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# 2 dimensional liquid chromatography-mass spectroscopy: A review

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**Abstract**
Through LC, the segments of a specimen are isolated by how they seep into the fixed and mobile phases. The info gotten by mass spectroscopy discovers and teaches about the structure of each part. The method called Comprehensive 2-Dimensional liquid chromatography (LC×LC) is new. The first instance that LC x LC was remarkable to the effect that it pointed out the sample's separations using two different columns, unlike the classical multidimensional chromatography. When met with the mixtures that are too hard to be resolved by other means, LC technicians like to this technique. This instrument has gained power when a mass of a tertiary dimension that a LC x LC system involves is added to a mass spectrometer. This article puts the 2D LC-MS equipment under the microscope, as well as the differences between LC and 2d LC and their applications are the objectives of this study.

**Keywords:** Liquid chromatography, interface, mass spectroscopy, detectors, mass analysers

## Introduction
The chromatography technique, derived from the Greek word "chroma" (colour), is widely used for separating mixtures and identifying unknown chemicals [1-3]. It involves the separation of components in a mixture based on differences in their affinity for a stationary phase while being carried by a mobile phase [4-6]. The stationary phase can be solid or liquid, while the mobile phase can be a gas or liquid [7-9]. The process of chromatography includes sample application, elution, and detection [10-12]. The sorbent with an orthogonal stationary phase and superficially porous stations provides insights into kinetics and thermodynamics of the isotherm [12-14]. The gas transfer process involves desorption and adsorption, leading to differential attachment/reattachment of the components [15]. Small changes in the partition coefficient due to modifications in the stationary phase can slightly affect the separation [16-17].

## Liquid Chromatography/LC/1D LC
**Liquid Chromatography (LC):** Separates and analyses components in a mixture using a liquid mobile phase and stationary phase. Substances are separated based on size, charge, or affinity. Detection methods like UV light can visualize components. Widely used in research and industries for identification and quantification. Four types:

**1. Reversed-Phase Chromatography:** In reverse-phase chromatography, hydrophobic molecules stick to the non-polar stationary phase, while hydrophilic ones move through the polar mobile phase and are eluted.

**2. Normal Phase Chromatography:** It uses a polar stationary phase and a non-polar mobile phase, ideal for separating water-sensitive compounds, isomers, and asymmetric molecules. Polar solvents like ethyl acetate pair with non-polar ones like hexane for separation.

**3. Ion Exchange Chromatography:** In ion exchange chromatography, ions are separated by their affinity to the ionic stationary phase, with a water-based buffer as the mobile phase. Strongly bound ions are retained longer.

**4. Size Exclusion Chromatography:** Size Exclusion Chromatography (Gel Filtration) separates molecules by size, with larger ones eluting first. The stationary phase's pore size is key, and it's often used for proteins and polymers.

**2D LC /LC×LC:** Liquid Chromatography in Two Dimensions (LC×LC) improves separation efficiency using two columns. A multiport valve automates transferring fractions from the first column to the second, ensuring analysis timing aligns with modulation periods for immediate processing of retained fractions.

**2D LC-MS:** LC×LC-MS combines liquid chromatography with mass spectrometry for improved compound identification. It uses two columns linked by a thermal modulator, enhancing detection limits and selectivity, especially for small compounds.

