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Comprehensive Analysis of C2–C8 PFAS Using a Novel LC Column

Restek Corporation

While not currently regulated, ultrashort-chain (C2–C3) per- and polyfluoroalkyl substances (PFAS) are of great interest. Current testing methodologies using reversed-phase liquid chromatography (LC) columns cannot be used because of a lack of retention, so either a separate method or a different column is required.

A unique, hybrid ion-exchange/HILIC column (Raptor Polar X) was used to develop a comprehensive LC–MS/MS method for the analysis of ultrashort-chain through long-chain, and alternative PFAS in water sources (tap, river, groundwater, and sewage effluent). The Raptor Polar X’s multimode retention mechanisms allow for retention with a single isocratic run.

Experimental

Chromatographic conditions are reported in Figure 1.

To avoid introducing background contamination, polypropylene vials and caps were used during sample preparation.

Each water sample of 250 μL was mixed with 250 μL of methanol and 5 μL of internal standard solution (10 ng/mL of 13C2-PFHxA, 13C2-PFOA, 13C3-PFBS, 13C4-PFOS in methanol).

 Calibration standards were prepared by using deionized water and fortified with 14 analytes (see Figure 1) at a range of 10–800 ng/L. The calibration standard solutions were diluted 1:1 as above.

A Restek tap water sample, along with three water samples (river, ground, and sewage effluent) supplied by the United States Environmental Protection Agency were fortified at 40 and 160 ppt. Blank and fortified water samples were diluted 1:1 in methanol as above for chromatographic analysis. For TFA measurement in groundwater, the sample was diluted fivefold with deionized water before fortification due to its high TFA concentration.

Results and Discussion

All analytes were eluted in 4 min with good peak shapes (Figure 1). The overall analytical cycle time was 8 min to ensure no matrix-related interferences.

<table>
<thead>
<tr>
<th>Peaks</th>
<th>tR (min)</th>
<th>Conc. (ng/L)</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 5-Chloroheptadecafluoro-3-oxo-1-sulfonate (5C-PF7ONS)</td>
<td>1.34</td>
<td>400</td>
<td>530.78</td>
<td>350.85</td>
</tr>
<tr>
<td>2 Perfluoroheptanoic acid (PF7DA)</td>
<td>1.38</td>
<td>400</td>
<td>498.84</td>
<td>79.97</td>
</tr>
<tr>
<td>3 Perfluorooctanoic acid (PF8A)</td>
<td>1.49</td>
<td>400</td>
<td>398.9</td>
<td>79.92</td>
</tr>
<tr>
<td>4 Perfluoroundecafluorooctanoic acid (PFODA)</td>
<td>1.64</td>
<td>400</td>
<td>298.97</td>
<td>79.97</td>
</tr>
<tr>
<td>5 Perfluorobutanoic acid (PF4BA)</td>
<td>1.73</td>
<td>400</td>
<td>248.97</td>
<td>79.97</td>
</tr>
<tr>
<td>6 Perfluorobutanoic acid (PF4BA)</td>
<td>1.86</td>
<td>400</td>
<td>198.98</td>
<td>79.92</td>
</tr>
<tr>
<td>7 Perfluorooctanoic acid (PF8A)</td>
<td>2.06</td>
<td>400</td>
<td>284.97</td>
<td>168.92</td>
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<tr>
<td>8 PFOS</td>
<td>2.11</td>
<td>400</td>
<td>412.9</td>
<td>268.91</td>
</tr>
<tr>
<td>9 Ammonium 4,8-dioxo-1H-perfluoroanion (ADONA)</td>
<td>2.15</td>
<td>400</td>
<td>376.9</td>
<td>250.93</td>
</tr>
<tr>
<td>10 Perfluorooctanoic acid (PF8A)</td>
<td>2.36</td>
<td>400</td>
<td>312.9</td>
<td>268.91</td>
</tr>
<tr>
<td>11 Perfluorobutanoic acid (PF4BA)</td>
<td>2.76</td>
<td>400</td>
<td>212.97</td>
<td>168.97</td>
</tr>
<tr>
<td>12 Perfluorooctanoic acid (PF8A)</td>
<td>3.06</td>
<td>400</td>
<td>163.03</td>
<td>119.01</td>
</tr>
<tr>
<td>13 TFA</td>
<td>3.77</td>
<td>400</td>
<td>113.03</td>
<td>69.01</td>
</tr>
</tbody>
</table>

