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# Peptide Mapping

The benefits of an automated dual protease digest



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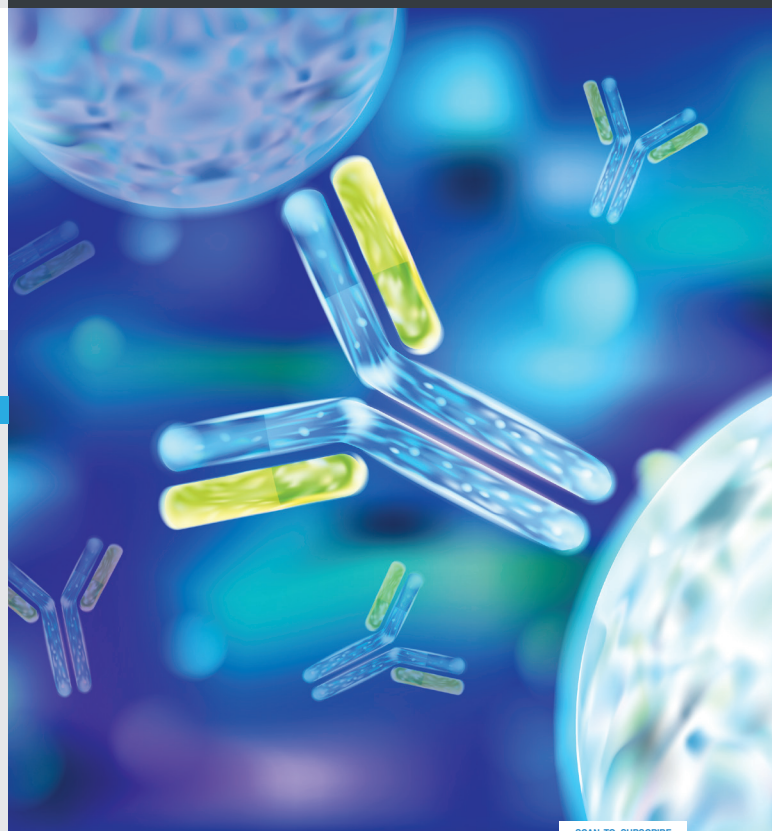
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## HPLC INTERVIEW

### Fundamentals of Chromatography: An HPLC Interview with Deirdre Cabooter

At HPLC 2023, LCGC spoke with Deirdre Cabooter of KU Leuven in Belgium, about her talk "Detailed Investigation of Intra-Particle Diffusion in LC".



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## RISING STAR

### Rising Stars of Separation Science: Leo Wang Hantao

We spoke to Leo Wang Hantao from the University of Campinas about his work investigating the influence of ion management parameters on the data produced by GC×GC–HRMS.



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## SUPPLEMENT

### Advances in (U)HPLC

*Advances in (U)HPLC* is a special supplement from LCGC and guest-edited by a pioneer of modern chromatography and member of LCGC's editorial advisory board, Gert Desmet from Vrije Universiteit Brussel.



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## July/August Update

Welcome to your July/August issue of *LCGC*

*Europe*! In the world of biopharmaceutical analysis, peptide mapping is a widely used technique in the characterization of monoclonal antibodies (mAbs), helping to understand the abundance of site-specific modifications and confirming the primary protein sequence. Our cover story for this issue discusses how to enhance traditional peptide mapping protocols using dual proteases with an automated digestion technique—where will this evolve in the future?

Where does one begin with scouting gradients? In "LC Troubleshooting", Dwight Stoll discusses how to design them, and how the data generated can be used to make decisions about the next steps to take.

We go back to basics with a look at using detectors in gas chromatography (GC). Nick Snow explores the challenges involved in detecting the effluent from a capillary GC column and summarizes the detector options on offer.

The team at RIC group delve into the analytical toolbox and examine polysorbate analysis in the biopharma field. A plethora of methods have been developed in recent years, and the team look at its diversity, variability, degradation, and structure/function relationship.

In "Sample Preparation Perspectives", Doug Raynie surveys the practical applications of partition coefficients as they are commonly applied to chemical extractions.

I would like to close by extending a warm welcome to our new associate editorial director, Caroline Hroncich, who will lead our editorial staff in print and online.

Happy reading!

### Mike Hennessy Jr

President and CEO, MJH Life Sciences®

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# Enhancing Peptide Mapping Sequence Coverage Through an Automated Dual Protease Digest

Craig Jakes<sup>1</sup>, Silvia Millán-Martín<sup>1</sup>, Dan Bach Kristensen<sup>2</sup>, Ken Cook<sup>3</sup>, Jonathan Bones<sup>1,4</sup>, and Sara Carillo<sup>1</sup>, <sup>1</sup>National Institute for Bioprocessing Research and Training, Blackrock, Co. Dublin, Ireland, <sup>2</sup>Symphogen, Ballerup, Denmark, <sup>3</sup>Thermo Fisher Scientific, Hemel Hempstead, UK, <sup>4</sup>School of Chemical and Bioprocess Engineering, University College Dublin, Dublin, Ireland

Peptide mapping is routinely used in the characterization of monoclonal antibodies (mAbs) for confirmation of the primary sequence and for the detection of post-translational modifications (PTMs). Trypsin is one of the most commonly used proteases in peptide mapping protocols due to its high level of specificity. However, it has been observed that trypsin alone is not always sufficient for full sequence coverage because of the presence of long sequences of hydrophobic amino acids that lack trypsin-specific cleavage sites. In this article, trypsin was combined with chymotrypsin to overcome this loss of sequence coverage. Through the immobilization of these proteases on magnetic beads, and by performing the digestion using an automated platform, a rapid and reproducible digest was achieved with low levels of nonspecific peptides (< 1.3%) and a low number of unique peptides generated across technical replicates (< 6). By using a ratio of 50:50 (v/v) trypsin–chymotrypsin, full sequence coverage was achievable.

Monoclonal antibodies (mAbs) are Y-shaped glycoproteins that consist of two identical heavy chains and two identical light chains that amount to a molecular mass of approximately 150,000 Da (1–3). The light and heavy chains are connected through a series of intra- and intermolecular disulfide bonds that stabilize the structure (4). For immunoglobulin G (IgG) mAbs, the heavy chain consists of four domains, one variable domain ( $V_H$ ), and three constant domains ( $C_H1$ ,  $C_H2$ ,  $C_H3$ ), while the light chain consists of just one variable domain ( $V_L$ ) and one constant domain ( $C_L$ ).  $V_L$  and  $C_L$  pair with the  $V_H$  and  $C_H1$  to form the fragment antigen binding (Fab) domain. The location of the two variable domains in the Fab is considered the Fv region. In this region, there are six complementarity-determining sites, which are unique depending on the target antigen for binding; as such, the number of amino acids and the sequence of the complementarity-determining region (CDR) varies (5).

Peptide mapping is one of the most commonly applied analytical tools for the characterization of monoclonal antibodies because it provides site-specific information on post-translational modifications (PTMs), such as the relative abundance levels of oxidation, deamidation, N- and C-terminal composition, glycosylation, isomerization, and glycation. It is also applied for the confirmation of the primary amino acid sequence. Confirmation of the primary sequence and the determination of PTMs present are both characterization requirements under the International Council for Harmonization (ICH) guidelines Q6B (6). Traditional peptide mapping protocols involve reduction and alkylation of disulfide bonds followed by digestion of the mAb using a protease—typically trypsin—before peptide separation using reversed-phase liquid chromatography (RPLC) coupled

## KEY POINTS

- Combining chymotrypsin with traditional trypsin digest can improve sequence coverage in the presence of long hydrophobic peptides.
- The trypsin–chymotrypsin ratio needs optimization to maintain low levels of nonspecific cleavages.
- Immobilizing proteases on magnetic beads allows for a higher reproducibility.



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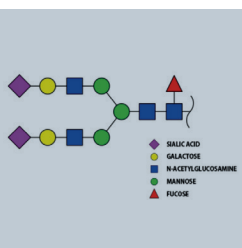
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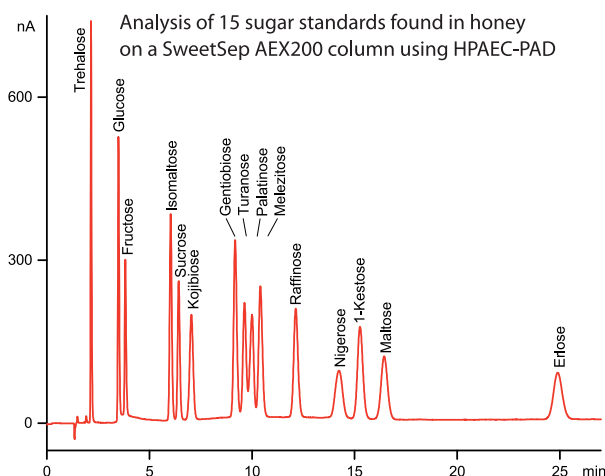
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with mass spectrometry (MS) (7,8). The acquired data are then analyzed offline using software algorithms. A drawback with the traditional methods used for peptide mapping is that they are typically time-consuming and can result in sample preparation-induced modifications. A number of studies have been performed to optimize the time required and reduce the input of process-induced modifications by making the sample preparation process more automated (9–12). Traditional tryptic digests can be insufficient for the characterization of some proteins because of an abundance or lack of tryptic cleavage sites within the primary sequence. Trypsin is a highly specific protease that cleaves proteins at the C-terminal of lysine and arginine (13). However, if these residues are not present in regions characterized by higher hydrophobicity, the resulting peptides might be difficult to track using reversed-phase chromatography for LC–MS analysis because of either the size or resulting hydrophobicity of the peptides formed following protease digestion. As a consequence, the sequence coverage obtained through peptide mapping might be incomplete, preventing the monitoring of critical quality attributes (CQAs) (14). To mitigate this effect, different strategies can be undertaken, including optimizing mobile phase strength and temperature or choosing alternative stationary phase chemistries, such as C4 rather than the widely used C18. Other strategies could involve alternative sample preparation procedures, for example, with the use of other proteases or a combination of more proteases, which could help to obtain smaller peptides that could be more easily monitored. Numerous studies have been published that utilize a combination of proteases in a single digest reaction to improve peptide mapping efficiency. One study compared

the result of a double trypsin digest to a surfactant-assisted digest and a combined LysC and trypsin digest. It was found that combining LysC and trypsin improved the digestion efficiency and reduced missed cleavages, despite the proteases sharing a common specificity in cleavage site (15). The combination of trypsin and chymotrypsin has been successful in the detection of sequence variants in recombinant human mAbs and in the identification of the location of epitopes for a number of mAbs that were shown to react with proteins detected in the brains of Alzheimer's patients (16,17).

In this study, trypsin and chymotrypsin were combined in a single digestion step using an automated workflow. Chymotrypsin is less specific compared to trypsin. Chymotrypsin preferentially cleaves the C-terminal of aromatic amino acids tryptophan, tyrosine, and phenylalanine; however, it has additional activity on leucine, methionine, and histidine amino acids (18). Due to its lower level of specificity, peptide mapping protocols using chymotrypsin may suffer from a lack of reproducibility and the digestion pattern can be altered when the digestion time is increased. Here, the proteases were immobilized on magnetic beads to allow for a time controlled, robust, and reproducible digest. The primary benefit is that during the immobilization process the proteases are chemically modified in such a way that they are stabilized while maintaining their specificity, and the selectivity of the cleavage site is not affected. On the other hand, the immobilization of the protease prevents them from autolysis, contrary to what is often observed using in-solution digestion (19,20,21). Using an optimized digestion protocol for the automated trypsin digestion (11), the implementation of chymotrypsin was evaluated using a human recombinant IgG1 mAb with known hydrophobic stretches within

the amino acid sequence of its CDR. The sequence coverage and relative PTMs abundance were investigated by comparing a tryptic digestion to a dual protease digest containing various ratios of trypsin and chymotrypsin. Different ratios of trypsin to chymotrypsin were investigated to determine at what ratio full sequence coverage could be obtained while minimizing the levels of nonspecific modifications generated from chymotrypsin. The optimized digest was then assessed to prove robustness and reproducibility of the sample preparation.

## Experimental

**Materials:** The human IgG1 monoclonal antibody investigated in this study was kindly provided by Symphogen. Thermo Scientific Smart Digest kits (magnetic resin option) for both trypsin and chymotrypsin and Thermo Scientific Bond Breaker Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) solution neutral pH were acquired from Thermo Fisher Scientific. LC–MS-grade 0.1% (v/v) formic acid in water was used as mobile phase A, while LC–MS-grade 0.1% (v/v) formic acid in acetonitrile was used as mobile phase B. Both mobile phase solutions were sourced from Thermo Fisher Scientific. Trifluoroacetic acid was acquired from Sigma Aldrich.

**Peptide Mapping Protocol:** Peptide mapping was performed using a Kingfisher Duo Prime Purification System under the control of BindIt software (Thermo Fisher Scientific).

A 100 µg measure of mAb was diluted to 1 mg/mL using high purity water from a Sartorius Arium Purification system, Smart Digest Buffer, and 5 mM TCEP (final concentration). A total volume of 15 µL of magnetic protease beads was used at different ratios of trypsin to chymotrypsin. The following ratios were investigated: 100:0, 75:25, 50:50, and 25:75 (v/v) trypsin–chymotrypsin,

respectively. Protease beads were diluted in 100  $\mu$ L of digest buffer and washed in a 1:4 (v/v) ratio of trypsin digestion buffer in water before being added to the mAb sample. Digestion was performed at 70 °C for 30 min at medium mixing speed. At the end of the digest, the beads were removed from the samples and the reaction was acidified to a final concentration of 0.1% trifluoroacetic acid (TFA) (Sigma Aldrich) before LC–MS analysis.

#### LC–MS Instrumentation and

**Parameters:** LC–MS analysis was performed on a Thermo Scientific Vanquish Flex Binary UHPLC system coupled online to a Thermo Scientific Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer using a heated electrospray ionization (HESI) source (Thermo Fisher Scientific). Peptide separation was performed

using a 2.1  $\times$  250 mm, 2.2- $\mu$ m Thermo Scientific Vanquish Acclaim C18 column. The peptide mapping gradient was as follows: 2% B to 45% B in 45 min, increased to 90% B at 46 min, and held at 90% B for four min. This was then increased to 2% B at 51 min until 53 min, and increased to 90% B at 54 min until 56.5 min. Requilibration was then followed at 2% B from 56.5 min until the end of the run at 60 min. The mobile phase flow rate was maintained at 0.300 mL/min for the duration of the run and the column temperature was 25 °C. The first two min and the last 12 min of the gradient were diverted to waste using an external valve.

MS tune parameters were set as follows: sheath gas flow rate was 40 AU, auxiliary gas flow rate was 10 AU, spray voltage was 3.80 kV, capillary temperature was

320 °C, S-lens RF voltage level was set to 50.0, and the auxiliary gas heater temperature was 400 °C.

Full MS was acquired at 70,000 resolution (at 200  $m/z$ ), with an acquisition gain control (AGC) target of  $3 \times 10^6$  ions and a maximum injection time of 100 ms. One microscan was performed and a scan range of 200–2000  $m/z$  was used. Data-dependent MS/MS was performed at 17,500 resolution (at 200  $m/z$ ), with an AGC target of  $1 \times 10^5$  ions and a maximum injection time of 200 ms. One microscan was performed and a loop count of five, MSX count of one, and a topN of five were also applied. Normalized collision energy of 28 and an isolation window of 2.0  $m/z$  were also used.

**Data Acquisition and Analysis:** Data were acquired using Thermo Scientific

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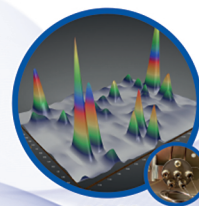
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**TABLE 1:** Parameters used for peptide mapping analysis in the software

| Component Detection  |   |
|--|---|
| Absolute MS Signal Threshold                                     | 8.00E+4   |
| MS Noise Level   | 8000.00   |
| S/N Threshold  | 10.00   |
| Beginning Peak Width (min)                                       | 0.30  |
| Typical Chromatographic Peak Width (min)                         | 0.30  |
| Ending Peak Width (min)  | 0.30  |
| Maximum Chromatographic Peak Width (min)                         | 0.50  |
| Use Restriction Time   | ✓   |
| Time Limits (min)  | 0.00–50.00  |
| Relative MS Signal Threshold (% of base peak)                    | 1.00  |
| Relative Analog Threshold (% of highest peak)                    | 1.00  |
| Width of Gaussian Filter   | 3   |
| Minimum Valley to be Considered as Two Chromatographic Peaks (%) | 80.00   |
| Minimum MS Peak Width (Da)                                       | 1.20  |
| Maximum MS Peak Width (Da)                                       | 4.20  |
| Mass Tolerance   | 4.00  |
| Maximum Retention Time Shift (min)                               | 1.00  |
| Maximum Mass (Da)  | 30,000.00   |
| Mass Centroiding Cut-off (% from base)                           | 15.00   |
| Identification   |   |
| Maximum Peptide Mass   | 7000  |
| Mass Accuracy (ppm)  | 5   |
| Minimum Confidence   | 0.80  |
| Enable Mass Search for Unspecified Modifications                 | ✓   |
| Mass Changes for Unspecified Modifications                       | -58 to 162  |
| Glycosylation  | Human   |
| Variable Modifications   | Gln→Pyro-Glu (N-Term)<br>Lys (C-Term)<br>Deamidation (N)(Q) (Side Chain)<br>Oxidation (MW) (Side Chain) |

Chromeleon Chromatography Data System (CDS) software 7.2.9 (Thermo Fisher Scientific). Peptide sequence coverage analysis and PTM detection was performed using Thermo Scientific BioPharma Finder software version 4.1. The parameters used are highlighted in Table 1. Peptides were filtered to include a confidence score of  $\geq 95\%$ , to be within a delta of  $\pm 5$  ppm and to exclude peptides containing  $\text{Na}^+/\text{K}^+$  adducts, gas phase ions, nonspecific, and unknown modifications. For trypsin, an additional filter of  $\leq 1$  missed cleavage was

applied because of the high specificity of trypsin. For the combined proteases this was not a viable filter, as there were additional cleavage sites to consider, even within traditional tryptic peptides. For example, DTLMISR, a commonly investigated peptide for the calculation of oxidation abundance, would have two potential missed cleavages when using chymotrypsin. To overcome this, a best overall average structural resolution (ASR) filter of  $\geq 1.0 \leq 1.6$  was applied, which resulted in peptides with good quality MS/MS spectra

containing sufficient b- and y-diagnostic ions for confirmation of identity. This value can be observed in the fragment coverage map for any detected peptide that contains MS2 scans. However, to assess N-glycosylation, the used filters were based on the peptides EEQYNSTYR, TKPREEQYNSTYR, and EEQYNSTYRVVSVLTVLHQDWLNGK, as they contain N-glycan sites and the signals showed good intensity values. A confidence score of  $\geq 95\%$  and accuracy within  $\pm 5$  ppm were again selected.

