Information

NMR analysis of mixtures using hyphenation techniques and software

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Two approaches to NMR analysis of mixtures used in Bruker instruments are considered: (1) a combination of high-performance liquid chromatography, NMR, and mass spectrometry, (2) the use of specialized software and spectral databases.

NMR over many years has developed into the main tool for structure elucidation in chemistry. For a chemist using NMR, the main task always was to determine the molecular structure of a single carefully isolated pure compound. Mixture analysis by NMR needs a completely different approach. In a mixture each component has its own NMR spectrum, which overlaps with spectra of other substances to form a mixture spectrum containing up to several thousands of NMR lines. These are two ways of analyzing those mixtures by NMR.

The first method is a combination of high-performance liquid chromatography (HPLC) with NMR (LC—NMR) and additionally with mass spectrometry (LC—NMR—MS). Special coupling devices and flow-through microprobeheads have been developed for this purpose. Stop flow and solid phase extraction lead to a sensitivity increase of almost two orders of magnitude and allow one to use all the informative and sensitive methods of modern multidimensional NMR Fourier spectroscopy. With high magnetic fields and cryoprobeheads the range of picograms can be approached for low-molecular compounds. The ozonolysis of α-pinene and metabolism of paracetamole are considered as examples in the present review.

The second method for mixture analysis is the combined use of specialized software together with spectral databases. We have developed the AMIX program for analysis of one- and multidimensional spectra of mixtures. Its most important applications presently lie in the field of combinatorial chemistry and toxicity screening of medical preparations in the pharmaceutical industry. An important medical application is screening of newborn infants for inborn metabolic errors.

Hardware development for increasing resolution and sensitivity

NMR is normally used by chemists for the elucidation of structures of newly synthesized and carefully purified compounds. Since in a mixture individual NMR spectra of all constituents overlap, no straightforward assignment of the lines to individual compounds is possible. For a chemist the usual way to deal with such a problem is to separate compounds and record individual spectra. An alternative approach (LC—NMR) is similar to the GC—MS and GC—IR methods widely used for the characterization of components of a mixture. For high-resolution NMR in solutions, HPLC would be the separation

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method of choice. However, the sensitivity of NMR is rather low compared to those of MS and optical spectroscopy. Therefore, the maximum sensitivity and resolution are vitally important prerequisites for coupled LC—NMR.

A high spectral resolution is necessary to resolve overlapping lines from signals with similar chemical shifts and multiplet structures. The distance between multiplets increases proportionally to the magnetic field strength, whereas the couplings do not depend on the field strength. Therefore, at higher fields not only the resolution increases but the spectra also become much simpler. Only superconducting magnets can provide these field strengths, and the progress of the highest available NMR frequency is closely related to the discovery of new superconducting materials and improvements in manufacturing technology. Beginning from a field strength of 18.790 T (800 MHz), magnet coils are wound from the Nb-Sn-Ta-multifilament wire and work in a persistent mode at a liquid helium temperature of 2 K. For such high fields, stray fields become a great problem and siting in existing buildings becomes difficult and often impossible. Now up to 800-MHz self-shielded magnets are available (supershielded, patented by Bruker), reducing the stray field by a factor of more than two.

The sensitivity increases more than linearly with the field strength (Table 1). For a given frequency, the optimal design of the probehead is crucial and it often has to be tailored to specific application. For an LC—NMR experiment, a small-diameter flow-through probes have to be used and microprobeheads (diameter 1 mm) are offered for minimum sample amounts. Cryoprobes for which the NMR coil and preamplifier are kept at helium temperature offer a fourfold increase in the sensitivity. They are supported by a special cryoplatform and allow normal NMR measurements at ambient and variable temperatures of the sample. In cases where a high sensitivity is required at medium resolution, a cryoprobehead at a lower frequency (500 MHz) has a better sensitivity (S/N ratio 570 : 1) than a conventional probehead at a higher frequency (800 MHz, S/N ratio 245 : 1) and may offer the best economical solution. Triple resonance probehead at 800 MHz can measure nanograms of low-molecular compounds. The picogram range can be approached with a selective probehead at higher frequency.

Table 1. Sensitivity and dispersion of chemical shifts at high fields

ν_0/MHz[a]	S/N[b]	ξ[c]
600	1.200 : 1	1.00
700	1.512 : 1	1.17
800	1.847 : 1	1.33
900	2.400 : 1	1.50
900[d]	7.300 : 1	1.50

[a] ^{1}H frequency.
[b] Signal/noise ratio (0.1% ethylbenzene).
[c] Relative dispersion of chemical shifts.
[d] Cryoprobe.

