were linear for all 16 PAHs for amounts from 39 to 5000 pg on column for the first 13 PAHs, and from 313 to 5000 pg on column for the last three. Calibration curves for PAHs 9–16 are shown in Figure 5. Correlation coefficients were all >0.994. LOD values were 20 pg o.c. for analytes 1–10, 80 pg o.c. for analytes 11–13, and 300 pg o.c. for analytes 14–16, using a signal-to-noise ratio of 3:1. Precision was acceptable at <5% RSD for the majority of analyte concentrations.

One unique aspect of this method includes the use of gradient elution, which is unusual for HPLC-ECD methods. The BDD working electrode is less sensitive than other electrodes (e.g., porous graphite, glassy carbon), but it enables the use of higher oxidation potentials needed for the measurement of these analytes, and it also allows for the use of gradient elution.

Although this ECD approach is sufficient for the analysis of aromatics in APIs, it is not sufficiently sensitive for environmental analyses, where lower LOD values are required.

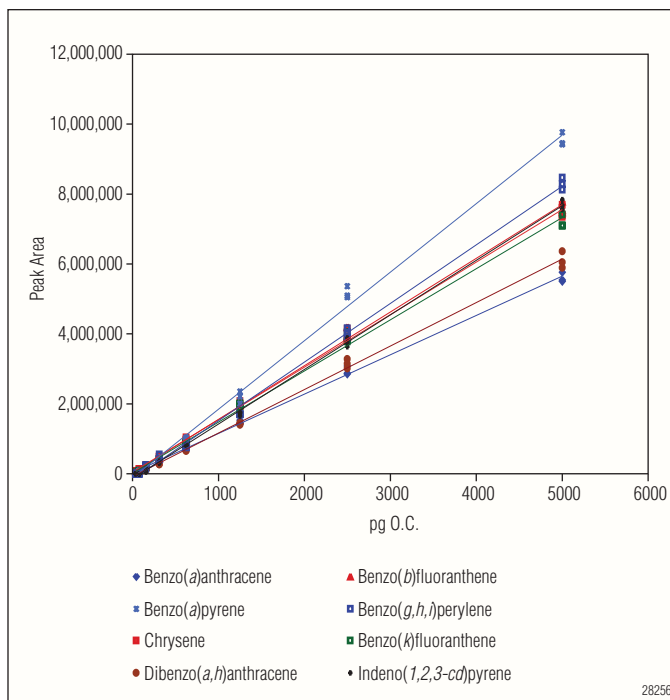


Figure 5. Calibration plots for eight late-eluting PAH compounds by ECD from LOQ to 5000 pg o.c.

Analysis of PAH by Gradient FLD

Improved sensitivity can be achieved by gradient elution with FLD using a 50 min HPLC-FLD method and a C18 column.⁹ LOD values, shown in Table 1, are improved significantly over those by ECD.

Table 1. Typical LOD and LOQ Values for PAH Analytes by a 50 min HPLC-FLD		
Analyte	LOD (pg)	LOQ (pg)
Naphthalene	0.276	0.83
Acenaphthene	0.167	0.50
Fluorene	0.378	1.13
Phenanthrene	0.011	0.03
Anthracene	0.004	0.01
Fluoranthene	0.440	1.32
Pyrene	0.018	0.05
Benzo(a)anthracene	0.041	0.12
Chrysene	0.066	0.20
Benzo(b)fluoranthene	0.032	0.10
Benzo(k)fluoranthene	0.005	0.02
Benzo(a)pyrene	0.015	0.05
Dibenzo(a,h)anthracene	0.011	0.03
Benzo(g,h,i)perylene	0.011	0.03
Indeno(1,2,3-cd)pyrene	0.011	0.03

Further enhanced sensitivities were found with use of the Method Conditions 4, which uses ultrahigh performance LC and fluorescence (UHPLC-FLD) to separate 17 PAHs in under 6 min, as shown in Figure 6. For this UHPLC method to work in this period of time, rapid wavelength changes are required by the detector. This is easily accomplished by the UltiMate® 3000 FLD RS detector that can change wavelengths in <250 ms.

Detection of PAHs by fluorescence offers greater sensitivity than by ECD-BDD. The FLD provides LOD values in the range of 380 to 5 fg on column, which is a significant improvement over ECD and is ideal for environmental samples where concentrations for these compounds are as low as ppt. One serious disadvantage to this approach, however, is the need to specify analyte wavelengths, both excitation and emission, to obtain these low-level sensitivities. Furthermore, not all PAHs (e.g., acenaphthylene) respond well to fluorescence detection.

Electrochemical detection, on the other hand, provides a more consistent response for all of the PAHs evaluated here, as the resulting phenols formed by electrotagging respond similarly. With the current ECD method conditions, the analysis of unknown PAHs or other compounds that are not normally fluorogenic or electrochemically active under traditional conditions is possible. This can be an important tool for process development when a new API is being developed.

