Recent Progress in LC-NMR

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LC-NMR has been noted as a practical method for mixture analysis in recent years. Technical backgrounds of high performance LC-NMR are discussed from the point of view of NMR, chromatography and related technologies. Constituent profiling and LC-2D NMR are introduced as practical applications. Further hyphenated techniques such as LC-NMR/MS and chiral LC-CD-NMR are also described.

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Introduction

Analytical methods that connect chromatographs and spectrometers online are called hyphenated techniques,[1] and they have attracted attention in recent years as high-throughput analytical methods that provide separation of mixtures at the same time as the spectra of the various components. Since, among these, detailed structural information can be obtained from LC-NMR (**Fig. 1**) that combines high-performance liquid chromatographs (HPLC) and nuclear magnetic resonance spectrometers (NMR), they have been applied widely in the analysis of complex mixtures that contain unknown components, such as impurities and metabolites in pharmaceuticals, natural products and synthetic polymers,[3), 4)] ever since they were first reported in 1978.[2)] On the other hand, looking at this from the standpoint of applications in research and development at manufacturers, it cannot be said that they have been sufficiently practical in terms of sensitivity and operability of equipment up to now, and they are not a widespread technique compared with the LC-MS.

Since the 2000s, however, there were important favorable changes in the situation, and currently, LC-NMR has become a highly practical analytical method because of increased sensitivity in the NMR devices, such as highly magnetic field magnets and highly sensitive probes, and maturation of peripheral technologies, such as solvent elimination technology and automatic measurement software suitable for multicomponent analysis.

Along with describing the fundamental technology for improving the performance of the most recent high-performance LC-NMR (**Fig. 2**) in this article, we will introduce examples of applications for research and development in fine chemicals: pharmaceuticals and agricultural chemicals. In addition, further combination technologies that link LC-NMR with other detectors have appeared in recent years, and it has become possi-

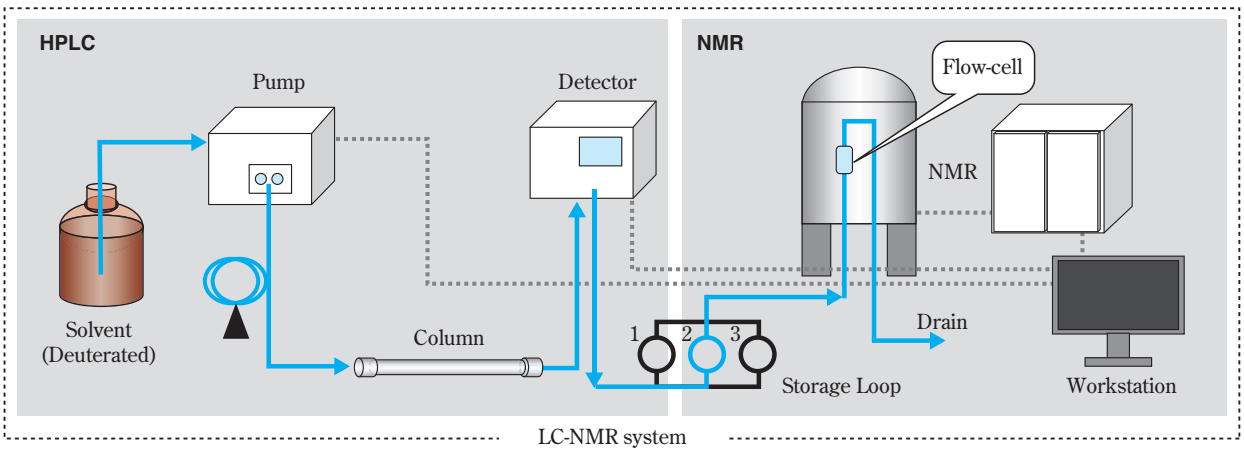

Fig. 1 Schematic view of conventional LC-NMR system

800 MHz magnet

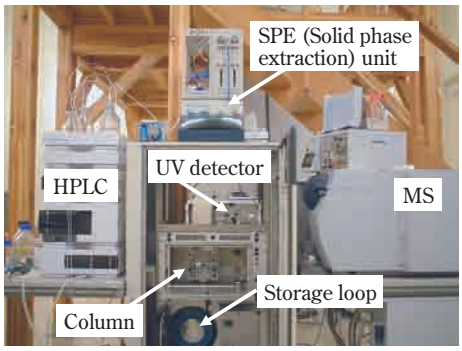

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Fig. 2 High performance LC-NMR system (800 MHz LC-SPE-NMR/MS equipped with cryogenic probe)

ble to acquire multiple spectra simultaneously. In the latter half of this article, we will introduce the recent trends in these technologies.