Table 2. Sensitivity of heteronuclear experiments

Experiment	Sensitivity		Time of measurement	
	^{13}C	^{15}N	^{13}C	^{15}N
Direct observation	1.0	1.0	1.0	1.0
INEPT	4.0[a]	9.9[a]	16.0[a]	98[a]
Inverse INEPT	7.9[a]	31[a]	624[a]	961[a]
	2.0[b]	3.1[b]	4.0[b]	9.6[b]
Inverse correlation	31.6[a]	306[a]	998[a]	93636[a]
	7.9[b]	31[b]	624[b]	961[b]

[a] Relatively to direct observation.
[b] Relatively to INEPT.

For experiments on low-abundant and low-sensitivity nuclei such as ^{13}C and ^{15}N, polarization transfer methods like INEPT[1] and DEPT[2] and inverse correlation methods[3,4] offer an enormous increase in the sensitivity and shortening of the measurement time (Table 2).*

Mixture analysis by combined LC—NMR and LC—NMR—MS methods

Once the sensitivity and resolution in the NMR itself have been optimized, the next step is to develop an optimal coupling of liquid chromatography and NMR using specialized high-sensitivity probeheads. The Bruker company, as the only manufacturer of NMR, MS, and HPLC instruments altogether, has pioneered in the development of the new LC—MS—NMR analytical method, which may be regarded as one of the most powerful analytical techniques.[5—8] It combines the high separation power of HPLC with the highly sensitive detection methods using a diode array UV detector (DAD) and a MS detector along with unsurpassed specificity of NMR for molecular structure determination.

The design of an LC—NMR—MS combined spectrometer (Fig. 1) is very compact and needs the same space as a single standard NMR spectrometer. The LC—NMR interface is mounted into the NMR console. The HPLC unit, LC fraction collector, and MS unit can be placed on the top of the NMR spectrometer. A special LC—NMR—MS software controls the experiment and

* INEPT is Insensitive Nuclei Enhanced by Polarization Transfer, DEPT is Distortionless Enhancement by Polarization Transfer.

Fig. 1. LC—NMR—MS spectrometer combination. *1*, B-NMI Bruker NMR—MS interface; *2*, HP1100+ autosampler; *3*, LC—NMR interface, UV DAD; *4*, Esquire 3000 mass spectrometer; *5*, Avance 500 NMR spectrometer; *6*, vacuum pump of the mass spectrometer.

displays the elution trace (UV detection, DAD) and UV, MS, and NMR spectra (Fig. 2).

A special interface (Fig. 3) makes it possible to trigger NMR measurements from the MS and UV (DAD) detectors on the flow. A computer-controlled valve switches between different operation modes. In the waste position (*a*) the liquid flow is directed to the waste or to a fraction collector if available. In the direct or trigger position of the valve (*b*), if a LC peak is detected, the liquid is directed to the MS and/or to the NMR spectrometer in parallel. The storage loop position (*c*) is the normal operation mode for NMR spectra recording. Low concentrations cannot be measured "on the fly" by the NMR method for reasons of sensitivity. Under the conditions of the limited time between two LC peaks, no sufficient signal can be accumulated. The stopped flow leads to peak broadening and, hence, to loss of the LC resolution and NMR sensitivity. A better alternative is the collection of chromatographic peaks into 36 loops for later detailed analysis using all possibilities of multidimensional NMR methods. Then the samples are transferred to the NMR spectrometer, and any one- and multidimensional NMR measurements can be executed without time limitation. In the valve position (*d*) in parallel with storage accumulation a portion of the sample is transferred to the MS spectrometer for more time-consuming experiments of tandem mass spectrometry (MS^n). Using this technology, LC peaks can also be collected outside the NMR laboratory and then transferred to the NMR spectrometer for analysis.

The most important device of the NMR side is the flow-through probehead (Fig. 4). Probeheads are available down to volumes of 1.5 μL. 2D spectra for low-molecular samples in the nanogram range (12.4 ng of

