Hyphenation of Liquid Chromatography and Nuclear Magnetic Resonance

The progress of systems where a Nuclear Magnetic Resonance detector has been coupled to Liquid Chromatography separation.

by

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04 October 2014, Amsterdam
Abstract
Nuclear magnetic resonance hyphenated liquid chromatography is a powerful but expensive tool that originally was very limited in its applications due to the state of technology at the time. Nowadays nuclear magnetic resonance hyphenated liquid chromatography is a powerful tool used to separate and unambiguously identify and characterize components inside mixtures. The method is used most efficiently in screening purposes and analysis of complex samples. Fields of applications range quite wide from analysis and identification of impurities in drug discovery studies in the pharmaceutical chemistry to the characterization and identification of complicated extracts from plants (natural products) in phytochemistry or creating reference libraries in combinatorial chemistry. The system is quite versatile and powerful but requires quite some knowledge to use properly and is costly to purchase and maintain.

This literature report describes the progress of hyphenating the popular separation method liquid chromatography with the powerful identification method nuclear magnetic resonance. A variety of applications of the method is discussed as well, including the the advantages and disadvantages over conventional liquid chromatography and nuclear magnetic resonance operations and its limitations. Attention has also been given to the development of the required instrumental components, the potential of relatively new separation methods and the additional hyphenation of the hyphenated systems to other secondary detectors.
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>LC, HPLC</td>
<td>Liquid Chromatography, High Pressure Liquid Chromatography, High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid Phase Extraction</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra(high) performance Liquid Chromatography</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>FT</td>
<td>Fourier Transformation</td>
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<tr>
<td>2D</td>
<td>Two Dimensional</td>
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<tr>
<td>INEPT</td>
<td>Insensitive Nuclei Enhancement by Polarization Transfer</td>
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<tr>
<td>NOE</td>
<td>Nuclear Overhauser Effect</td>
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<tr>
<td>NOESY</td>
<td>Nuclear Overhauser Effect Correlation Spectroscopy</td>
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<tr>
<td>COSY</td>
<td>Correlation Spectroscopy</td>
</tr>
<tr>
<td>TOCSY</td>
<td>Total Correlation Spectroscopy</td>
</tr>
<tr>
<td>INADEQUATE</td>
<td>Incredible Natural Abundance Double Quantum Transfer Experiment</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared Spectroscopy</td>
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<tr>
<td>MS</td>
<td>Mass Spectrometry, Mass Spectrometer</td>
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<tr>
<td>SPEC</td>
<td>Solid Phase Extraction Chromatography</td>
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<td>SFE</td>
<td>Supercritical Fluid Extraction Chromatography</td>
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<tr>
<td>SFC</td>
<td>Supercritical Fluid Chromatography</td>
</tr>
<tr>
<td>CapLC</td>
<td>Capillary Liquid Chromatography</td>
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<tr>
<td>UV</td>
<td>Ultraviolet light Spectroscopy</td>
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<tr>
<td>VIS</td>
<td>Visible Light Spectroscopy</td>
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<tr>
<td>RI</td>
<td>Refractive Index</td>
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<tr>
<td>PDA, DAD</td>
<td>Photodiode Array Detector, Diode Array Detector</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>RF</td>
<td>Radiofrequency</td>
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<td>DI</td>
<td>Direct Injection</td>
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<tr>
<td>FIA</td>
<td>Flow Injection Analysis</td>
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<tr>
<td>FFF</td>
<td>Field Flow Fractionation</td>
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<tr>
<td>IFFF</td>
<td>In Flow Flow Fractionation</td>
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<tr>
<td>ThFFF</td>
<td>Thermal Field Flow Fractionation</td>
</tr>
<tr>
<td>CFFF</td>
<td>Centrifugal Field Flow Fractionation</td>
</tr>
<tr>
<td>SFFF</td>
<td>Sedimentation Field Flow Fractionation</td>
</tr>
<tr>
<td>AF4</td>
<td>Asymmetry Flow Field Flow Fractionation</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
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<td>CEC</td>
<td>Capillary Electrophoric Chromatography</td>
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<tr>
<td>iCTP</td>
<td>Capillary Isotachophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size Exclusion Chromatography</td>
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<tr>
<td>GPC</td>
<td>Gel Permeation Chromatography</td>
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<tr>
<td>GFC</td>
<td>Gel Filtration Chromatography</td>
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<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>PRESAT</td>
<td>Transmitter Presaturation</td>
</tr>
<tr>
<td>WATERGATE</td>
<td><em>Water Suppressions By Gradient Tailored Excitation</em></td>
</tr>
<tr>
<td>WET</td>
<td>Water Suppression Enhancement Through T₁ (spin-lattice relaxation) Effect</td>
</tr>
<tr>
<td>BPPSTE</td>
<td>BiPolar Gradient Pulsed STimulated Echo</td>
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<tr>
<td>DOSY</td>
<td>Diffusion Ordered Spectroscopy</td>
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<tr>
<td>TOF</td>
<td>Time Of Flight Mass Spectrometer</td>
</tr>
<tr>
<td>TQ</td>
<td>Triple Quadruple Mass Spectrometer</td>
</tr>
<tr>
<td>ICR</td>
<td>Ion Cyclotron Resonance Mass Spectrometer</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>ICP</td>
<td>Inductively Coupled Plasma</td>
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<tr>
<td>CD</td>
<td>Chiral Dichroism</td>
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<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>MMD</td>
<td>Molecular Mass Distribution</td>
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<tr>
<td>CCD</td>
<td>Chemical Composition Distribution</td>
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<tr>
<td>FTD</td>
<td>Functional End Type Distribution</td>
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<tr>
<td>LAC</td>
<td>Lectin Affinity Chromatography</td>
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<td>LCCC</td>
<td>Liquid Chromatography Under Critical Conditions</td>
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1. Introduction

1.1. Liquid Chromatography

Liquid Chromatography (A term that reads “color writing” when translated from Greek or what might be a spin on the Russian translation of the creators surname (Tswett). Tswett means color in Russian giving the translation of “Tswett’s writing”), is the separation of compounds by means of a liquid. First conceived by the Russian Botanist, Mikhail S. Tswett [1], as part of an plant pigment study between 1896-1906 to separate colorful compounds extracted from plant life (plant pigments) by means of solvents extraction. Tswett experiments relied heavily on the use of gravity and the affinity interaction that the compounds of interest had with the solid particles he used to retaining said compounds on to the particles. When liquids were introduced in to the column that had a better affinity to a compound of interest then that compound had to the particles, they would elute together with the liquid. Due to big differences between liquid affinities in-between compounds of interest a separation could be established. Chromatography can be divided in two types of techniques. The first type involves chromatography done on a two-dimensional plane, known as Thin Layer Chromatography (TLC). In TLC compounds are separated on coated plate drenched in liquid of opposite polarity (compared to the polarity of the coating). Due to difference in compound polarity compounds that that has more affinity to the liquid will be carried further on the coated plane by the liquid, while compounds with more affinity to the coating would not be carried as far. In such a way a separation is established between compounds. This type of chromatography is quite time consuming and only has limited applications and resolving power. The second type of chromatography utilizes column or cartridge devices filled with an adsorption material and is known as column chromatography. Column chromatography is the type Tswett used to separate his plant pigments. This type of chromatography produces better separations and allows for a fractionated approach so that compounds can be separated from the mixture one at a time. The mechanism is the same, but the compounds of interest are retained instead by the adsorption material (packing particles). When the solute-liquid interaction becomes strong enough the compound of interest desorbs from the packing material in to the liquid. In order to create a liquid flow that pushes the liquid through the column either pressure or gravity is used. The higher separation prowess in column chromatography comes from the larger contact area with the coating (packing material) when compared to the two dimensional surface in TLC. When the degree of packing (amount of adsorption material) increases so does the resolving power of this method. By replacing the adsorption material inside the column with an adsorption filter the same kind of separation is achieved. The latter is still used as frequent clean-up step in many analyses and is known under the name of Solid Phase Extraction (SPE). To obtain even higher resolving power the contact area with the adsorbent needed to be increased, which ultimately meant higher pressure were needed to create good liquid flows. [2] In the 1970 this was achieved by High Pressure Liquid Chromatography (HPLC), using tubular columns packed with very small adsorbing particles (diameters <10 µm) that use high pressures from 35 bar up to 400 bar to create proper liquid flows. Due to the great resolving power of the improved technique Horváth and Lipsky [3] changed the name from High Pressure Liquid Chromatography to High Performance Liquid Chromatography (HPLC). Additions of several mechanical components, such as an oven, pumps and better detectors decreased analysis time and increased the quality of the analysis. The oven gave the ability to run temperature programs. The pumps meant gradient analysis could be done online by mixing solvent. Use of spectroscopy as a detection method meant low concentrations could be measured; compounds could easily be identified, separated and quantified.
with Mass Spectrometry (which is a much more sensitive detector that allows one to detect only specific masses) even incomplete separations of different compounds eluting at the same time could be quantified. It became one of the most predominant used analytical techniques in analytical chemistry. Nowadays extraordinary high pressures (1000 bar) and small particles (diameters < 1.7 µm) are used and we are able to work with such ultrahigh resolving power that we can reach amazing performances. The additional resolving power made people rename this form of HPLC to Ultra Performance Liquid Chromatography (UPLC). Throughout the years many different modes of separations have been developed using different polarities of adsorbents and different affinity principles to allow for separations of virtually every liquid dissolvable compound. The most common mode, Reverse Phase Chromatography uses nonpolar adsorbents to separate compounds based on their polarity. Other common modes include Normal Phase, Ion Exchange, HILIC/HIC, Size Exclusion and Bio-affinity Chromatography. Which allow respectively for the separation of polar, charged, hydrogen-bonded, differently sized, and biologically active complex-forming compounds. Two-dimensional liquid chromatography combines multiple modes with each other to allow even further separation that was previously impossible. [4]