High-performance LC-NMR

1. Fundamental technology for high-performance LC-NMR

The features of LC-NMR have already been discussed several times up to now. Chemists will be fully satisfied with the useful structural information obtained from NMR detectors. On the other hand, it is widely recognized that the sensitivity of NMR is low. The breakthrough for the sensitivity aspect of LC-NMR was not achieved by a single innovative technology; it was brought about by building up various technologies for increasing sensitivity. First of all, we would like to look at the aspects of both chromatography and NMR equipment regarding these technologies for increasing sensitivity.

(1) Technology for increasing sensitivity on the chromatography side

With LC-NMR, the effects on measurement sensitivity by the chromatographic separation must be considered. The observation part of LC-NMR is called a flow-cell (see **Fig. 1**), and components separated by HPLC are introduced to this part and NMR measurements are carried out. The highest sensitivity is provided when all of the separated components are introduced to the flow-cell (**Fig. 3** (A)).[5] However, the peak volume separated by HPLC is greater than the flow-cell capacity (normally about 30 µL to 120 µL); therefore, only part of the component is actually the target of measurements. At the normally used flow rate of 1 mL/min. with a conventional column having an inside diameter of 4.6 mm, the peak width corresponding to a 120 µL flow-cell is only 8 seconds. Even with a peak width of around 40 seconds, only about 60% of the component of interest is introduced to the flow-cell[6] (**Fig. 3** (B)). Therefore, the point of high-sensitivity measurements is making the peaks as acute as possible and being able to make the introduction to the flow-cell as efficient as possible.

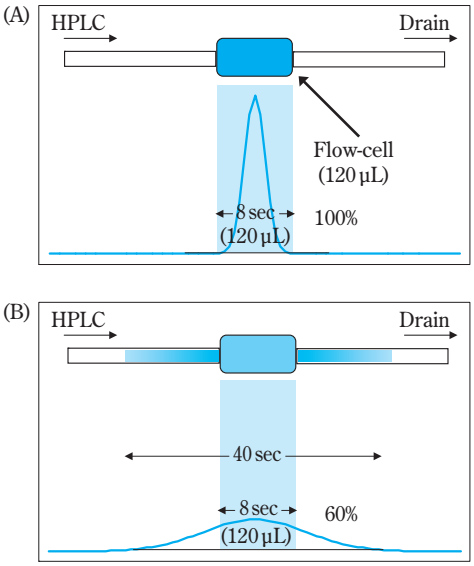


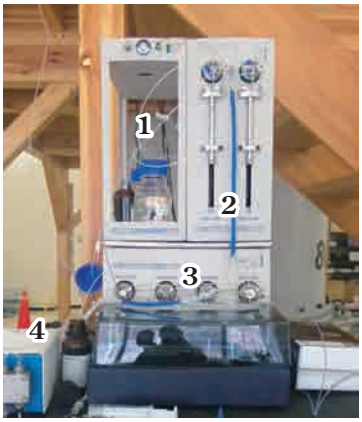
Fig. 3 Peak width and sample concentration in a flow-cell

The method of using columns with an internal diameter of around 2 mm, known as semi-micro columns, is a peak concentration method suited to LC-NMR. The volume of a semi-micro column is around 1/5 of a conventional column, and since the required amount of solvent is reduced in proportion to the elution, highly concentrated sample solutions can be introduced to LC-NMR. When there is a flow rate of 0.2 mL/min., which is typically used with a semi-micro column, the peak width corresponding to a 120 µL flow-cell expands to around 40 seconds, and it is possible to put the entire

amount for the peak into the flow-cell. However, with a semi-micro column, the sample load capacity is smaller than with a conventional column, and if the sample quantity of the conventional column is introduced as-is, separation deteriorates because of overloading, and the expected concentration may not be obtained.

What is important here is eliminating unnecessary fractions by efficient pretreatment, introducing only the targeted component to the column and controlling overloading. In terms of pretreatment methods, targeted components are partially purified offline by preparative HPLC and solid phase extraction (SPE) before LC-NMR measurements have been used, but at present, these are mainly implemented online.

The online SPE method is a method that is widely used in LC-NMR as a method for concentrating trace components.[7] The Spark Holland SPE system (**Fig. 4**) can be controlled through the same interface as LC-NMR, and the SPE cartridge absorbs the desired peak. After the sample is dried with nitrogen gas, it is eluted by a small amount of solvent of several tens of microliters, and a highly-concentrated target component can be introduced to the flow-cell. It is possible to fractionate multiple components continuously in different cartridges, and it is compatible with simultaneous analysis such as composition profiling as we discuss later.