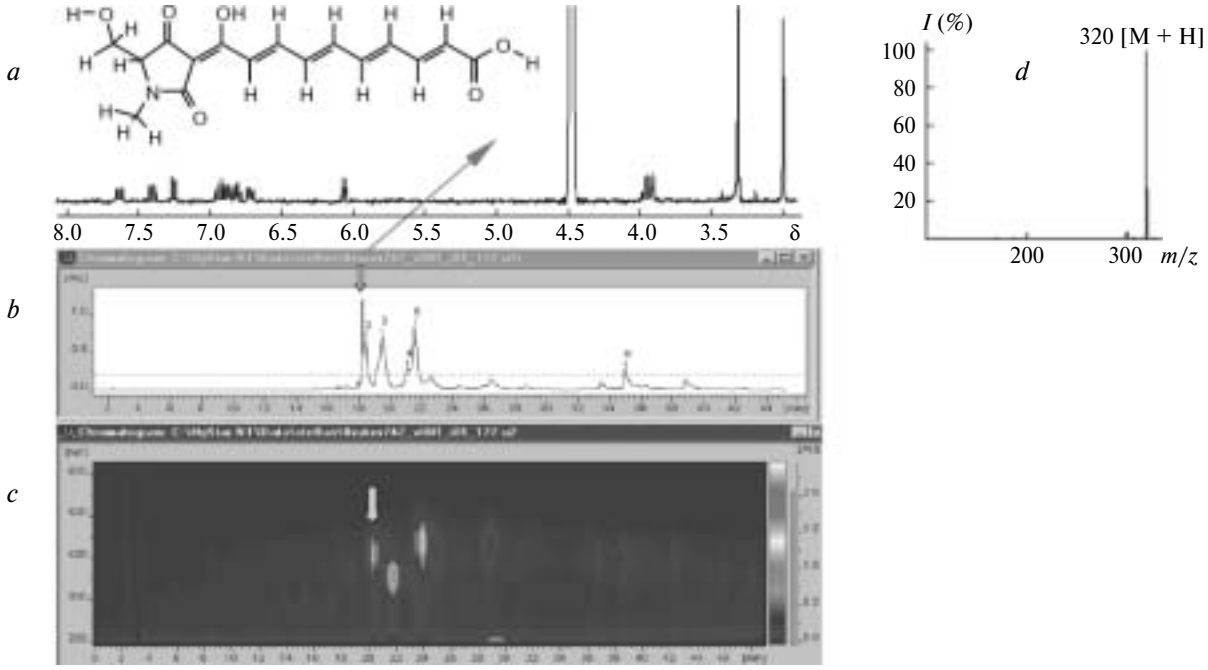

Fig. 2. Analysis of a mixture by the combination of the NMR, MS, and UV (with a diode array (DAD)) (the program displays simultaneously all data of all methods): (*a*) ^{1}H NMR spectrum (600 MHz) of the peak selected from the UV chromatogram (shown by arrow), (*b*) elution trace with UV detection at 390 nm, (*c*) elution trace with DAD detection, and (*d*) mass spectrum of the peak marked with arrow; detection of positive ions from an ion trap.