Columns: Raptor Polar X (cat.# 9311A52)
Detector: MS/MS
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 2.7 µm
Mode: MRM
Temp.: 40 °C
Instrument: UHPLC
Sample: Diluent: 50:50 Water:methanol
Conc.: 400 ng/L
Inj. Vol.: 10 µL
Mobile Phase
A: Water, 10 mM ammonium formate, 0.05% formic acid
B: 60:40 Acetonitrile:methanol, 0.05% formic acid

Figure 1: Chromatogram of a 400 ng/L standard
Method linearity from 20–800 ppt for trifluoroacetic acid (TFA) and 10–800 ppt for all other analytes provided $r^2$ values >0.996 and deviations <20% using a 1/x weighted quadratic regression.

Samples were fortified at the low and high concentrations of their calibration ranges and run in duplicate for each analytical batch. A total of three batches were measured on different days. Concentrations of fortified samples were adjusted to account for any observed background contamination in sample blanks. Results are presented in Table I.

**Conclusions**

These results demonstrate that switching to a mixed-mode LC column provides the capability to analyze currently monitored and emerging PFAS contaminants in a single, short, isocratic run, preparing laboratories for the future of PFAS testing.

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**Table I: Method accuracy and precision**

<table>
<thead>
<tr>
<th>Matrices</th>
<th>Tap Water</th>
<th>River Water</th>
<th>Groundwater**</th>
<th>POTW Water</th>
<th>Deionized Water</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conc. (ng/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TFA</td>
<td>40</td>
<td>160</td>
<td>40</td>
<td>160</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>106 (16.9)</td>
<td>97.9 (7.10)</td>
<td>97.4 (10.8)</td>
<td>97.6 (6.12)</td>
<td>97.5 (14.5)</td>
</tr>
<tr>
<td>PFPPrA</td>
<td>105 (3.48)</td>
<td>94.5 (6.85)</td>
<td>104 (2.36)</td>
<td>103 (9.37)</td>
<td>105 (8.34)</td>
</tr>
<tr>
<td>PFBA</td>
<td>106 (6.80)</td>
<td>117 (3.18)</td>
<td>105 (7.40)</td>
<td>114 (4.91)</td>
<td>111 (2.48)</td>
</tr>
<tr>
<td>PFHxA</td>
<td>93.3 (7.41)</td>
<td>111 (2.61)</td>
<td>91.8 (11.34)</td>
<td>103 (4.55)</td>
<td>102 (6.62)</td>
</tr>
<tr>
<td>PFOA</td>
<td>100 (4.24)</td>
<td>107 (3.14)</td>
<td>103 (6.71)</td>
<td>105 (2.64)</td>
<td>92.6 (3.85)</td>
</tr>
<tr>
<td>HFPO-DA</td>
<td>95.7 (11.9)</td>
<td>108 (9.05)</td>
<td>86.6 (8.97)</td>
<td>104 (5.45)</td>
<td>94.1 (18.6)</td>
</tr>
<tr>
<td>ADONA</td>
<td>106 (3.75)</td>
<td>116 (2.38)</td>
<td>100 (8.86)</td>
<td>110 (4.59)</td>
<td>104 (4.91)</td>
</tr>
<tr>
<td>PEEIS</td>
<td>94.8 (9.68)</td>
<td>110 (5.39)</td>
<td>89.4 (7.43)</td>
<td>102 (9.76)</td>
<td>96.5 (4.09)</td>
</tr>
<tr>
<td>PFPtrS</td>
<td>104 (4.97)</td>
<td>115 (4.19)</td>
<td>95.0 (3.87)</td>
<td>107 (4.26)</td>
<td>106 (10.6)</td>
</tr>
<tr>
<td>PFBS</td>
<td>97.4 (10.1)</td>
<td>113 (3.97)</td>
<td>93.6 (5.24)</td>
<td>104 (4.19)</td>
<td>97.8 (4.47)</td>
</tr>
<tr>
<td>PFHxS</td>
<td>99.4 (15.7)</td>
<td>114 (3.56)</td>
<td>94.3 (9.79)</td>
<td>104 (5.28)</td>
<td>95.2 (5.63)</td>
</tr>
<tr>
<td>PFOS</td>
<td>104 (7.54)</td>
<td>107 (7.69)</td>
<td>103 (8.43)</td>
<td>105 (7.23)</td>
<td>97.3 (14.9)</td>
</tr>
<tr>
<td>9CI-PF3ONS</td>
<td>98.7 (3.52)</td>
<td>105 (8.35)</td>
<td>91.8 (7.66)</td>
<td>103 (5.68)</td>
<td>94.7 (9.83)</td>
</tr>
<tr>
<td>11CI-PF300Us</td>
<td>106 (10.1)</td>
<td>113 (3.54)</td>
<td>95.0 (3.52)</td>
<td>113 (8.15)</td>
<td>107 (6.61)</td>
</tr>
</tbody>
</table>