## Results and Discussion

**Sequence Coverage:** A high level of sequence coverage in peptide mapping protocols is vital to ensure accurate detection of site-specific modifications and for confirmation of sequence in critical regions such as the CDR (10). The results from this investigation demonstrate that for the IgG1 used, the use of trypsin alone was not sufficient to obtain full sequence coverage (Figure 1). To overcome this loss of sequence coverage, an increasing amount of chymotrypsin was combined with trypsin to yield ratios of 75:25, 50:50, and 25:75 (v/v) trypsin–chymotrypsin, respectively. The results from this investigation show that when using ratios containing  $\geq 50\%$  chymotrypsin, full sequence coverage was obtained (Figure 1). Further investigation into the generation of nonspecific peptides with different ratios of trypsin to chymotrypsin showed that when the percentage of chymotrypsin was  $\geq 50\%$ , the relative abundance and number of nonspecific-generated peptides did not exceed those generated in trypsin alone (Figure 2). The levels of nonspecific cleavage were lower than those previously reported in our interlaboratory study using immobilized trypsin alone (11).

As smaller peptides can be more hydrophilic and thus more difficult

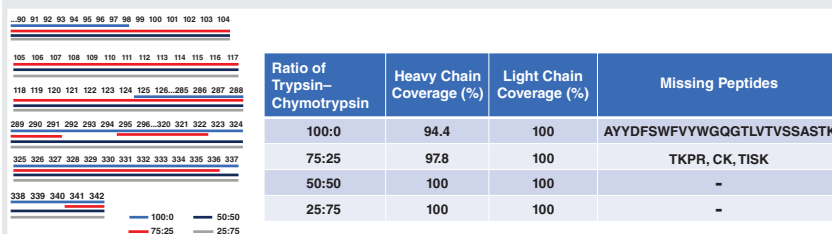


to retain on reversed-phased chromatographic supports, excessive digestion of the sample should also be avoided to achieve full sequence coverage. In this study, when adding chymotrypsin, a sharp increase of peptides consisting of three or four amino acids was observed while full sequence coverage was achieved; this was the result of overlapping peptides and good retention of the short peptides. Digestion conditions may need optimization for different analytes or when using a different separation setup.

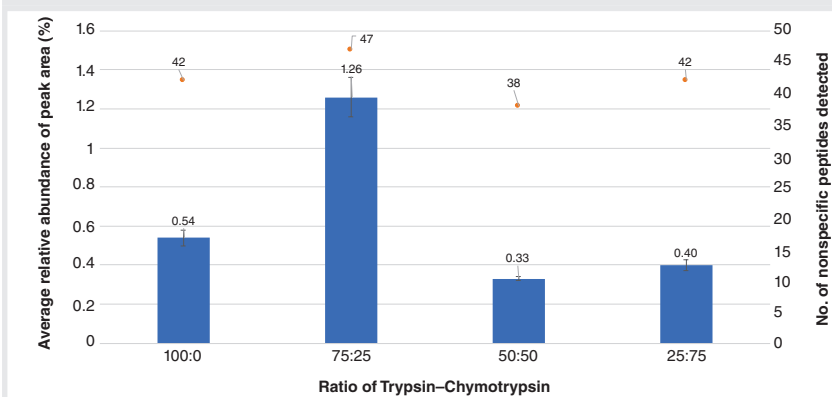
**PTM Abundance:** As previously mentioned, peptide mapping is a powerful analytical technique used for the detection of site-specific modifications. Using the different ratios of proteases, the average relative abundance ( $n = 3$ ) of a number of CQAs was investigated using a tryptic digest as a reference for comparative purposes. Figure 3 shows the average relative abundance for methionine oxidation (Figure 3[a]), asparagine and glutamine deamidation (Figure 3[b]), and N-terminal pyroglutamate formation and C-terminal lysine content (Figure 3[c]).

Oxidation is an important CQA to monitor because it can induce structural changes within the mAb and can result in decreased efficacy, safety, and stability (22). Oxidation commonly occurs in methionine residues, in particular at sites approximately located at M252 and M428 in the constant heavy chain region; however, oxidation has also been observed in tryptophan residues in the CDR, which can result in reduced or even abolished antigen binding (23,24). The average relative abundance of oxidation was lower in samples digested with a ratio of chymotrypsin  $\geq 50:50$  compared to the control for M48 and M254, while only a slight increase was recorded for M430. For all samples—including the control—the

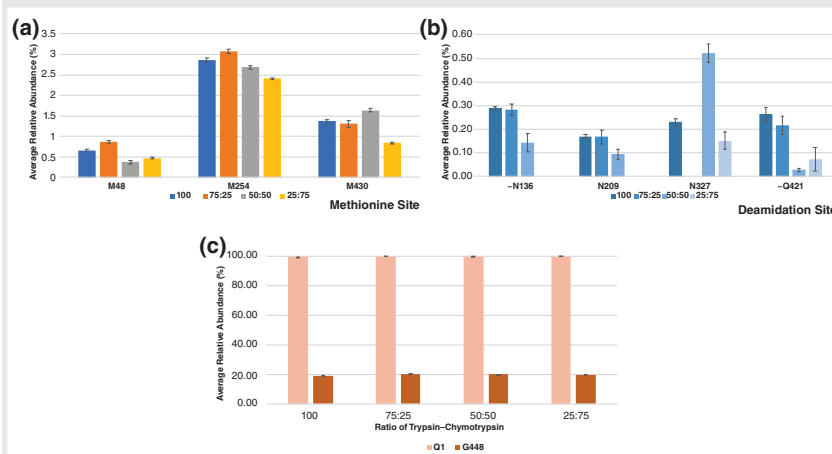
**FIGURE 1:** Sequence coverage at different ratios of trypsin–chymotrypsin. The numbers on the left correspond to the amino acid residue while the table on the right outlines the missing peptides. The missing peptides in the 75:25 ratio are small hydrophilic peptides and they may be lost as a result of the diverting of the valve to waste for the first two minutes.



**FIGURE 2:** Average relative abundance of nonspecific generated peptides at different ratios of trypsin–chymotrypsin.



**FIGURE 3:** Average relative abundance of (a) oxidation, (b) deamidation, and (c) pyroglutamate formation and lysine concentration at different protease ratios.



average relative abundance of each site was below 4%. The relative standard deviation (RSD) for all sites was below 10%, except for M48 using 50:50 where an RSD of 11.29% was determined.

Deamidation of mAbs can result in protein degradation and limit shelf life. Deamidation rates are susceptible to factors such as buffer concentration, pH, and storage temperatures (25).





210 peptides in common and that the number of unique peptides generated was low; no more than four unique peptides were detected in a single run, demonstrating a high level of reproducibility across the digestion procedures based on automated workflows.

## Conclusions

Peptide mapping remains the gold standard for understanding the abundance of site-specific modifications and for the confirmation of the primary protein sequence. This study has examined the applicability of using dual proteases with an automated digestion technique. First, different ratios of trypsin to chymotrypsin were investigated and full sequence coverage was achieved when using protease ratios containing  $\geq 50\%$  chymotrypsin. The findings from this study correspond well with

previously published studies; the first found that a combination digest of trypsin and chymotrypsin improved sequence coverage from  $\sim 88\%$  when using trypsin alone to  $98.5\%$  (17). The ratio of trypsin to chymotrypsin was supported by the second study, which used a similar ratio of proteases and was able to improve the detection of hydrophobic proteins (14). The average relative abundance of PTMs, such as oxidation, deamidation, and N-glycosylation, was measured in all protease ratios and compared to the levels observed when using trypsin alone as a reference. It was found that when using a protease ratio of 50:50, the levels of PTMs detected were comparable to those in the control. Method reproducibility was investigated next using six technical replicates. The method was shown to have a high level of reproducibility with

regards to retention time. The very low level of nonspecific peptides generated highlights its suitability for workflows such as the multi-attribute method (MAM) for molecules, where digestion with trypsin alone is not sufficient. Automation of the digestion further paves the way for dual protease digestion to be applied in quality control (QC) environments.

## References

- 1) Beck, A.; Liu, H. Macro- and Micro-Heterogeneity of Natural and Recombinant IgG Antibodies. *Antibodies (Basel)* **2019**, *8* (1), 18. DOI: 10.3390/antib8010018
- 2) Xu, Y.; Wang, D.; Mason, B.; et al. Structure, Heterogeneity and Developability Assessment of Therapeutic Antibodies. *MAbs* **2019**, *11* (2), 239–264. DOI: 10.1080/19420862.2018.1553476
- 3) Graf, T.; Heinrich, K.; Grunert, I.; et al. Recent Advances in LC-MS Based Characterization of Protein-Based Bio-Therapeutics - Mastering Analytical Challenges Posed by the Increasing Format Complexity. *J. Pharm. Biomed. Anal.* **2020**, *186*, 113251. DOI: 10.1016/j.jpba.2020.113251

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- 4) Vidarsson, G.; Dekkers, G.; Rispens, T. IgG Subclasses and Allotypes: From Structure to Effector Functions. *Front. Immunol.* **2014**, *5*, 520. DOI: 10.3389/fimmu.2014.00520
- 5) Vlasak, J.; Bussat, M. C.; Wang, S.; et al. Identification and Characterization of Asparagine Deamidation in the Light Chain CDR1 of a Humanized IgG1 Antibody. *Anal. Biochem.* **2009**, *392* (2), 145–54. DOI: 10.1016/j.ab.2009.05.043
- 6) ICH, Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products, (1999).
- 7) Qu, M.; An, B.; Shen, S.; et al. Qualitative and Quantitative Characterization of Protein Biotherapeutics with Liquid Chromatography Mass Spectrometry. *Mass Spectrom. Rev.* **2017**, *36* (6), 734–754. DOI: 10.1002/mas.21500
- 8) Rathore, D.; Faustino, A.; Schiel, J.; et al. The Role of Mass Spectrometry in the Characterization of Biologic Protein Products. *Expert Rev. Proteomics* **2018**, *15* (5), 431–449. DOI: 10.1080/14789450.2018.1469982
- 9) Richardson, J.; Shah, B.; Xiao, G.; Bondarenko, P. V.; Zhang, Z. Automated In-Solution Protein Digestion Using a Commonly Available High-Performance Liquid Chromatography Autosampler. *Anal. Biochem.* **2011**, *411* (2), 284–91. DOI: 10.1016/j.ab.2011.01.019
- 10) Mouchahoir, T.; Schiel, J. E. Development of an LC-MS/MS Peptide Mapping Protocol for the NISTmAb. *Anal. Bioanal. Chem.* **2018**, *410* (8), 2111–2126. DOI: 10.1007/s00216-018-0848-6
- 11) Millan-Martin, S.; Jakes, C.; Carillo, S.; et al. Inter-Laboratory Study of an Optimised Peptide Mapping Workflow Using Automated Trypsin Digestion for Monitoring Monoclonal Antibody Product Quality Attributes. *Anal. Bioanal. Chem.* **2020**, *412* (25), 6833–6848. DOI: 10.1007/s00216-020-02809-z
- 12) Qian, C.; Niu, B.; Jimenez, R. B.; Wang, J.; Albarghouthi, M. Fully Automated Peptide Mapping Multi-Attribute Method by Liquid Chromatography-Mass Spectrometry with Robotic Liquid Handling System. *J. Pharm. Biomed. Anal.* **2021**, *198*, 113988. DOI: 10.1016/j.jpba.2021.113988
- 13) Schmid, I.; Bonnington, L.; Gerl, M.; et al. Assessment of Susceptible Chemical Modification Sites of Trastuzumab and Endogenous Human Immunoglobulins at Physiological Conditions. *Commun. Biol.* **2018**, *1*, 28. DOI: 10.1038/s42003-018-0032-8
- 14) Vermachova, M.; Purkrtova, Z.; Santrucek, J.; et al. Combining Chymotrypsin/Trypsin Digestion to Identify Hydrophobic Proteins from Oil Bodies. *Methods Mol. Biol.* **2014**, *1072*, 185–198. DOI: 10.1007/978-1-62703-631-3\_14
- 15) Glatter, T.; Ludwig, C.; Ahn, E.; et al. Large-Scale Quantitative Assessment of Different In-Solution Protein Digestion Protocols Reveals Superior Cleavage Efficiency of Tandem Lys-C/Trypsin Proteolysis Over Trypsin Digestion. *J. Proteome Res.* **2012**, *11* (11), 5145–56. DOI: 10.1021/pr300273g
- 16) Ksiezak-Reding, H.; Chien, C. H.; Lee, V. M.; Yen, S. H. Mapping of the Alz 50 Epitope in Microtubule-Associated Proteins Tau. *J. Neurosci. Res.* **1990**, *25* (3), 412–9. DOI: 10.1002/jnr.490250319
- 17) Yang, Y.; Strahan, A.; Li, C.; et al. Detecting Low Level Sequence Variants in Recombinant Monoclonal Antibodies. *MAbs* **2010**, *2* (3), 285–98. DOI: 10.4161/mabs.2.3.11718
- 18) Williams, K. R.; Stone, K. L. Enzymatic Cleavage and HPLC Peptide Mapping of Proteins. *Mol. Biotechnol.* **1997**, *8* (2), 155–67. DOI: 10.1007/BF02752260
- 19) Morellon-Sterling, R.; Tavano, O.; Bolivar, J. M.; et al. A Review on the Immobilization of Pepsin: A Lys-Poor Enzyme that is Unstable at Alkaline pH Values. *Int. J. Biol. Macromol.* **2022**, *210*, 628–702. DOI: 10.1016/j.ijbiomac.2022.04.224
- 20) Sharma, S.; Gupta, S.; Princy, Kumar Arya, S.; Kaur, A. Enzymes Immobilization: Implementation of Nanoparticles and an Insight into Polystyrene as the Contemporary Immobilization Matrix. *Process Biochem.* **2022**, *120*, 22–34. DOI: 10.1016/j.procbio.2022.05.022
- 21) Tacias-Pascacio, V. G.; Morellon-Sterling, R.; Castañeda-Valbuena, D.; et al. Immobilization of Papin: A Review. *Int. J. Biol. Macromol.* **2021**, *188*, 94–113. DOI: 10.1016/j.ijbiomac.2021.08.016
- 22) Pavon, J. A.; Li, X.; Chico, S.; et al. Analysis of Monoclonal Antibody Oxidation by Simple Mixed Mode Chromatography. *J. Chromatogr. A* **2016**, *1431*, 154–165. DOI: 10.1016/j.chroma.2015.12.068
- 23) Stracke, J.; Emrich, T.; Rueger, P.; et al. A Novel Approach to Investigate the Effect of Methionine Oxidation on Pharmacokinetic Properties of Therapeutic Antibodies. *MAbs* **2014**, *6* (5), 1229–42. DOI: 10.4161/mabs.29601
- 24) Dashivets, T.; Stracke, J.; Deng, S.; et al. Oxidation in the Complementarity-Determining Regions Differentially Influences the Properties of Therapeutic Antibodies. *MAbs* **2016**, *8* (8), 1525–1535. DOI: 10.1080/19420862.2016.1231277
- 25) Pace, A. L.; Wong, R. L.; Zhang, Y. T.; Kao, Y. -H.; Wang, Y. J. Asparagine Deamidation Dependence on Buffer Type, pH, and Temperature. *J. Pharm. Sci.* **2013**, *102* (6), 1712–1723. DOI: 10.1002/jps.23529
- 26) Lu, X.; Machiesky, L. A.; De Mel, N.; et al. Characterization of IgG1 Fc Deamidation at Asparagine 325 and Its Impact on Antibody-Dependent Cell-Mediated Cytotoxicity and FcγRIIIa Binding. *Sci. Rep.* **2020**, *10* (1), 383. DOI: 10.1038/s41598-019-57184-2
- 27) Badgett, M. J.; Boyes, B.; Orlando, R. The Separation and Quantitation of Peptides with and without Oxidation of Methionine and Deamidation of Asparagine Using Hydrophilic Interaction Liquid Chromatography with Mass Spectrometry (HILIC-MS). *J. Am. Soc. Mass Spectrom.* **2017**, *28* (5), 818–826. DOI: 10.1007/s13361-016-1565-z
- 28) Boune, S.; Hu, P.; Epstein, A. L.; Khawli, L. A. Principles of N-Linked Glycosylation Variations of IgG-Based Therapeutics: Pharmacokinetic and Functional Considerations. *Antibodies (Basel)* **2020**, *9* (2), 22. DOI: 10.3390/antib9020022
- 29) Carillo, S.; Pérez-Robles, R.; Jakes, C.; et al. Comparing Different Domains of Analysis for the Characterization of N-Glycans on Monoclonal Antibodies. *J. Pharm. Anal.* **2020**, *10* (1), 23–34. DOI: 10.1016/j.jpba.2019.11.008
- 30) Lex, A.; Gehlenborg, N.; Strobel, H.; Vuilleumot, R.; Pfister, H. UpSet: Visualization of Intersecting Sets. *IEEE Trans. Vis. Comput. Graph.* **2014**, *20* (12), 1983–92. DOI: 10.1109/TVCG.2014.2346248

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# Initiating Method Development with Scouting Gradients—Where to Begin and How to Proceed?