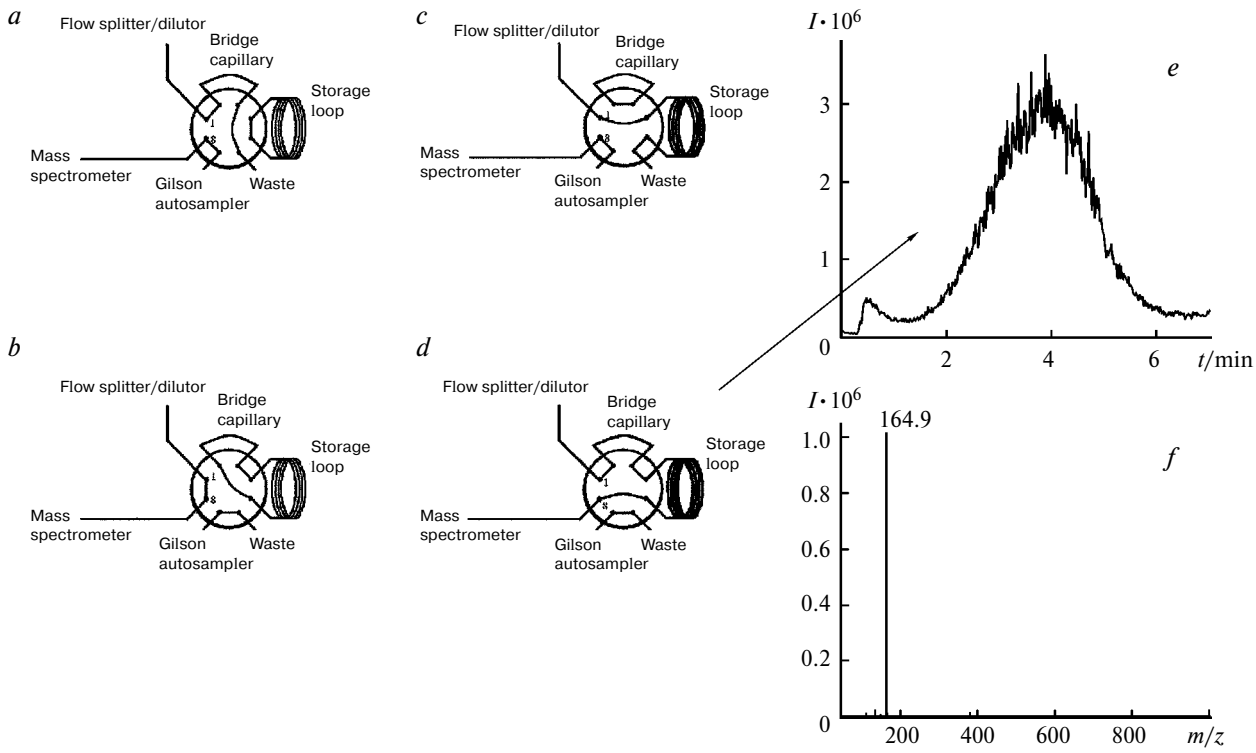

Fig. 3. LC—NMR—MS interface: *a*—*d*, valve position; initial (waste, ready for the introduction of a new sample) (*a*), direct (trigger) (*b*), storage loop position (NMR spectrum detection) (*c*), transfer to the ion source of the mass spectrometer (Gilson autosampler) (*d*); *e*, MS trace during transfer; and *f*, mass spectrum.

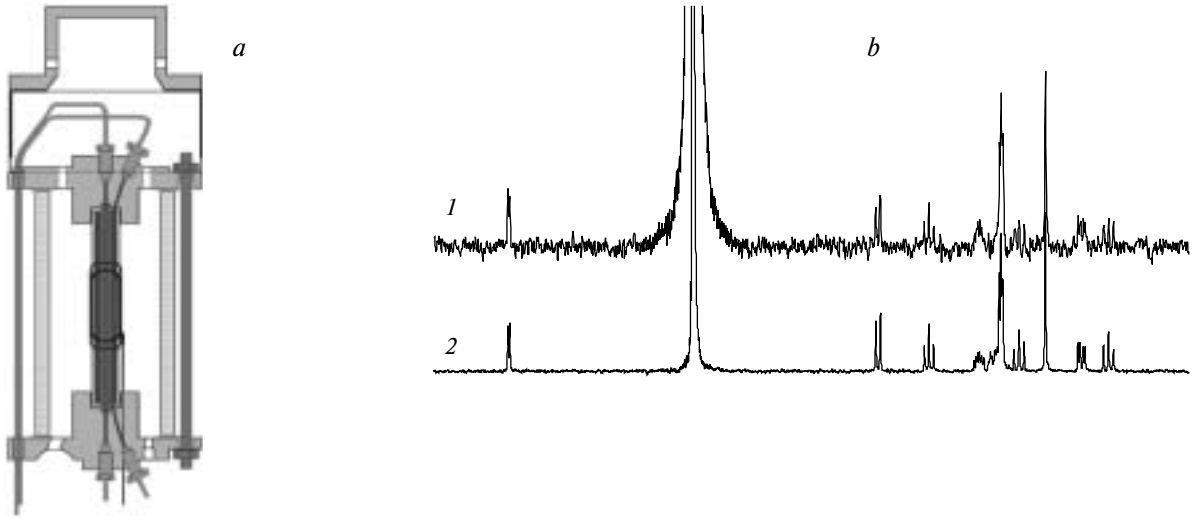

Fig. 4. Flow-through probehead (*a*) and ^{1}H NMR spectra of sucrose (*b*): *1*, sample 1: 25 μ*M*, volume 1.5 μL => amount 12.4 ng, number of scans NS = 3072, time ~3 h, S/N ratio = 5.5 : 1; *2*, sample 2: 5.13 μg of sucrose, number of scans NS = 1.

sucrose) can easily be measured on a 600-MHz instrument. The sensitivity can be increased significantly using cryoprobes at higher fields.[9] Flow-through probeheads provide the highest throughput for NMR experiments and are used for many applications even without LC coupling. This technique allows the complete automation of sample preparation, its transfer through a Gilson autosampler, and NMR spectra measurement.

A more significant increase in the sensitivity (by almost two orders of magnitude) is possible with pre-concentration of the sample by solid phase extraction. Special cartridges are used instead of loops for collection of

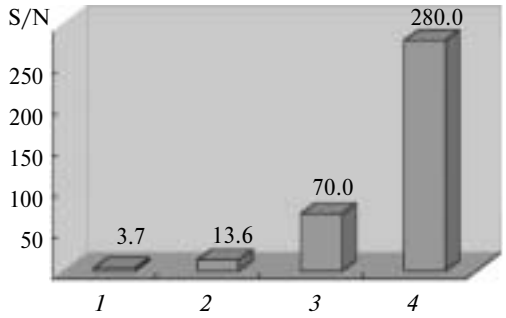

Fig. 5. Sensitivity increase (S/N) with solid phase extraction, depending on the method of sample preparation: *1*, direct loop collection; *2*, single solid phase extraction; *3*, quadruple extraction; and *4*, the same in combination with a cryoprobehead.