1: Organizer
2: HPD LC-SPE-NMR Dispenser
3: ACE LC-SPE-NMR Interface
4: Dilution pump

SPE cartridge
(Spark Hyspher Resin GP10, 96 cartridges, volume 30 µL)

SPE unit (96 catridges × 2)

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Fig. 4 Spark PROSPEKT·2™ SPE system

Online column trapping is also used as a peak concentration for LC-NMR. In this method, after separation using a conventional column, concentration is first done in a trap column, and the sample is separated again using a semi-micro column then introduced to NMR. Concentration by this technique is highly effective, and two-dimensional NMR measurements (DQF-COSY and HMBC), which are usually attended with much difficulty caused by low sensitivity, can be achieved[8] (discussed later). At present, conventional columns and semi-micro columns with identical filler can be obtained easily from various column manufacturers, and since the separation pattern does not differ from the conventional columns, this method is easy to use from the standpoint of not needing additional investigations into conditions other than matching the flow rate. The method of components being separated by preparative columns and concentrated in trap columns, then being introduced to LC-NMR after separation using a conventional column is also known as online column trapping.[9]

SPE and semi-micro columns reduce the amount of usage of expensive deuterated solvents, and interference due to signals originating in non-deuterated solvents is held to a minimum. It also has an advantage in improvement of solvent suppression efficiency.

(2) Technology for increasing sensitivity on the NMR equipment side

Next, we would like to take a look at the progress on the NMR equipment side. Highly magnetic field magnets and high-sensitivity probes have made a large contribution to the equipment aspect in the achievement of high-performance LC-NMR in recent years. NMR detection sensitivity is proportional to the magnetic field strength to the 3/2 power, and the stronger the external magnetic field is, the higher the sensitivity is.[10]

Therefore, the improvement of magnetic field strength is also a central problem for LC-NMR, so research has been progressing continuously (**Fig. 5**). Currently, the magnetic field strength has reached 800 MHz[11] or more, and compared with the 60 MHz equip-

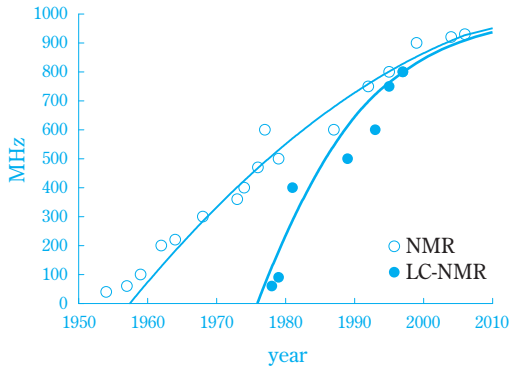

Fig. 5 Magnetic field strength in NMR and LC-NMR

ment in 1978, and this has made for a 50-fold improvement in sensitivity.

In addition, sensitivity and improvements on the probe side are important technology that has not been forgotten. If a high-sensitivity probe known as a cryogenic probe[12] that reduces the heat noise arising during NMR signal detection by cooling the coil using superconductor materials is used, an approximately fourfold improvement effect on sensitivity is obtained. Highly magnetic field magnets and cryogenic probes are fundamental technologies that are indispensable for achieving the current high performance LC-NMR.

Just what kind of performance can be obtained with an 800 MHz LC-NMR equipped with a cryogenic probe? Here, we will give results of measurements using an actual sample (**Fig. 6**). The elapsed time required for acquiring the ^{1}H NMR spectrum of a trace component with a content of 0.1% (approximately 1 μg in the sample) with the current 500 MHz equipment is 20 hours or longer, while with 800 MHz equipment it is only around 30 minutes. The improvement in sensitivity has made for a large reduction in measurement time. If we have this sensitivity, the experimental time required for measuring components on the level of several percent is within 1 minute. Even when complex mixtures are measured without partial purification, all components can also be analyzed together within a practical time. In addition, structural analysis is easily possible even with compounds that give more complex spectra with the improvement in signal resolution that accompanies increases in the magnetic field.

(3) Technology for solvent suppression

Along with the progress in chromatography and NMR equipment, the progress in solvent suppression technology has also greatly broadened the possibilities