1.2. Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) is a technique based upon the discovery of the physicist Bloch [5] and Purcell [6] in 1945 that a condensed phase in a magnetic field creates a magnetic resonance moment that can be detected. This magnetic moment is the result of energy transitions that occur because nuclei change their spin orientations in a magnetic field. However the nuclei do need to have a spin (quantum magnetic moment) of their own in order to be influenced by the external magnetic (radiofrequency) field, which is only the case for atoms that have unequal amount of protons and neutrons (equal protons and neutrons give a quantum spin number of zero). Those compounds tend to be mostly isotopes. The most used nuclei are $^1$H, $^2$H(D), $^{13}$C, $^{14}$N, $^{19}$F, $^{35}$Cl, $^{31}$P and a large amount of (isotopic) metals. In order to measure nuclei a high sensitivity is desired. The sensitivity is determined by two things, namely the strength of the applied external magnetic field (the higher the field the better) and the susceptibility of the nuclei. The latter depends on the amount of the nuclei present within the sample and the magnetic moment of the nucleus. When the nuclei have quantum spin numbers with larger deviations from zero, they will have a stronger field and as such are more easily influenced by another field. The amount of nuclei present is directly relatable to the abundance nature. More nuclei within the detection cell will create stronger signals. [7] While originally only condensed phases could be analyzed due to lack of understanding of the process and mechanical restraints, liquid phases and diluted phases become viable samples as technology and research on the field progressed. A few years after 1945 research showed that neighboring nuclei (which have spin orientation of their own) influence each other’s magnetic moment (coupling constant) and that the relative frequency (chemical shift) of the moment in comparison to the applied magnetic (radiofrequency) field is determined by the amount of electron shielding present around the nuclei. This gave the technique good potential to be used in structure elucidation of (complex) organic compound. [8] Further research on scalar coupling showed how nuclei (spins) influenced each other through bonds. And by means of double resonance (also known as spin-spin decoupling) the signal splitting induced by neighboring nuclei with individual orientations could be undone. Double resonance relied on a secondary external magnetic (radiofrequency) field that forces all nuclei in to the same spin orientation, which diminishes the signal splitting significantly. [9] Nuclei sensitivity was increased by use of polarization transfer processes such as
INEPT (Insensitive Nuclei Enhancement by Polarization Transfer) that rely on transfer of polarization energy from very sensitive nuclei to insensitive nuclei by means of scalar coupling (nuclei-interaction through bonds). This became the basis for the nowadays applied pulse sequences. These pulse sequences use a combination of long and short pulses to create the strongest possible magnetic moments, which generated higher signals. Better computers and superconductive magnets made the technique even more applicable and allowed for complicated computer-solved algorithms, like Ernst and Anderson’s Fourier Transform technique [10], to be used resolve the complicated NMR-spectrum in manner that more information could be extracted from it. Despite all of this the technique remained complicated and elucidating a structure was generally like solving complicated puzzles; A fierce task. The use of FT allowed Aue, Ernst and Bartholdi [11] to implement two-dimensional NMR (2D-NMR), which is a NMR technique that gives us an absolute structure elucidation. The high resolving power that arises from combining individual 2D-NMR techniques that correlate signals-to-bonds and signals-to-space makes any structure elucidation a simple problem. The principle of 2D-NMR is applying two separate radiofrequency pulse-sequences with different pulse-times on the sample that create complex spectra that after FT give a grid where each bond that influences a signal creates an individual peak separated from the other signals. This gives chemist a map that shows how the puzzle-pieces are connected. This is known as correlation spectroscopy (COSY) and has several variations depending on the type of nuclei or the amount of types of nuclei used. A different type of NMR created by Overhauser [12] to improved the signal-to-noise ratio ended up being used to measure the dipolar coupling (spin-spin coupling through space). This became known as the Nuclear Overhauser Effect (NOE), which like INEPT increases the sensitivity of insensitive Nuclei. The difference with INEPT is that NOE uses the transfer of polarization energy over space (by means of dipolar moments between nuclei) and not the polarization energy through bonds. By NOE protons with distances of 5 angstrom could be distinguished. The 2D-NMR that uses NOE Nuclear Overhauser Effect Correlation Spectroscopy (NOESY) gives us the ability to identify what kind of stereochemistry the molecules have. Many different forms of NOESY and COSY were made for different nuclei combinations. Each of these techniques has their own respective sensitivity. Incredible Natural Abundance Double Quantum Transfer Experiment (INADEQUATE) needs special mentioning, because it shows how the carbon skeleton is build-up in a single experiment. This technique does that by defining all the carbon-carbon bonds within the experiment, but due to the low abundance of the suitable carbon-nuclei the signal is very low and the experiment requires a lot of sample and a lot of time to be performed successfully. [9] An overview of the most common 2D-NMR detection modes, including detection times is given in figure 1.1.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Nuclei</th>
<th>Coupling type</th>
<th>Correlation range</th>
<th>Remarks</th>
<th>Spectrometer time typically needed</th>
</tr>
</thead>
<tbody>
<tr>
<td>COSY</td>
<td>$^1\text{H}$</td>
<td>Bond mediated scalar coupling</td>
<td>Usually over 2–3 bonds</td>
<td>COSY spectra are often crowded and hard to interpret</td>
<td>&lt;1 h</td>
</tr>
<tr>
<td>TOCSY</td>
<td>$^1\text{H}$</td>
<td>Relayed bond mediated scalar coupling</td>
<td>All $^1\text{H}$ nuclei within a scalar coupling network</td>
<td>TOCSY spectra are often crowded and hard to interpret</td>
<td>&lt;1 h</td>
</tr>
<tr>
<td>NOESY, ROESY</td>
<td>$^1\text{H}$</td>
<td>Dipolar coupling through space</td>
<td>Typically &lt;5 Å. Absent correlations must not be understood as $^1\text{H}/^1\text{H}$ distances &gt;5 Å.</td>
<td>NOESY works best with small molecules and is replaced with ROESY for higher molecular weights.</td>
<td>Several hours</td>
</tr>
<tr>
<td>HSQC, HMQC</td>
<td>$^1\text{H}$, $^{13}\text{C}$</td>
<td>Bond mediated scalar coupling</td>
<td>Strictly one $^1\text{H}/^{13}\text{C}$ bond</td>
<td>Correlates the $^1\text{H}$ network with the $^{13}\text{C}$ scaffold</td>
<td>Several hours</td>
</tr>
<tr>
<td>HMBC</td>
<td>$^1\text{H}$, $^{13}\text{C}$</td>
<td>Bond mediated scalar coupling</td>
<td>Usually over 2–3 bonds</td>
<td>Correlates the $^1\text{H}$ network with the $^{13}\text{C}$ scaffold</td>
<td>Over night</td>
</tr>
<tr>
<td>HSQC-TOCSY</td>
<td>$^1\text{H}$, $^{13}\text{C}$</td>
<td>Bond mediated scalar coupling</td>
<td>All $^1\text{H}$ and $^{13}\text{C}$ nuclei within a scalar coupling network</td>
<td>Combines the $^1\text{H}/^{13}\text{C}$ correlation with the $^1\text{H}$ coupling network</td>
<td>Over night</td>
</tr>
<tr>
<td>INADEQUATE</td>
<td>$^{13}\text{C}$, $^{13}\text{C}$</td>
<td>Bond mediated scalar coupling</td>
<td>One bond</td>
<td>Correlates the $^{13}\text{C}$ scaffold</td>
<td>Several days</td>
</tr>
<tr>
<td>ADEQUATE</td>
<td>$^{13}\text{C}$, $^{13}\text{C}$</td>
<td>Bond mediated scalar coupling</td>
<td>One bond</td>
<td>Correlates the $^{13}\text{C}$ scaffold</td>
<td>One to two days</td>
</tr>
</tbody>
</table>

* For any NMR experiment, the data acquisition time depends on the concentration and complexity of the analyte as well as on the field strengths of the employed magnet. Given figures are approximations for about 3 mg of a 500 Da compound (~10 mM solution in 500 µl NMR solvent) measured on a 600 MHz spectrometer in conventional 5 mm NMR tubes.

*Figure 1.1 An overview of the most common two-dimensional NMR detection modes. [13]*
2. Liquid Chromatography hyphenation with Nuclear Magnetic Resonance (LC-NMR)

2.1. Introduction
For a long time chemists have searched for alternative detectors that can replace the traditional LC UV-detector. While UV-detectors are very sensitive detectors they are unfortunately very limited in giving information about the structure of the eluting compound. Detectors that provide great inside in to the chemical structure of the compound, such as Infrared-spectroscopy (IR), Mass Spectroscopy (MS) and Nuclear Magnetic Resonance (NMR) would be preferred. From these three detection techniques NMR is the technique that allows for the best structure elucidation and can detect basically any compound. [13] Further strengths of NMR are that it can differentiate between isotopes, regardless whether they differ in an optical, conformational or structural manner and give an definite identity to the compound that cannot be argued about.

Another big strength of NMR is that it can be used as a quantitative technique, because the signals in NMR can be assigned to a specific kind of nuclei (with their own specific environment). The disadvantages of the technique is that in comparison to other spectroscopic techniques the intensity of the signals are quite low and that structure elucidation of mixtures for certain nuclei (such as $^1$H) that are abundant within the components of the mixture are so complex that it is virtually impossible to solve, unless some kind of sample preparation is applied. The sensitivity problem can be easily solved by employing strong superconducting magnets as the signal is proportional to the strength of the magnetic field. Regarding the complexity of the sample the only real choice is to use separation methods to turn a single complex sample mixture in to multiple simple samples. The logical choice of separation method falls on liquid chromatography, because this technique has many forms of applications (modes), is universally apply-able and can separate the sample in to multiple different manners depending on the mode of separation chosen. Once the mixture is separated all components need to be individually analyzed by the NMR. To do this offline requires a lot of manpower and time. The most efficient would be to analyze the compound directly as they separate from the mixture. This form of on-line analysis requires the two instruments to be connected to each other. This process can be rather tricky and challenging as both machines operate on completely different bases that might contradict each others. The process of connecting the individual instruments in such a manner that both techniques produce improved resolving prowess, while sorting out any (practical) problem that may arise from the connection is known as hyphenation or coupling of techniques. Besides that we also use the term hyphenation and coupling to refer to the research done were the combined techniques are being improved to higher standards that are unreachable with just the individual techniques. The first LC-NMR hyphenation experiments were conducted in the late 1970s, only a few years after the introduction of High Performance Liquid Chromatography [14]. But the insufficient strength of the magnets back then along with the dynamic range problem and the loss of separation efficiency due to the long distances that the liquid needed to be transported over between instruments created big difficulties in connecting the two techniques. The size/shape of the detector cell, which is larger than traditional liquid chromatography detectors, had to be specifically designed in order to reduce these problems. The dynamic range problem is basically a solvent interference problem. The solvent interference comes from the fact that in order to properly analyze a NMR spectrum a homogeneous magnetic field is required over the entire sample, but because most HPLC solvents contains a lot of nuclei of interest
(protons) that are influenced by the external magnetic field and make the field become heterogeneous. This makes obtaining a usable spectrum difficult, and for that reason isotopic proton solvents, such as heavy water (D₂O), are used instead. The isotopic solvents influence the desired signals significantly less allowing for the obtaining of usable NMR spectra. Originally researchers tried to use different pulse sequences to solve the problem, but those were not enough to obtain viable results. Because of these problems both “offline” and online LC-NMR was investigated. In offline LC-NMR the sample would be first separated in a commercial LC device, after which the separated components are collected separately and analyzed individually in a commercial NMR device. The main problem with this is that sample purification is usually needed as the collected fraction has very low concentrations. [15]

2.2. “Offline” coupling of LC-NMR

Conventional “offline” LC-NMR consist out of using commercially available LC devices to separate problematic mixtures into its components. These components are then collected in containers as they elute from the separation device. After a possible purification step these pure compounds are placed into the NMR and are analyzed as any other sample. Note that offline is written with hyphens to indicate that it is not really a hyphenated technique, because hyphenated techniques eliminate the human element from the equation. In “offline” LC-NMR we’re basically performing a sample preparation and a sample analysis step, which can only be justly described as a normal analysis. The term “offline” is however used in literature to avoid confusion with online LC-NMR. While nowadays online LC-NMR is superior to “offline” LC-NMR in the past the opposite was true. The “offline”-method is a very time-consuming approach, mainly because it involves a lot of steps and depending on sample, the separation mode and the NMR analysis mode both the separation and NMR-detection steps could be very time-consuming. This is especially the case for 2D-NMR methods, where the detection time is directly proportional to the quality of the analysis, which tends to take quite long per sample and is essential when a compound structure needs to be elucidated. [15] Using Solid Phase Extraction (SPE) devices as a separation column the concentration of the sample can be increased, which is needed since the sensitivity of NMR is so bad that unless pure compounds are measured no good signals are obtained. As shown by Wilson and Nicholson [16].