chromatographic peaks.[10] After peak trapping, nitrogen is passed through the cartridge to remove all residual solvents. This saves costs by using nondeuterated solvents and prevents the H—D exchange of the OH, NH, and NH_2 groups with D_2O, which is also substantial for mass spectrometry. A pure deuterated solvent is used only for peak elution from the cartridge into the NMR spectrometer. This creates very sharp elution bands (30—50 μL eluting volume) and enhances the sensitivity of the experiment (Fig. 5). The S/N ratio gain for NMR in this variant depends on the ratio of LC peak volumes eluted into the NMR spectrometer with and without peak trapping, probe volume, and peak broadening during transfer.

In a typical LC—NMR spectrum the ordinate (y) corresponds to the time scale, and the abscissa (x) corresponds to the chemical shifts in the NMR spectrum. The spectrum of human urine after administration of the anti-inflammation drug paracetamole is presented in Fig. 6.[5,11] In addition to the permanent peaks of the solvent, the NMR spectrum changes over time due to the appearance

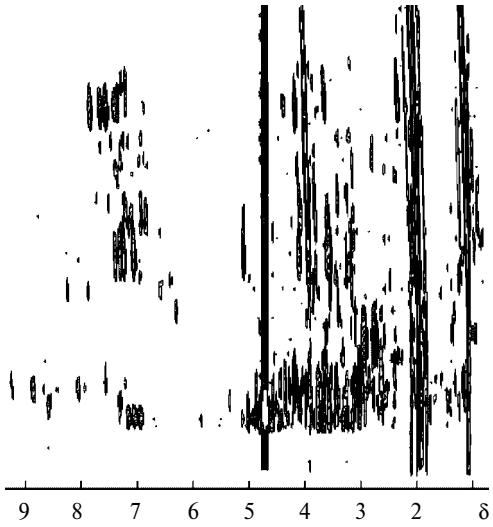

Fig. 6. LC—NMR spectrum of paracetamole in human urine (500 MHz, 16 scans/time slice in 16 s) 4 h after administration of 500 mg (sample volume 100 μL).

of various metabolites (see Fig. 6). Four cross-sections through this LC—NMR trace show the spectra of the main metabolites of paracetamole (Fig. 7). The structures of the metabolites can be determined from these individual NMR spectra (Fig. 8). The LC—NMR has become an extremely important analytical tool in the pharmaceutical industry.[12,13]

Another field of LC—NMR application is environmental research.[14,15] We studied the ozonolysis of α-pinene in cooperation with the Max Planck Institute (Max-Planck Institut für Kohleforschung in Muhlheim, Germany). α-Pinene is released from pine trees to the atmosphere, and the so-called "blue haze" is formed at high ozone concentrations. The ozonolysis of α-pinene

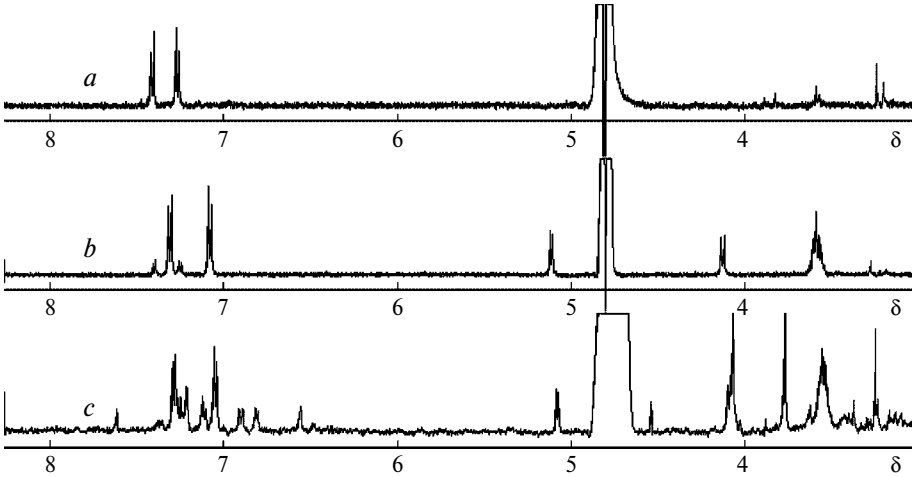

Fig. 7. Cross-sections through the LC—NMR trace of human urine after paracetamole administration. Stop flow detection in a flow-through cell using a cryoprobehead. Paracetamole metabolites: *a*, paracetamole sulfate; *b*, paracetamole glucuronide; and *c*, methoxylated paracetamole glucuronide.