Dwight R. Stoll, LC Troubleshooting Editor

**With so many options for method parameters to adjust during method development, identifying a starting point can be intimidating. Starting with scouting gradients can simplify the process and yield rich results that inform subsequent method development steps. In this instalment, we discuss how to design scouting gradients, how to use the resulting data to make decisions about next steps (including whether to use isocratic or gradient elution), and how to improve the separation once an elution mode has been chosen.**

One of the characteristics of the development process for chromatographic methods that I think keeps many chromatographers so engaged in the work is that there are many “knobs to turn”. Of course, modern instruments usually don’t actually have any knobs to turn anymore, but the point is that there are a large number of variables that can influence a chromatographic result. For example, a short list includes column chemistry, particle size, pore size, flow rate, mobile phase pH, temperature, and so on.

I think for many chromatographers it is enjoyable to think about the ways in which these variables influence the results. Method development is a puzzle waiting to be solved, some might say. However, the other side of the coin is that with so many variables in play, how does one know where to start, especially in situations where there is time pressure to get the method developed, without the luxury of being able to play with the variables

to satisfy our curiosity? In answer to this question, I would highlight two important skills: 1) the ability to prioritize the variables that are likely to have the most influence on your chromatographic results; and 2) the ability to “fail fast”, to borrow a concept from the business world. There is no point in spending a lot of time fine-tuning a method that is never going to work anyway.

I think the skill of prioritizing variables benefits from equal measures of experience and learning from others. Across the history of this “LC Troubleshooting” column, John Dolan wrote extensively about different aspects of the method development process. There is a lot that can be learned from these articles, even if some of them might seem dated now, because many of the concepts are still useful today. Indeed, in the LC Troubleshooting Bible tag cloud (<https://lcts bible.com/lc-troubleshooting-bible/postsbytags/>), “method development” is one of the

most prominent keywords. A great example of useful reading in this vein is John’s series of seven articles on “The Perfect Method” from 2007 (1,2). Readers interested in a comprehensive resource for development of gradient elution liquid chromatography (LC) methods are referred to Snyder and Dolan’s book on the subject (3).

As for “failing fast”, I think the use of scouting gradients is an incredibly powerful approach. These gradients, if designed properly, can yield rich information that can guide subsequent steps in the development process—either to a successful conclusion, or to the conclusion that the initial parameter choices should be abandoned in favour of trying something else. In this instalment of “LC Troubleshooting”, I discuss the concept of scouting gradients, how to design them, and how the information they yield can be used to make decisions about next steps in the development process.

## The First Scouting Gradient

The following guidance and discussion is primarily relevant to reversed-phase separations of relatively small molecules (that is, less than 500 Da). In principle, the same ideas can be extended to other separation modes and larger molecules; however, the details would all be different, so we need to save those discussions for a different day.

**Designing the Gradient:** When working with molecules whose chromatographic behaviour is unpredictable or unknown, gradient elution is particularly attractive because it increases the likelihood that analytes will be both retained to some degree, and eluted from the column during the analysis without being carried over to the next analysis. In other words, it is the closest we can get to a “fool-proof” elution program. Following are considerations for the starting and ending mobile phase compositions used in the first scouting gradient. It is important here to distinguish between %B and  $\phi$ , which are both commonly used to quantify the amount of organic solvent in the mobile phase.  $\phi$  is defined as the volume fraction of organic solvent in the mobile phase on a 0–1 scale. %B is defined as the volume fraction of the B solvent in the mobile phase on a 0–100% scale. If the B solvent is 100% organic solvent, then saying 100% B is the same as saying  $\phi = 1$ . Things get a bit more complicated when B is not 100% organic, and we have to do a little algebra to translate between %B and  $\phi$ .

- Initial composition ( $\phi_i$ ): Since we are discussing reversed-phase separations, we should use as little organic solvent as possible in

the mobile phase without causing problems. One problem that could occur here if we use too little organic solvent is “dewetting” of the stationary phase, where the mobile phase is literally expelled from the hydrophobic pores of the stationary phase particles (4). Many sources recommend starting with 5% organic solvent in the mobile phase, which is adequate for many applications. In my lab, we routinely go down to 2% organic solvent without any known problems.

- Final composition ( $\phi_f$ ): At the end of the gradient, we should use as much organic solvent as possible without causing problems. One major concern here is that many buffers in common use for reversed-phase separations are not highly soluble in the organic solvents used for reversed-phase LC (RPLC)—for example, acetonitrile. If the buffer salts precipitate from the mobile phase upon mixing with the organic solvent, this can have very serious negative consequences, including ruining pumps and valves. The precipitates can act as abrasives that scratch surfaces, and occlude filters and frits in the mobile phase path. The upper limit of organic solvent concentration that can be used depends on the type (for example, sodium phosphate behaves very differently from ammonium acetate) and concentration of the buffering agents. Readers interested in learning more about these details are referred to a *LCGC* article on the topic (5). In my laboratory, we try to avoid using more than 70% acetonitrile with phosphate buffers

for this reason. When working with mobile phase additives such as formic acid or dilute ammonium acetate, it is possible to go up to 95% acetonitrile.

Once the initial and final compositions have been chosen, the only major decision that remains is to choose the gradient time. The bad news here is that the “right” gradient time depends upon several other factors, but the good news is that accounting for these relationships is relatively straightforward. In isocratic elution, the retention factor ( $k$ ) of an analyte is only dependent on the mobile phase composition ( $\phi$ ), and independent of parameters like column length and diameter, flow rate ( $F$ ), and gradient time ( $t_g$ ). In isocratic elution, we generally try to adjust elution conditions so that retention factors are in the range  $2 < k < 10$ . In gradient elution things are different in the sense that  $k$  depends on  $\phi_i$  and  $\phi_f$ , column dimensions,  $F$ , and  $t_g$ . However, in developing the scouting gradient we have a similar target for  $k$  of around 5. Here, though, we have to use a different type of retention factor because local retention factor changes as the solvent gradient proceeds through the column. For this purpose we use  $k^*$ , which is defined as the local retention factor of the analyte when the midpoint (from inlet to outlet) of the column is reached. The relationship between  $k^*$  and the other variables is given in equation 1. Since we are interested in choosing a gradient time for our first scouting gradient, we can rearrange equation 1 to solve for  $t_g$ , as shown in equation 2. In these expressions,  $\Delta\phi = \phi_f - \phi_i$ , and  $S$  is

the slope of a plot of  $\ln(k)$  vs.  $\phi$  (from isocratic experiments) (3).

$$k^* = \frac{F \cdot t_g}{0.5 \cdot V_m \cdot \Delta\phi \cdot S} \quad [1]$$

$$t_g = \frac{0.5 \cdot k^* \cdot V_m \cdot \Delta\phi \cdot S}{F} \quad [2]$$

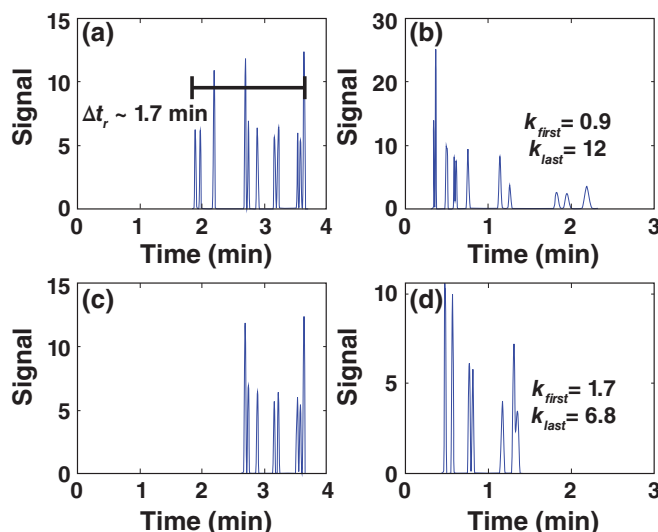
To see how this works, let's use a 50 mm  $\times$  2.1 mm internal diameter (i.d.) column as an example, and see what gradient time we come up with for our first scouting gradient. We will use the following input parameters:

- $k^* = 5$ ; as in isocratic chromatography, we want to aim for something in the range  $2 < k < 10$ .
- $V_m = 0.087$  mL; this is the column dead volume, which can be estimated using the volume of the empty column ( $\pi \cdot r^2 \cdot L$ ) times a total porosity of 50%, or calculated using a HPLC simulator (for example, [www.multidlc.org/hplcsim](http://www.multidlc.org/hplcsim))
- $\Delta\phi = 0.75$ ; this is the change in the fraction of organic solvent in a gradient that runs from 5 to 80% B, where the B solvent is 100% organic solvent.
- $S = 12$ ; this is the slope of  $\ln(k)$  vs.  $\phi$  for the molecules we are working with. For this calculation, we have to pick a representative value, and the value of 12 is a generally representative number for small molecules.
- $F = 0.5$  mL/min; this is the flow rate. Since this is a narrow column (2.1 mm i.d.) this is a modest flow rate.

Using these values, the calculated gradient time is 4 min. Given the relationships in equation 2, it is easy to see how the gradient time changes when we change some

**FIGURE 1:** Simulated chromatograms for separations of small molecule mixtures.

(a) Full mix–gradient, (b) full mix–isocratic with 48% B, (c) abbreviated mix–gradient, and (d) abbreviated mix–isocratic with 54% B. Chromatographic conditions: column: 50 mm  $\times$  2.1 mm, 3.5- $\mu$ m C18; flow rate: 0.5 mL/min.; temperature: 40 °C. Simulations were performed using freeware ([www.multidlc.org/MultiSimLC](http://www.multidlc.org/MultiSimLC)).



of the parameters. For example, if we double the column length to 100 mm,  $t_g$  goes to 8 min. If we double the flow rate to 1.0 mL/min., the  $t_g$  decreases to 2 min.

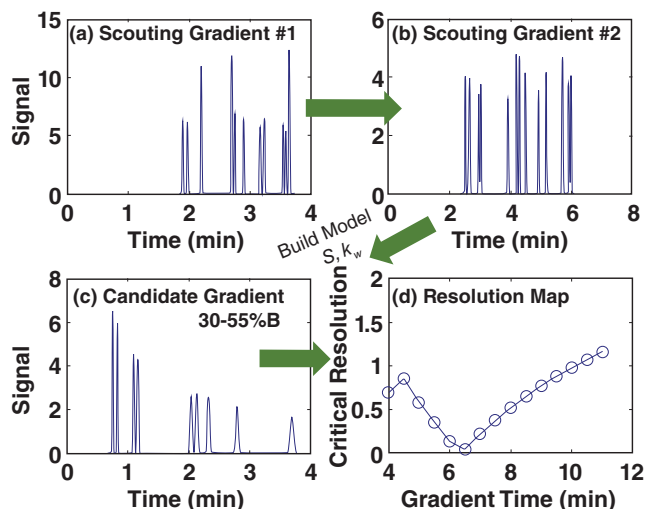
#### What Do the Results Tell Us?

Figure 1 shows two examples of chromatograms we might obtain from such scouting gradients. In Figures 1(a) and 1(c), the scouting gradient conditions are the same (as described above), but the sample mixtures are different. In both cases, the analytes are all small ( $< 500$  Da), representing both neutral and ionogenic molecules that might be encountered in pharmaceutical analysis. In Figure 1(a), the analytes are chosen such that they elute over a relatively large fraction of the gradient time. In Figure 1(c), the analyte set is restricted such that they are eluted over a narrower range. In Figure 1(a), we see that the analytes elute over a span of

about 1.7 min, which is 43% of the gradient time of 4 min. Here we can apply Dolan's "25/40% rule" (6), which says that if the span over which the peaks are eluted is more than 40% of the gradient time, then developing a gradient elution method will likely be most appropriate for the sample. An isocratic method could be developed, but it may have less desirable characteristics than the gradient method. Figure 1(b) illustrates three of the challenges associated with isocratic methods for samples that span a large fraction of the scouting gradient range. First, the early peaks are eluted at very low  $k$ . These peaks will be highly susceptible to extracolumn dispersion (7), and may be coeluted with other matrix peaks from the sample that simply "get in the way". Second, the late peaks elute at very high  $k$ . These peaks



**FIGURE 2:** Simulated gradient elution separations. (a) Scouting gradient #1, (b) scouting gradient #2, (c) predicted gradient based on a model building using data from the scouting gradients with 30–55% B, (d) resolution map for separations with a gradient of 30–55% B and different gradient times. All other conditions are as in Figure 1.



will be much broader, and thus much lower in height, which may cause trouble with quantitation for low abundance compounds. Such high  $k$  values will also translate to very long analysis times when larger volume columns are used.

Moving on to Figure 1(c), we see that the peaks from the abbreviated analyte mixture elute over a much narrower fraction of the gradient time (24%). Figure 1(d) shows an isocratic separation of this mixture using a %B that roughly corresponds to the middle of the elution window for those peaks in the scouting gradient. Here we see that the  $k$  range is much narrower (1.7 to 6.8), and thus the range of peak heights is not so broad as in Figure 1(b). Although there is coelution in the isocratic separation at 54% B, it is very likely that this coelution can be resolved through slight adjustment of the mobile phase without dramatically increasing the analysis time (8). This mixture is much more amenable to analysis

under isocratic conditions (as would be suggested by the 25/40% rule).

Generally speaking, isocratic methods are preferred over gradient methods for small molecules when they provide the separation needed in a reasonable time. Isocratic methods tend to be more robust, can be implemented with a wider range of instrumentation, and are easier to transfer between instruments.

### The Second Scouting Gradient

The first scouting gradient can tell us a lot about the number of compounds in the sample, the chemical diversity of those compounds (as measured by the range over which they elute), and whether or not we should proceed by aiming for an isocratic or gradient elution method. However, doing a second scouting gradient adds additional power to our method development strategy because it enables us to build a

model of the dependence of the retention of our compounds on many of the variables we discussed above, including column dimensions, flow rate, and mobile phase composition.

**Designing the Gradient:** Once we have chosen the conditions for the first scouting gradient, choosing the second one is much simpler. In short, we just need to change one of the parameters that affects retention time under gradient elution conditions. This could include the column dimensions, or the starting or ending points in the gradient (that is,  $\phi_i$  and/or  $\phi_f$ ), but it is most common by far to change the gradient time. The big question is “How much of a change is enough?” In our first scouting gradient, we used a gradient time of 4 min. A typical approach

would be to increase the gradient time threefold in the second scouting gradient, to 12 min. In a recent research paper (9), we pushed on this question a bit and explored the consequences of significantly varying the factor by which the gradient time is varied from as low as 1.5-fold to as much as 18-fold. To make a very long story short, we found that only modest fold increases in the gradient time are needed to build useful models, so long as the models are not used very far outside of the range of the data used to build models. In the example shown in Figure 2, I've used a gradient time of 8 min for the second scouting gradient. This reduces the time needed for the second gradient while maintaining a reasonable scope of applicability for the model.

### What Do the Results Tell Us?

Figures 2(a) and 2(b) show the chromatograms from the first and second scouting gradients we've designed here. The retention times for each compound in these two



chromatograms can be fit to a retention model using linear solvent strength (LSS) theory (or other nonlinear models) to obtain the retention parameters  $S$  and  $k_w$  for each compound. With these in hand, one can then predict an expected chromatogram for any elution conditions. An example of such a prediction is shown in Figure 2(c), where I've adjusted the starting mobile phase composition to 30% B and flattened the gradient to end at 55% B, which enables spreading the peaks across more of the gradient time. Since we get the retention times of all compounds from each prediction like this, we can also calculate the smallest resolution of any pair of neighbouring peaks in the chromatogram—we call this the *critical resolution*. Then, by changing a variable such as the gradient time, and calculating the critical resolution for each prediction, we can construct a resolution map as shown in Figure 2(d). This shows us that if we are happy with a minimum resolution of 0.9, then we can choose a gradient time of 4.5 min, and we are done with method development. However, if we want more resolution for the least resolved pair of peaks, then we need to go to longer gradient times, or change one or more other variable. This process of carrying out scouting gradients and then building resolution maps is what Snyder, Dolan, and coworkers started with in 1989 when they introduced DryLab for computer-driven method development (10,11), and this is still the principal process used by modern commercial method development packages today. The resolution map in Figure 2(d) shows the dependence of critical resolution on the single variable gradient time.

Contemporary software provides multidimensional resolution maps that enable exploration of multiple variables (for example, gradient time and temperature) simultaneously.

## Summary

Choosing a starting point for method development can be intimidating because there are so many possible places to start. Scouting gradients provide a simple, relatively fool-proof starting point. The chromatogram from a single scouting gradient can help make the decision about whether to proceed with development of an isocratic method, or if a gradient elution method is required to accommodate more diverse mixtures. The results from a second scouting gradient can be used to build a retention model that can in turn be used to predict separations with variations in gradient time and the mobile phase compositions used at the starting and ending points in the gradient. The concepts discussed here are the basic foundation of approaches and software used for modern method development. Understanding these foundational ideas is the first step towards more advanced approaches and more streamlined and effective method development workflows.