2.2.1. Solid Phase Extraction Chromatography-NMR (SPEC-NMR)

Solid Phase Extraction Chromatography uses a simple polypropylene syringe shaped tube filled with adsorbent materials that retains all analytes of interest. Usually the adsorbent applied is similar to the stationary phases found in reverse phase LC or TLC, namely C₁₈-coated silica material, since most analytes are nonpolar organical compounds. In order to elute the compounds a nonpolar solvent is used that has stronger interaction with the compound of interest than the adsorbent. In case when multiple compounds are present a sequence of solvents is eluted to the syringe (which differ slightly in polarity from each other). Starting with a polar solvent such as water and ending with a nonpolar solvent such as methanol. This type of gradient elution sequence will elute each compound one-by-one, but only when the analytes differ in polarity from each other. By using as little liquid as possible the sample is pre-concentrated at the same time and is pure enough to be directly put into the NMR and get usable results. The procedure has been applied successfully for drug metabolites in urine [16] and since then was common practice until online-LC-NMR become superior to “offline” LC-NMR. This was when “offline” LC-NMR could not provide the resolving power required to solve mixtures with more than 3 components. The main cause of this was that the degree of purity needed could not be obtained using this method, and as such the NMR sensitivity was too low to create viable
NMR-spectra that could be used for compound identification. Nowadays many online-LC-NMR have a SPE device between the two instruments in order to reduce unwanted longitudinal diffusion, concentrate the analyte and allows for simultaneous analysis of low concentration samples by Mass Spectrometry and NMR after LC separation. [17]

2.2.2. Supercritical Fluid Extraction Chromatography-NMR (SFE-NMR)

Supercritical fluid extraction chromatography (SFE) is simply SPEC where a supercritical fluid is utilized to obtain a separation instead of basic fluid. Supercritical fluids are fluids that exist in an intermediate phase between the liquid and gas-phase once the temperature and/or pressure exceed the critical point. The critical point is the pressure-temperature point after which no phase distinction can be made between the liquid and the gas phase of a compound (see figure 2.1 the phase diagram).

Supercritical liquids have properties belonging to both the liquid-phase and the gas-phase of the compound. Most notably advantages of supercritical fluids are higher diffusion coefficients than the liquid phase, and that the transport-processes within supercritical fluids are proportional to its density. The density of the supercritical fluid in turn is very sensitive to the applied pressure and temperature. Since the solubility is an exponential function depended on the density of the compound, we can easily manipulate the solubility of a supercritical fluid by changing either the pressure and/or the temperature slightly. Another great advantage is that supercritical fluids leave no contamination or residue once it has been fully forced in to its gas-phase (allowed to evaporate). The results are faster separations and higher quality extractions. [18] From all the possible supercritical fluids the most commonly used fluid is carbon dioxide (CO₂). This has two reasons. The first is that has an low critical temperature (31°C) and a critical pressure (73 bar) that can be easily utilized without needing much special equipment as opposed to other liquids (see figure 2.2 an overview of critical properties of some liquids). The second is that CO₂ only reacts too few compound and for most applications is a solvent that does not alter the chemical make-up of your components of interest. Since CO₂ is a non aqueous solvent an organic modifier must be added to the solvent to make it suitable for dissolving more polar compounds. The most common modifier used is Methanol.

<table>
<thead>
<tr>
<th>Critical constants*</th>
<th>p/ atm</th>
<th>V/m³/mol</th>
<th>T/K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia, NH₃</td>
<td>111</td>
<td>73</td>
<td>406</td>
</tr>
<tr>
<td>Argon, Ar</td>
<td>48</td>
<td>76</td>
<td>181</td>
</tr>
<tr>
<td>Benzene, C₆H₆</td>
<td>46</td>
<td>260</td>
<td>563</td>
</tr>
<tr>
<td>Bromine, Br₂</td>
<td>102</td>
<td>135</td>
<td>594</td>
</tr>
<tr>
<td>Carbon dioxide, CO₂</td>
<td>7.3</td>
<td>94</td>
<td>304</td>
</tr>
<tr>
<td>Dichlorine, Cl₂</td>
<td>76</td>
<td>124</td>
<td>417</td>
</tr>
<tr>
<td>Ethane, C₂H₆</td>
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<td>148</td>
<td>305</td>
</tr>
<tr>
<td>Ethene, C₂H₄</td>
<td>51</td>
<td>124</td>
<td>293</td>
</tr>
<tr>
<td>Hydrogen, H₂</td>
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<td>65</td>
<td>33</td>
</tr>
<tr>
<td>Methane, CH₄</td>
<td>46</td>
<td>99</td>
<td>191</td>
</tr>
<tr>
<td>Oxygen, O₂</td>
<td>50</td>
<td>74</td>
<td>155</td>
</tr>
<tr>
<td>Water, H₂O</td>
<td>218</td>
<td>55</td>
<td>647</td>
</tr>
</tbody>
</table>

*The critical volume V is the molar volume at the critical pressure and critical temperature.

Figure 2.1 (left) The phase diagram of a compound, showcasing the position of the critical point. [18]

Figure 2.2 (right) An overview of the critical points (critical pressure and temperature) of some liquids. [18]
“Offline” SFE-NMR is sometimes preferred over regular “offline” SPEC-NMR, because solvent problems are less apparent. There is no need to deal with expensive isotopic organic solvents, such as isotopic chloroform (CDCl₃) that can’t be disposed in normal manners, and because CO₂ evaporates quickly after decompression very pure compounds can be obtained. Any remaining CO₂ will have little influence in the NMR so that no solvent suppression method has to be applied, especially in ¹H-NMR analysis modes. [15]

2.3. **Online coupling of LC-NMR**

Watanabe and Niki were the first to try and hyphenate liquid chromatography with Nuclear Magnetic Resonance using a self-designed flow-probe utilizing stop-flow operation to analyze a mixture of three components. [19] Which was quite the accomplishment considering the limitations that Nuclear Magnetic Resonance had at the time. The self-designed flow probe had a sample volume of 15 µl and a length of 1 cm. The acquisition per separated component in the NMR did not last longer than 2 hours to ensure no excess broadening was present in the peaks. Solvent suppressions techniques were not available at the time and severely limited the experiments that they could perform. They concluded that further technological development was required to make LC-NMR a useful technique. [19] Bayer et al used an altered flow probe design to conduct stop-flow and on-flow LC-NMR experiments and realized that the resolution of the hyphenated NMR was much lower than the same NMR used unhyphenated. This made the measuring of the vital coupling constants needed for structure deduction hard as barely could be noticed. This changed later on (figure 2.3).

[20] Use of the common reverse phase LC-NMR was difficult because of three reasons. The first was that these separations used more than one protonated solvent; the second reason was that during gradient separations the change in solvent resonance caused huge interference; the third reason was that the signals were very small in comparison to the solvent signals. [20] Smallcombe et al created the WET-solvent suppression technique in 1995, which has the ability to significantly reduce the solvent interference so that LC-NMR became a useful technique. [21] Since then LC-NMR has been further hyphenated by hypanating it to mass spectrometry and other detectors. Nowadays people are able to detect compounds with nuclear magnetic resonance simultaneously with mass spectrometry as the compounds elute out of the chromatograph one by one. Recently further hyphenation with the newer generation of chromatography such as Size Exclusion Chromatography (SEC-NMR) for polymers, Solid Phase Extraction for trace analysis (SPE-NMR) and Capillary Electrophoresis (CEC-NMR and CE-NMR) and Field Flow Fractionated (FFF-NMR) for expensive or nanoliter volume applications. Capillary LC-NMR (capLC-NMR) is traditional liquid chromatography performed on capillaries that just like capillary electrophoresis applications uses micro coils instead of the traditional LC-NMR flow probe detectors and can measure multiple components at the same time. [9]

*Figure 2.3 The change of the NMR magnetic field strength in LC-NMR and unhyphenated NMR over the years. [22]*
The common online LC-NMR system consists out of a standard LC-device connected to a NMR-detection device where a flow-probe is inserted. The LC device consists out of a pump system that pushes liquid solvent through the system, a column where the separation takes place and a detector with flow cell that utilizes light to measure the components as they elute. This light-detector can either be an Ultraviolet-/Visible light (UV/VIS)-detector, a refractive index (RI)-detector or Infrared-light (IR) detector. Most common nowadays is the use of Diode Array Detector (DAD), a type of UV/VIS-detector that can measure multiple light-wavelengths at once. Basically any type of detector can be used, as long as it does not alter or destroy the sample. The NMR system consists out of a huge radiofrequency (RF) - magnet where a non-rotating flow cell has been put oriented vertically. This orientation allows for laminar flow and gets rid of bubbles in the mobile phase easily. The RF coil is wrapped around the cell so that a good filling factor is obtained and the difference in detection volume and coil volume is only the glass that comprises the flow cell. [13] Figure 2.4 illustrates this setup. [22]

Often forgotten but the NMR also allows for compound quantification. Quantification through NMR can be obtained using the following equation, provided an internal standard is added to the mixture:

\[ C = \frac{C_s \times n_s \times F \times M}{F_s \times M_s \times n} \]  

(1)

Where:

- \( C \) : Concentration of the analyte (mol/l).
- \( C_s \) : Concentration of the internal standard (mol/l).
- \( n_s \) : Amount of protons that generate the selected signal of the internal standard (protons).
- \( n \) : Amount of protons that generate the selected analyte signal (protons).
- \( F \) : Area of the analyte signal (signal units).
- \( F_s \) : Area of the internal standard signal (signal units).
- \( M \) : Molecular mass of the analyte (Da).
- \( M_s \) : Molecular mass of the internal standard (Da).