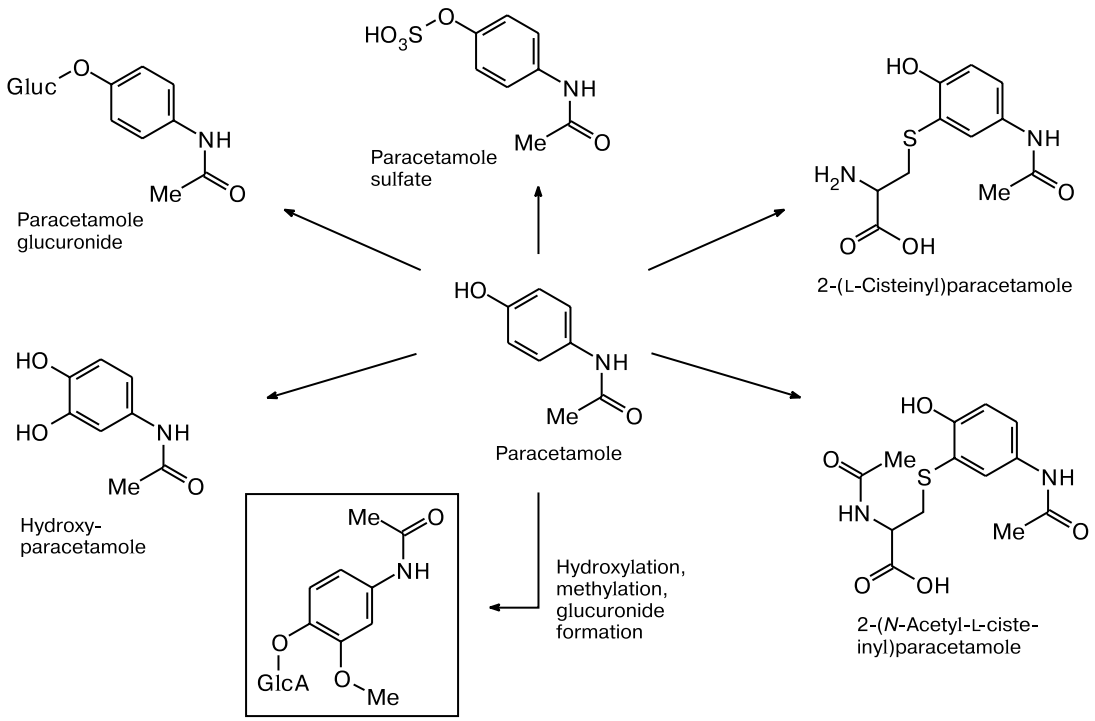

Fig. 8. Structures of paracetamole metabolites obtained from LC—NMR spectra analysis.

was simulated at the laboratory. The UV—LC traces along with the MS traces (positive and negative ions) contain a set of peaks. The most intense peak in both the UV and MS detections has a molecular mass of 184. Tandem MS/MS experiments with additional fragmentation made it possible to identify pinonic acid (Fig. 9). The NMR spectra (1D, COSY, NOESY) allowed the determination of the exact spatial structure of the molecule (Fig. 10). The corresponding pinonaldehyde (molecular mass 168) and pinic acid (molecular mass 186) were identified in the same way, and the complete chemical reaction scheme was established.

