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Advancements in Two-Dimensional Liquid Chromatography for Biological Samples Enhancing Peak Capacity and Speed through High-Temperature Techniques

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ABSTRACT

The need for improved separation power in Liquid Chromatography (LC), particularly for complex multi-constituent biological samples, is becoming increasingly critical. Recent advancements have led to a growing interest in Two-Dimensional Liquid Chromatography (2DLC), which offers significant advantages over traditional One-Dimensional Liquid Chromatography (1DLC) by enabling higher peak capacities and resolution. This review explores the potential of 2DLC, highlighting its ability to enhance separation by combining orthogonal separations and offering peak capacity that, under ideal conditions, is the product of the peak capacities of both dimensions. Despite its impressive separation power, comprehensive 2DLC is hindered by long analysis times, often extending several hours. Recent innovations in high-temperature LC techniques have addressed this challenge by drastically reducing the time required for the Second Dimension of 2DLC to approximately 20 seconds, enabling rapid separations with improved peak capacities exceeding 2000. These developments have led to significant reductions in analysis time, allowing complex biological samples with over 200 detectable peaks to be analyzed in under 30 minutes. This shift toward fast, high-temperature LC is transforming the capabilities of 2DLC, making it an invaluable tool for the analysis of biological samples. Additionally, the review touches on complementary techniques like NMR spin relaxation spectroscopy, which aids in characterizing protein dynamics, offering further insights into protein conformational changes on the picosecond-nanosecond timescale.

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Introduction

Overview of Liquid Chromatography (LC)

Liquid Chromatography (LC) utilizes a liquid mobile phase for separation, offering diverse solvating properties but facing kinetic limitations. Optimization involves selecting the appropriate separation mode, stationary phase, and mobile phase.

Separation Modes & Techniques

- **Liquid-Liquid Chromatography (LLC):** Based on partitioning between mobile and stationary liquid phases (e.g., paper chromatography).
- **Liquid-Solid Chromatography (LSC):** Uses adsorbents like silica or alumina for separation (e.g., thin-layer chromatography).
- **Ion-Exchange Chromatography:** Separates compounds via ionic affinities using resins or zeolites.
- **Size-Exclusion Chromatography:** Separates molecules based on size using a polymer gel [1].

Advancements

Modern LC has evolved from open-column techniques to High-Performance Liquid Chromatography (HPLC), improving resolution, speed, and sensitivity with high-sensitivity detectors

Importance of Two-Dimensional Liquid Chromatography (2D-LC)

2D-LC is a highly effective analytical technique that significantly enhances separation capabilities for complex samples. By integrating two distinct separation mechanisms, it achieves a peak capacity in the thousands, greatly surpassing conventional One-Dimensional Liquid Chromatography (1D-LC). This allows for the resolution of intricate mixtures with numerous analytes, a challenge for 1D-LC. Compared to other analytical methods like Gas Chromatography (GC) and Capillary Electrophoresis (CE), 2D-LC offers superior selectivity and sensitivity, even though those techniques may provide high efficiency or faster analysis. While 2D-LC has some limitations, such as extended analysis times and potential compatibility issues, its ability to achieve high-resolution separations, minimize peak assignment errors, and handle complex mixtures makes it invaluable in pharmaceuticals, biotechnology, and environmental analysis.

Challenges in Biological Sample Analysis

Analyzing biological samples is complex due to interfering compounds like proteins and phospholipids. The goal is to develop a reliable method to quantify target analytes (e.g., drugs or biomarkers) in biological matrices.

- **Sample Preparation & Clean-up:** Essential for minimizing interferences.
- **Chromatographic Separation:** HPLC or UHPLC is used

for high-resolution analysis.
- **Mass Spectrometry (MS):** Requires careful optimization of parameters for accuracy.
- **Data Processing & Calibration:** Critical for ensuring reliability [2].