A big advantage of NMR-quantification is that the internal standard does not need be chemically similar to the analyte. As long as you can assign one signal to a compound of known concentration and another signal to your analyte, then you can accurately quantify your component of interest. The only real limitation is that the molecular mass of both your analyte and internal standard must be known values. But this can be easily solved by running your mixture through a liquid chromatograph with a mass spectrometer as detector. Preferably it is better to use this type of quantification after the molecular formulas of the components have been identified. [23]
2.3.1. Nuclear Magnetic Resonance detector flow-probe designs

2.3.1.1. Traditional LC-NMR flow-probe (saddle type)

Standard NMR detection in "offline" LC-NMR was conceived in the 1950s and involved filling a tube 5 mm with sample and suspending this tube in iron coil magnets, while measuring with continuous wave magnetic fields. In order to obtain uniform magnetic fields the sample is rotated. Fast forward 30 years and today we use cryogenic cooled magnets with strong magnetic fields (> 600 MHz) that employ Fourier Transformation and pulse techniques for mostly two-dimensional applications. For those applications the sample can no longer be rotated as it introduces side-bands (distortions) in the obtained NMR-spectra. The first flow-probes appeared around 1980 and had a severely altered design. The flow cells had a U-shaped design with a container that allows for temperature adjusting and was placed upside down inside the NMR. This prevented rotation from taking place. A Helmholtz-type coil is “wrapped” as close as possible to the glass wall. Good results were obtained for this type of cell despite its inability to rotate. This is because the increased quality of the cryogenic magnets provided a better uniform magnetic field compared to the iron magnets. [24] The shape had the added benefit that it promoted laminar flow and eliminated bubble-forming. The material that the first flow cells were comprised of was silanized glass. This was to diminish adsorption and prevent carry-over effects from taking place. The bubble glass had a diameter of 2-4 mm and was placed parallel to the proton detection coil (18 mm). Both ends are tapered with PTFE-tubings fit (0.25 mm ID). The glass cell is connected to the small PTFE-tubing by means of shrink-fit tubing. Between the glass cell and the radiofrequency coils lies a cylinder which can be filled with deuterated liquid, allowing the detection of non-deuterated solvents. Figure 2.5 and 2.6 illustrates schematic sketches of the described NMR cells. [14]

![Image](image.png)

Figure 2.5 (left) The comparison of a traditional (Solenoid) NMR-cell (left) and a common commercial NMR- saddle flow cell (right). [25]

Figure 2.6 (Right) The dimensions of a common commercial saddle NMR-flow cell. [26]

The volume of these cells are generally between 40-120 µl, much larger than the standard 8 µl cells employed within other liquid detection applications. There are two reasons why this size is needed. The first is that the residence time (τ), the time that a part of the liquid containing a nuclei of interest is within the detection cell, must not come below 5 seconds. If it does then the flow will induce line-
broadening and the half-width of the signal will broaden. This in turn lowers the NMR resolution. The residence time, \( \tau \), is defined by the following equation:

\[
\tau = \frac{\text{detection volume}}{\text{flow rate}}
\]  

(2)

Where the detection volume is in \( \mu l \) and the flow rate is in \( \mu l/s \). The second reason is that NMR is a spectroscopic technique, where the signal is proportional to the amount of analyte present in the flow cell. Higher detection volumes will contain more analyte and produce better signals. Unfortunately chromatographic separations require small tubes because the separation quality is reduced over time due to longitudinal diffusion. Therefore later eluting peaks will be spread over a volume bigger than the detection volume, meaning less of it will be inside the flow cell (lesser signals). That is unless a bigger detection volume is used, which in turn ruins separation capacity. For an ideal NMR-resolution this detection volume should be in the order of several millilitres. The diameter of the bubble would end up somewhere between 10-20 mm. In order to maintain some degree of separation, while having a decent NMR-signal a compromise between separation and detection is made resulting in the described design. [25]

The broadening that occurs when the detection volume is less than 120 \( \mu l \) and flow rates are as high as 1 millilitre per minute can be assigned to the decreasing spin-spin relaxation time from its original value \( T_2 \) to its new value \( T_{2,\text{obs}} \). [15] In equation form this is seen as:

\[
\frac{1}{T_{2,\text{obs}}} = \frac{1}{T_2} + \frac{1}{\tau}
\]  

(3)

Where \( T_2 \) describes the spin-lattice relaxation time. Once the spin-lattice relaxation time decreases one is able to use more rapid pulsing (as is the case for higher flow rates). The more rapid the pulsing, the higher the quality factor of the radiofrequency –coil becomes. As such more rapid pulsing can contribute to better sensitivity in LC-NMR. [26]

\[
\frac{1}{T_{1,\text{obs}}} = \frac{1}{T_1} + \frac{1}{\tau}
\]  

(4)

Most liquid chromatography applications are of Reverse Phase nature, usually employing a gradient between two solvents which are mixed in different ratios throughout the analysis. The problem is that the solvent used in these applications usually have a lot of protons, which forces the use of Nuclear Overhauser Effect Spectroscopy (NOESY) pre-saturation techniques (\( T_1 \) decreasing effect) to eliminate solvent interference from the signal. [27] Additional RF-coils are added to the design in order to detect \(^{13}\text{C}\)-nuclei.

When creating a good flow cell the following criteria need to be taken in to consideration: [28]

- The shape of the cell should be suited to flow characteristics which provides a good spectral resolution. The resolution should be at such a level that it properly shows the multiplets (that form due to scalar coupling) in such a way that the structure of the component can be deduced from it.
- Line-broadening due to magnetic susceptibility of the cell-material and other spectra distortions (that take place because of the material which makes up the cell) should be minimized.
- The design and properties of the cell should give the highest NMR sensitivity.
The sensitivity of the NMR is proportional to the signal-to-noise ratio of the NMR flow probe. The signal-to-noise ratio follows the equation below in saddle-type flow probe:

$$\frac{signal}{noise} = \frac{\gamma N(N+I)f \sqrt{\left(B_1/T\right)^3} \frac{Q*V_s}{b}}{n} \frac{\gamma_e N \gamma^3 B_0^3 B_1}{\sqrt{4k_bTb(R_cT_c+T_a) - R_s(T_c+T_a)}}$$

(5)

Where:

- \(N\) : Number of detected nuclei
- \(\gamma\) : Gyro magnetic ratio of the nuclei with subscript \(e\) being the excited nuclei and \(d\) the detected nuclei.
- \(I\) : Spin quantum number of the nuclei
- \(I_{coil}\) : Coil current
- \(B_0\) : Strength of the applied static magnetic field
- \(B_1\) : Radiofrequency pulse
- \(R_c\) : Resistance of the coil
- \(R_s\) : Resistance of the sample
- \(f\) : Fill factor (ratio between the sample volume and the inner volume of the coil)
- \(V_s\) : Sample volume
- \(Q\) : Quality factor of the radiofrequency coil
- \(T\) : Temperature of detection
- \(T_c\) : Coil temperature
- \(T_a\) : Temperature of amplifier
- \(k_b\) : Boltzmann constant
- \(b\) : Receiver bandwidth
- \(n\) : Noise figure related to preamplifiers

Formula 5 shows us that we can increase the signal-to-noise ratio by reducing the operation temperature, increasing the fill factor, increasing the strength of the magnetic field, increasing the sample volume and reducing the receiver bandwidth and adjusting the noise figure by improving the preamplifiers. Due to the requirements of liquid chromatography the only real ways to improve the sensitivity is by improving the fill factor and using magnets of higher magnetic fields. Through means of shimming, adjusting the homogeneity of the magnetic field, the optimization of the settings of mechanical parts in the NMR can be achieved easily. Shimming becomes easier when lower sample volumes are used and is done automatically by the computer in most cases. The Q factor is lower for saddle flow probes in comparison to a horizontal solenoid coil cells (and flow probes). [15]

The standard (saddle) type was vastly improved by creation of cryogenic flow probe which consist out of the probe, a Helium compressor and cryogenic cooling unit. The standard 3 mm cryogenic probe (used for offline NMR spectroscopy with cryogenic cooled magnets) was altered to a 60 µl saddle type (sample volume of 40 µl) flow probe which had a fill factor ~8 times larger than the conventional 3 mm saddle type (sample volume of 120 µl) fill factor for the same amount of sample in the detection cell. The temperatures of the coils are usually brought down to 20 K while the samples are maintained at ambient temperatures. Application of this cell in the analysis of human urine allowed for identification of a new metabolite in just 16 scans per slice using timed-sliced flow mode opposed to the long stop flow mode experiment which was previously needed to identify the same metabolite. These cryo-flow probes vastly improved the LC-NMR resolution. The scientist proceeded by using two-dimensional Nuclear Overhauser Effect Correlation spectroscopy to identify the position of the methoxy-group in the metabolite. [29] The mass sensitivity is defined as the signal-to-noise ratio divided by the amount of moles and the root of the residence time. [30]
2.3.1.2. **Capillary NMR flow probes (Solenoid type)**

The “probes” used in the newer generation separation methods hyphenated with LC-NMR, namely capillary liquid chromatography-nuclear magnetic resonance (capLC-NMR), capillary electrophoresis-nuclear magnetic resonance (CE-NMR and CEC-NMR) and Field Flow Fractionation-nuclear magnetic resonance (FFF-NMR) that utilize very narrow capillaries, are capillaries where micro coils are wrapped tightly around. These flow cells with severely reduced diameters have their center of their magnetic field overlapping with the length of the capillary which enhances the sensitivity for small volume sample. The signal-to-noise ratio, which is proportional to the NMR-sensitivity, depends on the diameter of the capillary in a solenoid probe according to equation 6 (when the diameters are equal or greater than 100 µm). [31] When the diameter increases the sensitivity decreases, meaning this type of probe design is very suitable for use in capillaries. When compared to a saddle-type the solenoid type sensitivity is several times more sensitive. Because of this the solenoid-type generally needs a volume of 0.02-5 µl of sample to obtain resolutions similar or higher as the 120 µl flow probes. In figure 2.7 a schematic sketch is given of the solenoid detector-type.

![Diagram of solenoid probe design](image)

**Figure 2.7** Solenoid probe design used in capillary LC-NMR modes, such as CEC-NMR, CE-NMR and FFF-NMR. Adjusted from [26] and [32].

A big advantage of this type of detector is that it can quite easily detect multiple detection windows, so that multiple components can be measured at the same time. And it is quite easily to add additional coils so that other nuclei (such 13C nuclei) can be measured simultaneously. This type of flow cell is not suitable for large volumes and for applications utilizing large volumes the traditional saddle-type must be used. [33]

\[
\frac{\text{signal}}{\text{noise}} = \frac{\omega_0^2 \left( \frac{n}{d_c} \sqrt{1 + \left( \frac{h}{d_c} \right)^2} \right)}{1 + \left( \frac{h}{d_c} \right)^2} \times \frac{\sqrt[4]{\omega_0^2}}{d_c} \times \frac{n^2 \cdot d_c \cdot \sqrt{\omega_0}}{h} \]

(6)

Where:
- \(\omega_0\) : Nuclear precession frequency
- \(n\) : number of turns in the RF-coil
- \(d_c\) : Outer capillary diameter
- \(h\) : Capillary length

These kinds of probes are placed horizontally in the NMR. The applied magnetic field is perpendicular to the flow direction of the liquid. Electromagnetic field theory states that a radiofrequency coil wrapped tightly around an infinitely long cylinder creates a uniform and static magnetic field. However line broadening can occur near the edges of the capillary due to the difference in magnetic
susceptibility as result of the capillary material. This worsens as the coil is wrapped more
closer/tighter around the capillary. So in order to reduce this effect the space between the coil and
the capillary is surrounded with liquid that has a magnetic susceptibility matching that of the
capillary wall. The small volume allows for greater pulsing and decoupling which partially negates the
heterogeneity of the magnetic field. The current induced magnetic field inside the capillary is
described by equation 7: [31]

\[ B_1 = \frac{2\mu_0 * I_{\text{capp}} * r}{\pi d_i^2} \]  

(7)

Where:
\( \mu_0 \) : Permeability constant
\( I_{\text{capp}} \) : Current through the capillary
\( r \) : Radial distance from the centre of the capillary
\( d_i \) : Inner diameter of the capillary