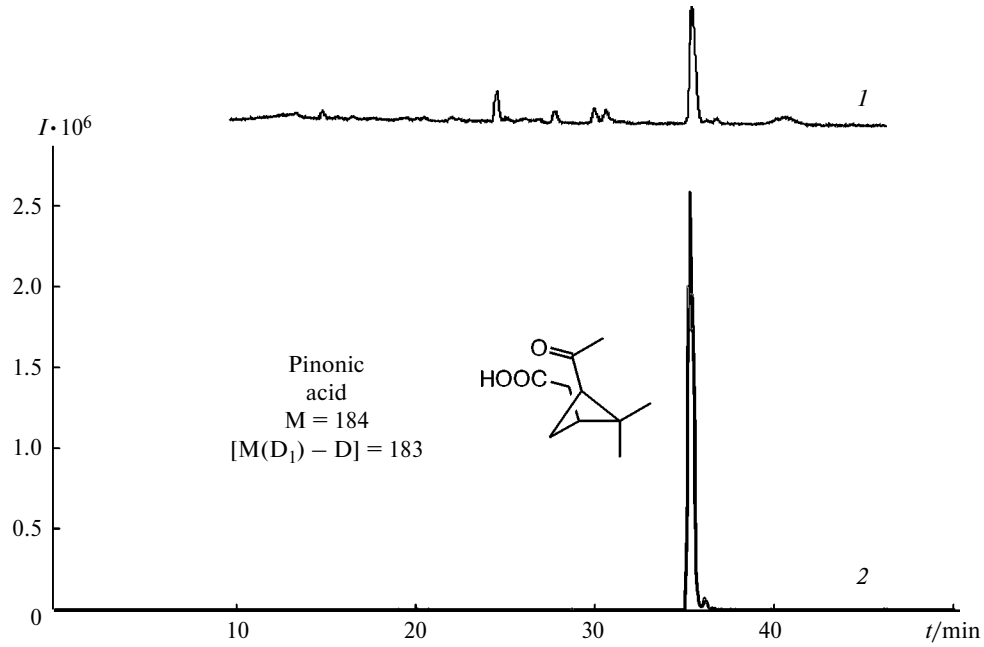

Fig. 9. UV and extracted ion chromatograms of pinonic acid after α-pinene ozonolysis. Elution trace: UV detection at 200 nm (*1*), MS detection by the extracted ion current *m/z* 183 (*2*).

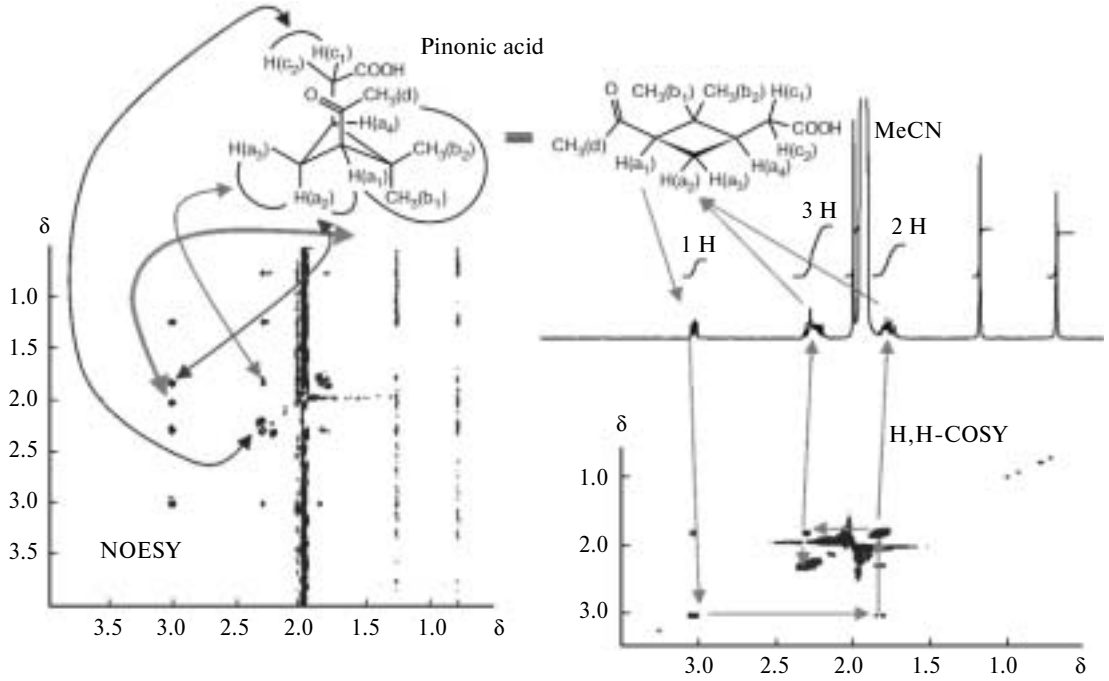

Fig. 10. Determination of the pinonic acid structure using 2D NMR spectroscopy (COSY is Correlation Spectroscopy, NOESY is Nuclear Overhauser Effect Spectroscopy).

Direct mixture analysis using databases and pattern recognition

To determine the constituents and their relative concentrations in a mixture, spectra of individual components must be identified. This can be accomplished with the help of databases and known spectral patterns for individual compounds. Combinatorial chemistry is a good example to show the principles of analysis of mixtures using the AMIX program. Following the principles of combinatorial chemistry, molecules are regarded as combinations of different substructures. Correspondingly, 2D HSQC spectra ($^1H—^{13}C$) are regarded as a sum of spectra of substructures, which determine a structure code. The assigned structure codes can be verified by the "proper" determination and recognition of subspectra. Different ligand groups can be connected to the core of a molecule at various sites (Fig. 11). Even a small number of binding sites and possible ligands can create a great number of reaction compounds.