Both methods provide a reliable means of evaluating a sample for genotoxins and aromatics. ECD, being less sensitive than FLD for PAH compounds, offers a more generalized response with the ability to detect any aromatic compound without a specific wavelength. The FLD is much more sensitive for PAH compounds but requires analyte specific excitation and emission wavelengths.

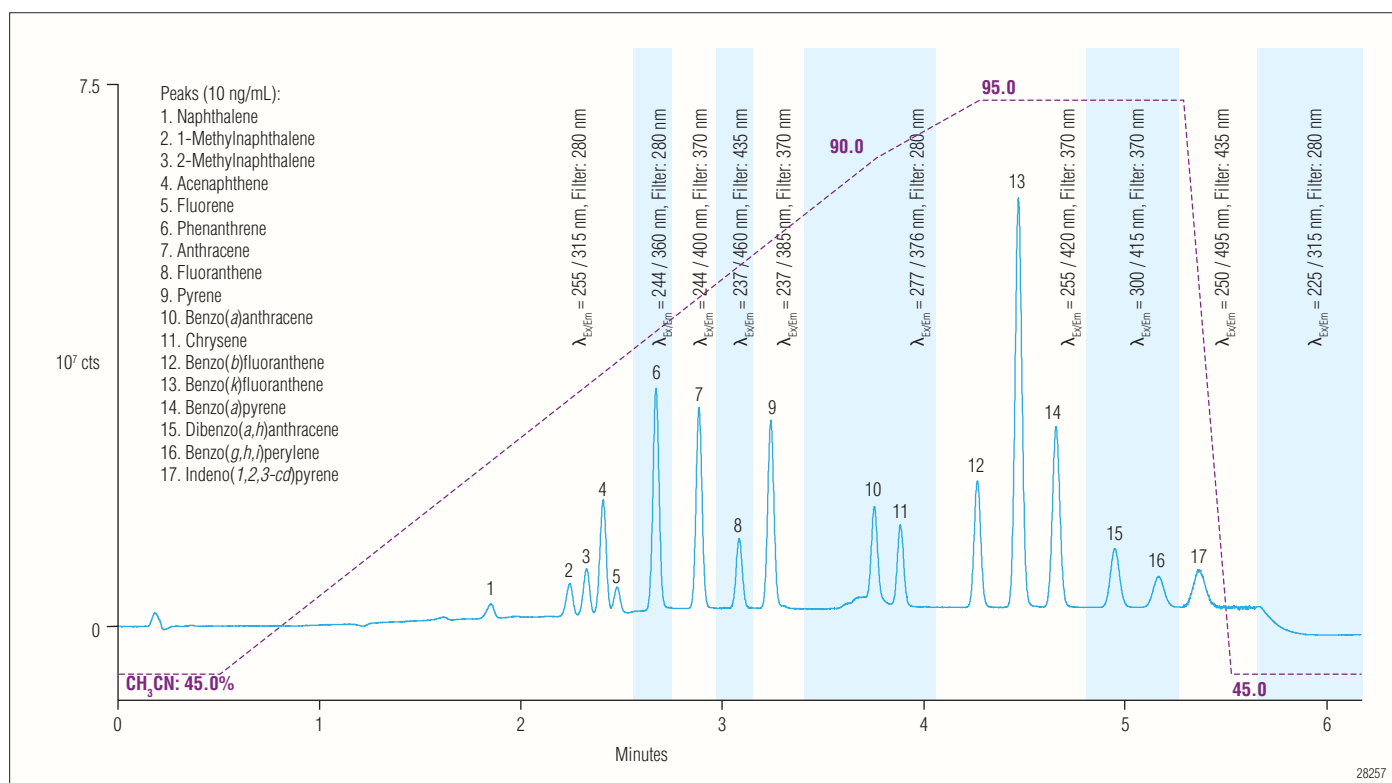


Figure 6. UHPLC-FLD chromatogram of 17 PAH analytes at 125 pg o.c.

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CONCLUSION

The BDD working electrode, with its ability to be used with gradient elution and at high potentials, extends the range of compounds that can be measured electrochemically. By generating HO• free radicals, aromatic compounds can both be hydroxylated in situ, or electrotagged, and detected with the BDD electrode. This unique approach for detection can be used to measure, at ppb sensitivity, numerous aromatic compounds such as drugs, genotoxic impurities, and environmental pollutants that cannot be analyzed by traditional ECD approaches.

Fluorescence detection is a more sensitive approach for PAH compounds, and it can be used for routine environmental testing. Use of the UltiMate 3000 FLD RS detector, with its unique capability of rapid wavelength and filter changes, allows for rapid analyses of PAHs and other fluorescent analytes.

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