*20 ng/L LLOQ for TFA
**Groundwater was diluted fivefold for TFA only
Cannabis and its products are on the road to being legalized throughout the United States. Because of the risk of pesticide exposure through inhaled and consumed cannabis products, the pesticides in the plant material and its products must be monitored. As matrices, cannabis and its related products have proven to be difficult to extract, and more traditional pesticide extraction methods, like QuEChERS, have been shown to perform poorly with these products. Thus, a better extraction method is needed. In this application note, in collaboration with SCIEX, the EDGE® was utilized to extract the Oregon pesticide list from cannabis flower and including edible cookies, chips, chocolate, hard candy, and granola bars. The average recoveries obtained were between 60% and 101%, and the average matrix effects were found to be between 70% and 130%. Thus, the EDGE is an excellent choice for laboratories extracting pesticides from cannabis and its products.

The use of cannabis and its products is growing more popular. With its continued legalization for recreational and medicinal use, it is subject to regulation, including monitoring its pesticide content. The presence of pesticides in cannabis is a concern because cannabis products are often inhaled or eaten, and with their consumption pesticides can bioaccumulate, causing potentially deleterious effects on human health. Because of these concerns, the list of pesticides monitored gets longer each year, and there is a need for good methods to extract and quantitate these compounds. Traditionally, the QuEChERS method has been used to extract pesticides from plants and food matrices, but cannabis and its products have shown time and again that cannabis is a difficult matrix to work with. Because of this, a simple, efficient method that produces high recoveries and repeatable results is still needed.

The EDGE is an extraction system that utilizes automation, heat, and solvent to extract samples quickly and effectively. It was used in a collaboration with SCIEX to extract pesticides from the Oregon list from cannabis edibles, including hard candy, chips, chocolate, cookies, and granola, and the cannabis flower. The average recoveries obtained for most pesticides in all the matrices were between 60% and 101%. The matrix effects were also assessed and found to be in the range of 70% to 130%. The EDGE offers cannabis laboratories an automated option for high recoveries with good matrix effects.

### Materials and Methods

#### Extraction

Cannabis flower and cannabis cookies, chips, chocolate, hard candy, and granola bar were milled using a small coffee grinder. A portion of 300 mg of each matrix was weighed directly into a Q-Cup® containing a S1 Q-Disc® stack. The S1 Q-Disc stack is a sandwich of three filters including a glass-fiber filter surrounded by two cellulose filters. The samples were spiked with 150 ng of the Restek Oregon Pesticide Standards 1-6 (Catalogue numbers 32586, 32587, 32588, 32589, 32590, and 32591). A Q-Screen® was placed on top of each sample after spiking. The samples were loaded into an EDGE rack containing glass collection vials to collect the resulting extract. The samples were extracted using the indicated EDGE method. Five replicates of each sample type were extracted.

#### EDGE Method for Pesticides from Cannabis Flower and Edibles

Q-Disc: S1 Q-Disc stack (C9+G1+C9 sandwich)

**Cycle 1**

Extraction Solvent: Acetonitrile with 1.0% formic acid (v/v)

Top Add: 25 mL Bottom Add: 0 mL

Rinse: 5 mL Temperature: 40 °C

Hold Time: 02:00 (mm:ss)