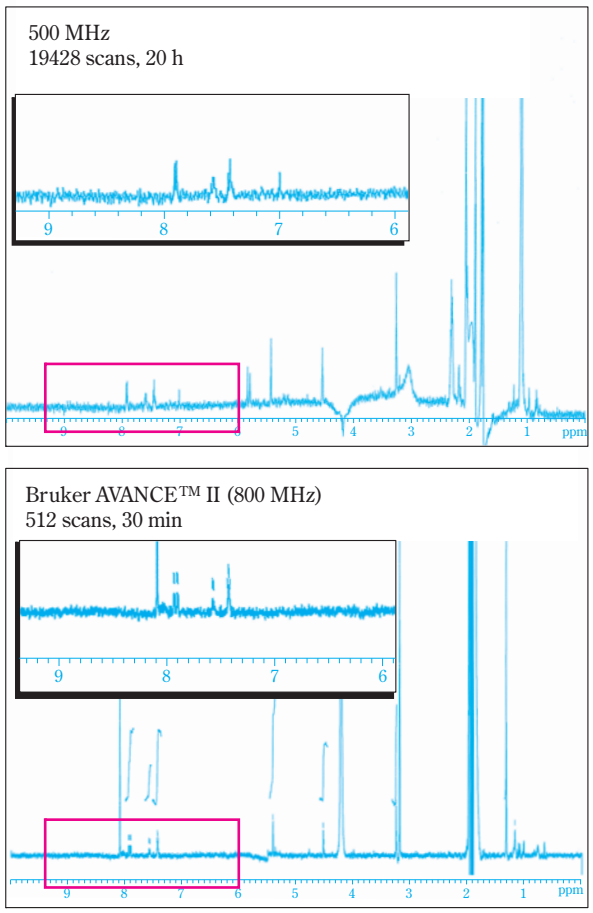

Fig. 6 ^{1}H NMR spectrum of a 0.1% component with an 800 MHz LC-NMR equipped with cryogenic probe

for LC-NMR. At the initial stages of LC-NMR, the proton signals disturbed the measurements, so the analysis was limited to low polarity compounds by normal phase mode separation that used solvents such as carbon tetrachloride, tetrachloroethylene and Freon which do not contain hydrogen atoms in a mobile phase. After that, reversed phase mode began to be applied to LC-NMR with the wide spread of versatile ODS columns in typical HPLC analysis. During the first half of the 1990s, however, most reports were on aromatic compounds having little overlap with the signal from mobile phase solvents: residual water in deuterium oxide, acetonitrile and methanol.

Since the WET method[13] which combines selective excitation pulses and a pulsed field gradient (PFG) can suppress multiple solvent peaks, it is a solvent elimination method that is currently widely used with LC-NMR. With the advent of this technique, it became possible to make measurements with little effect from solvent signals even when reversed phase ODS columns were used, and LC-NMR became a general-purpose analytic

method. Even when the solvent signal does not directly overlap the signals of the target component, the signal from trace components is not detected because of the dynamic range when the large solvent signal is not eliminated. Therefore, solvent suppression is also important technology for high-sensitivity measurement.

In addition, along with suppresing the solvent, the solvent elimination efficiency can be increased by using highly deuterated solvents for SPE and column trapping elution, which was discussed previously.

(4) Progress in multicomponent measurement technology

With the accumulated technology for increasing sensitivity as described above, the measurement time per component has been greatly reduced, and it has become practical in terms of machine time to measure all of the components in mixtures at the same time. On the other hand, in this high throughput analysis, innovations are indispensable for increasing the efficiency of processing for a large number of samples. We would like to describe this point from here.

A measurement mode known as the loop storage method is used in the simultaneous analysis of multiple components. This method is one where all of the components separated by column chromatography are first fractionated in sample loops, and then they are transferred from the loops to the flow-cell in order and measured (**Fig. 1**). It is easy to combine this with automatic measurements because separation can be done without stopping the chromatography flow and the various components that have been separated are handled individually. In addition, even if the measurement time increases, it is not affected by diffusion, so it is suitable for analyzing trace components by accumulation. Since measurements can be carried out automatically with commercially-available automatic measurement software if the measurement method for each component and the number of scans is set first, testing time can be greatly reduced.

2. Examples of high-performance LC-NMR application

(1) Composition profiling

With the advent of high-performance LC-NMR, composition profiling that clarifies the content and structure of all components contained in a mixture reached a practical level. If this is applied in the chemical industry and the mechanisms for generating impurities and causes inhibiting reactions can be identified from exhaustive analysis of impurities in the development of pharmaceuticals and agricultural chemicals, then we can expect to obtain knowledge which is useful for the development