## References

- 1) Dolan, J. The Perfect Method, Part 1: What Is Your Goal? *LCGC Eur.* **2007**, 20 (9), 438–443.
- 2) Dolan, J. The Perfect Method, Part 7: The Gradient Shortcut. *LCGC Eur.* **2008**, 21 (3), 138–140.
- 3) Snyder, L. R.; Dolan, J. W. *High-Performance Gradient Elution: The Practical Application of the Linear-Solvent-Strength Model*; John Wiley, 2007.
- 4) Stoll, D. R. Reversed-Phase Liquid Chromatography and Water, Part 1: How Much Is Too Much? *LCGC Eur.* **2019**, 32 (2), 72–78.
- 5) Schellinger, A. P.; Carr, P. W. Solubility of Buffers in Aqueous–Organic Eluents for Reversed-Phase Liquid Chromatography. *LCGC North Am.* **2004**, 22 (6), 544–548.
- 6) Dolan, J. Making the Most of a Gradient Scouting Run. *LCGC North Am.* **2013**, 26 (1), 18–22.
- 7) Broeckhoven, K.; Stoll, D. R. But Why Doesn't It Get Better? Kinetic Plots for LC, Part 1: Basic Concepts. *LCGC Eur.* **2022**, 35 (2), 52–56. DOI: 10.56530/lcgc.na.sm2490k6
- 8) Stoll, D. R. What Are Options to Improve My Separation? Part 2: Likelihood of Separation, Adjusting Selectivity for Simple Mixtures. *LCGC Eur.* **2023**, 36 (5), 163–168. DOI: 10.56530/lcgc.na.qz2990k6
- 9) den Uijl, M. J.; Schoenmakers, P. J.; Schulte, G. K.; Stoll, D. R.; van Bommel, M. R.; Pirok, B. W. J. Measuring and Using Scanning-Gradient Data for Use in Method Optimization for Liquid Chromatography. *J. Chromatogr. A* **2021**, 1636, 461780. DOI: 10.1016/j.chroma.2020.461780
- 10) Dolan, J. W.; Lommen, D.; Snyder, L. R. DryLab Computer Simulation for High-Performance Liquid Chromatographic Method Development: II. Gradient Elution. *J. Chromatogr. A* **1989**, 485, 91–112. DOI: 10.1016/S0021-9673(01)89134-2
- 11) Snyder, L. R.; Dolan, J. W.; Lommen, D. C. Drylab Computer Simulation for High-Performance Liquid Chromatographic Method Development: I. Isocratic Elution. *J. Chromatogr. A* **1989**, 485, 65–89. DOI: 10.1016/S0021-9673(01)89133-0

## ABOUT THE EDITOR

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# The Future of Chromatography Sample Preparation: Trends in Solid-phase Extraction Methods

## *Challenges and advancements in solid-phase extraction, as well as tips for the workflow*

**S**olid-phase extraction (SPE), a sample preparation technique, is a useful addition to any chromatographer's analytical toolkit. Much of the scientific community, however, still finds that the technique poses notable challenges and is difficult to master. To dive further into this topic, LCGC sat down with James Edwards (Application Scientist – Chromatographer, Porvair Sciences) to discuss how Porvair Sciences is developing technology and working with customers to streamline SPE and unearth all its potential.



**James Edwards**  
Application Scientist  
Chromatographer  
Porvair Sciences

### **LCGC:** What is solid-phase extraction and how has it evolved over the years from when it was first used?

**EDWARDS:** SPE is a sample preparation technique for chromatography. It's where you have an interaction between a compound of interest with a solid or stationary phase, and you put a liquid phase through it, which is hopefully interacting with the compound that you want to remove from that matrix and leaving it bound to the stationary phase. Then, you're basically washing off the matrix or contaminants to gain your clean solution that you can run on your system.

Regarding how it's evolved over the years, silica was the original base material for SPE and now it's moved to new resins that are used in addition to silica for SPE, such as polymeric, carbon or molecularly imprinted polymers. This became possible due to scientific advances over the years. They typically offer different selectivity than silica and they're also more stable at different conditions, especially at the extreme ends of the pH range. I also see people moving to smaller bed weights for their SPE, mainly due to the environmental and social governance (ESG) or green nature of that type of smaller bed weight. You're saving your solvent, reducing your prep time and the volume of sample that's needed to perform your prep work. Finally, like most industries, SPE is moving to automation. Automating your sample preparation is going to give you a lot of advantages, such as reproducibility, and it's also one of the key principles of green sample preparation that people are looking to move to.

### **LCGC:** What makes SPE so challenging?

**EDWARDS:** SPE gained this reputation of being challenging to people. Some of the reasons may include the so-called complexity of SPE, how all the steps of the methods will be developed, the risk of error, and what equipment and setup may be needed for it. However, I feel like with any new skill or tool, it's always daunting to take on that task of tackling the initial learning curves and challenges that come with it. I think the benefits of SPE will outweigh this in the long term, and with the right support and guidance, it's definitely a valuable technique that you can add to your analytical toolkit. Porvair Sciences looks to work with customers to help make SPE less challenging to them. We are analysts as well; we understand the issues that people have, and we have the experience of dealing with those issues.

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**LCGC:** Can you explain how a typical SPE product is made?

**EDWARDS:** The traditional form of SPE is that you have two frits, which are plastic discs typically made of polyethylene (PE), polypropylene (PP), or sometimes polytetrafluoroethylene (PTFE). Then you have resin that's sandwiched between those two frits, forming a packed bed of resin. This has been used since SPE was first developed. It does have some downsides that can occur. This includes voiding, which is the gap between the resin and the top frit, channeling, where the solvent flow through the packed bed takes a path of least resistance, and variable bed weight or compaction of the resin, where you've added different weights or pressed it down to different heights in the product. This all leads to variability in the flow rate through that packed bed, and flow has quite a large impact on how your solid phase is going to work, because that's what typically will interact with the compounds.

If you have a variable flow, you can get less-reproducible results, which can mean that you might need to retest samples or add more replicates to your testing, which means you're wasting your sample and you may also have less confidence in the data. Overall, this can cause a lot of frustration and stress for an analyst because you're unsure if it's the product, the system, or the work that you've done yourself, and you're also reducing the efficiency of that work that you're performing.

**LCGC:** How has Porvair looked to innovate on the current products out there?

**EDWARDS:** Porvair Sciences has looked at the downsides of these traditional products and how we could improve on that traditional design. We've developed what we call a hybrid technology that's the core of our Microlute® sample preparation range. It takes the same components as the traditional product, the material from the frits and the active resin, blends that together into one master blend, and sinters that into one solid porous frit without degrading the performance of that resin. We purposely designed it to reduce the issues with loosely packed products. The frit is a single piece, so there's no voiding or compression when you're putting it into the plate or cartridge. The resin is supported in place so you can't get any of the channeling with the particles shifting aside. Because of that bulk blend, we spread the error of weighing out over the course of a larger scale. We're reducing that error. All of this leads to an improved flow and interaction through our product. This is both batch-to-batch and sample-to-sample. We've designed it to give analysts a product that gives them more confidence in their data and to reduce the sample and time wasted during their sample preparation.

*Porvair Sciences has looked at the downsides of these traditional products and how we could improve on that traditional design. We've developed what we call a hybrid technology that's the core of our Microlute® sample preparation range.*

**LCGC:** What are your top tips for getting started with SPE?

**EDWARDS:** My first tip would be to understand your compound, because the more you know about this, the easier it makes it to troubleshoot and develop an SPE method. Typically, you'd look at your chemical properties for the compound or compounds of interest, such as the logP or pKa. My next tip would be to choose the right solid phase for your SPE. Using those properties of the compound, you can pick the right phase for that compound. You want a phase that will interact with a compound of interest and not your interferences. My final tip would be to take your time to optimize your SPE method. It's going to save you time in the long run. If you get it right, you can reduce the issues and frustrations down the line. It may also help you understand why an issue is cropping up when you are doing the analysis further down the line, or maybe when you're doing a completely new method in the future.

**LCGC:** Can Porvair Sciences help with other parts of the SPE workflow too?

**EDWARDS:** Of course! We offer a range of instruments, such as evaporators and plate sealers for people in the lab. We also have a range of filtration and sample collection plates for people doing SPE. We also want to give people the best opportunity to get started with their SPE work, so we're always happy to give advice on issues that people might be having with their SPE. What I really enjoy about working at Porvair is how we develop products with customers. Porvair is there to make life in the lab easier.

# Basic Care and Feeding of Your Detector

Nicholas H. Snow, GC Connections Editor

**Detectors in gas chromatography (GC) are unique in all analytical science. The detector performs several difficult functions that make sensitive and selective analysis possible. In this instalment, we will explore the challenges involved in detecting the effluent from a capillary gas chromatographic column, and summarize the many detector options. We will then consider basic ideas and tasks that allow detectors to function properly. Finally, we will examine some specific troubleshooting and preventative maintenance tips for common detectors. We will see that, with some simple preventative thinking and action, detectors in gas chromatography can provide excellent sensitivity and selectivity for years of use.**

In capillary gas chromatography (GC), the detector plays a role that is unique in all analytical science. It must be small, sensitive, and fast, with selectivity varying based on the type of detector used. First, consider the effluent that is eluted from the detector end of a typical capillary column. This effluent is a stream of vapour moving at velocities of usually more than 50 cm/s, and sometimes over 1 m/s. With a typical volumetric flow rate at the column exit of about 2 mL/min, this generates about 30  $\mu$ L of carrier gas per second, which, during peak elution, contains analytes with masses usually measured in nanogram or picogram. Therefore, the detector must be of sufficiently small volume to not allow significant additional expansion of the carrier gas effluent as it leaves the column. Too large a detector volume would lead to extracolumn peak broadening, reducing both resolution and peak height. The detector must be sensitive enough

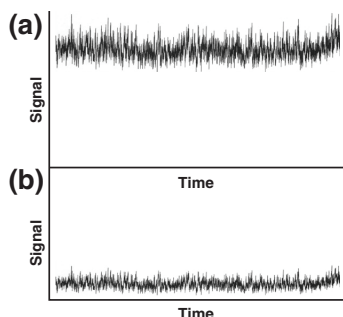
to record the passage of nanogram/picogram of vapour phase analyte dispersed in the above-mentioned 30  $\mu$ L of carrier gas. Finally, the detector must have a very rapid response time, producing individual signals fast enough to generate at least 20 data points across peaks that may be 1 s or shorter in width. Therefore, small, sensitive, and fast are the three fundamental requirements for a detector.

*Selectivity*, the ability to discriminate between different analytes or to detect some compounds or compound classes but not others, is another critical detector characteristic. A detector may be universal, as in a thermal conductivity detector (TCD) or full-scan mass-selective detector (MSD), detecting any analyte other than the carrier gas, or it may be selective, as in a flame ionization detector (FID), which detects only carbon-containing compounds; an electron capture detector (ECD), which detects only compounds with

highly electronegative functional groups; or a selected ion monitoring mass selective detector (MSD), which detects only the molar masses set by the user. Since gas chromatography is very widely used in many industries, and not just by specialized analytical chemists, detectors must also be easy to use, operate, and maintain.

Based on these characteristics, Table 1, adapted from Schug, McNair, and Hinshaw, provides grades and a summary for several common gas chromatographic detectors (1). We can see there is often a trade-off between characteristics such as selectivity and *sensitivity*. More selective detectors, such as ECD, detect fewer analytes, but are more sensitive than more universal detectors, such as FID or TCD, which detect more analytes but are not as sensitive. There is another clear trade-off between sensitivity and ease of use. More sensitive detectors often require

**FIGURE 1:** Expanded chromatograms showing baseline noise with no sample injected. (a) Elevated background signal with greater noise; (b) Original noise with system running properly. Reprinted with permission of the author from reference 4.



greater care, particularly to avoid contamination that may generate spurious signals or excessive noise at lower detection levels.

Finally, a detector may be *concentration* or *mass sensitive*. Among classical detectors, TCD and ECD are concentration sensitive, while FID is mass sensitive. This is a subtle difference that may not be observed until troubleshooting or optimizing, and is noticed when looking at raw peak heights and peak areas. If optimizing for speed by increasing the flow rate, we would initially think that the faster flow rate would result in faster, sharper peaks, therefore greater peak height. This is true for a mass sensitive detector; the peak height is higher, but since the total mass of analyte stays the same, the peak area is the same. For a concentration sensitive detector, the peak area may decrease, as the height is unchanged (volume of the detector is constant), but the peak area can decrease, as the peak will be sharper, with the same height. If calibrating using standards or a calibration curve, these differences may not be noticeable unless working at or near the limit of detection.

## Troubleshooting and Preventative Tips for All Detectors

There are some common ideas for preventative maintenance and troubleshooting for all detectors. In the first American Chemical Society Short Course on capillary GC that I took in 1990, Stuart Cram of Hewlett-Packard (now Agilent Technologies) and Milos Novotny of Indiana University stated succinctly the most important truism in gas chromatography—"Capillary GC is clean GC". Most preventative maintenance and troubleshooting revolves around keeping the detector clean and avoiding contaminants in the carrier gas stream that can either foul the detector or elevate the baseline.

### Keep the Detector Powered-Up and Heated:

On any gas chromatograph, the detector is one of three separately heated zones, along with the inlet and the column oven. Generally, the detector should be heated to a temperature above that of the highest temperature of the methods being run. It is heated for the sole purpose of keeping it clean. Also, when initially heating the detector, it is a good idea to

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**TABLE 1:** Performance grade sheet for modern gas chromatographic (GC) detection methods (Reprinted from reference 1 with permission from the author)\*

| Parameter              | TCD | FID | ECD | PID | FPD | BID | MS | VUV |
|------------------------|-----|-----|-----|-----|-----|-----|----|-----|
| LOD                    | C   | A   | A+  | A   | A   | A   | A+ | A   |
| Qualitative speciation | D   | D   | C   | C   | C   | D   | A  | A   |
| Linear range           | A   | A   | C   | A   | B   | A   | B  | B   |
| Universal response     | A   | B   | D   | B   | D   | A   | B  | A   |
| Specificity            | D   | D   | A   | C   | B   | D   | B  | A   |
| Robustness             | A   | A   | C   | A   | B   | A   | C  | A   |

\*This ranking cannot fully account for all manifestations of each detector system

allow it to equilibrate for a couple of hours at the set temperature prior to use. Remember that the temperature sensor is in one place, not over the whole detector.

#### Ensure That the Column End

**Is Correctly Installed:** Most detectors require a specific distance that the column must extend into the detector from the fitting inside the column oven. Unfortunately, this is a blind connection. The fitting must be installed onto the column outside the column oven and then carefully installed into the detector. When troubleshooting problems of excessive noise, small signals, no peaks, or poor peak shapes, column installation problems are often the cause. Also, ensure that the column end is properly cut. Improper column cutting leads to active sites at the column end that cause poor peak shapes, and can also cause small pieces of fused silica or polyimide coating to foul the detector, usually resulting in increased noise.

**Use High Purity Gases:** Gas purity is critical for reducing detector noise and contamination. Carrier gas contaminants can include air and water from leaks to the atmosphere or water and organic contaminants

from the instrument, gas lines, or the gas cylinder itself. Ensure that you begin with high purity gases either at the point of purchase or by using appropriate gas scrubbers. Your instrument vendor can assist you with ensuring that your gas supply is highly pure. Then, ensure that the system is free of leaks. Even though the gas lines are pressurized, contaminants can back diffuse into the gas flow. Finally, ensure that your inlet and sampling devices are clean and properly maintained.

#### Comments About Using

**Hydrogen Generators:** Many laboratories today have foregone classical cylinders for hydrogen generators. Although a capital expense item, significant long-term operating savings and improved safety can result. When choosing a hydrogen generator, similar to purchasing cylinders, double-check the purity of the delivered gas, along with volumetric flow and output pressure, and ensure that these are sufficient for providing fuel gas to the detector. If hydrogen carrier gas is used, ensure that the maximum capacity of the generator will provide sufficient gas flow for the inlet, especially when operating under split injection with high split ratios.

#### Signs That the Detector Needs

**Attention:** The most common evidence that the detector requires attention, cleaning, or maintenance is a change in the baseline. On my own systems, I check the baseline daily. On a modern data system, this involves starting a blank run, and then monitoring the real-time signal. The baseline should look the same as the previous day. Figure 1 shows a normal baseline and an elevated baseline with increased noise, indicating that the detector needs attention. If the rapid noise has a greater amplitude, this usually indicates some change in the environment or the electronics; perhaps another instrument is working on the same circuit as the GC system. If the overall baseline is elevated, the usual problem is contamination in one or more of the gas streams. If there is no signal at all, check that the detector is operating. Checking the baseline is one of the simplest of all tests for proper detector operation.

#### Some Specific Advice for Common Detectors

##### Flame Ionization Detector (FID):

The FID requires three gases. The carrier gas can be helium, nitrogen,



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or hydrogen; in previous articles, I have advocated using nitrogen with capillary GC and temperature programming, as it is the least expensive and the most green choice (2,3). The fuel gas is hydrogen, and the oxidant is air. Take care to ensure that the fuel and oxidant gases are pure and have stable flow rates. Since hydrogen is being combusted in the detector, the product of the reaction of hydrogen and oxygen is water. Most modern FIDs include an automatic ignitor, but take care to ensure that the detector is fully heated prior to ignition, or water can form on the metal surfaces. When installing the column, take care with the insertion distance, and ensure that the column end does not contact the flame jet. If the column end is inserted too far, it can contact the flame jet and ignite, causing excessive noise and possible loss of signal. If not inserted far enough, the void space may cause peak broadening, or there may be a gas leak.

#### **Mass Selective Detector (MSD):**

MSDs provide both qualitative and quantitative data, and have become straightforward to operate. Daily tuning or optimization are important when operating an MSD, as the ion source changes over time as samples are run and eventually contaminate metal surfaces and lenses inside the source. In a previous article, we discussed a wealth of information obtainable from the tune report (4). The simplest measures of whether the ion source needs maintenance are the electron multiplier voltage and the peak resolution between the main and isotope peaks from the tuning compounds. Increasing electron multiplier voltage or loss of resolution between the adjacent mass isotope

peaks are a strong indication that the ion source requires cleaning.