### Table I: Recoveries for each matrix

<table>
<thead>
<tr>
<th>Compound</th>
<th>Flower Average</th>
<th>STDEV</th>
<th>Cookie Average</th>
<th>STDEV</th>
<th>Chips Average</th>
<th>STDEV</th>
<th>Chocolate Average</th>
<th>STDEV</th>
<th>Hard Candy Average</th>
<th>STDEV</th>
<th>Granola Bar Average</th>
<th>STDEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbofuran</td>
<td>85.91</td>
<td>3.27</td>
<td>94.96</td>
<td>3.53</td>
<td>78.92</td>
<td>7.23</td>
<td>88.79</td>
<td>4.90</td>
<td>88.66</td>
<td>8.19</td>
<td>95.27</td>
<td>3.14</td>
</tr>
<tr>
<td>Cyantranilprole</td>
<td>83.04</td>
<td>10.36</td>
<td>112.85</td>
<td>12.02</td>
<td>99.83</td>
<td>2.19</td>
<td>98.30</td>
<td>7.25</td>
<td>105.32</td>
<td>7.25</td>
<td>103.65</td>
<td>5.38</td>
</tr>
<tr>
<td>Cypermethrin</td>
<td>99.17</td>
<td>2.30</td>
<td>100.65</td>
<td>3.52</td>
<td>85.04</td>
<td>5.24</td>
<td>96.40</td>
<td>0.99</td>
<td>96.90</td>
<td>5.24</td>
<td>100.88</td>
<td>3.71</td>
</tr>
<tr>
<td>Fenproximate</td>
<td>107.80</td>
<td>2.60</td>
<td>91.93</td>
<td>3.13</td>
<td>75.40</td>
<td>8.68</td>
<td>82.81</td>
<td>3.29</td>
<td>86.90</td>
<td>6.43</td>
<td>89.83</td>
<td>6.06</td>
</tr>
<tr>
<td>Methiocarb</td>
<td>80.07</td>
<td>10.50</td>
<td>91.21</td>
<td>6.01</td>
<td>74.71</td>
<td>2.92</td>
<td>85.93</td>
<td>2.35</td>
<td>86.22</td>
<td>4.65</td>
<td>91.42</td>
<td>1.68</td>
</tr>
<tr>
<td>Permethrin trans</td>
<td>95.22</td>
<td>3.63</td>
<td>83.00</td>
<td>5.88</td>
<td>70.42</td>
<td>4.37</td>
<td>83.11</td>
<td>4.35</td>
<td>82.65</td>
<td>3.23</td>
<td>84.20</td>
<td>2.92</td>
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<tr>
<td>Piperonyl Butoxide</td>
<td>92.99</td>
<td>2.99</td>
<td>87.46</td>
<td>2.09</td>
<td>72.36</td>
<td>1.85</td>
<td>80.36</td>
<td>3.05</td>
<td>84.78</td>
<td>3.63</td>
<td>84.04</td>
<td>1.50</td>
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<tr>
<td>Pralethrin</td>
<td>74.13</td>
<td>3.07</td>
<td>113.83</td>
<td>8.00</td>
<td>73.16</td>
<td>5.70</td>
<td>79.79</td>
<td>8.38</td>
<td>86.38</td>
<td>4.66</td>
<td>82.38</td>
<td>4.42</td>
</tr>
<tr>
<td>Spirotetramat</td>
<td>84.46</td>
<td>6.98</td>
<td>92.92</td>
<td>6.79</td>
<td>82.29</td>
<td>5.47</td>
<td>91.55</td>
<td>3.80</td>
<td>90.31</td>
<td>9.75</td>
<td>92.30</td>
<td>3.66</td>
</tr>
<tr>
<td>Thiacloprid</td>
<td>83.92</td>
<td>6.06</td>
<td>93.29</td>
<td>3.67</td>
<td>79.20</td>
<td>3.44</td>
<td>87.78</td>
<td>6.41</td>
<td>91.28</td>
<td>3.25</td>
<td>90.92</td>
<td>4.89</td>
</tr>
</tbody>
</table>
The recoveries were determined using solvent-matched calibration curves. An abbreviated set of recoveries with their respective standard deviations can be found in Table I. The average recoveries for all matrices were mostly within the range 60% to 101%, which is generally the ideal range for recoveries. The standard deviations of the recoveries for almost all compounds were below 15%, which is also ideal.

The matrix effects were also assessed by comparing the extract of each matrix effects, with signal enhancement and suppression extremes. Propiconazole, spirofensulfuron, and acequinocyl were found to be suppressed in the flower matrix. The cookie, chocolate, and chip matrices had the least variability, with a tighter distribution overall.