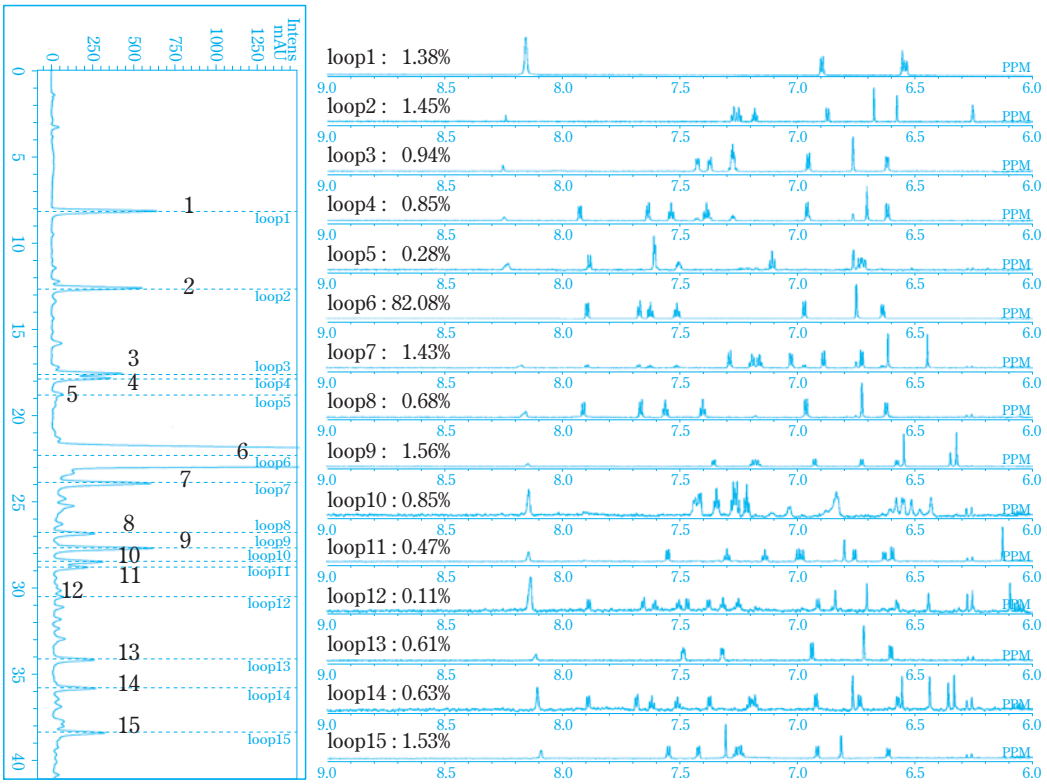

Fig. 7 Constituent profiling by ^{1}H LC-NMR (aromatic region)

of manufacturing processes. In the following, we will introduce the results of our measurements using high-performance LC-NMR. After a fractionation of a total of 15 components containing unknown impurities at the 0.1% level into a sample loop by only one HPLC separation, we set the number of scans for each component according to the content and carried out automatic ^{1}H NMR measurements. The time required up to the start of these measurements was approximately one hour for both the separation by chromatography and the setting of the measurement conditions. The ^{1}H NMR spectra for all of the components were acquired in approximately 20 hours (**Fig. 7**), and the spectra obtained were analyzed and the structure of each of the components was obtained.

In the past, unknown impurities on the level of 0.1% were been purified for several weeks to several months per component and their structures were thereafter analyzed by NMR measurements. However, high-performance LC-NMR can greatly speed up the measurement as in this example.

(2) LC-2D NMR (two-dimensional NMR measurements using LC-NMR)

There are many compounds, such as heterocycles and ring-fused compounds, with a small number of protons that causes a less connected spin system. Two-dimensional NMR (HSQC, HMBC, etc.) spectra, which give observations of ^{1}H-^{13}C correlations are useful for analysis of these compounds. HSQC spectra are a method for observing correlation between carbons ($^{1}J_{CH}$) that directly bond protons to protons, and HMBC spectra for observing correlation between carbons ($^{2}J_{CH}$ and $^{3}J_{CH}$) that are more remote. The carbon shift information and bonding information that is obtained is extremely useful for structural analysis. In particular, since correlation is observed even when sandwiching a heteroatom, HMBC is used for connecting partial structures to each other. However, these methods have lower sensitivity than ^{1}H NMR, so they have not been popular in LC-NMR.

In our study, it was possible to measure HMBC spectra of components at concentrations of a few dozen percent (approximately 300 µg) in actual samples by the high-performance LC-NMR and SPE by trapping three times (**Fig. 8**). From these results, HMBC spectra of components at concentrations of several percent or less can be obtained with repetition of concentration by SPE or combination with a semi-micro column.

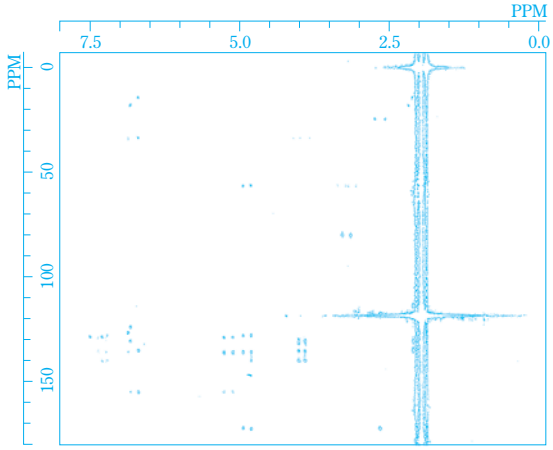

Fig. 8 HMBC spectrum with LC-SPE-NMR (800 MHz, cryogenic probe)