**Instrumentation**

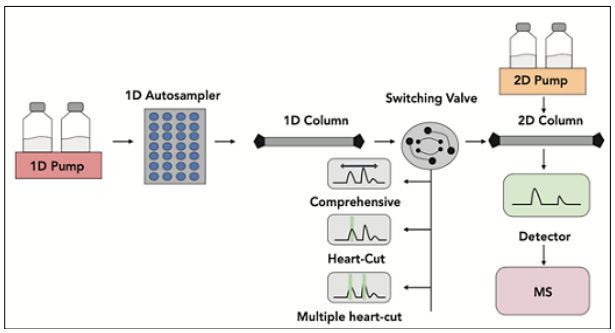


**Fig 1:** 2D LC-MS Instrument

**LC×LC/2D LC**

**Elements that make up 2D LC**

1. Sample Injector
2. Columns
3. Detector
4. Pump
5. Modulation Device
6. Data system and Software

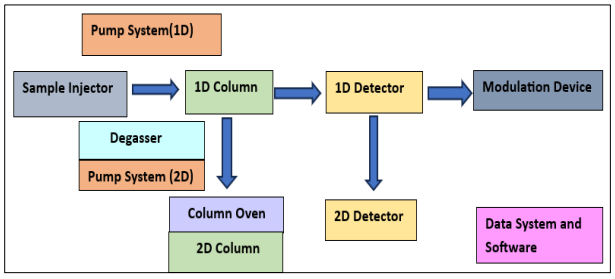


**Fig 2:** LC×LC Instrument

**1. Injector-** The 2D-LC injector introduces a precise sample amount into the chromatographic system for separation. It first injects the sample into the 1D column, and after initial separation, fractions are transferred to the 2D column for further separation.

**Injection techniques**

1. Loop Injection
2. Flow-Through Needle Injection

**Loop Injection**

Introduces samples into 1D column of 2D chromatography using a sample loop and switch. Improves quantification, resolution, and automation, enhances throughput and separation of complex mixtures.

**Flow-Through Needle Injection**

Rapidly introduces samples into 1D column of 2D LC, minimizes sample loss, accommodates various sample types, and can be automated for accurate and precise analysis.

**2. Column**

Two-dimensional liquid chromatography (2D LC) is an advanced method for separating complex mixtures using two columns with distinct separation mechanisms.

**Column considerations**

**Phase selection:** Separates components in a mixture using a liquid mobile phase and stationary phase. Relies on interactions like ion exchange and adsorption. Effective for coloured and colourless substances. Uses visualization methods like UV detection.

**Column dimensions**

**Length:** 50-250 mm (shorter for faster analysis, longer for better resolution).

**Internal Diameter:** 1.0-4.6 mm (wider for larger samples, narrower for high sensitivity).

**Packing Material Particle Size:** 1.7-5 µm (larger for reduced backpressure, smaller for enhanced resolution but increased backpressure).

**Types of columns**

1. Inverse-Phase (RP) Column.
2. Phase Normal (NP) Column.
3. Ion transfer Column.
4. Chromatography by Size-Exclusion Method Column.
5. Column of Affinity.

**Inverse-Phase (RP) Column:** Essential in 2D-LC was high resolution and versatility. Stationary phase is non-polar silica with hydrophobic groups. Mobile phase is Polar aqueous organic solvents Separation is effective for non-polar to slightly polar compounds Applications Analysis of complex biological samples, pharmaceuticals, and environmental studies

**Normal Phase (NP) Column:** Essential in 2D-LC Separate polar compounds. Mobile phase was Non-polar hexane or chloroform. Stationary phase wasPolar alumina or quartz. Analysis: Effective for polar analytes Orthogonal separations: Combined with RP columns for enhanced efficiency

**Ion transfer Column:** Essential in 2D-LC: Separate charged molecules like nucleotides and proteins. Stationary phase: Charged. Mobile phase: Aqueous buffer with salt gradients Separation: Separate complex mixtures in the first dimension, followed by enhanced resolution in the second dimension using mechanisms like reversed-phase (RP) chromatography

**Chromatography by Size-Exclusion Method Column:** Essential in 2D-LC: Separate molecules by size. Stationary phase: Porous polymer beads or silica. Separation: Larger molecules elute first, smaller molecules retained longer. Applications: Analysis of macromolecules, paired with RP

or IEX chromatography in the second dimension for enhanced separation efficiency in polymer characterization and biopharmaceutical analysis

**Column of affinity:** Essential in 2D-LC: Enable selective separations based on specific biological interactions. Stationary phase: Ligands that bind target molecules, such as antibodies. Application: Commonly used for purification of monoclonal antibodies. Separation: Isolate analytes from complex mixtures in the first dimension, followed by further resolution in the second dimension using SEC or RP chromatography. Benefits: Enhanced separation efficiency and specificity, valuable for proteomics, biomarker discovery, and biopharmaceutical analysis.