This magnetic field might influence the uniformity of the applied magnet field by causing distortions.
This can be partially reduced by shimming. Even the slightest differences in temperature over the
capillary can create small deviations in the conductivity of liquids with ionic particles. Because of this
there is a \( B_1 \) gradient over the cross-sectional area of the capillary. The radial gradient depends
linearly on the current in the \( B_0 \)-direction. Significant NMR signal degradation occurs when the
capillary configuration is not parallel to \( B_0 \), as illustrated in figure 2.8. [31]

Figure 2.8 NMR signal distortion from a capillary configuration A) parallel to B) not-parallel compared to \( B_0 \). [31]

2.3.2. Flow modes

HPLC-NMR is one of few chromatographic coupled techniques where the flow is stopped mid
experiment. This is because certain NMR applications, such as 2D-NMR require significantly more
measuring time (residence time) in order to obtain useable spectra. The downside of this approach is
that longitudinal diffusion might ruin some of the separation while the flow is zero or that the
component of interest diffuses out of the detection volume. Depending on the NMR application
(one-dimensional or two-dimensional, high abundance nuclei (\(^1\)H) or low abundance nuclei (\(^13\)C)) it
might be better to stop the flow mid experiment (stop-flow mode or time-sliced flow mode) or to
just keep a constant flow (on-flow). The time-sliced stop-flow mode become very useful in cases
where the chromatographic separation was of low resolution and compounds of interest did not
adsorb UV light. By stopping the flow for short intervals as the peak passes through the NMR
detection window higher signal can be obtained that is more suited for quantification or structure
elucidation. Peaks can also be temporary stored in loops. Where one by one each loop is eluted
through the NMR (loop storage flow mode). [34] Finally LC-NMR systems have been used for other
flow-modes where no chromatographic separation takes place and instead just a sample is detected
in whole in the NMR. Such mode are Flow Injection Analysis (FIA) and Direct Injection (DI) and these
type of flow-modes are very useful for earlier separated compounds that have been collected in
fractions. (figure 2.12 illustrates all flow modes in a single figure) Solid Phase Extraction –NMR (SPE-
NMR) is one of the applications where these column less flow modes prove very useful.
2.3.2.1. **On-flow mode**

In the on-flow mode the eluents is measured live as it passed through the detection cell. This type of flow mode is suitable for components of interest that are present in high concentration in the sample, where the measured nucleus is abundant in nature (such as $^1$H and $^{19}$F NMR spectroscopy). The advantages of this flow mode are the rapid data acquisition and that all peaks are detected in a uniform manner. The applications for these modes are very limited, because high signal-to-noise ratio one dimensional-NMR spectra can only be obtained when concentrations are relatively quite high. [35] The limit of detection is 10 µg (for a magnetic field of 300-500 MHz), residence time is generally ~5 seconds (at flows of 1 ml/min) and only one dimensional-NMR applications can be used. Because of this it is mostly used for screening purposes. By screening the sample in this way a quick insight in to the identity of the major component of the mixture is obtained. [36] Most of the online flow-mode applications utilize reverse phase C$_{18}$-chromatography involving gradient analysis between the solvents CH$_3$CN and D$_2$O. Flow rates can go as low as 0.1 L/min with probe volumes as low as 60 µl. Others use isocratic elution with mixtures of CH$_3$CN and D$_2$O. [32] The flow mode has been used in the analysis of food chemistry, such as drinks [38] and in higher capacity chromatography involving C$_{30}$-columns. [39] Recently some development has been made in conducting two dimensional NMR with this flow mode by using one or a few temporary signals. Two kind of rapid 2D-NMR techniques have been developed. The first developed by Frydman [40] et al and the second by Kupce et al. [41] Frydmans rapid two dimensional acquisition involves heterogenic initial excitation and is based on the position-dependant evolution of the spins. The method gives the average concentration of the compound in the detection cell. The Frydman method requires no prior pre-knowledge of the unknown sample and can be used on any sample. [40] The Kupce method utilizes multichannel excitation and detection of NMR signals in the Hadamard matrix frequency domain. Prior knowledge of the needed NMR signal frequencies is required for the Kupce method as it excites all frequencies at once. Disentangling of these signals occurs by referencing to the encoding scheme used. This method has a higher sensitivity than the Frydman method, but requires prior knowledge of the analyte spectrum so that the proper encoding scheme can be defined. [41] The lower limit of detection are generally in the order of 2 - 85 µg for $^1$H spectroscopy (S/N = 3) performed on NMR with magnetic fields of 500 MHz utilizing a saddle flow probe. [42]

2.3.2.2. **Stop-flow mode**

Stop-flow modes become an option when the retention time of the analyte is known or the other detectors connected to the system have pinpointed a unique trait regarding the identity of the compound (e.g. chromophore in UV/VIS spectroscopy, specific vibration in IR spectroscopy, specific mass signal in the mass spectrometer etc.). In stop-flow mode the other detectors beside the NMR are used to locate the spatial position of a component in the system so that the flow can be brought to zero when the component of interest has entered the detection cell. While the flow is stopped no further chromatographic separation can take place but this allows for the NMR detector to obtain the needed signal-to-noise ratio to conduct (high resolution) two-dimensional NMR spectra by optimization of the field homogeneity and acquisition settings. (High resolution) two dimensional NMR requires very large detection times that can vary from hours to days. Because the main goal of using LC-NMR is structure elucidation (which requires two dimensional NMR in most cases) most applications use stop-flow modes. [35] Longitudal diffusion that occurs when the flow is stopped causes the analyte to diffuse outside of the detection cell and gives band broadening of the signal. This effect is illustrated in figure 2.9. This effect is limited by the small diameter PTFE-tubing that
connect to the detection cell (figure 2.10) that keeps most of the volume inside the detection cell for a long time. Because of this very good spectra are obtained using (high resolution) two-dimensional NMR. Through stop-flow mode higher resolutions are obtained for one-dimensional \(^1\)H and \(^{19}\)F NMR spectroscopy where detection limits of less than 100 ng have been reported for applied magnetic fields of 600 MHz for solenoid flow probes. Peak shaped can be improved by taking data from multiple injections, but this is only possible if enough sample is available. [35] For a general magnetic field of 500 MHz utilizing \(^1\)H spectroscopy (S/N = 3) the limit of detection is between 0.7 – 20 µg for a saddle type flow probe.

Another big limitation of stop-flow mode analysis is that the loss in chromatographic resolution is proportional to the time that the separation has been stopped. For this reason most (high resolution) two-dimensional NMR spectra analysis usually involves the use of storage loops to store peaks and prevent loss of component purity. Two-dimensional NMR modes using stop mode are mostly of TOCSY and NOESY kind, which require less than an hour to acquire. Time sliced stop-flow is an alternate version of stop-flow. [32]

**Figure 2.9 The longitudinal diffusion that forces the volume outside of the bulk of the flow cell that occurs during stop flow. A) the original state of the volume at short residence time (on-flow) and B) the volume dispersion at higher residence times (stop-flow). [22]**

**2.3.2.3. Time sliced stop-flow mode**

In time sliced stop-flow the flow is stopped several times at regular interval during the elution of the peak. This flow mode is used when two peaks elute closely together or have retention times in close proximity to each other. It is also used when the separation is poor to obtain as much data as
possible from each section of the overlapping peak. It also eliminates the need to rely on the secondary detector to position the bulk of the peak inside the RF-coil wrapped detection cell. [15]

2.3.2.4. **Loop storage flow mode**

Loop storage flow mode is a combination of on-line and stop-flow operation modes. In this mode the flow control and peak-sampling units plays an important role. As the chromatographic peaks elute from the column they are stored on the capillary loops of the flow modes. Afterwards the content of a capillary loop is pumped in to the NMR detection cell allowing for time-consuming high resolution two-dimensional NMR spectra acquisition and less time-consuming one-dimensional/two-dimensional spectra. Once the first capillary loop acquisition is done the second capillary loop is pumped in to the cell and detected and this process is repeated until all capillary loops have been drained. The operator defines the order that these loops are eluted in to the NMR. The major advantage of this approach compared to stop-flow mode is that no diffusion takes place so that the separation of the compound is not lost. [32] Other advantages are that the loops can be used to flush out the detection cell to eliminate carry over effects and that the amount of sample inside the detection cell can be increased by collecting multiple fractions of several injections. The limitation of this approach is that the numbers of components that can be analyzed in this manner are limited to the number of loops. Other disadvantages are that the component arrives diluted in the storage loop and that long-term storage can potentially dissociate or degrade the components of interest. [35]

2.3.2.5. **Other flow modes (flow modes involving no chromatographic separation)**

The newer and less known flow modes skip the chromatographic separation and instead insert the sample directly in to the detection cell by pump systems. This category can be further divided in to two subgroups based upon the fluid used, namely Flow Injection Analysis (FIA) mode and Direct Injection (DI) mode and is mainly used in combinatorial chemistry. [43]

In Flow Injection Analysis (FIA) flow mode the sample is injected as a sample plug in the solvent stream that carries the plug from injector to the detection cell. Afterwards the flow is stopped if this high resolving power NMR acquisition is needed. FIA has proven itself quite useful in the acquisition of High-throughput NMR. The allure of this mode is it simplicity and speed. A FIA system can rapidly, automatically and reliably create a NMR reference library and accurately quantify compounds by NMR (provided the molecular mass of the components and the internal standard are known values). [44]

In Direct Injection (DI) flow mode the system is further simplified by removing the mobile phase, the pump to leave only the detectors and connecting tubes. The sampling and spectra acquisition is run by an auto sampler controlled by the NMR detector. This system allows for rapid, robust NMR automation since the worst that can happen is the blockage of the injection port. Direct Injection mode offers high sensitivity (no solvent means no dilution) and makes sample recovery easy. The sample can be stored on disposable plates or vials as opposed to the precision glass sample tubes and cartridges that are associated with normal offline liquid state NMR. The sample volumes that can be run on these systems vary from 150 to 350 µl. The sample concentration themselves usually range from 1 mM to 50 mM. Analysis time of these systems is usually between 2-7 min for one-dimensional NMR but vary depending on the sample viscosity and concentration. Direct Injection flow mode systems are used primarily in fields such as pharmaceutical chemistry to create combinatorial
chemical libraries and to screen samples, but are also used to accurately quantify compounds by NMR detection. [44]

FIA is sometime used with segmented flow, where small plugs of immiscible liquids carry the plug across the detector. This allows for accurate positioning of the sample within the detection cell as it prevents longitudinal diffusion. No equilibrium is required, making it highly suitable for high resolution NMR. This flow mode however is problematic with the conventional saddle flow probe, but has proven very effective in combination with the solenoid type flow probe. By sandwiching the plug with immiscible fluorocarbon FC 43 fluid even smaller sample sizes are required than when sandwiched with air. While degradation of the NMR signal line is no problem with air, it still frequently occurs when FC 43 is used. Sample is still frequently lost by segmented flow due to degradation and carry-over effects occurring between (fittings of) the connecting tubes within the system. Sometimes the sample plug brakes apart in smaller plugs, though this usually only occurs at low pressures. The throughput rate for FIA is significantly better, because the samples can be loaded at a small distance from each other instead from sampler to detector. In order to fix the problem with the carry-over zero dispersion segmented flow was created, which besides enhancing the throughput of FIA by at least a factor of 2 also virtually eliminated all the carry-over. This was done by wetting the Teflon transfer lines by fluorocarbon liquid and putting plugs of wash solvents between samples. At the moment zero-dispersion segmented flow is only available for micro-coil solenoid type detection cell and allows for sample volumes up to 1 µl instead of the conventional 8 µl (dead volume) of the solenoid type flow probe (figure 2.11 provides a schematic sketch of the setup). [45] The lowest reported limit of detection for these kind of zero-dispersion segmented flow capillary FIA injections on a 600 MHz NMR solenoid flow probe device is 50 ng. [46]

Figure 2.11 An schematic sketch of the use of zero-dispersion segmented flow in library creation [45]

Direct injection- and flow injection analysis flow mode systems are both suitable for generating high resolution \(^1\)H spectra of 1 mg/ml samples regardless of whether they are pure or diluted. Direct injection systems are used more for high resolution NMR spectra acquisition and library creation in cases where the amount of the sample is limited. Flow injection analysis systems on the other hand
offer small volumes and higher throughput rates. Both systems are used for screening purposes and creating reference libraries, but one focuses on speed and the other on sensitivity. [43]

Online Solid Phase Extraction-NMR (LC-SPE-NMR) is usually done on either standard/partially hyphenated LC systems that allow for fractionation. During these fractionations components are stored at cartridges after elution from the column. Then an auto sampler injects the content of cartridges on to a LC-NMR system running on FIA-flow mode or DI-flow mode (after solvation). Because the FIA- and DI-flow system allow for automation it was possible to develop online SPEC-NMR. Recently zero-dispersion segmented flow-systems have entirely taking over FIA injection systems as it offers better sample placement in detection cell. Since NMR is concentration sensitive, it offers higher resolution signals than FIA-systems were able to provide before.