To view the entire set of pesticides extracted from this study please download the full application note at https://cem.com/en/the-extraction-of-pesticides-from-cannabis-flower-and-edibles.

**Conclusion**

With the expanding cannabis industry within the United States, testing laboratories are monitoring cannabis and its products for pesticide content. Traditional pesticide extraction methods, like QuEChERS, have shown to work poorly with cannabis plant and cannabis edibles. Therefore, new and better extraction methods are needed. Within this work, in a partnership with SCIEX, the EDGE was used to extract pesticides from the Oregon List from cannabis flower and the edibles cookies, chips, chocolate, hard candy, and granola bar. The determined recoveries found for all pesticides, except spinosad, in all matrices were between 60% and 101%, which is well within the desired range, and all the recoveries, except for that of daminozide, methyl parathion, and imidacloprid, had standard deviations below 15%. The matrix effects for each matrix were found to be 70% to 130%. Thus, these data indicate that the EDGE is an excellent choice for the extraction of pesticides from cannabis material and edibles.

We would like to thank our collaborators at SCIEX for extracting these samples on the EDGE and analyzing the results.
The Chiral Separation of Remdesivir and Several of its Key Starting Materials

Weston J. Umstead, Chiral Technologies, Inc.

The start of 2020 saw the outbreak of the COVID-19 pandemic, and almost immediately, the search for a viable vaccine candidate, and effective treatments for those diagnosed with the virus, began. One of the compounds that emerged early on for the treatment of COVID-19 patients was remdesivir, an antiviral drug manufactured by Gilead Sciences. Studies conducted since its initial implementation have confirmed its effectiveness in shortening the recovery times of patients hospitalized, and in lowering the overall mortality rate from the disease.

The synthesis of remdesivir was well documented in the literature, however there are a few key chiral intermediates in the synthesis that were not previously well resolved under high-performance liquid chromatography (HPLC) conditions. The chiral separations of these compounds (shown in Figure 1 along with remdesivir itself) is shown on two Daicel immobilized chiral stationary phases (CHIRALPAK® IA-3 and CHIRALPAK IG-U), as well as on one of Daicel’s achiral columns, DCpak® PTZ. The separation of remdesivir is also shown on CHIRALPAK IA-3 under normal-phase HPLC conditions.

Discussion

The two phosphoramidite starting materials contain two chiral centers, one on the phosphorus and the other on the carbon alpha to the phosphoramidite functional group. Therefore, the goal was to develop a separation, that could quantitate all four potential isomers. For the pentafluoro analog, a normal phase screening across Daicel’s library of immobilized chiral stationary phases (CSPs) yielded a very nice resolution on CHIRALPAK IA-3 (Figure 2). No additional optimization from this initial screening was required.

The 4-nitro analog was a bit more challenging, as no viable separations on normal phase were initially found. However reversed-phase screening using a water/MeOH gradient

Figure 1: Remdesivir and its key starting materials

<table>
<thead>
<tr>
<th>Table I: Chromatographic conditions for the separation of remdesivir pentafluoro phosphoramidite KSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
</tr>
<tr>
<td>Mobile Phase</td>
</tr>
<tr>
<td>Flow Rate</td>
</tr>
<tr>
<td>Temperature</td>
</tr>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>Injection Volume</td>
</tr>
</tbody>
</table>
yielded a partial separation for two of the four expected peaks. The addition of acetonitrile as a mobile phase component resulted in better selectivity and thus a separation of the final two, earlier eluting isomers (Figure 3).

The separation of the triol and tri o-benzyl nitrile starting materials is a chiral/achiral separation, which can be accomplished on a Daicel polysaccharide CSP. However in this case, Daicel’s HILIC DCpak PTZ column was used. The initial screening was performed without the addition of any additives, however the peak shape was less than desirable. The addition of 0.01% o-phosphoric acid to the mobile phase A component had a significant improvement to the peak shape, and optimization of the gradient led to a baseline resolution of all four isomers (Figure 4).