**Table 1:** Difference between phase inverse (RP) column, phase normal (NP) column, ion-transfer column, size-exclusion method chromatography column, column of affinity

| Parameter | Phase Inverse | Phase Normal | Ion transfer | Size Exclusion method | Column of Affinity |
|---|---|---|---|---|---|
| Stationary Phase | Non-polar | Polar | Charged groups | Porous particles | Specific ligands |
| Mobile Phase | Polar solvents (e.g., water, acetonitrile) | Non-polar solvents (e.g., hexane) | Buffered solutions | Aqueous or organic solvents | Buffer with specific elution conditions |
| Separation Mechanism | Hydrophobic interactions | Polar interactions | Electrostatic interactions | Size-based separation | Biochemical interactions |
| Typical Applications | Non-polar compounds | Polar compounds | Ionic species | Macromolecules | Specific biomolecules |
| Column Material | Silica-based or polymer-based | Silica-based | Polystyrene-divinylbenzene or silica | Silica or polymer-based | Agarose or silica-based |
| pH Range | 2-8 | 3-7 | 2-12 | 2-8 | 3-10 |
| Temperature Range | Up to 60 °C | Up to 40 °C | Up to 60 °C | Up to 40 °C | Up to 40 °C |

**Detector:** There are numerous detectors that can be used in LC. Every detector has unique qualities, benefits, and disadvantages.

**Ultraviolet (UV) Absorbance Detectors:** It measure UV radiation absorbed by analytes, identifying and quantifying chemicals based on their specific absorption wavelengths. They are highly sensitive and widely used in various fields. However, they are ineffective for non-UV-absorbing compounds and can be affected by UV-absorbing contaminants in the mobile phase.

**Advantages:** UV detectors are essential in various fields for identifying UV-absorbing substances. They offer high sensitivity for qualitative and quantitative analysis, and are easy to use and maintain.

**Disadvantages:** Cannot identify non-UV-absorbing substances, impurities in mobile phase can cause false positives, temperature and lamp variations affect baseline drift, and standard wavelength range may not suffice for all analytes.

**Mass Spectrometry (MS) Detectors:** Ionizes analytes eluting from chromatography column, calculates m/z using techniques like ESI and APCI. Mass analyzer separates ions by m/z, and their abundance is measured to produce a mass spectrum.

**Advantages:** Excel in trace analysis, providing detailed information on molecular weight and structure. They can analyze various chemicals, including non-UV-active ones, and offer both quantitative and qualitative data.

**Disadvantages:** Non-portable, high cost and maintenance, requires specialized knowledge, and may struggle to distinguish between isomers.

**Diode Array Detectors (DADs):** Provide extensive spectral data in 2D LC, measuring absorbance across multiple wavelengths. They can detect contaminants and degradation products, but require specialized tools for data interpretation and are generally more expensive than single-wavelength detectors, though less sensitive than mass spectrometers.

**Advantages:** DADs simultaneously monitor multiple wavelengths, providing comprehensive spectral information for each analyte. They offer both quantitative and qualitative data and assess overall quality. DADs are ideal for applications in food safety, environmental studies, and pharmaceuticals.

**Disadvantages:** Higher noise levels due to low light at each photodiode, sensitivity to light source variations, and difficulty in interpreting extensive spectral data without advanced software and expertise.

**Fluorescence Detectors:** Fluorescence detectors measure emitted light after stimulating analytes, detecting naturally fluorescent substances or those tagged with fluorescent labels. They enhance sensitivity and selectivity in 2D liquid chromatography for analyzing pharmaceuticals, metabolites, peptides, and proteins.

**Advantages:** Fluorescence detectors excel in trace analysis, detecting low analyte concentrations with high selectivity. They ensure minimal interference from non-fluorescent substances and are suitable for biochemistry, environmental analysis, and pharmaceuticals.

**Disadvantages:** It can only identify naturally fluorescent analytes or those needing derivatization (time-consuming), and measurement accuracy may be affected by quenching agents and environmental changes.

**Refractive Index (RI) Detectors:** Refractive Index (RI) detectors measure non-UV-absorbing substances by tracking refractive index changes. They are less sensitive, affected by temperature changes, and require consistent mobile phase composition for stable operation.

**Advantages:** RI detectors can identify any substance with a different refractive index from the mobile phase, allowing for broad chemical detection.

