Short Topic

Hexafluoroisopropanol in Ion Pair Chromatography

Maximilian Boehm
TCI AMERICA

All progress in all chemical disciplines, such as organic chemistry, materials science and biochemistry has been reliant on ever improving analytical and separation technologies. Without reliable methods to detect and distinguish one component from another, as well as fully characterize individual compounds, any progress in furthering our understanding of how molecules interact with each other could not be measured. As all scientific disciplines progress towards an increasingly complex understanding of our world, the need for accurate detection and separation of individual compounds within complicated mixtures increases as well. Among the many available analytical techniques, liquid chromatography has become an indispensable tool especially in the biotech sector. As we increasingly discover how biomolecules interact in complicated ways on the cellular level, we have become more and more dependent on highly purified molecules.

While the demands on analytical labs keep increasing, it is of paramount importance to keep procedures simple. Established methods like reversed phase chromatography have proven themselves to be extremely versatile and have been able to accommodate our ever increasing demands through coupling to various detection systems as well as simple modifications of analytical protocols. One such simple modification has been the use of ion pair technology.

Foundations

While historically chromatography has been developed utilizing a polar, stationary phase (usually silica) and an apolar mobile phase, the opposite combination has shown itself as more versatile and robust for general analytical use. Through modifications on the surface of the silica column, the stationary phase can be turned into a hydrophobic surface allowing for strong retention of most organic analytes, while eluting with water provides advantages in terms of compatibility with coupled detection methods, like mass spectrometry. LC-MS has long been the standard for separation and detection of a multitude of organic molecules.

It is at the interface between HPLC and MS, that ionization properties become a crucial factor in analytical method development. For instance, reversed phase chromatography depends on reasonable retention of the analytes on the stationary phase, requiring the presence of apolar analytes. On the other hand, modern mass spectrometry using soft ionization methods like ESI (electrospray ionization) requires a chemical environment that allows for an easy conversion of analytes into detectable ions at a sufficiently high concentration to enable a high sensitivity.

Biomolecules have been a prime target for developing robust LC-MS protocols. Most biomolecules as well as most developed drugs contain ionic functional groups. To enable a high resolution during the separation step it has been found that basic additives to the mobile phase provide an easy fix.

HPLC/MS for Oligonucleotides

A prime challenge for LC-MS methods have been the separation and detection of long oligonucleotides, especially when present in more complex mixtures and when they are of the same length, but containing different base sequences.

It was in 1997 that Apffel *et al*. at Hewlett-Packard presented a novel method that allowed them to separate mixtures of long (up to 75 bases) oligonucleotides with high resolution and analyze them with high sensitivity via ESI-MS.[1)] The challenges that needed to be overcome were finding a balance between high resolution in the separation step and high concentration of ionic species for the MS detection.

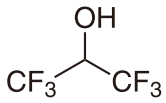
At that time it has already been established that addition of strong, organic bases like triethylamine (TEA) prevent the binding of sodium and potassium ions to the phosphate backbone of the oligonucleotides. This cation adduction has been a cause of lower detection sensitivity in the past and TEA improved that situation.

Unfortunately, this optimization at the detection step leads to reduced resolution in the separation step. On the other hand, the use of buffers containing carboxylic acids to enable better resolution leads to a lower production of analyte ions and thus sensitivity. This resulted in a quest to find a different approach that enables both, high resolution of

complex biomolecules in the separation step as well as a high concentration of ions to enable a higher sensitivity in the detection step.

HFIP

This search for an improved method that improves both sides of the procedure (good separation and high detection sensitivity) resulted in the discovery of hexafluoroisopropanol (HFIP) as an ideal additive together with TEA.



The physicochemical properties of HFIP make it a good candidate as an additive to chromatography systems:

1. Miscibility with water, methanol, 2-propanol and even hexane.
2. Boiling point of 58°C, allowing for high volatility during electrospray ionization
3. High level of fluorination increasing acidity to a pK_a of 9.3 compared to a pK_a of 16.5 for non-fluorinated propanol.
4. No UV absorption

The approach of employing a HFIP/TEA buffer eliminates the need for low volatility buffer additives while preserving their resolution enhancing benefits.
The mixture of TEA and HFIP stabilizes the pH during the separation step. The TEA pairs with the oligonucleotides to allow better retention on the stationary phase which in turn leads to higher chromatographic resolution. It was initially hypothesized that during the ionization step, HFIP's lower boiling point leads to a rapid elimination from the droplet surface, leading to an increase of the pH within the droplet. With an increased pH, the TEA-oligonucleotide pair dissociates leading to a higher availability of free oligonucleotides in the gas phase which in turn enhances the sensitivity during detection. Since then, a more extensive study of this phenomenon by Chen *et al.*[2] has shown that it is not only the high volatility of HFIP, but also its moderate acidity that contributes to an enhancement of the MS signal. According to this study, running ESI in negative ion mode leads to the reduction of protons in the solution, thus driving the equilibrium of protonated species like this:

The anion A^- stemming from the analyte will compete with the anion generated by deprotonation of HFIP. In the case of carboxylic acids that used to be part of buffer solutions, their low pK_a leads to a higher amount of carboxylate ions thus suppressing the analyte signal. HFIP on the other side is much less acidic and provides much less competition with the deprotonated oligonucleotides.

Since this discovery, HFIP/TEA has established itself as the prime buffer system for HPLC/ESI-MS applications in the nucleotide space.

The TCI Advantage

TCI has been providing labs with HFIP of the highest quality for many years. Our stringent QC requirements and our commitment to customer satisfaction above all else has won the trust of many leading biotech companies that rely on the consistent purity of our materials every day in R&D as well as production environments. The accuracy of your analytical results should not be limited by the purity and consistency of available solvents and additives. This is why TCI is committed to continue providing the industry with the highest quality ion pair reagents on the market. For more information on TCI's ion pair portfolio, visit: https://www.tcichemicals.com/assets/brochure-pdfs/Brochure_A1084_E.pdf

References

1) A. Apffel, J. A. Chakel, S. Fischer, K. Lichtenwalter, W. S. Hancock, *Anal. Chem*. **1997**, *69*, 1320.
2) B. Chen, S. F. Mason, M. G. Bartlett, *J. Am. Soc. Mass Spectrom*. **2013**, *24*, 257.

Related Products

Triethylamine (TEA)	25mL	100mL	500mL	T0424
1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP)	25g	100g	500g	H0424