#### **Electron Capture Detector (ECD):**

ECD is among the most sensitive and selective of all detectors. As seen in Table 1, it gets the highest grade for sensitivity, but the lowest for qualitative speciation, as it is very selective for a subset of electronegative species. It also has the lowest grade for robustness because it is easily contaminated, even by the room air. ECD was reviewed in a recent column, including a discussion that many originally felt that it would not be a useful detector because it is so easily contaminated (5). Users should ensure that the carrier and makeup gases are of the highest purity and extremely dry. The laboratory environment must also be contaminant-free; I have witnessed elevated baseline on an ECD in the presence of cigarette smoke residue on the operator's clothing.

#### **Conclusions**

In gas chromatography, the detector is the third critical heated zone on the instrument. The overriding consideration for ensuring long-term effective operation is cleanliness and preventing contamination. Some considerations, such as ensuring pure carrier and detector gases, sufficient heating and temperature equilibration, and proper column installation are common to all detectors. Beyond these, each detector has its own operating and maintenance principles. With simple preventative maintenance and care for the entire system, from gas sources to detector, detectors for gas chromatography are sensitive, selective, and rugged.

#### **References**

- 1) Schug, K. A.; McNair, H. M.; Hinshaw, J. V. GC Detectors: From Thermal Conductivity to Vacuum Ultraviolet Absorption. *LCGC Eur.* **2015**, *28* (1), 45–50.
- 2) McCann, S. P.; Rana, H.; Handzo, B. A.; Snow, N. H. Go With the Flow: Thinking About Carrier Gas Flow in GC. *LCGC Eur.* **2020**, *33* (3), 127–134.
- 3) Snow, N. H. Green Chemistry: What Is It (and What Is It Not)? And How Does It Apply to Gas Chromatography? *LCGC Eur.* **2023**, *36* (5), 169–173. DOI: 10.56530/lcgc.na.az3979e4
- 4) Snow, N. H. Stopping GC and GC–MS Problems Before They Start. *LCGC Eur.* **2019**, *32* (1), 22–27.
- 5) Snow, N. H. Selectivity and Sensitivity: The Electron Capture Detector (ECD), Its Unique Inventor James Lovelock (1919–2022), and GAIA. *LCGC Eur.* **2022**, *35* (10), 454–458. DOI: 10.56530/lcgc.na.dm6277r4

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# Polysorbate Analysis in Biopharmaceutical Applications—A Snapshot of the Analytical Toolbox

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**Polysorbates (PS) are added to biopharmaceutical formulations to stabilize proteins and monoclonal antibodies (mAbs) and to prevent aggregation, denaturation, and surface adsorption. Polysorbate analysis can be challenging because of their inherent complexity, polydispersity, and their presence in matrices containing high concentrations of proteins and other excipients such as sugars, amino acids, salts, and buffers. This instalment of “Biopharmaceutical Perspectives” provides an overview of different approaches for the qualitative and quantitative analysis of polysorbates, its impurities and degradants in raw material, intermediate dilutions, drug substance, and drug products. Various gas (GC) and liquid chromatography (LC) methods and accompanying detection techniques are briefly discussed and illustrated with results obtained at the authors’ laboratory.**

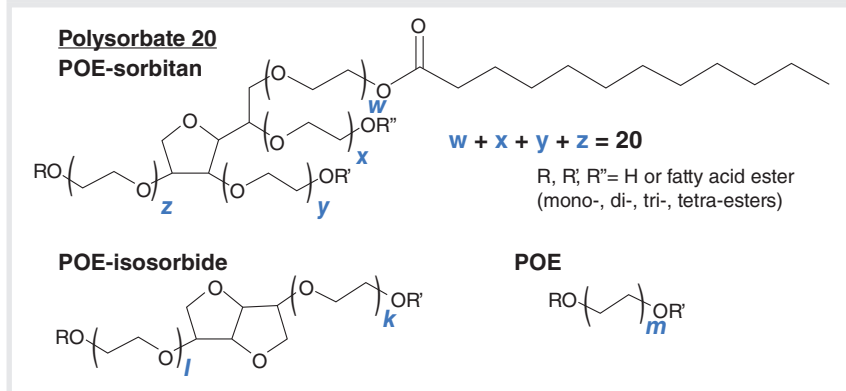
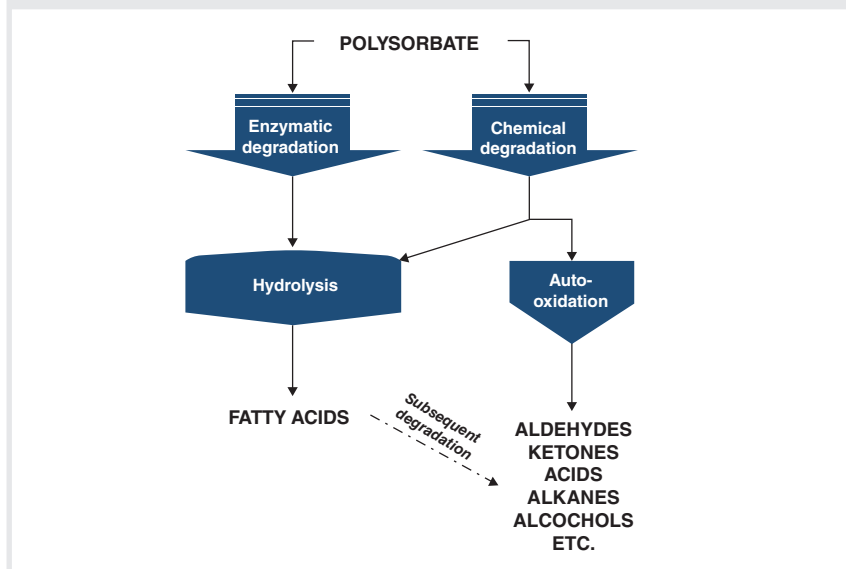
Polysorbates (PS) are a class of nonionic surfactants mainly used in the food and pharmaceutical industry. In the biopharmaceutical field, polysorbate 20 (PS20) and 80 (PS80) are widely accepted and prepared at concentrations between 0.01–0.05%—well above the critical micelle concentration (1–4). These key excipients have low toxicity and feature stabilizing properties, thereby countering aggregation, denaturation, and surface adsorption of proteins and monoclonal antibodies (mAbs) in solution. As a result of their chemical nature, polysorbates are prone to degradation, which is influenced by several factors (1–8). Polysorbate degradation has emerged as a hot topic in the industry in recent years, predominantly because of fatty-acid mediated particle formation in PS-containing formulations, and has been identified

as one of the main challenges during formulation development of protein biopharmaceuticals. Understanding the origin of degradation and the implementation of control and mitigation strategies have been widely discussed (3,4,6). Analyzing PS is therefore fundamental, and has resulted in the development of different methodologies that are becoming an essential part of the analytical arsenal for protein biopharmaceuticals.

## Structural Heterogeneity of Polysorbates

Polysorbates are heterogeneous amphiphilic substances composed of a hydrophilic polyoxyethylene (POE) sugar head group and esterified fatty acids that make up the hydrophobic tail (Figure 1). In contrast to other small molecules that are defined by a specific structure, its chemical synthesis generates a complex

mixture, with PS heterogeneity originating from the type of fatty acids (lauric, myristic, palmitic, stearic, oleic, linoleic, linolenic), esterification degree (0–4), sugar structure (sorbitan, isosorbide), and POE chain length (target ethylene oxide units equals 20), giving rise to well above a thousand possible species and a complex structure–function relationship (1,3,6). On top of this, the PS diversity among manufacturers and lot-to-lot variability adds to the complexity. The prevailing esterified fatty acid, lauric and oleic acid, respectively, differentiates PS20 and PS80. Remarkably, the target structure of the latter surfactants, that is, POE ( $x+y+w+z = 20$ ) sorbitan monolaurate and POE ( $x+y+w+z = 20$ ) sorbitan monooleate, is typically found to account for only 20% of the total PS species (1,3).

**FIGURE 1:** Chemical structure of PS20 and constituting species.**FIGURE 2:** Polysorbate degradation pathways.

### Stability of Polysorbates

While polysorbates are highly functional excipients, they are chemically and enzymatically labile and prone to degradation by oxidation and hydrolysis (Figure 2) (1–8). The oxidative pathway can be triggered by exposure of neat or formulated PS to air, light, and transition metals, and is influenced by the purity grade of the raw materials (for example, peroxide content). Hydrolysis can proceed in a purely chemical manner by heat or alkaline and acidic conditions, or by the presence of trace amounts

of host-cell hydrolytic enzymes (esterases, lipases) co-purified with the therapeutic protein. The chemically induced hydrolysis pathway is uncommon in a biopharmaceutical context, while enzyme-mediated hydrolysis has been identified as the most challenging degradation in industry and typically occurs at high protein concentrations (1,4).

Different risks are associated with degradation through oxidation and hydrolysis. To a certain extent, oxidized PS is able to maintain its protein-stabilizing properties (7).

Accumulation of degradation products, such as aldehydes, ketones, and peroxides, may nevertheless present a risk towards the chemical stability of the therapeutic protein, potentially impacting its efficacy (5). Peroxides can lead to oxidation of methionine, tryptophan, or histidine residues, while aldehydes react with primary amino groups (protein N-terminus or lysine) (1,7).

The hydrolytic degradation of PS can impact its functionality as a protein stabilizer (7). Furthermore, the main degradation products, free fatty acids (FFA), may form visible and subvisible particles when above their aqueous solubility threshold, drastically impacting drug product quality and shelf life (1,4,6). Particles in parenteral drug products carry risks associated with blood vessel occlusion during intravenous delivery and immunogenicity reactions during subcutaneous and intramuscular delivery.

### The Analytical Toolbox

Polysorbate analysis within a biopharmaceutical context aims at determining PS identity, content (quantity), heterogeneity, variability, impurities, and degradants in the raw materials, intermediate dilutions, drug substance, or drug product, in stability or purposely stressed samples. Given the range of questions to be addressed and the structural complexity and solute diversity (from aldehydes to polyesters) encountered, a multitude of analytical methodologies has been developed, with some focusing on one analytical target (content determination) and others with a broader utility. A crucial part in each methodology is sample preparation, which typically consists of a protein removal step,



enrichment, and derivatization of solutes to enable PS analysis.

The following sections will provide the reader with an overview of the chromatographic and nonchromatographic methodologies for PS analysis.

### Nonchromatographic Methods

The fluorescence micelle assay (FMA) is well embedded in industry for the determination of PS content (1,3,9). The principle of the methodology is based on increasing the fluorescence emission of N-phenyl-1-naphthylamine (NPN) once it distributes to the hydrophobic core of a PS micelle. The assay, originally developed for critical micelle concentration determination (10), can be implemented in plate format or can be based on flow injection analysis (FIA) using high performance liquid chromatography (HPLC), with the dye present in the mobile phase and the interaction with PS taking place in a knitted reaction coil prior to fluorescence detection (Figure 3[a]). Run times are fast, but throughput is hampered by extensive equilibration runs ( $\pm 50$ ) and the use of replicate sample and calibration runs to cope with the varying fluorescence signal. As NPN also interacts with other hydrophobic components, protein precipitation or dilution is usually required for protein concentrations  $> 50$  mg/mL. It should be noted that the assay has demonstrated stability indicating character that depends on the PS type and degradation mechanism.

Alternative nonchromatographic methods for PS quantification that do not rely on micelle formation, each with their own weaknesses and strengths towards sensitivity, matrix complexity, and stability-indicating character, have also been described (1).

These include spectroscopic assays based on the formation of coloured ferric or cobalt thiocyanate PS complexes or the differential emission of the amphiphilic fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate in polar and nonpolar environments (11). Colorimetric

and fluorometric assays are used to determine the content and formation of peroxides at early stages of the oxidation cascade (1).

### Chromatographic Methods

**Liquid Chromatography:** HPLC methods have been widely applied for PS analysis because of their versatility,



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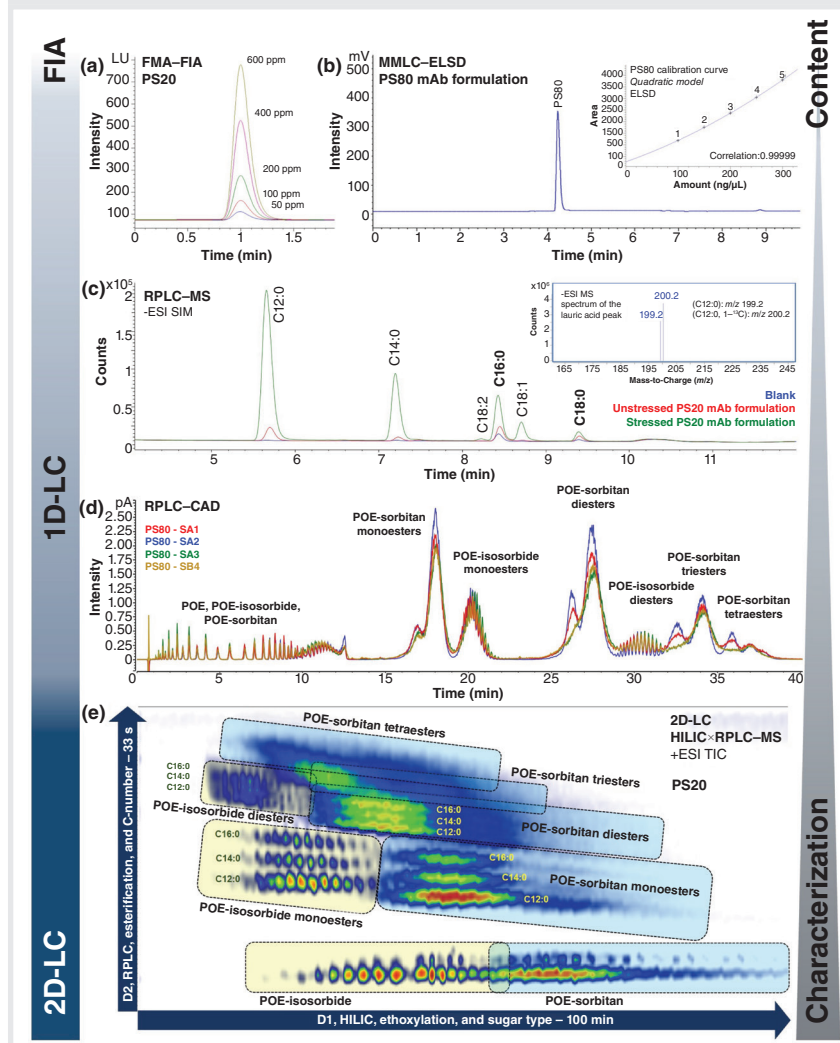
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**FIGURE 3:** Versatility of HPLC in PS analysis—from content determination to in-depth characterization. (a) PS20 calibration standards analyzed by FMA using a HPLC system operated in FIA mode. (b) MMLC–ELSD chromatogram of a mAb formulation containing PS80. A representative PS80 calibration curve (quadratic model) is included. (c) Analysis of FFAs by reversed-phase LC–MS in mAb formulations containing PS20. Increased levels of FFAs are observed in aged formulation. Low levels of palmitic and stearic acid contaminants are apparent in the blank. The single quadrupole ESI-MS spectrum for the peak at 5.7 min is provided, with  $m/z$  corresponding to lauric acid and isotopically labelled lauric acid-13C added as internal standard. (d) RPLC–CAD (inverse gradient) chromatograms of different PS80 raw materials (SA1-3) and PS80 of a different supplier (SB1). Figure kindly provided by, and edited with permission of, Frank Steiner and Mauro De Pra (Thermo Fisher Scientific) (18). (e) Comprehensive HILIC×RPLC–MS (single quadrupole, ESI positive) analysis of PS20.



simplicity, richness in information, and general acceptance within the pharmaceutical industry. The absence of a chromophore restricts detection by UV, hence universal detectors,

such as evaporative light scattering (ELSD) and charged aerosol (CAD) detectors, alongside mass spectrometry (MS), are implemented. All have similar requirements towards

mobile phase volatility, offer good sensitivity in the encountered concentration range, and show compatibility with reversed-phase LC (RPLC) and hydrophilic interaction liquid chromatography (HILIC).

The hydrophobic nature of PS renders this surfactant highly suitable for RPLC, and its excellent retentivity allows separation between different PS species to be achieved, facilitating characterization; alternatively, PS can be eluted into one single peak to simplify quantification (1,6,9,12–19). A protein removal step via solid-phase extraction (SPE) or precipitation is typically required. The latter can be circumvented to a certain extent using mixed-mode liquid chromatography (MMLC), which combines anion exchange and reversed-phase mechanisms, thereby enabling separation of nonionic PS from positively charged protein material and hydrophilic excipients (9,12,16). Applying a step gradient from an aqueous acidic mobile phase to one of higher organic content on a short mixed-mode cartridge results in the coelution of all PS species in the ELSD, as illustrated in Figure 3(b) for a PS80-containing mAb formulation. The implementation of a postcolumn switching valve to prevent ELSD contamination and increase method robustness hinders the detection of the mAb in the void volume. When using linear organic solvent gradients on state-of-the-art RPLC columns, PS species predominantly elute based on their esterification degree, thereby providing a fingerprint in comparability and stability studies as shown in Figure 3(d) for different PS80 raw materials (13–19). While degradation and batch inconsistencies can be monitored,

PS quantification from such complex chromatographic profiles can be challenging, especially given the latter batch variability. To obtain a uniform CAD response under gradient conditions, the chromatograms in Figure 3(d) were obtained using a postcolumn inverse compensatory gradient. In the absence of this correction, the mass balance was disturbed with an overestimation of di-, tri-, and tetra-esters and an underestimation of nonesterified components. When combining MMLC and RPLC in an online heart-cutting two-dimensional (2D)-LC setup, PS can automatically be separated from the protein in the formulation and further resolved in the second chromatographic dimension (16). The resolution added by MS further extends the characterization capabilities and allows unambiguous identification of the species observed (13–19). Spectra collected over the RPLC peaks display mass envelopes, with characteristic 44 Da spacings corresponding to the ethylene oxide building blocks. PS is commonly transferred in the gas phase using electrospray ionization (ESI) operated in positive mode and—as a result of a lack of basic functional groups—detected as singly, doubly, and triply charged ammonium or sodium adducts depending on the mobile phase composition.