A systematic approach to method development and validation enhances accuracy, robustness, and reproducibility in bioanalysis

Principles of Two-Dimensional Liquid Chromatography Fundamentals of 2D-LC

Three fundamental methods for identifying 2D-LC are

1. Incompatibility
2. Active Solvent Modulation (ASM)
3. Stationary-Phase-Assisted Modulation (SPAM)

Incompatibility

The modulation interface in 2D-LC transfers fractions from the First-Dimension (1D) effluent to the Second-Dimension (2D) column, using a 2-position valve with storage loops. Challenges include solvent incompatibility, which causes flow instability, peak deformation, or splitting; viscous fingering; and solvent strength mismatch, particularly when combining chromatographic modes like SEC and RPLC. Active modulation solutions, such as pH or solvent adjustments, additives, and specialized columns, improve separation, sensitivity, and analytical reliability.

Active Solvent Modulation (ASM) in 2D-LC

Introduced by Stoll et al. in 2017, ASM enhances 2D-LC by modifying a conventional valve to include added ports, a bypass capillary, and extra rotational positions. This technique facilitates the coupling of different modes, like HILIC and RP, and prevents breakthrough, improving separation efficiency. ASM is applied in protein and peptide analysis, target molecule quantification in polymers, and supercritical fluid chromatography for vitamins, offering improved separation precision in 2D-LC.

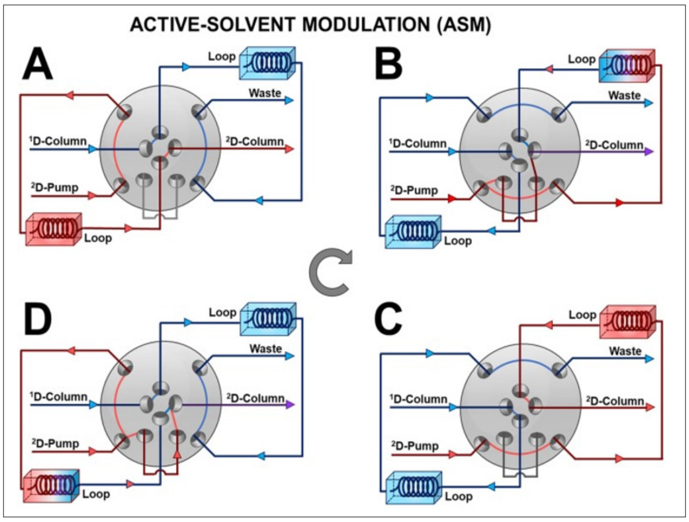

Figure 1: Active Solvent Modulation (ASM)

Stationary Phase Assisted Modulation (SPAM) in 2D-LC

SPAM, introduced by Vonk et al., is an active-modulation strategy in 2D-LC that uses low-volume trapping columns ("traps") to retain analytes and remove incompatible 1D solvents. It reduces solvent incompatibility, enhances detection sensitivity by focusing analytes, and lowers 2D injection volumes. However, it faces challenges like incomplete analyte retention and potential discrimination effects. SPAM has improved peak capacity, sensitivity, and resolution in peptide, protein, polyphenol, and procyanidin analysis, enabling high-loadability 1D columns and nanoscale 2D separations.

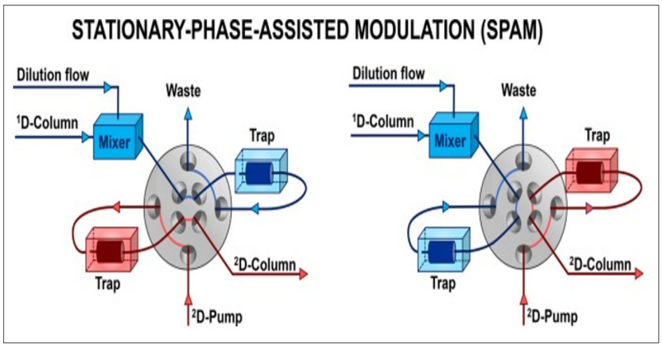

Figure 2: Stationary-Phase-Assisted Modulation (SPAM)

Advancements and Applications of 2D-LC

2D-LC is a powerful technique for separating complex mixtures, using various separation modes, modulation systems, and detection methods.