Table 1 HMBC and HSQC experiments by LC-NMR

Sample		Frequency (MHz)	Cryogenic probe	SPE	Other techniques	Experiment	Reference
Plant extract	Major component	500				NOESY, HSQC, HMBC	a)
Plant extract	Major component	600	✓	✓		HMQC, HMBC	b)
Plant exudate	Major component	500		✓		COSY, TOCSY, HSQC, HMBC	c)
Plant extract	Major component	600		✓		COSY, HSQC, HMBC	d)
Plant extract	Major component	400		✓		COSY, TOCSY, HMBC	e)
Synthetic peptides	Major component	600			Capillary LC-NMR	COSY, HSQC, HMBC	f)
Model mixture	Minor component	500			Semi-preparative LC-SPE-NMR	TOCSY, HMBC	g)
Drug degradation products	Minor component	600	✓		Column trapping	COSY, HMBC	h)

a) E. Garo, J. Wolfender, K. Hostettrnann, W. Hiller, S. Antus and S. Mavi, *Helv. Chim. Acta*, **81**, 754 (1998).
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In recent years, HSQC and HMBC for obtaining ^{13}C information have been less commonly reported along with the generalization of SPE (**Table 1**). Such studies will increase in the near future.

Current State of Technology Combining LC-NMR with Other Detectors

In addition to high-performance LC-NMR, integral structural analysis techniques that combine LC-NMR with other detectors have been practical. These methods give information that cannot be obtained by LC-NMR alone from different detectors, and as a result multifaceted structural analysis become possible. Here, we will give an introduction focusing on LC-NMR/MS which is a combination with MS, which is particularly useful in the analysis of fine chemicals, and chiral LC-CD-NMR, which is capable of distinguishing between optically active compounds.

1. LC-NMR/MS

If NMR and MS measurements can be made at the same time, it is possible to unambiguously determine the structures of most organic compounds. Therefore, the advent of LC-NMR/MS was comparatively early, and a research group at Pfizer reported on it in the latter half of the 1990s.[14] However, the first LC-NMR/MS was a system where a splitter was installed downstream of the LC, and the NMR spectrum and mass spectrum were obtained at the same time. Since deuterated solvents are present as the mobile phase, molecular ions where the hydrogen atoms of compounds having exchangeable protons were replaced by deuterium atoms were observed, and the interpretation of the obtained mass spectra was difficult.

Subsequently, this problem was solved by the development by Exarchou et al of LC-SPE-NMR/MS (**Fig. 9**) that incorporated SPE.[15] If this method is used, the deuterated solvent is only used in the elution from SPE to NMR, and a normal mass spectrum with no deuterium substitution can be obtained at the same time as the NMR spectrum.

There are not a few cases where structural information that could not be obtained by LC-NMR alone can be obtained by using this system. A fine example is the obtaining of useful information about compounds having NMR-silent halogen atoms from the mass spectrum. The species and number of the halogen atoms contained in the halogen compounds can be identified from the characteristic isotope patterns.[16] In addition, it is difficult to distinguish among compounds having repeated structures with NMR, but it is comparatively easy to make estimates from molecular weight information. Therefore, it is expected that the combination of NMR and MS will become the main current in hyphenated techniques.

2. Chiral LC-CD-NMR[17]

Finally, we would like to introduce our study on the chiral LC-CD-NMR method. With this method, LC-NMR and a circular dichroism (CD) detector are linked to provide a completely new effect.

Since stereoisomers of optically active compounds that have mirror images of each other (enantiomers in the following) are known to sometimes have undesirable or different strength of biological activity, the analy-