RPLC is valuable in measuring PS degradants or impurities such as FFAs or aldehydes and ketones—the latter entities following derivatization with 2,4-dinitrophenylhydrazine (DNPH) to facilitate detection by UV (1,20,21). The RPLC–MS analysis of FFAs in a PS20-containing mAb formulation is shown in Figure 3(c). Separation is based on carbon number, and degree of saturation and sensitivities down to 10 ng/mL can be achieved in negative ESI mode with a single quadrupole mass analyzer. FFA impurities and degradants can therefore be quantified in mAb formulations following protein precipitation. In the presented example, PS degradation is noticed in a stability sample because of the marginal presence of residual host-cell hydrolytic enzymes. To guarantee sensitive, precise, and accurate quantification, isotopically labelled fatty acid counterparts are typically used and precautions are taken to limit extrinsic fatty acid contamination. In the case presented, dedicated consumables and chemicals were used in addition to a reversed-phase isolator column installed prior to the autosampler to retain fatty acid contaminants, such as palmitic and stearic acid, originating from the mobile phase (21). While MS allows fatty acid measurement as such, early work reports on carboxylic acid derivatization using 1-pyrenyldiazomethane (PDAM) to render fatty acids

fluorescent (1). Adequate sensitivity can be obtained at the expense of additional sample preparation.

HILIC exploits the polar regions of PS and offers complementary selectivity. The technology has been used less frequently in one-dimensional (1D) separations; however, combining it with RPLC in a comprehensive 2D-LC setup provides an unsurpassed fingerprint where small compositional changes originating from batch variability or degradation can easily be revealed (Figure 3[e]) (22). While HILIC resolves polysorbates based on the degree of ethoxylation and type of sugar (isosorbide, sorbitan), RPLC separation is predominantly based on esterification degree and carbon number.

**Gas Chromatography:** Due to their molecular mass, polarity, and low volatility, polysorbates cannot be analyzed as such by gas chromatography (GC). Nevertheless, GC and GC–MS, in combination with various sample preparation methods, can provide highly relevant and complementary information in PS analysis.

PS quantification and characterization can be achieved by fatty acid analysis following transesterification. The raw material, intermediate dilution, drug substance, or



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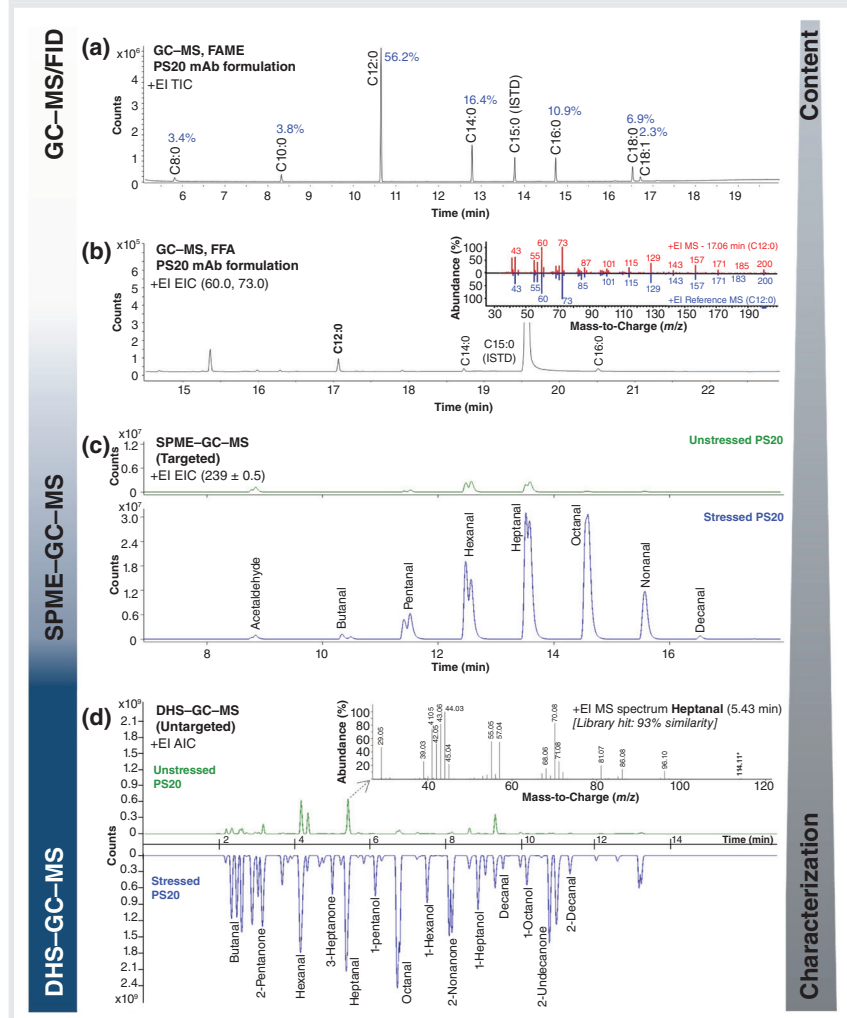
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**FIGURE 4:** The application of GC in PS analysis—from content determination to shedding light on fatty acid composition and measuring impurities and degradants. (a) GC–MS FAME analysis in a drug product containing PS20 with EI and single quadrupole mass analysis. Relative fatty acid composition is indicated in blue. Pentadecanoic acid methyl ester (C15:0) is used as the internal standard. (b) GC–MS analysis of FFAs in aged PS20 containing mAb formulation with EI and single quadrupole operated in SIM/scan mode. (c–d) Derivatization HS–SPME–GC–TOF–MS and DHS–GC–TOF–MS analysis showing EIC of saturated aldehydes and untargeted AIC in nonstressed and temperature-stressed PS20, respectively.



drug product is treated with a derivatization reagent such as boron trifluoride ( $\text{BF}_3$ ) in methanol. The PS is hydrolyzed and fatty acids are converted to the fatty acid methyl esters (FAMEs), which, following liquid–liquid extraction using hexane, are analyzed by GC in combination with flame ionization detection (FID)

or MS. A typical electron ionization (EI) total ion chromatogram (TIC) of a PS20-containing mAb formulation obtained on a polyethylene glycol (PEG)-based free fatty acid stationary phase (FFAP) is shown in Figure 4(a). Excellent separation according to carbon number and unsaturation is demonstrated and the relative fatty

acid composition can be assessed and compared against PS batches, PS types, and stability samples. Changes in relative composition or loss of unsaturated fatty acids can indicate PS oxidative degradation. Determining the relative composition is performed by GC–FID because of the nearly constant detector response for fatty acids in the C10–C20 range. Using proper calibration, preferably with an identical PS batch, both GC–FID and GC–MS can be used for absolute PS quantification. Beyond FFAP columns, cyanopropyl stationary phases can be used for FAME analysis, offering the benefit of resolving *cis/trans* double bond isomers. The former PEG-based phase, however, has the advantage that it can also be used for FFA analysis, as demonstrated in Figure 4(b) for an aged PS20-containing mAb formulation. Note that the above-described FAME analysis does not discriminate between free and esterified fatty acids in PS samples as all are converted to methyl esters. For FFA analysis by GC, sample preparation involves protein precipitation followed by liquid injection of the organic supernatants. To maximize sensitivity, the single quadrupole mass analyzer is operated in simultaneous scan and selected ion monitoring mode (SIM) transmitting the selective ions at  $m/z$  60 (McLafferty rearrangement) and 73.

Various PS degradants resulting from oxidation are volatile and amenable to GC analysis in a targeted or untargeted manner. A typical example of targeted analysis is the determination of C1–C12 aldehydes and ketones. The sensitive and selective analysis of the latter species can be performed using solid-phase microextraction (SPME) in headspace (HS) mode



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combining extraction and in-fibre derivatization. Briefly, the procedure includes loading the SPME fibre with derivatization reagent by sampling the headspace of an aqueous solution of O-(pentafluorobenzyl)hydroxylamine (PFBHA), followed by SPME sampling in the headspace of the sample, thermal desorption of the derivatized aldehydes and ketones in the GC inlet, and GC-MS analysis. This procedure is typically automated using a dedicated autosampler. Figure 4(c) presents the results obtained for fresh and temperature-stressed PS20 raw material. The overlaid extracted ion chromatogram (EIC) at  $m/z$  239, selective for saturated aldehydes, shows substantially increased levels of C4-C10 aldehydes due to stress.

Untargeted analysis allows a broader screening of degradants and impurities, and a highly sensitive approach is based on dynamic headspace sampling (DHS), where the headspace of a sample is purged with inert gas and the volatile organic compounds (VOCs) are exhaustively concentrated on an adsorbent trap (typically containing Tenax). The trap is subsequently thermally desorbed online using GC-MS analysis. Figure 4(d) shows the analytical ion chromatograms (AIC) obtained for unstressed and stressed PS20 material. Excellent separation of aldehydes, ketones, alcohols, and other VOCs was obtained using a polyethylene glycol GC column and untargeted detection facilitated by fast scanning time-of-flight (TOF)-MS and powerful deconvolution algorithms. An alternative complementary solventless extraction method that can be used is stir-bar sorptive extraction (SBSE) (5,23). SBSE allows the enrichment of apolar and semi-polar organic compounds from aqueous solutions,

without the need for protein removal. As the method is solventless, it can be considered as a green analytical sample preparation, and solvent blank issues are minimized. While DHS has a bias towards the more volatile species, SBSE will preferentially sample less volatile compounds, including lactones.

### Conclusions

With all eyes on this precious, yet moody, surfactant in the biopharmaceutical industry, a multitude of methodologies has been developed in recent years for PS analysis. This has substantially increased our understanding of PS structural diversity, variability, degradation, and structure/function relationship, and has led to various strategies to optimize its use in protein formulations. With dozens of creative analytical scientists being active in the field, more methods are expected to emerge in the coming years, further boosting our knowledge of this important subject.

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### References

- 1) Martos, A.; Koch, W.; Jiskoot, W.; et al. Trends on Analytical Characterization of Polysorbates and their Degradation Products in Biopharmaceutical Formulations. *J. Pharm. Sci.* **2017**, 106 (7), 1722-1735. DOI: 10.1016/j.xphs.2017.03.001
- 2) Brovč, E. V.; Mravljak, J.; Šink, R.; Pajk, S. Rational Design to Biologics Development: The Polysorbates Point of View. *Int. J. Pharm.* **2020**, 581, 119285. DOI: 10.1016/j.ijpharm.2020.119285
- 3) Wuchner, K.; Yi, L.; Chery, C.; et al. Industry



- Perspective on the Use and Characterization of Polysorbates for Biopharmaceutical Products Part 1: Survey Report on Current State and Common Practices for Handling and Control of Polysorbates. *J. Pharm. Sci.* **2022**, *111* (5), 1280–1291. DOI: 10.1016/j.xphs.2022.02.009
- 4) Wuchner, K.; Yi, L.; Chery, C.; et al. Industry Perspective on the Use and Characterization of Polysorbates for Biopharmaceutical Products Part 2: Survey Report on Control Strategy Preparing for the Future. *J. Pharm. Sci.* **2022**, *111* (11), 2955–2967. DOI: 10.1016/j.xphs.2022.08.021
  - 5) Kishore, R. S. K.; Kiese, S.; Fischer, S.; et al. The Degradation of Polysorbates 20 and 80 and its Potential Impact on the Stability of Biotherapeutics. *Pharm. Res.* **2011**, *28* (5), 1194–1210. DOI: 10.1007/s11095-011-0385-x
  - 6) Dwivedi, M.; Blech, M.; Presser, I.; Garidel, P. Polysorbate Degradation in Biotherapeutic Formulations: Identification and Discussion of Current Root Causes. *Int. J. Pharm.* **2018**, *552* (1–2), 422–436. DOI: 10.1016/j.ijpharm.2018.10.008
  - 7) Larson, N. R.; Wei, Y.; Prajapati, I.; et al. Comparison of Polysorbate 80 Hydrolysis and Oxidation on the Aggregation of a Monoclonal Antibody. *J. Pharm. Sci.* **2020**, *109* (1), 633–639. DOI: 10.1016/j.xphs.2019.10.069
  - 8) Dwivedi, M.; Buske, J.; Haemmerling, F.; Blech, M.; Garidel, P. Acidic and Alkaline Hydrolysis of Polysorbates under Aqueous Conditions: Towards Understanding Polysorbate Degradation in Biopharmaceutical Formulations. *Eur. J. Pharm. Sci.* **2020**, *144*, 105211. DOI: 10.1016/j.ejps.2019.105211
  - 9) Lippold, S.; Koshari, S. H. S.; Kopf, R.; et al. Impact of Mono- and Poly-Ester Fractions on Polysorbate Quantitation Using Mixed-Mode HPLC-CAD/ELSD and the Fluorescence Micelle Assay. *J. Pharm. Biomed. Anal.* **2017**, *132* (5), 24–34. DOI: 10.1016/j.jpba.2016.09.033
  - 10) Brito, R. M. M.; Vaz, W. L. C. Determination of the Critical Micelle Concentration of Surfactants Using the Fluorescent Probe N-Phenyl-1-Naphthylamine. *Anal. Biochem.* **1986**, *152* (2), 250–255. DOI: 10.1016/0003-2697(86)90406-9
  - 11) Martos, A.; Berger, M.; Kranz, W.; et al. Novel High-Throughput Assay for Polysorbate Quantification in Biopharmaceutical Products by Using the Fluorescent Dye Dil. *J. Pharm. Sci.* **2020**, *109* (1), 646–655. DOI: 10.1016/j.xphs.2019.10.013
  - 12) Hewitt, D.; Zhang T.; Kao, Y. H. Quantitation of Polysorbate 20 in Protein Solutions Using Mixed Mode Chromatography and Evaporative Light Scattering Detection. *J. Chromatogr. A* **2008**, *1215* (1–2), 156–160. DOI: 10.1016/j.chroma.2008.11.017
  - 13) Hewitt, D.; Alvarez, M.; Robinson, K.; et al. Mixed-Mode and Reversed-Phase Liquid Chromatography–Tandem Mass Spectrometry Methodologies to Study Composition and Base Hydrolysis of Polysorbate 20 and 80. *J. Chromatogr. A* **2011**, *1218* (15), 2138–2145. DOI: 10.1016/j.chroma.2010.09.057
  - 14) Borisov, O. V.; Ji, J. A.; Wang, Y. J.; Vega, F.; Ling, V. T. Toward Understanding Molecular Heterogeneity of Polysorbates by Application of Liquid Chromatography–Mass Spectrometry with Computer-Aided Data Analysis. *Anal. Chem.* **2011**, *83* (10), 3934–3942. DOI: 10.1021/ac2005789
  - 15) Zhang, R.; Wang, Y.; Tan, L.; Zhang, H. Y.; Yang, M. Analysis of Polysorbate 80 and its Related Compounds by RP-HPLC with ELSD and MS Detection. *J. Chromatogr. Sci.* **2012**, *50* (7), 598–607. DOI: 10.1093/chromsci/bms035
  - 16) Li, Y.; Hewitt, D.; Lentz, Y. K.; et al. Characterization and Stability Study of Polysorbate 20 in Therapeutic Monoclonal Antibody Formulation by Multidimensional Ultrahigh-Performance Liquid Chromatography–Charged Aerosol Detection–Mass Spectrometry. *Anal. Chem.* **2014**, *86* (10), 5150–5157. DOI: 10.1021/ac5009628
  - 17) Evers, D. H.; Schultz-Fademrecht, T.; Garidel, P.; Buske, J. Development and Validation of a Selective Marker-Based Quantification of Polysorbate 20 in Biopharmaceutical Formulations Using UPLC QDa Detection. *J. Chromatogr. B* **2020**, *1157*, 122287. DOI: 10.1016/j.jchromb.2020.122287
  - 18) De Pra, M.; Ispan, D. A.; Lovejoy, K.; et al. Polysorbate 80 Profiling by HPLC with Mass and Charged Aerosol Detection. Thermo Fisher Scientific Application Note, 2021.
  - 19) Liu, H.; Jin, Y.; Menon, R.; et al. Characterization of Polysorbate 80 by Liquid Chromatography–Mass Spectrometry to Understand Its Susceptibility to Degradation and Its Oxidative Degradation Pathway. *J. Pharm. Sci.* **2022**, *111*, 323–334. DOI: 10.1016/j.xphs.2021.08.017
  - 20) Honemann, M. N.; Wendler, J.; Graf, T.; Bathke, A.; Bell, C. H. Monitoring Polysorbate Hydrolysis in Biopharmaceuticals Using a QC-Ready Free Fatty Acid Quantification Method. *J. Chromatogr. B* **2019**, *1116*, 1–8. DOI: 10.1016/j.jchromb.2019.03.030
  - 21) Evers, D. H.; Carle, S.; Lakatos, D. Hydrolytic Polysorbate 20 Degradation - Sensitive Detection of Free Fatty Acids in Biopharmaceuticals via UPLC-QDa Analytics with Isolator Column. *J. Chromatogr. B* **2021**, *1174*, 122717. DOI: 10.1016/j.jchromb.2021.122717
  - 22) Vanhoenacker, G.; Steenbeke, M.; Sandra, K.; Sandra, P. Profiling Nonionic Surfactants Applied in Pharmaceutical Formulations Using Comprehensive Two-Dimensional LC with ELSD and MS Detection. *LCGC Eur.* **2018**, *31* (7), 360–370.
  - 23) David, F.; Ochialy, N.; Sandra P. Stir Bar Sorptive Extraction: A Versatile, Sensitive and Robust Technique for Targeted and Untargeted Analyses. In *Evolution of Solid Phase Microextraction Technology*; RSC, 2023; pp. 175–213.