Lastly, remdesivir was screened, and a good separation under normal-phase conditions was observed, with slightly undesirable peak shape. Because the compound contains both basic and slightly acidic functional groups, the addition of both ethanolamine and formic acid was able to sharpen the peaks, and the combination of isopropanol and ethanol gave a nice compromise between selectivity and retention.
Analysis of Redox and Bioenergetics Metabolites with Polymeric iHILIC®-(P) Classic HILIC Column and Mass Spectrometry

Susan Kim*, Wen Jiang†, and Joshua D. Chandler*‡,

*Department of Pediatrics, Division of Pulmonology, Allergy & Immunology, Cystic Fibrosis and Sleep Medicine, Emory University, Atlanta, Georgia †Hilicon AB ‡Children’s Healthcare of Atlanta, Atlanta, Georgia

Aerobic organisms use oxygen as the terminal electron acceptor in cellular respiration. Molecular oxygen is capable of accepting up to four electrons to become water through the reaction catalyzed by mitochondrial complex IV (1). However, intermediate reactive oxygen species (ROS) are formed by partial reduction of oxygen. Aerobes have evolved both physiological and biochemical strategies to mitigate molecular damage caused by ROS while maintaining oxygen availability for cellular respiration (2). Core metabolism and redox control depend on a number of metabolites, including nucleotides (NADP⁺/NADPH and NAD⁺/NADH) and redox-active amino acids and peptides (particularly glutathione, cysteine, and methionine). For example, reduced glutathione (GSH; γ-L-glutamyl-L-cysteinylglycine) is an important regulator of local hydrogen peroxide signalling by serving as a cofactor of glutathione peroxidases (3). In turn, oxidized glutathione (GSSG) is reduced back to GSH in an NADPH-dependent fashion by GSSG reductase (4). Both cysteine and methionine in proteins may act as redox switches linked to NADPH-dependent repair mechanisms (5,6). NADPH is generated by the pentose phosphate pathway that links to glycolysis, which generates ATP and relies on NAD⁺ as a cofactor. Multiple steps of the citric acid cycle generate NADH that supports chemiosmotic ATP synthesis.

Liquid chromatography–mass spectrometry (LC–MS)-based metabolomics present an opportunity to monitor many molecules simultaneously, relying on a combination of chromatographic and mass-based resolution to quantify hundreds to thousands of molecules in a typical experiment (7). However, many of the metabolites noted above could be challenging to profile simultaneously because of poor chromatographic separation or peak shape (8). We sought to develop a simple method using hydrophilic interaction liquid chromatography (HILIC) that could simultaneously profile the metabolites that are important in redox regulation and bioenergetics. By incorporating these metabolites into a nontargeted-compatible and derivatization-free approach, information about redox and bioenergetics can be gained as part of routine profiling.

**Experimental**

**LC–MS System:** A Vanquish Horizon binary pump was hyphenated to a Q Exactive HF (Thermo Fisher Scientific), set at 120,000 FWHM, 1e6 AGC target, and 200 ms max IT. Connecting tubings between the column, autosampler, and MS system were 100 µm i.d. PEEK-lined stainless steel MarvelXACT tubing (IDEX Health & Science) instead of original Viper MP35N. The HESI-II probe was held at 320 °C and +3.5 kV with 40 sheath gas, eight auxiliary gas, and one sweep gas flow (arbitrary units).

**Column:** 150 × 2.1 mm, 5-µm, 200 Å, iHILIC®-(P) Classic (P/N 160.152.0520, Hilicon AB), coupled to a 20 mm guard column via a PEEK coupler.

**Eluent:** A) 15 mM ammonium acetate pH 9.4, B) acetonitrile.

![Figure 1](image-url): Base intensity-normalized chromatograms of (a) GSH/GSSG; (b) Met/MetO/MetO₂; (c) NAD⁺/NADH/NADP⁺/NADPH; (d) AMP/ADP/ATP.
Gradient Elution: 0–15 min, gradient from 10–90% A; 15–17 min, 10% A (column re-equilibration)
Flow Rate: 200 µL/min
Column Temperature: 40 °C
Injection Volume: 2.5 µL

Metabolite Samples: Individual metabolites were prepared fresh from reference standards in water (50 µmol/L). We focused on the following 14 metabolites: oxidized/reduced nicotinamide adenine dinucleotide (NAD+/NADH), oxidized/reduced nicotinamide adenine dinucleotide phosphate (NADP+/NADPH), adenosine mono-di-/triphosphate (AMP/ADP/ATP), glutathione/glutathione disulfide (GSH/GSSG), methionine/methionine sulfoxide/methionine sulfone (Met/MetO/MetO\(^2\)), and cysteine/cystine (Cys/CySS). We also analyzed human plasma Standard Reference Material 1950 (MilliporeSigma) and metabolite extract of E. coli (Cambridge Isotope Labs), which were either reconstituted with a solution of 1:1:1 acetonitrile–methanol–water or extracted with addition of 2 vol 1:1 acetonitrile–methanol + 12.5 µM D5-hippuric acid followed by vortexing and incubation on ice for 30 min. Samples were then centrifuged at 20,000 \(g\) and 4 °C for 10 min. The supernatant was then applied for HILIC–MS analysis.