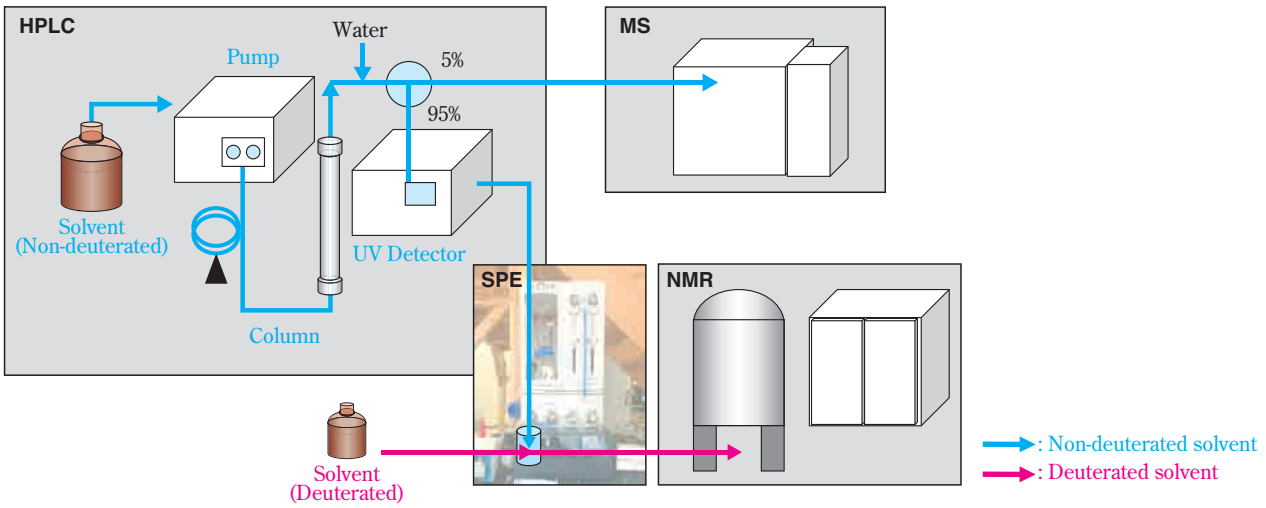

Fig. 9 Schematic representation of the LC-UV-SPE-NMR/MS

sis of optical isomers is very important from the standpoint of the safety and quality of pharmaceuticals and agricultural chemicals. In recent years, a chiral LC method (direct method) that carries out separation using a chiral column filled with an optically active stationary phase has been widely used as an analytical method for such compounds. Although chiral LC has the advantage of being able to analyze mixtures that contain impurities and isomers, it requires a large amount of effort to optimize analytical conditions by preparation of analytical standards and an identification of the peaks one by one.

Chiral LC-CD-NMR (**Fig. 10**) is an innovative analysis system that can simultaneously analyze stereoisomers and impurities without using analytical standards. In this method, trace amount of optical isomers that are included in bulk drugs (technical materials) as byproducts and impurities are identified from CD spectra and NMR spectra, and their elution positions are identified.

Here, we will describe our example of analyzing a technical material comprising a pyridylalanine derivative by chiral LC-CD-NMR. As is shown in **Fig. 11** (A), the main component and eight peaks from trace components are observed if the technical material is analyzed using normal UV detection. We can not identify what the peaks are, and we do not know whether each peak includes other components or not. Therefore, the elution positions must be identified by synthesized enantiomers, diastereomers and impurities to establish the separation conditions. On the other hand, with chiral LC-CD-NMR, the CD chromatogram and NMR spectrum are acquired at the same time as the UV chromatogram for the technical material. Using the characteristic of the enantiomer pair being detected from the CD chromatogram (**Fig. 11** (B)) that is obtained with the opposite sign, this can be narrowed down to one of peaks 5, 6 and 8 that have a negative for the opposite sign in contrast to the positive of the main peak being the enantiomer. Furthermore, the ^{1}H NMR spectrum (**Fig. 11** (C)) that is acquired simultaneously is found for each peak, and those components with spectral patterns that match the main component are identified. Ones for which the ^{1}H NMR spectrum is identical are identified by the opposite sign of the CD spectrum from