- **Separation Modes:** RPLC, HILIC, IEX, SEC.
- **Modulation Systems:** Passive modulation is dominant, with SPAM rising in popularity.
- **Detection Techniques:** UV and MS (quadrupole, TOF, Orbitrap, ion-trap).

Applications

2D-LC is crucial in pharmaceuticals, biopharmaceuticals, polymers, and food analysis.

- **Chiral Separation:** Key in separating enantiomers for pharmaceutical purity and efficacy.
- **Biopharmaceuticals:** Separates proteins and peptides based on charge, size, and hydrophobicity [3].
- **Synthetic Polymers:** Separates polymers by molecular weight, composition, and functionality, aiding in property analysis

Enhancing Peak Capacity in 2D-LC

Definition and Importance

Peak capacity is the maximum number of peaks resolvable in a chromatographic separation, calculated from the peak capacities in both 2D-LC dimensions. Higher peak capacity improves resolution, essential for proteomics, metabolomics, and pharmaceutical analysis.

Strategies for Improvement

Optimize chromatographic conditions, use longer columns or smaller particles, and employ orthogonal separation mechanisms.

High Temperature Techniques

HTLC and UHTLC above 100°C enhance resolution by lowering mobile phase viscosity, allowing faster flow rates and higher peak capacities for better separations.

Strategies to Accelerate 2D-LC Analysis

- **Optimized Conditions:** Shorter columns, smaller particles, and refined gradient profiles reduce analysis time.
- **High-Temperature Techniques:** Elevated temperatures improve kinetics, resolution, and speed.
- **Advanced Column Technologies:** Sub-2-µm and 2.7-µm particles enable faster separations.
- **Hybrid Separation Modes:** Combining heart-cutting and

comprehensive modes enhances efficiency [5].
- **Selective Comprehensive 2D-LC:** Focusing on specific regions reduces time and boosts peak capacity

Factors affecting Analysis Speed
Column Length and Diameter: Shorter columns with smaller diameters reduce analysis time.
Particle Size and Type: Smaller or specialized particles enable faster separations.
Flow Rate and Gradient: Optimized flow rates and gradient profiles speed up analysis.
Temperature and Pressure: Elevated conditions improve kinetic performance and speed up analysis.
Sample Complexity and Injection Volume: Complex samples or large volumes can slow down analysis [6].
Instrumentation and Hardware: Advanced tools, like ultra-high-pressure pumps and fast detectors, enhance speed

High Temperature Chromatography Mechanisms and Benefits
Mechanisms
Temperature-Dependent Retention: Elevated temperatures decrease the mobile phase's dielectric constant and surface tension, reducing retention times.
Viscosity Reduction: Higher temperatures lower mobile phase viscosity, reducing backpressure and allowing faster flow rates.
Improved Mass Transfer: Elevated temperatures enhance mass transfer, resulting in narrower peaks and better resolution.

Benefits
Faster Separations: Enables rapid separations for high-throughput analysis.
Improved Efficiency: Boosts column efficiency, improving resolution and peak shape.
Reduced Solvent Consumption: Minimizes the need for organic solvents, making it more eco-friendly [7].
Increased Selectivity: Temperature can be optimized to enhance separation selectivity

Instrumentation and Experimental Considerations
Instrumentation
High Temperature Columns: Columns with temperature-resistant materials and insulation.
Temperature Control Systems: Systems that ensure uniform temperature across the column.
High Pressure Pumps: Pumps that handle high pressures and flow rates at elevated temperatures.
Detectors: Detectors like UV-Vis or MS that function at high temperatures.