Results and Conclusion
The newly developed method provides excellent peak separation and overall good peak quality for all of the metabolites, as shown in Figure 1.
We analyzed all of the reduced metabolite standards to determine if autoxidation was rapidly occurring (Table I). Peak areas of most oxidized species from injections of reduced standards were <1% that of the reduced species at pH 9.4. However, in the case of the Cys standard, only oxidized CySS was detectible. When using neutral pH instead of 9.4, Cys was preserved and detected (though considerable autoxidation was still noted). Cys may be unstable at alkaline pH as a result of enhanced deprotonation to the nucleophilic thiolate form (\(pK_a = 8.3\)) (3).
We then determined whether the 12 metabolites shown in Figure 1 were well represented in standard reference materials for untargeted metabolomics experiments. Human plasma SRM 1950 and the unlabelled E. coli (Escherichia coli) metabolite extracts were used as probes. Across the two matrices, signals for all metabolites except NADP\(^+\) were observed (Table II).
This work demonstrates the feasibility of profiling core metabolites related to redox metabolism and bioenergetics, which might be incorporated into a nontargeted workflow for more extensive profiling.

We note that thiol metabolites are labile and nontargeted profiling of these should be experimentally validated with derivatization-based methods (9). A HILIC method with an acidic mobile phase may offer better stability for thiol compounds as well.

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References
Molecular Weight Determination of VLPs Using the LenS3 Multi-Angle Light Scattering Detector

Tosoh Bioscience LLC

Viruses and virus-like particles (VLPs) are multimeric protein structures that mimic native viruses but are non-infectious. VLPs are subjects of interest, as their potential continues to grow as candidates in new vaccines and gene therapy products. For example, commercially available VLP-based vaccines are available for hepatitis B and human papillomavirus. Robust analytical techniques are needed to not only ensure quality of final products but provide data for informed decision-making during the development process.

Size-exclusion chromatography (SEC) is an analytical technique that provides results on the size and purity of macromolecules. When coupled with multi-angle light scattering (MALS), it offers both molecular weight (MW) and radius of gyration ($R_g$) or size. Importantly, $AU_{280}$ detection is only concentration dependent, whereas MALS corresponds to both concentration and molecular weight. Thus, the large molecular weight characteristic of VLPs inherently provides MALS with a strong scattered-light response and enables VLP detection even in a dilute solution that is well below the $AU_{280}$ detection limit.

The primary challenge in the analysis of very large macromolecules by SEC is the selection of the appropriate analytical column. Here we explore the TSKgel® PWxL series of SEC columns, which includes a wide range of different pore sizes on a polymethacrylate stationary phase, for their utility in the analysis of large macromolecules such as VLPs. The protein calibration curves (Figure 1) show the separation range of TSKgel PWxL columns. The majority of VLPs have a molecular weight of >1 megadaltons (MDa), which make the TSKgel G5000PWxL (100 nm pore size), TSKgel G6000PWxL (>100 nm pore size) and TSKgel GMPWxL (mixed bed) ideal columns of choice for analysis of VLPs.

**Materials and Methods**

**Columns:** TSKgel GMPWxL, 13 μm, 7.8 mm ID × 30 cm
TSKgel G5000PWxL, 10 μm, 7.8 mm ID × 30 cm

**Instrument:** Thermo Scientific UltraMate® 3000

**Mobile phase:** 0.145 mol/L NaCl, 0.01 mol/L HEPES, 0.05% sodium azide, pH 7.4 (refractive index, 1.333)

**Flow rate:** 0.3 mL/min or as indicated

**UV:** UltraMate 3000 multiple wavelength detector

**RI:** Shodex RI-504 semi-micro RI detector

**MALS:** LenS3 MALS detector

**Sample:** Parvovirus VLP (MVM-MVP) (Cygnus Technologies), stock 1 x 10^{12} particles/mL (10–15 μL injection), (dn/dc = 0.19, dA/dc = N/A)