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# Practical Understanding of Partition Coefficients

Douglas E. Raynie, Sample Preparation Perspectives Editor

**Partition coefficients,  $\log P$  or  $K_{ow}$ , are often used in introducing and describing new extraction techniques. They can also be used in developing new separation methods. An understanding of partition coefficients, along with diffusion, is necessary to more completely guide separation processes. As with the application of any theoretical concepts, several assumptions may be applied. This month, we take a dive into the practical applications of partition coefficients as they are commonly applied to chemical extractions.**

With apologies to Mark Twain and other famous orators, one of my favourite quotes of all-time (excluding song lyrics and quotes from Yogi Berra) comes from the 50-year-old textbook used in my sophomore quantitative analysis class:

*"If two compounds are to be separated, we must, somewhere along the line, get them into two different and separable phases. At the heart of any chemical separation are the processes of (1) phase contact and equilibrium; and (2) phase separation. These steps occur in all separation techniques, and a key to understanding a given method is the identification and classification of the steps according to the nature of the phases involved and the mechanism of phase contact and separation. Similarly, if a particular method of separation is to be improved, these are the only processes worth adjusting (1)."*

This quote so eloquently places the fundamentals of chemical separations squarely in the understanding of equilibria. Thus, when developing new techniques or describing the methodology of chromatography and

extraction, one frequently encounters the terms *partitioning*, *distribution ratio*, *octanol-water partition coefficient*,  $\log P$ ,  $K_{ow}$ , and  $D$ . What do these terms mean, or are they synonymous? When do we use these concepts? Why are they important?

Let's start by defining these terms, at least for the most important versions of partition coefficients:

The *partition coefficient*, designated  $P$  (or, more commonly,  $\log P$ ), is a ratio of the concentration of a compound at equilibrium when disbursed between two immiscible phases. As an equilibrium constant, it is dependent on temperature. Partition coefficients may be estimated by the ratio of the solubilities of the solute in each solvent and the term generally, but not always, refers to liquid mixtures (such as two-phase solutions). The two immiscible phases are undefined by this definition, so  $\log P$  is generally used for more descriptive purposes, and more specific constants are used for individual situations. Some of these more important coefficients are described.

The *distribution ratio*, typically abbreviated as  $D$ , is similar to the

partition coefficient, with the important exception that all forms of the solute are taken into account; that is, if the compound is known to ionize, associate or dissociate, volatilize, or decompose, one may replace  $\log P$  with  $\log D$ . If the species of interest remains in a single chemical form, partition coefficients may be used. This is especially important, for example, when the pH of a system, relative to the compound's  $pK_a$ , may lead to protonation or deprotonation. Note, however, that the distribution ratio does not discriminate between the individual forms of a compound, but rather adds the concentrations of each version in a given phase. Distribution ratios are increasingly important when performing mass balance calculations.

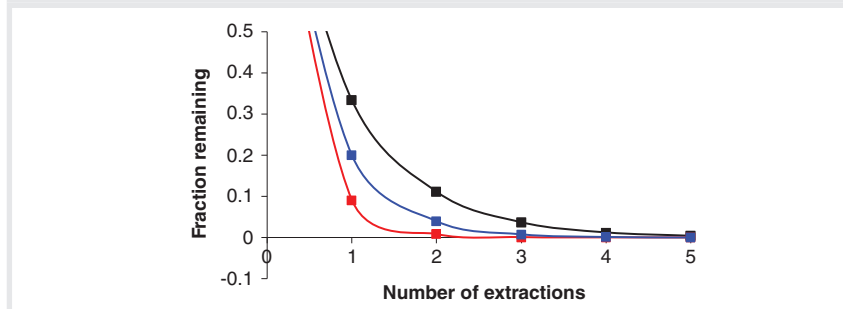
*Octanol-water partition coefficients*, or  $K_{ow}$ , are commonly used versions of partition coefficients used to describe the lipophilicity (fat solubility) or hydrophilicity (water solubility) of a compound. In this case, the immiscible phases are *n*-octanol and water.  $K_{ow}$  values are often used by environmental chemists, biochemists, or toxicologists to estimate environmental fate (or distribution),

cellular uptake, or bioaccumulation. For example, in the fields of pharmacokinetics and toxicokinetics (a compound's adsorption, distribution, metabolism [biotransformation], and excretion properties),  $K_{ow}$  values are used in predicting a compound's bioavailability according to the Lipinski Rule of 5. Since *n*-octanol is a good representation of nonpolar solvents in general,  $K_{ow}$  values are good predictors of suitable solvents for extraction from aqueous samples. This value is so important and so commonly used that  $K_{ow}$  tabulations are commonplace in electronic or physical databases.

Henry's law constants,  $K_H$ , or air-water partition coefficients express the ratio of a compound's partial pressure in air to the concentration of the compound in water, again dependent on temperature. If expressed in terms of atmospheres of pressure and mole per cubic metre of water, and if the compound is less than 200 daltons, the compound is generally considered volatile.  $K_H$  may, for example, be applied to headspace extractions. Extraction sensitivity, or analyte concentration in the gas phase, is related to  $K_H$  and the phase ratio ( $\beta$ ); that is, the ratio of water to headspace. Since temperature has minimal effect on phase ratio, sensitivity will be directly related to  $K_H$ .

Adsorption-desorption distribution coefficients,  $K_d$ , are used in studies to model or predict environmental fate because of partitioning of a compound between water, soil, sediment, or other compartments. More so than other partition coefficients,  $K_d$  is dependent on a variety of matrix and environmental factors.  $K_d$  values and their measurement often do not account for the shape of the adsorption isotherm—that is, the nature of the sorptive interaction between the solute and the matrix. Consequently, laboratory measurements of  $K_d$  can

**FIGURE 1:** The relationship between extraction efficiency and number of extraction steps as a function of partition coefficient and solvent volumes. The upper (black) trace indicates the situation where  $K = 2$  and the phase ratio ( $\beta$ ) is 1; in the middle (blue) curve,  $K = 2$  and  $\beta = 0.5$ ; and in the bottom (red) curve,  $K = 10$  and  $\beta = 1$ .



be difficult to extrapolate to the field, though general trends can be observed.

To overcome this issue,  $K_d$  is often normalized to the organic carbon content of the matrix to determine the organic carbon-water partition coefficient,  $K_{oc}$ .

### Uses of Partition Coefficients in Extraction

We've previously suggested that  $K_{ow}$  values can be used in solvent selection during analytical extractions. One would generally prefer a solvent with a log  $P$  value equal to or greater than the log  $K_{ow}$ . Of course, extractions are dependent on more than solute solubility. Diffusivity, compatibility with the analytical method, and green considerations are also important. We've also looked at the relationship between Henry's law constants and headspace concentrations, including the role of temperature.

Partition coefficients play an important role in understanding the quantitative nature of analytical extractions. Consider, for example, the situation with batch extractions. If we can define a partition coefficient as  $K = [A]_{org}/[A]_{aq}$ , where  $[A]$  is the analyte concentration in the organic (*org*) or aqueous (*aq*) phases, we can estimate the extraction efficiency following a certain number of batch extractions:

$$[A]_i = \left( \frac{V_{aq}}{V_{org}K + V_{aq}} \right)^i [A]_0 \quad [1]$$

where  $[A]_i$  is the concentration of analyte in the aqueous sample phase after  $i$  number of batch extractions,  $[A]_0$  is the analyte concentration in the original aqueous sample, and  $V_{aq}$  and  $V_{org}$  are the volumes of aqueous and organic solvent, respectively, in each batch. For example, in Figure 1, we can see an application of this concept. In Figure 1, we can use the green (upper) plot as our baseline case, where equal amounts of aqueous and organic solvents (say, 100 mL of each, for example) at a partition coefficient of 2 are used. This means that two units of analyte would move into the organic phase with one unit (or 33% of the original amount) remaining in the aqueous sample. Upon a second extraction, one third of the 33% of aqueous analyte remaining in the sample, or 11%, remains in the sample. If we define quantitative extraction as greater than 95% recovery, then a third extraction to yield just over 96% extracted, with 300 mL of solvent required. If we aim to improve the extraction method by doubling the amount of organic extracting solvent to 200 mL, then the first extraction would isolate 80% of the analyte into the

organic phase. A second extraction would remove 80% of the remaining 20% in the aqueous sample, or a cumulative 96% of the analyte is extracted. However, in this case, 400 mL of solvent is required. However, if a different extracting solvent with a larger partition coefficient ( $K = 10$ ) is used with the same phase ratio as our baseline situation, diagrammed in red, then in the first extraction, ten units of analyte extract into the organic phase and one unit, or 9%, of analyte remains in the aqueous sample. With a second extraction, just over 99% extraction yield is achieved with the use of only 200 mL of solvent. This example shows the importance of favourable partition coefficients during solvent selection.

The trend towards microscale extractions relies on the knowledge that these separations are equilibrium based, and are thus dependent on understanding partition coefficients. For example, in solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE), and related sorptive extractions, one can use  $K_{ow}$  to approximate the stationary phase–water partition coefficient. Then  $K_{ow} \approx [A]_{stat}/[A]_{aq}$  where  $[A]_{stat}$  is the analyte concentration in the stationary (extracting) phase. Breaking down the concentrations to their mass ( $m$ ) and volume components,  $K_{ow} \approx (m_{stat}/m_{aq})(V_{aq})/(V_{stat})$  or  $\beta(m_{stat}/m_{aq})$ . Thus, as long as the phase ratio is constant (that is, a constant volume is used for all samples and standards), the amount of analyte adsorbed directly correlates to the amount of analyte in the sample. Of course, one must be sure to extract for a long enough time to achieve equilibrium. For solvent-based microextractions, such as single-drop extraction, absolute phase immiscibility is required, since the extracting phase is not immobilized on a fibre or other support.

The above treatment of partition coefficients applied to extraction assumes a neutral solute, or one which does not hydrolyze, ionize, or otherwise degrade. For these more complicated situations, one must use distribution coefficients. For example, in the case of a weak acid, HA, which may dissociate in water, the acid dissociation constant,  $K_a$ , results from the aqueous reaction  $HA + H_2O \leftrightarrow H_3O^+ + A^-$ . Note that di- or tri-protic acids will have multiple, stepwise dissociations, each with its own  $K_a$ . We must also be concerned with the partitioning of the neutral species, HA, into the nonpolar organic phase. Thus, the distribution coefficient is  $D = \frac{P[H_2O_{aq}]}{[H_3O^+]_{aq} + K_a}$ , keeping in mind that  $P$  is the generalized partition coefficient for HA. The extent of protonation or deprotonation, and consequently  $D$ , is related to the pH of the aqueous sample relative to the  $pK_a$  or  $pK_b$  of HA. Thus, pH may be manipulated to “push” the acid into the neutral form (HA) to extract the acid into the organic extracting solvent.

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The more complicated example is the case of ionic salts, which must account for the aqueous-organic partition of both the nondissociated salt and the chelated ions, as well as the dissociation of the salt to its component ions and the equilibrium for the chelation of those ions. Although this situation may be somewhat more complex, its straightforward treatment is common in most analytical chemistry textbooks. Of course, the most complex situation is the real world, as these are mixtures containing more than one analyte type. Hence, assumptions

and simplifications are often made in the use of partition coefficients.

### Measurement of Octanol-Water Partition Coefficients

For this section, we will focus on the laboratory measurement of  $K_{ow}$ , perhaps the most widely used and applicable partition coefficient in analytical chemistry. The definition of octanol-water partition coefficients indicates that these values can be determined by calculating a simple ratio of the analyte solubility in *n*-octanol to its solubility in water.

However, we must keep in mind that this value is based on an equilibrium, so we must keep in mind the mutual solubility of the two solvents in each other. For example, water is quite soluble in *n*-octanol, up to around 20%.

Table 1 summarizes the laboratory methods for the determination of  $K_{ow}$  endorsed by the Organization of Economic Cooperation and Development (OECD) (1,2). Of these, the shake-flask methods are the simplest and most reliable, though time-consuming.

**TABLE 1:** Methods for determining  $K_{ow}$  from the Organization of Economic Cooperation and Development (2)

| Method – Equipment and Key Conditions  | Measured Endpoint   | Reported Value   | Advantages   | Disadvantages  |
|--|---|--|--|--|
| <b>OECD 107 – Shake flask</b><br>Water and octanol solubility. Neutral compounds.<br>Range $-2 < \log K_{ow} < 4$  | Concentration of test material in water and <i>n</i> -octanol | $\log K_{ow}$  | Reliable.<br>Can investigate low $\log K_{ow}$ values                                    | Slow.<br>Not suitable for ionizable compounds.<br>Not suitable for high $\log K_{ow}$ ( $>4$ ) substances.   |
| <b>OECD 107 – Shake flask at pH 5, 7, and 9</b><br>Water and octanol solubility.<br>Ionizable compounds.<br>Range $-2 < \log K_{ow} < 4$                             | Concentration of test material in water and <i>n</i> -octanol | $\log K_{ow}$<br>$\log D_{ow}$ at pH 5, 7, and 9         | Reliable. Can investigate low $\log K_{ow}$ values.<br>Suitable for ionizable compounds. | Slow.<br>Not suitable for high $\log K_{ow}$ ( $>4$ ) substances.  |
| <b>OECD 117 – HPLC method</b><br>Reversed-phase HPLC.<br>Reference substances.<br>Neutral compounds.<br>Range $0 < \log K_{ow} < 6$                                  | Retention time on reversed-phase column                       | $\log K_{ow}$  | Rapid. Small sample size.<br>Several compounds at once.                                  | Variable retention times.<br>Poor reproducibility.   |
| <b>OECD 122 – pH-metric method</b><br>Soluble compounds.<br>Range $-2 < \log K_{ow} < 7$   | Acid-base aqueous titration                                   | $pK_a$ , $\log D_{ow}$<br>across pH range, $\log K_{ow}$ | Rapid and convenient.<br>$\log D_{ow}$ data for entire pH range. $\log K_{ow}$           | $\log K_{ow}$ and $\log D_{ow}$ are estimates based on $pK_a$ titration.<br>Insoluble or neutral compounds cannot be measured.<br>Requires sophisticated analytical technology.<br>Limited availability.<br>OECD 122 has not been finalized. |
| <b>OECD 123 – Shake flask slow stirring method, option for pH 5, 7, and 9</b><br>Water and octanol solubility.<br>Hydrophobic substances.<br>Range $\log K_{ow} > 4$ | Concentration of test material in water and <i>n</i> -octanol | $\log K_{ow}$  | Suitable for hydrophobic ( $\log K_{ow} > 4$ ) compounds                                 | Slow   |
| <b>OECD 107 – EU A.8, option for pH 5, 7, and 9</b><br>Insoluble and/or multi-protic substance.<br>Surface active substance.   | Solubility in water and octanol. Turbidity measurements.      | $\log K_{ow}$ estimated                                  | Practical alternative for water insoluble, surface active, and multi-protic compounds    | $\log K_{ow}$ is an estimated value.<br>Correlation between solubility ratio and $\log K_{ow}$ is weak.  |

Related to the shake-flask method is a derivation called the *slow-stir method*, with the major difference being the glassware used and mode of agitation (phase contact and equilibrium). Following the shake-flask method, solute concentrations may be measured by any applicable analytical technique, commonly chromatography, optical spectroscopy, or proton-nuclear magnetic resonance spectroscopy. Reversed-phase liquid chromatography (RPLC) is used to determine  $K_{ow}$  values with the key assumption being that the stationary phase, generally octyl- or octadecyl-, mimics *n*-octanol. RPLC is mostly applicable to ionic samples.

### Conclusions

At its most fundamental level, chemical separations, both analytical and industrial scales, are based on the concepts of equilibrium and diffusion. Regarding equilibria, the equilibrium constants describing partitioning and other phase distribution processes are vital, including consideration of solute dissociation. To simplify this confusing multitude of terms, simple definitions, applications of partition coefficients to chromatographic sample preparation (such as extractions), and a summary of how to measure partition coefficients are given in this article.

### References

- 1) Peters, D. G.; Hayes, J. M.; Hieftje, G. M. *Chemical Separations and Measurements: Theory and Practice of Analytical Chemistry*, W. B. Saunders Co., 1974; pp. 468–469.
- 2) Ecetoc, Technical Report 123. <https://www.ecetoc.org/technical-report-123/appendix-b-measurement-of-partitioning-kow/> (accessed 2023-05-09).

### ABOUT THE EDITOR

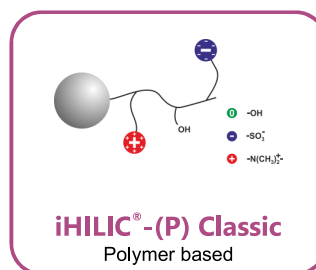
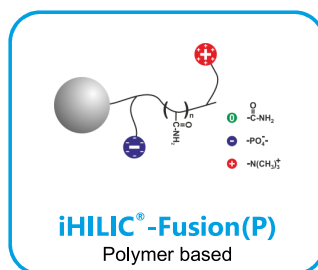
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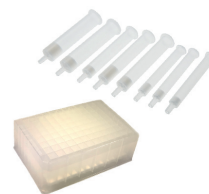


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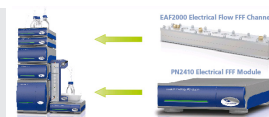


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ISSS symposia are itinerant scientific meetings organized annually, in turn, by each of the 11 member countries of the Central European Group for Separation Sciences, namely in membership order: Croatia (1998), Slovenia (1998), Slovakia (1998), Hungary (1998), Czech Republic (1999), Austria (2000), Italy (2000), Poland (2000), Ukraine (2007), Romania (2008), and Serbia (2019).