Experimental Considerations
1. **Column Selection:** Choosing appropriate stationary phases and particle sizes for high-temperature use.
2. **Mobile Phase Selection:** Using stable and compatible mobile phases for high temperatures.
3. **Temperature Programming:** Optimizing temperature to enhance separation and reduce analysis time.
4. **Pressure and Flow Rate Control:** Managing pressure and flow rates to protect equipment and optimize separations

Applications in Biological Sample Analysis
Proteomics
2D-LC enables protein separation and identification by extracting, purifying, and digesting proteins into peptides. These peptides are separated based on hydrophobicity and charge, analyzed by MS, and identified through database matching. It also facilitates Post-Translational Modification (PTM) analysis, including phosphorylation for signaling pathway insights and glycosylation for protein function and stability.

Metabolomics
2D-LC supports metabolite profiling by separating metabolites based on hydrophobicity and charge, offering insights into metabolic pathways and disease mechanisms. It also aids biomarker discovery by detecting and validating metabolites linked to diseases.

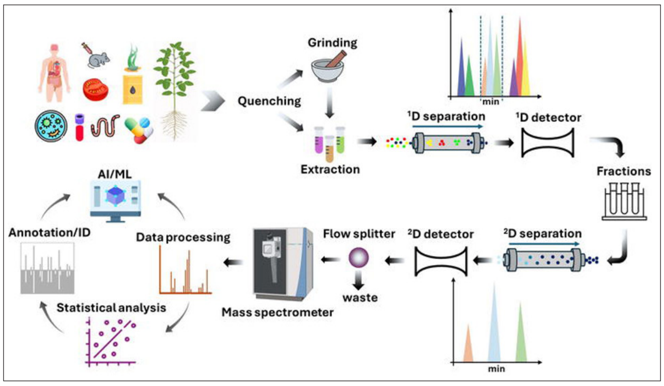

Figure 3: Major 2D-LC Applications Utilized in Metabolomics Research

Other Omics Approaches
Lipidomics analyzes the lipidome, separating lipids and providing insights into lipid metabolism and disease mechanisms [9].

Glycomics focuses on the glycome, analyzing glycosylation patterns essential for protein function and identifying disease biomarkers through glycosylation changes

Challenges and Limitations of High Temperature Techniques
High-temperature techniques face challenges such as column degradation, mobile phase instability, and stationary phase deterioration, affecting chromatographic performance. Sample degradation at elevated temperatures can alter chromatographic profiles, while large sample volumes may cause injection band broadening and peak splitting.

Potential Solutions
Temperature Responsive Liquid Chromatography (TRLC) enables reversed-phase separations in aqueous conditions. Specialized high-temperature equipment and alternative column materials improve stability [10]. Optimized mobile phases enhance thermal compatibility, and refined refocusing mechanisms minimize peak distortions

Stability and Thermal Degradation of Biological Samples
High temperatures in 2D liquid chromatography can degrade biomolecules like proteins and peptides, altering their structure, function, and binding properties. This may lead to inaccurate results and compromised sample integrity. Additionally, elevated temperatures accelerate chemical reactions, further degrading biological samples [11].

Thermal degradation affects analytes by causing structural changes, protein denaturation, and breakdown of biomolecules such as DNA and RNA. These effects can lead to loss of analyte integrity, making accurate analysis challenging. Optimizing temperature conditions and sample preparation is essential to minimize degradation and preserve sample stability [12].

Future Perspectives and Emerging Trends
Advancements in 2D-LC instrumentation will enhance speed, sensitivity, and robustness. Emerging column technologies, such as monolithic and nano-LC columns, will improve separation efficiency and resolution. Increased adoption is expected in pharmaceutical analysis, bioanalysis, and environmental monitoring.

Integration with Advanced Detection Techniques
Recent advances in detection methods have improved LC sensitivity and selectivity. Mass Spectrometry (MS) provides structural insights, while Tandem MS (MS/MS) enhances sensitivity by analyzing ion fragments. ICP-MS enables elemental analysis for environmental and pharmaceutical applications, and NMR spectroscopy aids structural elucidation [13].