**MALS calibrant:** BSA, 5 mg/mL (dn/dc = 0.185, dA/dc = 0.66)

In this application, parvovirus VLP was separately analyzed on both a TSKgel GMPWxL and TSKgel G5000PWxL. SEC column coupled with the LenS3 MALS detector. Either RI or UV can function as the concentration detector. RI was used with the right angle light scattering signal (RALS) to measure MW. Extreme low angle (LALS), right angle, and extreme high angle (HALS) signals were used to plot angular dissymmetry and to determine $R_g$. The MALS detector was calibrated with BSA prior to sample analysis and all data were processed and analyzed using SECview® software.

Analysis of parvovirus VLP by SEC-MALS using the TSKgel GMPWxL column revealed a MW of ∼4 MDa and $R_g$ of 12.8 nm (Figure 2). These results closely align with reported values for this VLP (Biotech. Prog. 34, 1213–1220, 2018).

As seen in Figure 3, parvovirus VLP was diluted up to 64-fold and injected at 10 μL onto a TSKgel G5000PWxL column. Approximately 3 x 10^{15} particles per mL can still be detected using the RALS signal from the LenS3 MALS detector, which allows for analysis of materials with low concentration or when working with limited sample.

**Conclusion**

Mass spectrometry is the most common method previously used for VLP size determination, but this technique is costly and impractical for frequent analysis. Inclusion of SEC–MALS as an analytical technique to determine the MW and $R_g$ is a preferred alternative, and allows for both routine analysis and process monitoring. The wide range in pore sizes and separation ranges of TSKgel PWxL SEC columns overcome challenges in analytical SEC where separations of large macromolecules require a larger pore-sized stationary phase. When these SEC columns are then combined with the greatly enhanced sensitivity of Tosoh Bioscience’s LenS3 MALS detector, fast and easy analysis of MW and $R_g$ with an improved level of detection (LOD) is provided.

![Figure 1: Protein calibration curves on TSKgel PWxL columns](image-url)
Figure 2: Analysis of parvovirus VLP and BSA on TSKgel GMPWxL mixed bed pore size SEC column

Figure 3: Limit of detection by RALS using TSKgel G5000PWxL at 0.5 mL/min

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Polyurethanes are typically analyzed by gel-permeation chromatography (GPC) or size-exclusion chromatography (SEC) using polymer standards and extensive column calibration. Besides being tedious, column calibration means that the resulting answers will be relative and not absolute, relying on the assumption that the reference standards have the same conformation, density and column interactions as the analyte of interest.

A far more effective means of characterizing polyurethanes involves adding multi-angle light scattering (MALS) to SEC, creating a SEC–MALS system that eliminates the need for column calibration and reference standards (1). SEC-MALS analyses derive from first principles; they are absolute and do not depend on such assumptions.

Materials and Methods
A polyurethane sample was dissolved in DMF with 0.01 M LiBr. The specific refractive index increment, dn/dc, was measured off-line by injecting aliquots at a series of carefully prepared concentrations into an Optilab differential refractometer (Wyatt Technology). The Optilab uses a light source at the same wavelength as the DAWN multi-angle light scattering detector (Wyatt Technology), for the measured dn/dc value to provide maximal accuracy in concentration and MALS analyses.

An aliquot was then injected onto the SEC column at a flow rate of 0.485 mL/min. The eluting fractions were characterized by the DAWN and Optilab to determine the molar mass of each one-second slice.

Results and Discussion
The following results—courtesy of Kolon Corp., Korea—display the absolute molar mass at each slice in the light scattering chromatogram (Figure 1) and the conversion to a differential distribution (Figure 2). The logarithmic relationship displayed between molar mass and elution volume provides clear indication that the separation is ideal that and no unexpected column interactions occur.

The results are further analyzed to produce the absolute weight-average molar mass $M_w$, other molecular weight moments of the peak, and the polydispersity $M_w/M_n$. These are shown in Table I, as are the uncertainties of each of the calculated quantities.

Conclusions
Although standard GPC is considered to be the primary technique for determination of molar mass distributions, only SEC–MALS provides unbiased and absolute molar mass measurements. Simply adding a MALS instrument to an existing GPC system reveals new horizons in absolute polymer characterization.

Reference