![Diagram](image)

Figure 2.12 An overview of all the flow modes available in LC-NMR systems. (MP stands for Measuring point; the entrance of the detection cell)

2.3.3. Liquid Chromatography hyphenated with Solid Phase Extracted NMR (LC-SPE-NMR)

Online liquid chromatography hyphenated with solid phase extracted NMR is a relatively new application in hyphenated liquid chromatography NMR. These systems are based upon automated “offline” SPEC-NMR. First chromatographic separation takes place, usually on reverse-phase columns (though basically any kind liquid chromatographic separation mode can be used) with non-deuterated solvents. As the peaks elute from the column the system puts them inside small cartridges (rough cartridge volume is 125 µl with 30 µl suitable for peaktrapping) filled with sorbent suitable for trapping the component. The system controls this process by monitoring signals from secondary detectors or on basis of pre-programmed retention times. Alternatively the operator can control the process manually. The flow modes available for this process are typically on-flow and time-sliced stop flow. The advantage of time-sliced stop flow is that it can prevent NMR-active compounds that have weak- or no-activity in the secondary detector from being inserted in the SPE-cartridges. Just before the component is eluted over the SPE-cartridge (but after it has already left the column) some water is added to bring the polarity-strength of the solvent closer to a neutral value. This in turns makes the solid phase extraction more effective. The cartridges are subsequently dried with nitrogen gas and slightly dissolved in deuterated solvent before being injected directly in to a NMR flow probe using either Direct Injection flow systems or (zero-dispersion segmented) FIA systems. The process is portrayed in figure 2.12. The major advantage of this hyphenated system in comparison to the normal LC-NMR system is the pre-concentrating that takes place before NMR-detection, which prevents peak broadening in the NMR while maintaining purity. This makes the system very suitable for high resolution NMR detection, such as two-dimensional NMR spectra acquisition that takes several hours to days and one-dimensional NMR-spectra such as $^1$H and $^{19}$F
The entire component on the SPE-cartridges fills the entire detection cell, which in turn gives very high NMR sensitivity. The concentration of the analyte is a factor 5 higher in comparison to normal LC-NMR. Another advantage is that the entire detection volume is filled with the LC-SPE-NMR method as opposed to the fraction of the detection volume in LC-NMR (fractionated through diffusion). Cartridges go as low as volumes of 8 µl suitable for peak-trapping, but lower detection volumes (smaller cryo-flow probes with volumes lower than the conventional 40-120 µl) are required to properly analyze samples using those sizes of SPE-cartridges. The lack of solvent removes the need for solvent repression. In case of low concentrations multiple injections in the LC-system using the same cartridges increases the concentration of the analyte placed in the detection cell. This in turn increases the signal-to-noise ratio and increases the sensitivity of the component in the NMR. LC-SPE-NMR has the potential to decrease the amount of work significantly and increases the usability of the NMR results. [47] [48]

LC-SPE-NMR solves a lot of difficulties encountered in LC-NMR, but the application can be tricky outside of routine applications. The fact that LC-NMR is mostly used for clearing up unknown (complex) mixtures makes the optimization of the analysis for a specific component difficult and time-consuming. This leads to two approaches. The first approach is time-consuming, where for every peak research needs to be conducted in finding a proper cartridge to trap the components on. This approach is required when the nature of the sample is unknown. To overcome this problem, a second approach is used. In this second approach variety of SPE-cartridges have been developed which provide decent trapping efficiencies for most classes of components. Usually one starts out with C_{18} adsorbent material and universal general purpose (GP) resin cartridges. If these prove insufficient one switches over to trays with several SPE-cartridges made for different varieties of compound classes. Limitations are found mostly for charged and polar analytes that are hard to trap on cartridges. Use of ion-exchange or porous carbon material will improve recoveries in these cases. Most LC-MS and LC-UV experiments can be easily converted in to LC-SPE-NMR experiments. Once a decent method has been developed, analysis of similar samples can be automated by means of an auto sampler. In cases where only a small limited amount of sample is available for analysis NMR detectors with micro coil solenoid type are used for capillary-NMR applications to produce concentrations profiles similar to the pure component (without presence of solvent and contaminants). And in other cases NMR detectors with saddle type flow probes are used, because they provide higher signal resolution. [48]
2.3.4. Supercritical Fluid Chromatography –NMR (SFC-NMR)

Nuclear magnetic resonance hyphenated supercritical fluid chromatography was developed before there were good ways to suppress solvent influence of protonated solvents in $^1$H NMR spectra of nuclear magnetic resonance hyphenated liquid chromatography. For this reason people searched for applications in LC-NMR that did not employ solvents with protons. One way to solve this is the use of supercritical fluids, since supercritical fluids such as carbon dioxide produced no significant solvent signal in $^1$H NMR-spectra. A technique resembling nuclear magnetic resonance hyphenated gas chromatography (GC-NMR) was obtained by using supercritical fluids. No further effort was put in developing true gas chromatography hyphenated with NMR spectroscopy, because the gaseous phase is not an appropriate phase to detect susceptible compounds, therefore development was halted. Instead the idea of supercritical fluid chromatography hyphenated with NMR spectroscopy was developed further. The increased diffusion that comes from the semi-gas phase makes chromatographic separations much quicker allowing for faster analysis than the normal LC-NMR could give in its earlier years. The flow probe used in SFC-NMR was developed in a similar manner as the first LC-NMR flow probes (section 2.3.1.1) with the inclusions of additional compartments that could be used to change the temperature and pressurize of the sample in the flow probe. The same experimental design used in Supercritical Fluid Extraction-NMR can be used for SFC-NMR. [49] Both the instruments within the SFC-NMR systems as well as the flow probe are shown in figure 2.14.

![Flow Diagram](image)

(a) Block diagram for on-line SFE-NMR and SFC-NMR. Key: (a) and (b) HPLC pumps, (c) cryostat for precooling of CO$_2$ and pump heads, (d) CO$_2$ cylinder with dip tube, (e) oven with mixing chamber and steel capillary, (f) injection valve, (g) UV detector, (h) air supply for probe-head heating, (i) NMR spectrometer, (k) back-pressure regulator and (l) computer. (b) Details of the NMR probe used for SFE-NMR and SFC-NMR. Key: (a) external lock container, (b) r.f. coils for $^2$H lock, (c) $^1$H coil, (d) flow cell, (e) air for probe-head heating and (f) capillaries containing client.

Figure 2.14 The experimental setup and flow probe used in nuclear magnetic resonance hyphenated supercritical fluid chromatography. [15]

Within the experimental design the pump heads of the CO$_2$ pump are cooled to roughly 275 K with cryogenic static carbon dioxide from a gas cylinder with dip tube, while the backpressure regulator keeps the pressure stable. Separations performed in supercritical chromatography utilizes pressure gradients and closely resemble reverse phase gradient analysis. The solvent has an organic modifier added and since the pressure determines the solubility of the compound the separation goes from being able to solvate very non polar compounds at low CO$_2$ pressures (resembling normal phase chromatography using aliphatic carbohydrates as mobile phase) to polar compounds at higher
pressures (resembling reverse phase chromatography using dichloromethane as mobile phase). Supercritical fluid chromatography is generally used to extract organic compounds from biological materials, such as plants. SFC-NMR can be done in both online- and stop-flow mode and has the ability to generate both one-dimensional spectra (such as $^1$H and $^{19}$F nuclei containing compounds) and two-dimensional spectra (COSY and TOCSY). The decreased viscosity makes the spin-lattice relaxation time ($T_1$) of protons several times larger compared to conventional old state of LC-NMR, making detection of $^1$H protons hard in online flow-mode. This was solved by adding a cartridge with immobilized free radicals before the probe and made the technique more viable for connection to capillary separation techniques. [49] The practical use of SFC-NMR has been reduced to being mostly redundant due to superior solvent suppression techniques using pulse sequences that can take most of the solvent peak out of the spectra. The development of SFC-NMR was therefore stopped and the application is nowadays barely used. Reverse phase LC-(SPE-)NMR now offers better analysis times, detection and far lower costs.