**Disadvantages:** Less sensitive than UV or MS detectors for low concentrations, sensitive to temperature changes causing baseline drift, typically require isocratic elution, and lack detailed analyte information like molecular weight or structure.

**Pump:** Pumps ensure precise and consistent mobile phase movement in 2D liquid chromatography (2D LC).

**Binary pump:** Binary pumps mix two solvents for precise gradients using separate pumps and a high-pressure mixing chamber. This allows quick adjustments in solvent composition, reducing dwell volume for rapid gradient changes, essential for high-resolution separations in analytical applications.

**Quaternary Pumps:** Quaternary pumps mix up to four solvents for gradient creation in liquid chromatography using a precise proportioning valve. This allows flexible solvent composition, ideal for complex separations and technique development.

**Modulation Device:** The modulation device in 2D liquid chromatography transfers fractions between dimensions for effective separation and analysis of complex mixtures.

**Valve-Based Modulators:** Transfers fractions between dimensions for better separation. Ensures accurate transfer for high-quality analysis. Synchronizes dimensions for smooth operation. Supports various 2D LC configurations. Enhances peak capacity and resolution.

**Thermal Modulator:** Uses temperature changes to collect and transfer fractions. Heats and cools sample to concentrate and release analytes. Concentrates sample at low temperature, then releases with rapid heating. Repeats process for continuous modulation.

**Data Systems and Software-Method Setup:** 2D LC software: Graphical interface for easy method setup. Simple mouse clicks for 2D methods and mode selection. Creates and modifies gradients for optimal separation.

**Data Analysis:** 2D data visualization software uses contour plots and linked chromatograms. Facilitates interpretation of complex results. Provides qualitative and quantitative analysis, including spectral data and repeatable quantitative outcomes.
**Reporting:** Automated Reporting: Produces detailed reports with customized calculations and advanced 2D-LC data analysis. Integration: Can be integrated with MS data systems for enhanced analysis

**What sets 1D LC apart from 2D LC**
**1D LC:** Single separation method, suitable for simpler samples, limited resolution for complex mixtures.
**2D LC:** Two orthogonal separation methods, improved peak capacity and resolution, ideal for complex samples, more complex setup and longer analysis time.

**Conclusion**
2D LC is preferred for complex samples due to its superior analysis, despite 1D LC being faster and simpler.