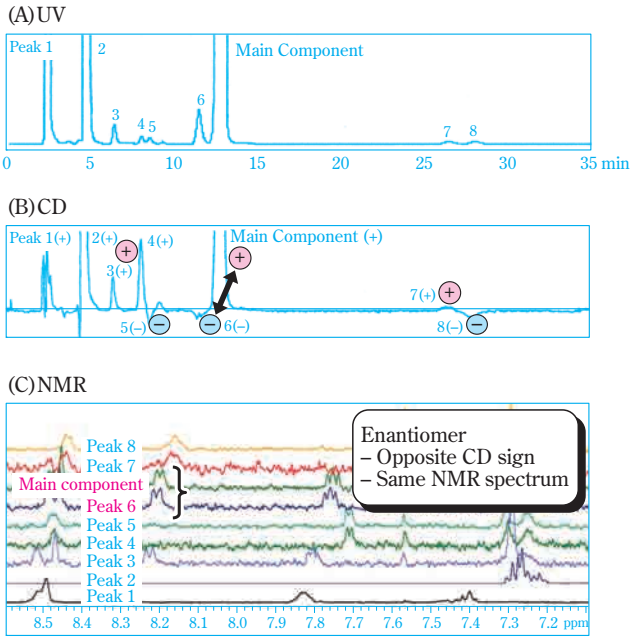

Fig. 11 Chiral LC-CD-NMR analysis of pyridylalanine derivative

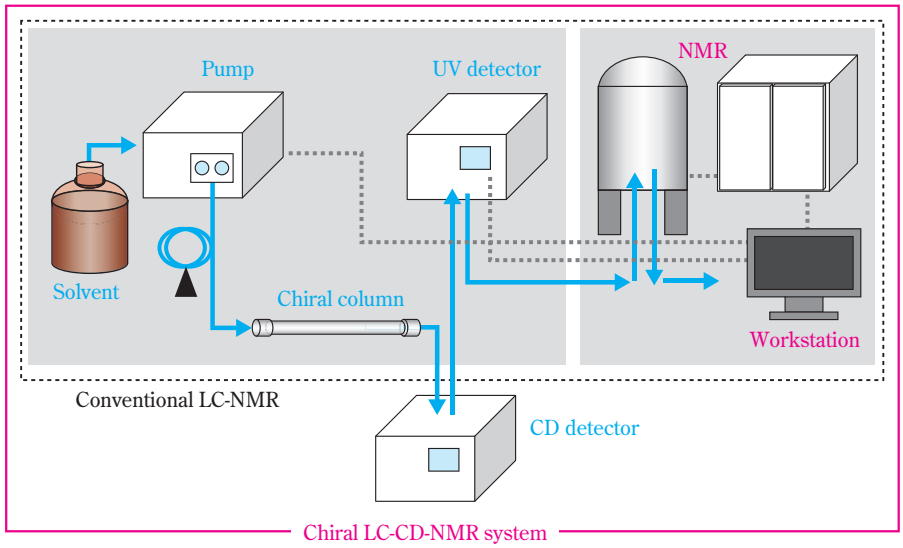

Fig. 10 Schematic view of chiral LC-CD-NMR system

the information obtained in this manner; peak 6 can be unambiguously identified as an enantiomer this time. With this method, it is also possible to determinate the structures of diastereomers, their enantiomers and impurities in the same manner based on the NMR spectrum. Although the separation of the enantiomers is not fully satisfied and they are detected as a single peak in UV and NMR detection, it is possible to determine enantiomers based on the presence or absence of reversal signs in the CD chromatogram.

If the chiral LC-CD-NMR is used, the elution times of the main component, enantiomers and other impurities can be identified for each condition just by a single analysis of the sample even if the chiral LC conditions are varied. In addition, this method can be efficiently used for optimization of the chiral LC conditions .

For example, we analyzed a technical material with three chiral columns, identified the elution positions of each of the enantiomers and calculated the degree of separation between the main component and its enantiomer (**Table 2**). As a result of comparisons, column C was the best separation condition and could easily be determined to be the optimal column. In the same manner, it is possible to vary the mobile phase, column temperature, flow rate and other conditions one after another and proceed with optimization with degrees of separation as an index. This achieves much greater efficiency than existing methods where confirmation of position using analytical standards is required for all conditions, one at a time.

Current and Future Outlook for LC-NMR

As has been discussed up to here, the current LC-NMR has matured to the point that it is provided with sufficient practicality in terms of both sensitivity and operation through a combination of highly magnetic field magnets, high-sensitivity probes, and techniques for increasing the sensitivity such as chromatography, solvent elimination techniques and automatic measurement software. At present, LC-NMR with these technologies built in is commercially available, and it will be used in multifarious ways.

In actual measurements, composition profiling that has not be achieved because of the sensitivity and LC-2D NMR measurements have reached a practical level. In addition, further hyphenated techniques of LC-NMR, such as LC-NMR/MS and chiral LC-CD-NMR, are not just online purification and spectroscopy techniques, but give new insights into structure information. In the near future, analytical methods that connect multiple detectors in parallel[18] which was considered in earliest days of hyphenated techniques may be realized. In addition, techniques of combining precolumn reactions and LC-NMR online[19] and connecting bioassay system and NMR directly by flow NMR[20] have been reported. These studies are not limited to analysis, but are a challenge of integrating reactions, separation, analysis and bioassays. In addition, the high resolution of high-performance LC NMR is suitable for analysis of the microstructures of synthetic polymers and biopolymers such as proteins, therefore the application fields will be expanded from low molecular weight compounds to polymers.

This time we were able to achieve high throughput analysis in composition profiling by increasing sensitivity and using automatic measurement software. It will be important to use spectral databases and structure analysis software[21] for acceleration of structure elucidation. Acquisition of know-how in measurements and a continuous effort in improving sensitivity will be indispensable, and particularly approaches from the chromatography side should increase. We would like to utilize our technical know-how on chromatography accumulated in our laboratory to maximize the performance of LC-NMR for our research and development.

Table 2 Comparison of *Rs* Values between Chiral Columns

	Major component		Enantiomer		
	Retention time (min)	Peak width (min)	Retention time (min)	Peak width (min)	*Rs*
Chiral column A	11.56	0.84	12.83	1.46	1.10
Chiral column B	25.98	0.87	28.34	7.87	0.54
Chiral column C	28.50	1.69	32.88	2.82	1.94

– Column
– Solvent
– Flow rate
– Column temperature etc.

Best separation with Chiral column C

Acknowledgments

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