This year, for the second time, the ISSS symposium is organized in Cluj-Napoca by the Separation Sciences' Section of the Romanian Chemical Society, under its auspices as well as those of the Central European Group for Separation Sciences, the European Society for Separation Science, the Babeş-Bolyai University, and the Romanian Ministry of Research, Innovation, and Digitization.

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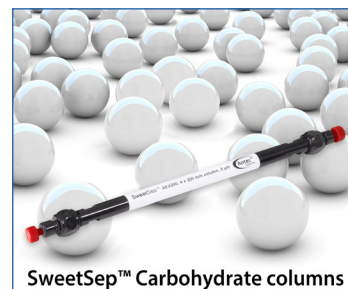
## LC PRODUCTS

**Carbohydrate Columns**

High performance anion-exchange chromatography (HPAEC) is the most powerful analytical technique for carbohydrate analysis due to its ability to separate all classes of alditols (polyols), aminosugars, mono-, oligo-, and polysaccharides including glycans, according to structural features such as size, composition, anomericity, and linkage isomerism.

HPAEC in combination with pulsed amperometric detection (HPAEC-PAD) is the method of choice for the analysis of carbohydrates. It combines superior selectivity with sensitive detection down to femtomole levels without the need for derivatization or complicated sample preparation.

A novel polymeric anion-exchange stationary phase SweetSep™ AEX200 has been developed by Antec Scientific. The stationary phase is based on a monodisperse 5 µm resin coated with quaternary amine functionalized nanoparticles. The high monodispersity of the resin allows for rapid, high-resolution separations of carbohydrates. The size and exchange capacity of the latex nanoparticles were optimized to enable the analysis of a wide variety of carbohydrate samples ranging from monosaccharides present in food, plants, and glycoproteins to oligosaccharides such as FOS (fructo-oligosaccharides) and N-linked glycans. SweetSep columns can be used with any IC system and most bioinert (metal-free) HPLC systems that can work at high pH and are equipped with PAD or MS.



**SweetSep™ Carbohydrate columns**

**Antec Scientific**

Alphen a/d Rijn, The Netherlands

**Email:** [info@AntecScientific.com](mailto:info@AntecScientific.com)

**Website:** [www.AntecScientific.com](http://www.AntecScientific.com)

## KNAUER WISSENSCHAFTLICHE GERÄTE GMBH

## LC PRODUCTS

**UHPLC System**

The AZURA® UHPLC System equipped with the new AZURA P 8.1L UHPLC pump is the ideal choice for running challenging chromatographic applications, where system performance is key to success.

The system offers a maximum delivery pressure of 124 MPa/1240 bar and very low dispersion for ultra-fast and high-resolution applications. The adaptive pulsation compensation of the pump is independent of flow rate, back pressure, and eluent type through real-time eluent compressibility monitoring. The variable piston stroke, together with the ultra-precise piston movement thanks to KNAUER's proprietary advanced piston drive technology, results in outstanding flow reproducibility at any working conditions.

The ultra-precise and independent piston movement and the variable stroke is translated in very low pulsations. The system is ideal for running applications with mobile phases containing trifluoroacetic acid (TFA), without the need of larger mixers. The ultra-low pump pulsation allows lower limits of detection to be reached, without sacrificing the gradient delay volume. The pump technology guarantees high reproducibility and confidence in the retention times in any conditions; this is also true when running shallow gradients or gradients starting with very low % B—commonly applied for peptide mapping or separation of structurally similar compounds.


**KNAUER Wissenschaftliche  
Geräte GmbH**

Hegauer Weg 38, 14163 Berlin,  
Germany

**Email:** sales@knauer.net

**Website:** www.knauer.net/p81l

## PEAK SCIENTIFIC INSTRUMENTS LTD

## LC PRODUCTS

**Nitrogen Generator**

PEAK Scientific's Genius XE is a cutting-edge nitrogen generator designed with advanced technology and robust engineering. It offers stress-free nitrogen gas generation for high-performance LC–MS in laboratories, providing a premium standalone solution.

The Genius XE incorporates multi-stage purification with five stages. A Triplex Particulate Filter ensures clean and high-purity nitrogen. The AirMax™ air intake maximizes compressor efficiency, and a two-stage moisture removal system delivers ultra-dry nitrogen gas.

Using a custom advanced polymer hollow fibre membrane technology, Genius XE ensures consistently high purity, even at high flow rates. It also includes NMHC Capture, removing ambient hydrocarbons to enhance efficiency and performance.

With Genius XE, laboratories have a constant nitrogen gas supply produced on-demand from compressed air, reducing reliance on cylinders or dewars. It offers a sustainable and environmentally friendly option for greener labs. Genius XE is engineered with refined components, developed through years of experience manufacturing laboratory nitrogen generators. It revolutionizes gas generation, providing labs with independence, efficiency, and a sustainable source of gas for instruments.



*Your local gas generation partner*


**PEAK Scientific Instruments Ltd**

Fountain Crescent, Inchinnan Business  
Park, Inchinnan, PA4 9RE, Scotland, UK

**Email:** discover@peakscientific.com

**Website:** www.peakscientific.com

## TOSOH BIOSCIENCE GMBH

## LC PRODUCTS

**Multi-Column Chromatography System**

Octave BIO is a comprehensive and versatile multi-column chromatography (MCC) system with a modular design and added functionalities that support a range of process scales, implementations, and applications, including continuous purification. The implementation of the established Octave MCC technology enables process resin savings, buffer savings, and other tangible multi-column chromatography benefits.

Alongside the Octave BIO instrument, Tosoh Bioscience has also launched SkillPak BIO, a set of pre-packed columns designed with standard shorter bed heights optimized for the fast flow rates and short residence times of MCC. The two products together offer a holistic approach to intensifying and optimizing purification steps, ultimately helping companies to reduce operating and capital costs while improving efficiency—both prerequisites for lowering biologic drug costs for patients.

**Tosoh Bioscience GmbH**

Im Leuschnerpark 4

64347 Griesheim, Germany

**Email:** sales-marketing.tb@tosoh.com

**Website:** www.tosohbioscience.de

## YMC EUROPE GMBH

## LC PRODUCTS

**(U)HPLC Columns**

Sensitive substances such as oligonucleotides, (phospho)peptides/proteins, phospholipids, or coordinating small molecules tend to interact with standard column hardware. Adsorption of analytes by metallic surfaces typically leads to peak tailing, carryover, and reduced recovery.

For these substances, YMC provides YMC-Triart (U)HPLC columns in different hardware options. The recently introduced YMC-Accura columns feature a bioinert surface coating of both column body and frits. The robust bioinert coating used on YMC-Accura hardware is 130- to 320-fold thicker, making it more durable than other similar hardware concepts. A long-term inertness against sensitive substances is ensured. YMC-Accura Triart columns can be used without preconditioning, which is usually necessary for the analysis of sensitive substances with standard column hardware.

YMC-Triart stationary phases are based on organic-inorganic hybrid silica with virtually no metal impurities. Combined with the inert YMC-Accura column hardware, excellent peak shapes for sensitive compounds are obtained due to the elimination of carryover and good recovery. This makes YMC-Accura Triart columns ideal for highly sensitive LC–MS analyses. All YMC-Triart stationary phases are available as inert YMC-Accura columns, with 1.9- $\mu$ m for UHPLC and 3- or 5- $\mu$ m for HPLC. Widepore YMC-Accura Triart Bio C18 is the preferred choice for oligonucleotides for example, while YMC-Accura Triart C18 is ideal for (phospho)peptides.

**YMC Europe GmbH**

Schöttmannshof 19

43549 Dinslaken, Germany

**Email:** support@ymc.eu

**Website:** www.ymc.eu

## MARKES INTERNATIONAL LTD

## SAMPLE PREP PRODUCTS

**Sample Preparation Platform**

Markes International has expanded its line of award-winning Centri® sample preparation platforms, all versions of which automatically extract and concentrate samples, resulting in greatly enhanced GC–MS sensitivity.

Markes' new Centri 90 platform provides automated high-sample throughput coupled with high sensitivity for both headspace and SPME-based workflows. It delivers rapid and efficient extraction and preconcentration of VOCs and SVOCs from liquid, solid, and gaseous samples, and can be easily upgraded to the newly introduced Centri 180. Centri 180 offers additional techniques of thermal desorption and high-capacity sorptive extraction, all on a single platform.

All Centri instruments use “gold-standard” robotics for high-throughput automation, and a back-flushed, electrically cooled, trap for optimized analyte trapping, which gives enhanced analytical sensitivity. Advanced water management eliminates water to enhance the quality and quantity of information obtained from each sample analyzed.

The Centri instruments can be interfaced with all GC–MS systems whilst allowing conventional GC injection modes to remain available. Centri 90 and 180 are also “multi-gas enabled” meaning that they can work with He, H<sub>2</sub>, and N<sub>2</sub> carrier gases—a future-proofing measure that many laboratories consider essential to deal with the increasingly high costs and dwindling supplies of helium.

# MARKES

international

**Markes International Ltd**

1000B Central Park, Western Avenue,  
Bridgend, CF31 3RT, UK

**Email:** [enquiries@markes.com](mailto:enquiries@markes.com)

**Website:** [www.markes.com](http://www.markes.com)

# MARKES

international

A breakthrough in  
automated sample  
preparation,  
extraction and  
concentration  
for GC–MS



**NEW**  
Centri 90

## High-sensitivity, high-throughput analysis by Headspace, SPME and SPME Arrow with trap

- Robotic automation for unattended, rapid & efficient extraction.
- Dedicated to routine analysis.
- Fast processing of both Headspace and SPME samples.
- Analyte trapping to maximise sensitivity.
- H<sub>2</sub> carrier gas – benefit from faster chromatographic speed and lower cost of ownership.
- Add on thermal desorption & high-capacity sorptive extraction (Centri 180).
- Compatible with all major brand of GC/GC–MS.
- Classical GC injection modes remain available.

## Explore the Centri series

[chem.markes.com/Centri90](http://chem.markes.com/Centri90)

## PORVAIR SCIENCES

## SAMPLE PREP PRODUCTS

**Positive Pressure Manifold**

The UltraPPM LITE from Porvair Sciences is the latest in positive pressure manifold technology, offering improved usability, reliability, and reproducibility. By applying consistent pressure above the solvent of a multiwell processing plate, this instrument reduces sample processing time for reproducible analyte recovery. The instrument has a front panel facing the user that allows precise control of its air flow and pressure through the microplate. Controlled flow through the plate maximizes interactions between sample and plate allowing for consistent, reliable results.

Features:

- Simple to set up and use. Only gas source required.
- Ideal for viscous and difficult samples
- Suitable for all process plates
- Consistent pressure application
- Ergonomically designed for easy pressure control
- Two hand operation to ensure safe use

Applications:

- Packed bed and composite-based SPE
- Packed bed and composite-based SLE, PLR, protein precipitation, and filtration.

**porvair**  
sciences

**Porvair Sciences**

Unit 73, Clywedog Rd South  
Wrexham Industrial Estate  
LL13 9XS, Wales

**Email:** [int.sales@porvair-sciences.com](mailto:int.sales@porvair-sciences.com)

**Website:** [www.porvair-sciences.com](http://www.porvair-sciences.com)

## UCT

## SAMPLE PREP PRODUCTS

**Solid-Phase Extraction**

HydraFlow® is the latest addition to the UCT's family of SPE manifolds geared towards efficient sample processing. The system's unique design provides enhanced precision and streamlined sample extractions for large-volume water analysis. While the assembly is simple, the compact footprint is perfect for fume hood setup and the overall weight of only 12 kgs makes it easy to relocate the unit in the laboratory. HydraFlow is equipped with four independent channels that allow the user to process the samples separately or simultaneously without the risk of cross-contamination during extraction. PEEK, PTFE, and stainless steel in the sample flow pathways ensure minimal to no corrosion from exposure to organic solvents such as dichloromethane and acids.

The distinctive feature of diverting the organic solvents and the aqueous solvents in separate collection bottles contributes to an effective waste management system and long-term cost savings. The waste and eluent collection channel switching valve adds convenience to the overall user experience.

Compared to traditional manifolds, HydraFlow eliminates the need to turn off the vacuum pump, drain the waste liquid out, and place the collection vials in the chamber. The precision control valves are another excellent feature used to achieve the desired flow rates of waste and elution solvents.

**UCT**

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**Email:** [info@unitedchem.com](mailto:info@unitedchem.com)

**Website:** [www.unitedchem.com](http://www.unitedchem.com)



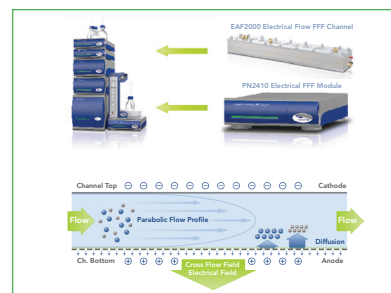
## POSTNOVA ANALYTICS GMBH

## FFF PRODUCTS

**Electrical/Asymmetrical Flow Field-Flow Fractionation**

Traditional separation technologies for biopharmaceutical and nanoparticle applications provide particle size or molar mass distributions as the final result. However, it is clear that particle and molecular charge play a primary role in many applications such as protein aggregation, polymer flocculation, particle agglomeration, and in pharmaceutical formulations in general. The Postnova EAF2000 instrument using electrical asymmetrical flow field-flow fractionation (EAF4) technology allows the particle size or molar mass distributions to be further differentiated and transformed into charge distributions. This identifies charge heterogeneities present within the different size and molar mass fractions and will help to aid research or establish more efficient product development processes.

The instrument works by combining the principle of electrical and AF4 in one system. This instrument is a key tool, particularly for protein research, because existing techniques for zeta-potential are limited by concentration and are batch techniques giving an average value for all components in the solution. The EAF2000 can determine the zeta-potential of each individually separated component, such as protein monomer and dimer (or higher aggregates) or antibody monomer and fragments–aggregates.

**Postnova Analytics GmbH**

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## ECOM spol. s r.o.

## PREPARATIVE CHROMATOGRAPHY PRODUCTS

**Chromatography Separation System**

ECOM has introduced a complete flash/preparative/pilot-scale/production modular system with an excellent price and performance ratio. The system can be used, for example, within high-capacity systems or with centrifugal liquid/liquid chromatography systems, such as in pharmaceutical purification of plant extracts (cannabinoids [CBD, CBN, CBG, CBC, THC]), peptides and oligonucleotides, algae extracts, and oleoresins, etc. The system can also be used for new molecule development or synthesis.

The device has three pumps. The two main ones are intended for “high” pressure gradient (parallel pumps connection), with one of them featuring a six-solvent selection valve. An optional third pump offers a repetitive sampling of high-volume samples. The unit also has a loop sample injection valve and a switching valve for CCC/CPC applications (ascending/descending mode). The unit’s universal PDA detector measures absorbance at four wavelengths at the same time at up to 800 nm.

The ECOMAC software or Clarity software (FDA 21 CFR Part 11 compliant) is internally installed in the PC controlling the system. The unit is completely customizable to the application and can meet any scale criteria—from small flash sample separation testing through to pilot-scale prediction and high-capacity production. It can work from 10 to 6000 mL/min and can be easily and automatically switched between these scales.

**ECOM spol. s r.o.**

Chrastany u Prahy, Czech Republic

**Email:** [info@ecomso.cz](mailto:info@ecomso.cz)**Website:** [www.ecomso.com/prepsystem-chromatography-separation-system](http://www.ecomso.com/prepsystem-chromatography-separation-system)

## ACD/LABS (ADVANCED CHEMISTRY DEVELOPMENT, INC.)

## SOFTWARE PRODUCTS

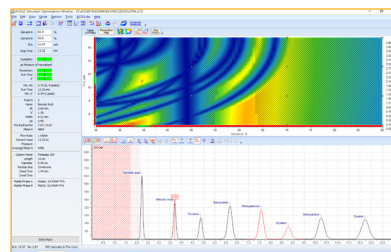
**Software Application**

Method Selection Suite is a software application that is designed to help scientists optimize and accelerate method development. Use the comprehensive set of tools available in the software to create efficient and systematic method development workflows and achieve more reliable results in the laboratory.

- Quickly and rationally identify and select the most promising starting conditions (pH, solvent, temperature)
- Fine-tune methods using the robust optimization algorithms in the software, saving time and resources by eliminating unnecessary experiments and ensuring optimal separation, resolution, and sensitivity
- Visualize and interpret experimental data using the software's advanced data analysis capabilities and generate informative reports to confidently present findings

Recent enhancements facilitate the effective transfer of methods between instruments and laboratories with the following capabilities:

- Vary column optimization parameters (flow rate, column length, column diameter, dwell volume)
- Automatic generation of methods that include all combinations of selected parameters to ensure coverage of the relevant method domain.

**ACD/Labs (Advanced Chemistry Development, Inc.)**

8 King Street East, Suite 107, Toronto,  
Ontario, M5C 1B5, Canada

**Email:** [info@acdlabs.com](mailto:info@acdlabs.com)

**Website:** [www.acdlabs.com](http://www.acdlabs.com)

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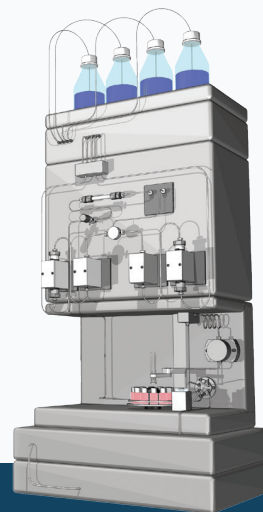
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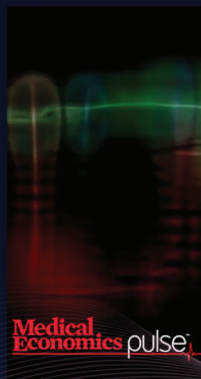
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up to 99,999%

Integrated collision gas option

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(> 20,000 hrs between  
preventative maintenance cycles)

Exclusive patented features for  
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with the collision gas option are  
running successfully