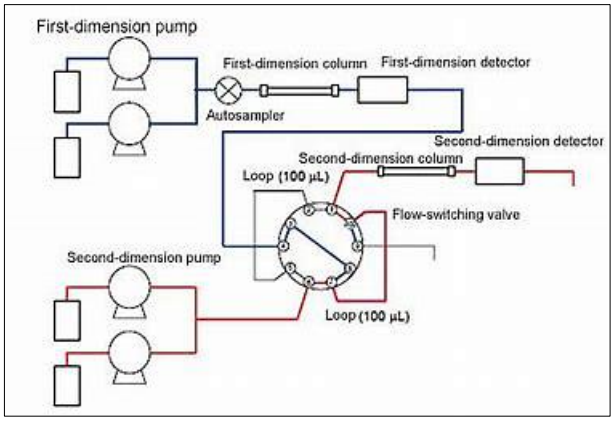


**Fig 3:** Schematic representation of both 1D and 2D LC

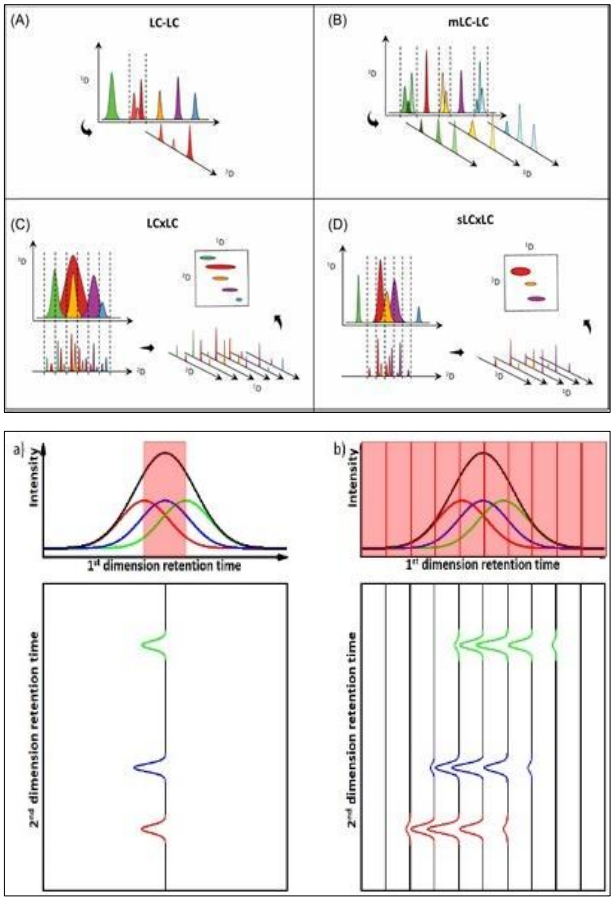


**Fig 4:** Difference between both 1D and 2D LC

**Advantages of Two-dimensional LC:** Improved resolution using two orthogonal separation methods, increased peak capacity, cleaner sample analysis, enhanced sensitivity, applicable to various applications, ensures accurate component identification.

**Applications of 2D LC**
**Case studies 1**
**Title:** A Standardized 2D-LC Screening Platform for Peak Purity Determination in Pharmaceutical Analysis.
**Journal:** The Journal of Chromatography A
**Author name:** John T.
**Columns:** C18 (2.1x50 mm, 2 µm)
**Mobile Phase:** Mobile phase A-0.1% Trifluoroacetic acid, 25 mM ammonium acetate pH 4.5 or 25mM ammonium acetate pH 6.8.
Mobile phase B-Acetonitrile and 6-minute gradient elution

**Wavelength:** 280 nm
**Injection volume:** 3 µL
**Flow rate:** 1 mL/min.

**Conclusion**
**2D-LC screening:** Determines API purity, successfully separated API/impurity mixtures in all ten test cases, evaluates peak purity analysis effectiveness, analyzes screening parameters (column and mobile phase choice).

**Case studies 2**
**Title:** Development of an online capillary comprehensive 2D-LC system for the analysis of proteome samples.
**Journal:** Journal of Separation Science
**Author name:** Eduardo sommella
**Columns:** D1 separations-C18 column (150 0.3 mm, 2.7 mm i.d), D2 separations-18 column (100 0.32 mm, 5 mm)

**Mobile Phase**
**D1 mobile phase:** D1-water/acetonitrile 90:10 v/v having pH 9, D2-water/acetonitrile 10:90, v/v having pH 9.
**D2 mobile phase:** D1-0.1% Trifluoro acetic acid in water/acetonitrile 90:10 v/v having pH 2, D2-0.1% trifluoro acetic acid in water/acetonitrile 10:90v/v having pH 2.
**Wavelength:** 214 nm
**Flow rate:** D1- 1.5mL/min, D2-12 mL/min
**Sample:** proteome

**Conclusion**
RSD values: 0.8-6.0% for primary dimension, 1.0-3.0% for secondary dimension. High retention time reproducibility. Peak area RSD < 9.5%.

**Case studies 3**
**Title:** Cross Referencing 2D-LC Determined of Intact Gliptins in Urine.
**Journal:** Journal of chromatographic science
**Author name:** M Amal Mahamad
**Column:** Gemini C18 column having 250.0 × 4.6 mm i.d., 110 A0, 5.0 µ.
**Mobile Phase:** Methanol & Aqueous solution of 10.00mMo-PA solution
**Wavelength:** 212, 225, 237 and 250 nm
**Flow rate:** 1 ml/min
**Sample:** Urine

**Conclusion**
**Calibration curves:** Linear over 0.10-100.00 µg/mL, good regression coefficients. Method applicability: Useful for forensic medical case screening and therapeutic drug monitoring of gliptins.