2.3.5. Solvent suppression

When online LC-NMR analysis are performed special care has to be given when choosing the mobile phase since they will almost always produce signals in the NMR spectra because of their molecular structure. It is quite annoying when solvent signals overlap analyte signals. This can be solved partially by using deuterated solvents, but these can be quite expensive when they are used for chromatographic purposes. For protonated solvents the $^1$H solvent signals might be so strong that the analytes might not even be detected. This issue is caused by the dynamic range problem. The dynamic range problem can be solved by using the proper solvent suppression technique. By using specifically designed pulse sequences the intensity of the solvent signals can be severely reduced or even fully suppressed. A variety of the most common solvent suppression techniques are described below. [50]

In transmitter presaturation (PRESAT) a low power pulse is performed before the main excitation pulse. This results in the compensation of the different occupation of Zeeman levels (splitting of static magnetic field in to multiple lines) for nuclei precessing at a given frequency. A multi frequency shifted laminar pulse is used for multi transmitter presaturation in the situation that multiple signals need to be suppressed. Since it takes some time for this method to be effective it is generally used for stop-flow mode experiments. [50]

Water suppressions by gradient tailored excitation (WATERGATE) is an alternative suppression technique that can be used in both online- and stop-flow mode experiments. By using a pulse sequence consisting out of a dephasing-, an inverse $180^\circ$, rephrasing gradient pulse that suppresses solvent signals. The transversal magnetization is put on a zero value by timing the time delays in the pulse sequence in such a way that it suited for the relaxation properties of the solvent. The shape pulses are much shorter than PRESAT making it usable in both flow modes and it can be used together with the $^{13}$C satellites suppression sequence. [50]

Water suppression Enhancement through $T_1$ (spin-lattice relaxation) effect (WET)—sequence are four solvent-selective shape pulses of different flip angles. Each shape pulse if followed directly by a dephasing gradient pulse with gradients strength ratios of 8:4:2:1. By doing this the magnetization of the solvent nuclei is nullified just before acquisition. Once shifted laminar pulses are added it can be used for multiple solvent suppression. This method is both usable in on and stop-flow modes and
allows for $^{13}$C decoupling of solvent satellites during the shape pulses by using band selective
decoupling schemes. WET suppression is the most commonly used suppression techniques in LC-
NMR applications and also one of the oldest. [50]

BiPolar gradient Pulsed STimulated Echo (BPPSTE) is a diffusion experiment that can be used in
solvent elimination by adjusting the diffusion delays to the detection of macromolecules. BPPSTE was
originally designed for diffusion-ordered Spectroscopy (DOSY), but can be used for solvent
suppression if the solvent and analyte diffusion coefficients vary greatly. [50]

2.3.6. Further online hyphenation with other secondary detectors (Hypernation)

Quite early on in the development of NMR hyphenated liquid chromatography the possibility of
further hyphenating (further hyphenation of a hyphenated system is called Hypernation) the system
to secondary detectors was explored. Hyphenated systems are the most powerful system to detect,
quantify and identify compounds with and are primarily used to study complex mixtures. However
the normal hyphenated LC-NMR system was able to provide the best stereo chemical information for
a compound, but not an undisputable identification. Furthermore it is quite hard to elucidate the
correct structure without knowing the molecular mass of the components in the mixture. NMR
quantification can only be used when the molecular masses of the component and the standard are
known values. Therefore without the molecular mass one has a very expensive system that can’t be
used for quantification or unambiguous compound identification, which is a huge waste. For this
reason two approaches were taken to obtain that the molecular mass information. The first was to
conduct both liquid chromatography experiments individually (LC-MS and LC-NMR) where one
experiment had a mass spectrometer as detector (LC-MS) and the other experiments of similar
nature had a nuclear magnetic resonance detector (LC-NMR). This approach means two systems are
required which are basically running the same experiments and double of everything (sample,
instruments, solvents, analysis time, space) is needed. Besides the additional expenses needed to run
the system, a problem will occur once we’re faced with a mass limited sample. The secondary
approach was to further hyphenate the LC-NMR system by adding an online mass spectrometer in to
the system. [51]

The mass spectrometry hyphenated LC-NMR systems are placed parallel to each other with a splitter
(flow ratio: 95% NMR and 5% MS) that redirect fractions of the volume to the mass spectrometer
(MS) and the NMR. The notation of this system uses a “/”-sign to illustrate that the systems are not
connected in series but are instead connected parallel. The MS in the LC-NMR/MS system provides
the molecular masses of the components along with some characteristic fragmentations (depending
on the MS settings). Complications in connecting the two systems were the fact that the vacuum
system had to handle large volumes and new ionizations methods were needed for non-volatile polar
compounds. The first was easily solved by using a nebulizer with an additional splitter; the second
problem was solved with the development of Electron Spray Ionization (ESI) and Atmosphere
Pressure Chemical Ionization (APCI). Afterwards the LC-NMR/MS system was easily fabricated. The
first of these systems were reported as early as 1997. [52] Since then many different kind of MS
systems have been hyphenated, including quadruple-, Time-of-Flight (TOF)-, triple quadruple (TQ),
ion Cyclotron Resonance (ICR)-, orbitrap- and Inductive Coupled Plasma (ICP) Mass Spectrometers.
Figure 2.15 illustrates a schematic sketch of these systems. These systems were both suited for on-
flow and stop-flow modes, but in cases of high residence times the resolution of the compound in
the MS could degrade significantly as result of loss of separation quality. Generally samples were first
measured in on-flow mode to ensure acquisition of the proper molecular mass before going over to stop-flow mode for two-dimensional measurements in the NMR. Either deuterated solvent were used or non-deuterated solvent with an additional D/H exchanger just before the entrance of the NMR detector. [51]

![Diagram of LC-NMR/MS system](image)

**Figure 2.16** A schematic sketch of an LC-SPE-NMR/MS system. [22]

Since then many applications have been developed for LC-NMR/MS including many forms of chromatography (reverse phase, ion exchange, normal phase, supercritical fluid extraction etc.) The biggest development was the creation of MS hyphenated LC-SPE-NMR system. The LC-SPE-NMR/MS system solved one of the biggest problems original present. This problem was that the deuterated species present in the solvent interchanged protons with some compound in the sample. The resulting MS spectra were hard to interpret. The solid phase extraction unit allows for regular chromatography with $^1$H solvents, while only deuterated solvents have to be used in the SPE-eluting step. (system illustrated in figure 2.16) [22]

![Diagram of LC-NMR/MS system](image)

**Figure 2.15** The earlier design of LC-NMR/MS systems. Adapted from [22]

Other noted examples of hyphenation are the creation of semi- and fully online FTIR hyphenated LC-NMR/MS systems to LC-IR-NMR [53] and LC-IR-NMR/MS [54] configurations that allowed for additional data acquisition on the characteristic groups in the components. [51]

More promising is the hyphenation of NMR with LC systems that have a chiral dichroism (CD) detector after a chiral column. These system have a CD detector before the photodiode array - detector. In these LC-CD systems an effect is obtained that allows for the separation and detection of stereo isomers of optically active compounds. Drug studies have shown that certain enantiomers of active compounds in medicine have bad influence on the health, which are required to be separated
and removed before the fabrication of the medicine. Chiral liquid chromatography (runs on columns which have optically active stationary phases) is used in a lot of applications involving the separation of chiral compounds. The use of CD-LC is hard because of the need to optimize analytical conditions by preparation of analytical standards and identification of the individual peaks. Once optimized it becomes applicable for the analysis of mixtures with contaminations and isomers. The hypernated chiral LC-CD-NMR system is a system that can analyze the contaminations and isomers directly without the requirements of standard needed for optimization. The contaminations (and enantiomers) can be easily identified using the combination of UV-chromatographs, chiral chromatographs and NMR data of each peak. Once identified retention time can be assigned to each specific component in the mixture. Another advantage of this system is that it can be used for optimization. [22]

The biggest difficulty in hypernating are the increased complexity in running the system, difference between sensitivities and finding a single eluent that is well suited for all spectroscopy applications and the mass spectrometer. The first two problems are easily overcome by the modern instrumentation making the only real challenge finding suitable solvents. Other more practical problems of hypernated system are the fact that the systems are expensive and that it is hard to utilize all the data the system generates. The focus of Hypernation remains obtaining as much data in as little time possible. Another way to describe the process of Hypernation is to create the most efficient analysis possible. [51]
2.3.7. Capillary Separations hyphenated with NMR (CapSep-NMR)

2.3.7.1. Capillary Liquid Chromatography-NMR (capLC-NMR)
Conventional LC-NMR columns require large volume of solvent in order to analyze components. The use of deuterated solvent is generally too expensive to be used in LC-NMR. But if non-deuterated solvents are used the solvent needs to be repressed so that better dynamic ranges of analyte are obtained in order to get viable NMR spectra. Solvent repression techniques will get rid of the solvent signals but it might affect the spectral feature of the signal neighbouring to the solvent resonances. As such the resolution of the spectra worsens, because important analyte signals are distorted. This effect is predominantly visible in components with low concentrations. By the use of capillary liquid chromatography (capLC-NMR) only a few millimetres of deuterated solvents are needed, significantly reducing the cost of the LC operation. The major advantage of using deuterated solvent is that the entire chemical range is usable for NMR detection and that the sample is diluted far less in comparison to conventional LC-NMR. [30] The dilution factor (D) is given by equation 8.

$$ D = \frac{C_0}{C_{\text{max}}} = \frac{d^2 \varepsilon \pi * (1 + k) \sqrt{LH\pi}}{\sqrt{8} * \text{Vinj}} $$

Where $C_o$ is the initial concentration and $C_{\text{max}}$ the final concentration at the peak maximum, $\varepsilon$ the column porosity, $k$ the retention factor, $L$ the column length, $H$ the plate height and $\text{Vinj}$ the injection sample volume. Even with the use of LC-SPE-NMR the dilution of the sample is proportional to the diameter of the column, meaning that capillary columns will provide relative pure components in the detection cell when compared to factor 100 bigger normal columns used in liquid chromatography. Combining small capillaries with capillary NMR flow probes will provide the best mass sensitivity possible. Because of these reasons capLC-NMR is mostly used for mass limited samples and acquisition of two dimensional NMR-spectra using loop-collected stop- and regular stop flow modes. Capillaries with diameters of 50 to 100 µm are used to bring the sample from the column to the flow probe (which is generally within 30 cm distance from the column in a well-shielded NMR). Saddle flow cells of 180-320 µl have been used in combination with capLC-NMR but the poor filling factor gives poorer sensitivity in comparison to normal LC-NMR. For those reasons bigger sample volume saddle flow probes are used. When samples are mass limited it is better to use soloidal flow probes and when this is not the case the use of the larger cryogenic saddle flow probes gives the best mass sensitivities. [30]

2.3.7.2. Capillary Electrophoresis-NMR (CE-NMR and CEC-NMR)
The strength of NMR spectroscopy hyphenated capillary electrophoresis (CE-NMR) is the same as capillary liquid chromatography. Namely, the requirement for little sample in combination with the fast and high resolution separations that are achieved. The improved separation and detection associated with capillary electrophoresis (higher efficiencies, peak capacities and better speed in comparison to LC) and capillary flow probes is the main reason that CE-NMR has been pursued. The lower volumes and shorter residence times make capillary electrophoresis hard to detect with NMR spectroscopy. Similar to capillary LC-NMR systems the column must be kept at quite a distance from the column unless the magnet is properly shielded. The outlet vial, detection cell and the capillary NMR flow probe are kept within the magnet while the inlet vial can either be inside or outside the magnet. Generally speaking most CE-NMR applications have inlet vials inside their magnetic field; the exception is when capillary electrophoresis chromatography hyphenated NMR (CEC-NMR) where a part of the capillary after the inlet vial has a packaging inserted with stationary phases. This
stationary phase creates the separation by means of partition chromatography. Insertion of this packaging creates additional heat that negatively affects the magnetic field. Each signal has its own migration rate and that in turn influence the NMR signal intensity and line width of the eluting species. Saddle coil NMR probes were made for CE-NMR so that online-flow applications could be applied. By using inserts in the traditional saddle flow probes the detection volumes could be adjusted to 250 – 400 nL. Stop flow-measurements are done in solenoid types flow probe, while online measurements are done in saddle type flow probes. Stop flow modes are used for small volumes to collect high resolution NMR spectra, while the online mode can be used for screening purposes, identification with one-dimensional NMR and to follow the separation inside the CEC-NMR. Online CE-NMR has a detection limit of 1 µg, while offline CE-NMR has been reported as low as 100 ng for simple 1H spectroscopy (S/N = 3). [55] One big advantage of CE-NMR is that capillary isotachophoresis (ICTP) can be used to stack positively and negative charged compound in a form of pre-concentration between the leading and trailing electrolyte. Figure 2.17 shows a schematic sketch of a double and single detection CE-system. Showcasing the potential of multiplex systems, where sample is detected in several detection cells at once reducing analysis time and producing higher signal-to-noise ratios. [30]

Figure 2.17 Schematic sketch of CE-NMR system using double micro coils for dual NMR-detection (left) and single micro-coil for single NMR-detection (right). [30]

2.3.8. Size Exclusion Chromatography hyphenated with NMR (SEC-NMR)

Size Exclusion Chromatography (SEC) is a type of chromatography that separated polymeric samples based upon their hydrodynamic size. There are two types of SEC: Gel Phase Permeation (GPC) that use non polar solvents and Gel Filtration Chromatography (GFC) that use polar solvents to carry the sample through the column. Inside the column, particles of larger size have less permeability in the gel, which limits the path length of the particles. The smaller path lengths give shorter retention times in comparison to the retention time of the smaller particles (that need to cover a longer path). The path length is the dependant on the permeability of the component in the gel. This process is ideally fully entropy driven.