Integration with LC
Combining LC with advanced detection techniques enhances sensitivity, selectivity, and accuracy, enabling the detection of lower analyte concentrations, differentiation of specific compounds in complex samples, and more precise quantification

AI and Machine Learning in 2D-LC Optimization
AI and ML are transforming 2D-LC by automating method optimization, reducing time, and improving accuracy. Predictive modelling helps optimize column and mobile phase selection, while ML algorithms refine operating conditions like flow rate and temperature. AI also enables automated method development, streamlining processes and enhancing productivity. Despite these advantages, challenges remain. Data quality is crucial, as poor input can lead to inaccurate models. The complexity of ML algorithms can make results difficult to interpret, limiting their practical application. Additionally, regulatory acceptance of AI-driven methods in chromatography is still evolving [14]. The integration of AI and ML in 2D-LC holds great potential, offering efficiency and precision while requiring careful consideration of data reliability and regulatory frameworks.

Next Generation High Temperature Approaches in 2D-LC
Advancements in HTLC have enhanced separation efficiency, speed, and analyte solubility. New column materials, such as polymer- and silica-based options, improve thermal stability, while high-temperature instrumentation ensures precise temperature control. Innovative 2D-LC methodologies, including comprehensive and heart-cut approaches, enable superior analysis of complex samples.

HTLC is widely applied in pharmaceutical analysis for impurity detection, bioanalysis for protein and peptide studies, and environmental monitoring of pollutants [15]. These next-generation approaches continue to refine 2D-LC, expanding its capabilities across diverse scientific fields.

Summary of Advancements and Benefits in 2D-LC
Advancements in 2D-LC
Recent improvements in instrumentation, such as enhanced column switching valves, high-resolution detectors, and automation, have increased the efficiency of 2D-LC. Methodological advancements, including comprehensive, heart-cut, and online 2D-LC, offer greater resolution, selectivity, and speed.

Benefits of 2D-LC
Compared to 1D-LC, 2D-LC provides superior resolution, peak capacity, sensitivity, and analytical flexibility by integrating two orthogonal separation mechanisms.

Applications
2D-LC is widely used in pharmaceutical analysis for impurity detection, bioanalysis for biomolecule studies, and environmental monitoring for pollutant analysis [16]. With continuous advancements, 2D-LC remains a crucial tool for high-resolution, high-sensitivity analysis across multiple scientific fields

Future Innovation in 2D-LC
2D-LC continues to advance, offering enhanced resolution, sensitivity, and peak capacity for complex sample analysis. Future innovations will focus on improved instrumentation, new column technologies like nano-LC and SFC, advanced data analysis through AI, and increased automation. Comprehensive and online 2D-LC, along with hyphenated techniques like 2D-LC-MS, will further expand its capabilities [17]. As the field evolves, 2D-LC will remain a vital tool across pharmaceutical, bioanalytical, and environmental applications.

Conclusion
In conclusion, Two-Dimensional Liquid Chromatography (2D-LC) has emerged as a powerful and versatile technique for the separation of complex mixtures. Through the use of various separation modes, modulation systems, and detection techniques, 2D-LC has significantly advanced the analytical capabilities in fields such as pharmaceuticals, biopharmaceuticals, and polymer analysis. Key methods like solvent incompatibility management, Active Solvent Modulation (ASM), and Stationary-Phase-Assisted Modulation (SPAM) have improved the efficiency and resolution of 2D-LC separations. Recent innovations, including high-temperature techniques and the application of machine learning, continue to enhance the sensitivity, speed, and precision of 2D-LC. With its ability to separate complex samples based on multiple properties, 2D-LC holds great promise for future applications in advanced fields, offering more accurate, reliable, and high-throughput analytical solutions.

Conflicts of Interest: None

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