**Mass spectroscopy**
**Instrumentation**

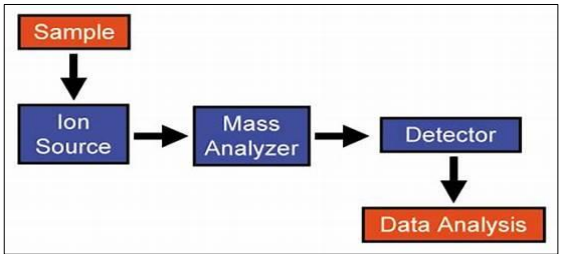


**Fig 5:** Diagrammatic illustration of mass spectroscopy

**1. Sources of Ion**
Ion sources are essential for liquid-phase analytes to become gas-phase ions in mass spectrometry. Used in both primary and secondary dimensional LC-MS.
1. Electrospray Ionization (ESI)
2. Atmospheric Pressure Chemical Ionization (APCI)
3. Photoionization of Atmospheric Pressure (APPI)
4. Direct Liquid Introduction (DLI)
5. Thermospray Ionization

**Electrospray Ionization (ESI):** ESI Produces ions by high voltage, "soft" ionization method, preserves molecular ion, minimal fragmentation. 2D LC-ESI Liquid sample introduced via capillary, high voltage applied, solvent evaporates, droplets break apart, ions released, ions analyzed by mass spectrometer.

**Atmospheric Pressure Chemical Ionization (APCI):** APCI Ionizes analytes using atmospheric pressure corona discharge, "soft" ionization method for less polar substances. 2D LC-APCI Liquid sample nebulized into droplets, heated nebulizer evaporates droplets, corona discharge ionizes molecules, ions analyzed by mass spectrometer.

**Photoionization of Atmospheric Pressure (APPI):** Ionizes analytes using VUV radiation, "soft" ionization method for non-polar molecules. 2D LC-APPI: Liquid sample nebulized into droplets, heated nebulizer vaporizes droplets, vaporized sample subjected to VUV radiation, analytes ionized directly or through dopant, ions analyzed by mass spectrometer.

**Direct Liquid Introduction (DLI):** DLI Direct connection between mass spectrometer and liquid chromatograph, reduces sample loss and simplifies UI. Liquid effluent directly enters mass spectrometer ion source for ionization. Capillary tube and nebulizing gas used. Ionized analytes analyzed by mass spectrometer.

**Thermospray Ionization:** Heats LC effluent to mist, ionizes for gas-phase ions (soft ionization). 2D LC-Thermospray-Liquid vaporized in capillary, ionized by electrode, solvent evaporates, ions to mass spectrometer.

**2. Mass analyzer**
Mass analyzers in 2D LC-MS Separate ions based on m/z. Quadrupole mass analyzer-Four cylindrical rods, ions separated by electric fields, specific m/z selection possible.
**Time of flight:** Ions accelerated by electric field, velocity determined by mass-to-charge ratio, time to reach detector measured, mass-to-charge ratio calculated.

**3. Detector**
The quantity of ions with a certain mass is counted using a detector. The quantity of different-mass ions that went through the mass analyzer is plotted on a graph called the mass spectrum.

**Electron multiplier:** Used for ion currents less than 10-15 amp. Detects ion signals from mass analyzer.
**Principle:** Works based on secondary electron emission. Charged ion/particle/electron collision causes surface atoms to emit secondary electrons. Number of secondary electrons

depends on impact particle type, energy, and surface properties.

**Detector design:** In mass spectrometry, electron multipliers are often designed with one of two fundamental designs:
1. The distinct electron multiplier for a –dynode.
2. Dynode electron multiplier in continuous mode.

**Faraday cup:** Conductive metal cup used to collect charged particles in vacuum. Measures current to determine ion/electron quantity. Ion impact: Neutralizes ions, generates small current, correlates with ion count. Bridge between electrons and ions: Relates electric current to ion charge. Ion count: N/t = I/e for continuous ion stream. Electron beam: Generates current when electrons strike cup. Accuracy: Highly accurate but less sensitive than electron multiplier detectors.

**Upgrades of 2D LC-mass spectrometry**

**Study of case 1**

**Title:** Development and validation of a multi-class analysis of pesticides in corn products by comprehensive 2D LC-MS
**Journal:** Chromatography Journal
**Author:** Laura Martín-Pozo
**Injection:** 1.0 µl
**Injection temperature:** 350 ℃
**Column:** 4.6 mm internal diameter of C18 column.
**Oven programme:** 30 degree celsius
**Sample:** corn

**Conclusion**

**LOQ values:** Lower than European law limits (500 µg/kg). Precision: Within-day < 12.9%, across-day < 15.1%. Recovery: > 70% for 70-120% range at 50, 500, 1000 µg/kg, SD < 20%. Matrix effect: 13-161%. Real sample analysis: Detected 3 pesticides in trace amounts. Future applications: Potential for handling complex matrices like corn products.