This method is generally considered one of the easiest and best ways to determine important properties of a polymer mixture. These properties are the weight-averaged and number-averaged molecular mass associated with the molecular mass distribution. The latter can be used to find the polydispersity that gives the heterogeneity of the polymers in the sample. [50] Quite early on in the developments applications for online NMR hyphenated SEC (SEC-NMR) were reported. [54]
2.19 showcases an example of a SEC-NMR separation with low separation resolution in regards different kind of polymers with similar hydrodynamic sizes). In order to utilize SEC systems they first need to be calibrated with well-defined polymer standards. While SEC is applicable in most heterogeneous polymer mixtures extended approaches are needed to analyze polymers with a different chemical make-up but a common hydrodynamic size. For this purpose UV light detectors, IR detectors, Refraction Index detectors and NMR detectors can be used to analyze the chemical makeup, provided that the signals obtained differ from each other. SEC-NMR is quite useful as it can define the chemical structure of the polymer to a very accurate extent in stop flow modes and allows for rapid screening of the compound in online modes. This basically creates a two-dimensional technique, as is illustrated in figure 2.19.

2.3.9. Field Flow Fractionation hyphenated with NMR (FFF-NMR)

Field Flow Fractionation (FFF) is an alternative separation method that can be used to fractionate polymer mixtures based upon molecular mass or chemical composition. FFF consists out of a two dimensional separation based upon applying a secondary field perpendicular to the inlet flow that allows for polymer separation similar to Size Exclusion Chromatography (SEC) but with higher resolutions. In the first dimension compounds are separated based upon the difference in diffusion of the particles. The convectional diffusion known as In-flow FFF (IFF) separates the components based upon the diffusion coefficient of the components. The second dimensional separation usually takes place in the cross section of the plate (asymmetry Flow-Flow Field Fractionation (AF4)) using an orthogonal separation method. The most common modes are described below.

- Thermal Field Flow Fractionation (ThFFF): Based upon differences in thermal diffusivity.
- Centrifugal Field Flow Fractionation (CFFF): Based upon density differences.
- Sedimentation Field Flow Fractionation (SFFF): Based upon density differences.

The instrumental setup of an AF4 plate can be seen in figure 2.18. [50] It consists out of a plate with an empty channel within a partially porous trapezoid plate. The asymmetry flow comes from the flow between the permeable (imbedded porous frit with semi-permeable membrane (accumulation wall)) bottom plate to the top plate (which is non permeable). The semi-permeable plate only allows solvent molecules to pass through the membrane. The molecular mass limit is 1000 Da for aqueous solvents and $10^6$ Da for organic solvents.

By hyphenating FFF with NMR more components were separated in polymer analysis (separation of homo polymers having similar hydrodynamic sizes) nano particles and micro particles can be separated and subsequently identified (see figure 2.19). This approach allows the identification of polymer nano composites. This was successfully performed by Hiller [50] and showcases the potential of FFF-NMR over SEC-NMR. FFF offers a variety of advantages over SEC:

- No stationary phase which minimizes shear degradation.
- Low accumulation wall surface area which minimizes adsorption and secondary effects.
- Open channels do not require filtration.
- Exclusion limit is 100 times higher allowing for more applications.
- Complex mixtures can be analyzed in one run.
- Working conditions are suited for highly degradable compounds.
Figure 2.18 Field Flow Fraction instrumental sketch (Instrument used for (polymer) separations). [50]

Figure 2.19 Contour plots showcasing that Thermal FFF-NMR is suited for separation of copolymers with similar hydrodynamic sizes, while the SEC-NMR of polymer standards only separated copolymers with different hydrodynamic sizes. In ThFFF the bottom plate is cold, while the top is hot forcing thermal diffusion from bottom to top plate. [50]

2.3.10. Two-Dimensional Liquid Chromatography hyphenated with NMR (2D-LC-NMR)

Complex polymer mixtures usually differ strongly in the distribution of three factors. These factors are the distribution of the molecular mass (MMD), Chemical makeup (CCD) and Functional type (FTD). Because in most cases these factors are not orthogonal to each other characterization is difficult. This can be solved by applying multiple analysis principles on the same sample, which brings us to the use of two-dimensional liquid chromatography (2D-LC). Once these systems are online hyphenated with NMR they provide a wealth of information as showcased by Hillet et al. [50] In this separation he analyzed polymer samples consisting out of polyethylene-oxides with different chain length and functional groups. He first developed a 2D-LC method suitable for analyzing these parameters, namely a Lectin Affinity Chromatography (LAC)-Liquid Chromatography under Critical Conditions (LCCC) combination experiment. Where LCCC was used to define the end group of the polymers and LAC supplied separation on basis of the chain length. For the hyphenation with NMR
the sample volume of the flow probe and collection loop volume for loop storage were increased and additional RP-columns were included to remove methanol for better spectra resolution. Finally a small amount of hydrochloric acid was added to facilitate the $^1$H and $^2$H interchanging between solvents that suppressed the solvent signal in the NMR. Secondly both methods were hyphenated to NMR as single dimension chromatographic experiments to optimize the separation conditions, which succeeded. The result of the LCCC-NMR can be seen in figure 2.20. The one-dimensional LAC-NMR was used on a similar sample which had the same component composition as the one used in LCCC-NMR, with the only change being that the components here had the same functional end group. The result can be seen in figure 2.21 compared to a similar purpose SEC-NMR experiment. The availability of this combinational data allowed for the chain lengths to be calculated for all the $C_{12}$-$C_{14}$ end group components (see figure 2.22). The final result was the creation of a three-dimensional separation scheme (figure 2.22). This showcases the great potential that NMR has in making three-dimensional polymer separations with the help of 2D-LC-NMR. In this way both the molecular mass distribution, chemical composition distribution and the functional end type could be defined and the components could be characterized. 2D-LC-NMR will most likely become a very important tool in complex polymer mixture identification and separation. [50]

Figure 2.20 (Left) The one-dimensional LCCC-NMR optimized experiments for complex poly-ethoxy-oxides polymer samples with A) showing the OCH$_2$ high field and B) OCH$_2$ chemical shift. The functional end groups are shown on the left of the A-side [50].

Figure 2.21 (Right) The one-dimensional LAC-NMR optimized experiment for complex poly-ethoxy-oxide polymer samples which are $C_{12}$-$C_{14}$ terminated. For illustration purposes the matching SEC-NMR experiment is shown. [50]

Figure 2.22 (Left) The molar amount per chain length of the $C_{12}$-$C_{14}$ end group poly-ethoxy-oxides components. (Right) The three-dimensional polymer separation grid in to three factors: molecular mass distribution, chain length distribution and functional end group. [50]
3. Conclusions and discussions

It is undeniable that liquid chromatography hyphenated with nuclear magnetic resonance spectroscopy is one of the most powerful and expensive analysis systems in the world when it comes to compound identification. However, its practical use remains limited because of the high cost of system. It does provide a powerful alternative when faced with complex samples that require identification because of guidelines in a specific field or purpose. LC-NMR has proved itself very useful in rapid screening purposes, where compounds can be identified online through the use of one-dimensional nuclear magnetic resonance spectroscopy or relatively short two-dimensional acquisition modes (TOCSY or COSY). Research on identities of components in complex (natural) samples in the fields of agricultural, food, environmental, pharmaceutical and phytochemical analysis have been proven to the main purpose of the technique as this fields deal with samples that are either bound by law to fully have each component identified or are chemically very complicated.

The technique has improved relatively fast considering that it slightly more than 30 years old. Since then applications for the technique have increased exponentially because of the advancements within nuclear magnetic resonance spectroscopy, including the creation of far stronger cryogenically magnetic field (micro coil) superconducting magnets that allows for much quicker high resolution NMR spectroscopy with better sensitivities. Other major factors were the research performed on further hyphenations and the creation of additional (cryogenic cooled) detection cells and chromatographic separations. The introduction of cryogenic flow probes and capillary separations have not only made the technique very useful for mass limited samples, such as trace compounds analysis (applications include impurity, metabolite and degradation products quantification and identification) which is now relatively simple with the combination of high resolution NMR and a variety of flow modes (allowing for screening and identification in on-flow detection modes and absolute identification using time-consuming high resolution in (storage loop) stop flow detection modes). The use of direct injection or flow injection flow modes allow for fast library creation and fast screening in combinatorial chemistry. With the latter (Flow injection Analysis) using zero-dispersion segmented flow nowadays, so that much faster throughput of far lower concentrations sample concentrations can be used. Most recently published limit of detections (LOD of 0.1-1 µg for capillary applications and 2-85 µg (on-flow detection mode), 0.7-20 µg (stop-flow detection modes) saddle flow probe applications using a 500 Hz magnet) are lower than ever before making the technique more applicable. Another major step in the hyphenation procedure was the coupling to mass spectrometer which allows for absolute identification and quantification of the samples, since these things are not possible without the molecular mass. The most important development was the online hyphenation with solid phase extraction NMR, which has automated many existing LC-MS and LC-UV chromatographic separation in combination with (zero-dispersion segmented flow) flow injection analysis and direct injection analysis. This approach gives the best sensitivity and throughput to date for LC-NMR and the pre concentration that takes place is essential when obtaining high resolution two-dimensional NMR spectra. The technique can however require a considerable amount of method development time when faced with complex unknown samples. While SPE-NMR/MS is limited by the available adsorbents and difficulties experienced in quantitatively eluting peaks from these traps it is a technique that keeps on improving. Size Exclusion Chromatography-NMR is excellently suited for complex polymer mixtures, but newly available two-dimensional liquid chromatography (2D-LC) separations and channel separations such as FFF have great potential in further developing the use of LC-NMR in polymer analysis as these provide better
and easier identification and separation. Other newly hyphenated techniques such as chiral separations combined to NMR have been proven to allow for easy optimization of the standard chiral LC analysis, because it get rid of the elaborate standard calibration needed to optimize these techniques. LC-CD-NMR/MS system are able to do this in short times with just the samples. Hypernation of LC-NMR/MS systems with younger generation separations along with the further development of both the SPE-NMR/MS systems and the use of multiplex (simultaneous detection of multiple) capillary micro coil NMR detectors will be the subject of LC-NMR research in the future and is likely to make the technique more applicable than it is already (by achieving lower detection times and limits of detections) in the earlier mentioned fields.
4. Bibliography