**Case studies 2**

**Title:** Enhanced sensitivity and multiplexing with 2D Liquid chromatography/MRM-Mass spectrometry and labeled standards for deeper and more comprehensive protein quantitation
**Journal:** Proteomics Journal
**Author:** Christoph H. Borchers
**Injection:** 1.5 µl
**Column:** BEH300 column having size 4.6 × 150 mm, 5 µm
**Mobility phase:** Water and ACN
**Sample:** Protein

**Conclusion**

2D LC/MRM-MS study demonstrates improved sensitivity for detecting mid-to-low abundance proteins in human plasma without antibodies or pre-fractionation.

**Case Studies 3**

**Title:** Determination of oxyntomodulin, an anorectic polypeptide, in rat plasma using 2D-LC–MS coupled with ion pair chromatography
**Journal:** Chromatography journal
**Author:** Matthew S. Halquist
**Injection:** 30 µl
**Column:** First dimension- Advance chromatographic technique column C8, 5 m, 2.1 mm × 50 mm
Second dimension-Waters C18 Column, 5 m, 2.1 mm × 100 mm
**Mobile phase:** First dimension- 0.1% NBA having pH 4.7 (v/v) in acetonitrile
Second dimension-80:20 acetonitrile: water
**Sample:** Plasma

**Conclusion**

**Method validation:** Reversed estimated residuals -8.6-6.0%, linear range 1-1000 ng/mL, average R2 0.992, intra- and inter-day precision/accuracy ±17%. Rat plasma application: Demonstrated method's usefulness for oxyntomodulin analysis.

**Case studies 4**

**Title:** Development and validation of an efficient automated method for the analysis of 300 pesticides in foods using two-dimensional liquid chromatography–tandem mass spectrometry
**Journal:** Chromatography journal
**Author:** Stefan Kittlaus
**Column:** Primary dimension- YMC Pack Diol, dimensions 2.1 mm x 100 mm, 5 m, 120. A second dimension- 2.1 mm × 100 mm; 2.7 m; 120˚ Agilent Poroshell 120 EC-C18
**Column temperatures**-30 ℃.
**Injection:** 5 µl.
**Mobility phase:** ACN/Water (90:10)
Primary dimension contains ACN/water in 90:10
**Sample:** lemon, cucumber, rocket, wheat flour, and black tea.

**Conclusion**

**Recovery rates:** 70-120% for most analytes, RSD < 20%. LOD: 0.01 mg/kg or lower for almost all chemicals, good sensitivity for > 50% analytes at 0.001 mg/kg. Matrix effects: Demonstrated strong results with tea and hops matrices.

**Case studies 5**

**Title:** Screening active anti-breast cancer compounds from Cortex *Magnolia officinalis* by 2D LC–MS
**Journal:** Journal of separation science
**Author:** Xiaofang Hou
**Column:** First dimension: Column 10 mm × 2.0 mm id, 5 m; MDA-MB-231/CMC
**Second dimension:** VP-ODS column (150 mm×2.0 mmid, 5 m).
**Mobile phase:** Water and Methanol
**Column temperature:** 37 ℃.
**Injection:** 5 µl.
**Wave length:** 254 nm
**Sample:** Honokiol, magnolol

**Conclusion**

**Inhibition rates:** Honokiol and magnolol inhibited MDA-MB-231 cell growth at 23 and 64 M, respectively. Conclusionis Combined analytical method could be valuable for drug discovery strategies.

**Conclusion**

The most common pairing of MS with LC × LC gives compounds additional dimension of classification. High selectivity is maintained throughout the chromatogram by MS, which also offers structural details for clear

identification. Numerous kinds of compounds exhibit distinct fragmentation patterns in the mass spectrum, providing insightful details regarding the substances that have spectral libraries connected to them in literature.

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