At three sites *x*, *y*, and *z*, 5, 4, and 4 fragments can be bound, and 80 compounds can be created from 14 fragments. In a minimum subset (for example, $A_1B_1C_1$, $A_2B_2C_2$, $A_3B_3C_3$, $A_4B_4C_4$, and $A_5B_1C_2$) each fragment

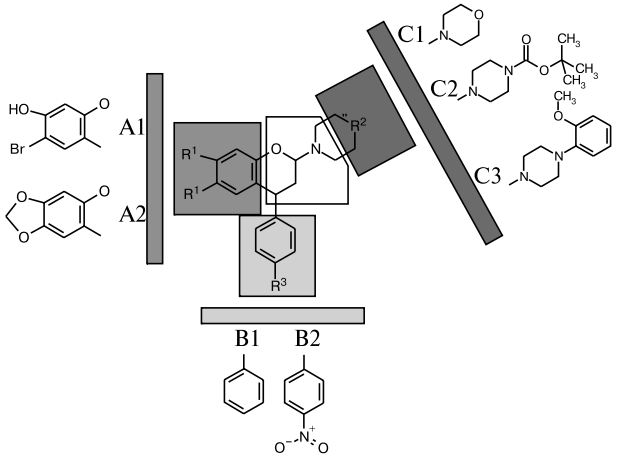

Fig. 11. Core of the molecule with different ligands. Partition into fragments.

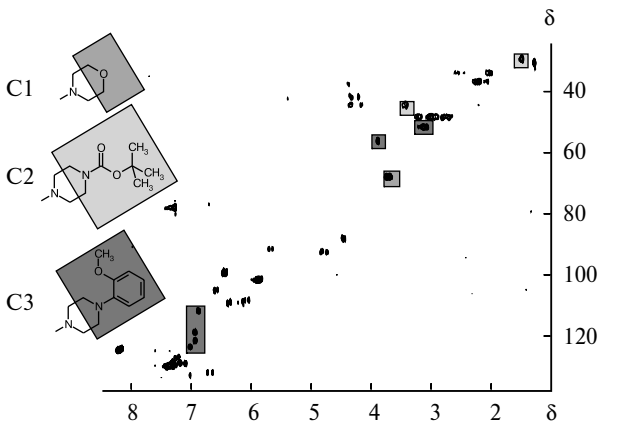

Fig. 12. Assignment of NMR patterns to different molecular fragments.

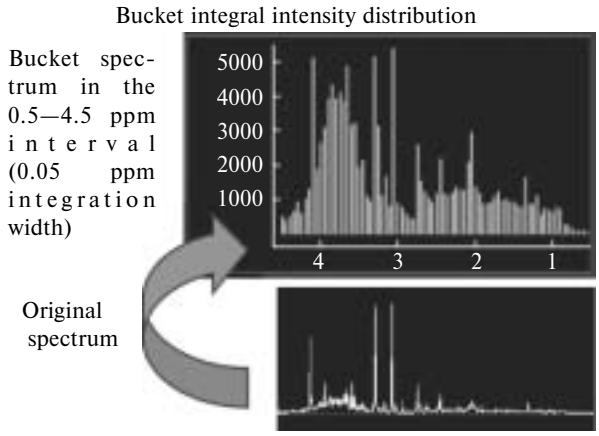

Fig. 13. Transformation of the NMR spectrum into the bucket spectrum.

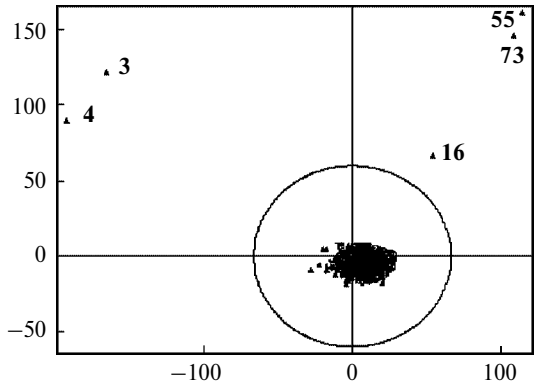

Fig. 14. Principal component analysis of the principal components for 200 urine spectra showing suspect for various pathologies: **3** and **4**, maple syrup disease; **16**, orotic aciduria; **55** and **73**, mevalonic aciduria.

should occur at least once. The NMR spectrum is checked for all possible spectral patterns, which are then assigned to the corresponding molecular fragments (Fig. 12).

Investigation of biofluids and screening of inborn metabolic errors in newborn infants

The investigation of biofluids is another important field of application of the methods considered.[16,17] The spectra of rat urine can be used for screening of toxic effects of medical preparations.[18]

Inborn metabolic errors present a big problem in modern medicine. Children often show retarded physical and mental development. However, when this becomes evident, it may be too late for a successful treatment. The urine composition reflects the metabolism of all organs and could reflect various diseases.[19] In cooperation with several medical institutions we checked if NMR can be used for screening of metabolic diseases.[18,20,21] The tech-

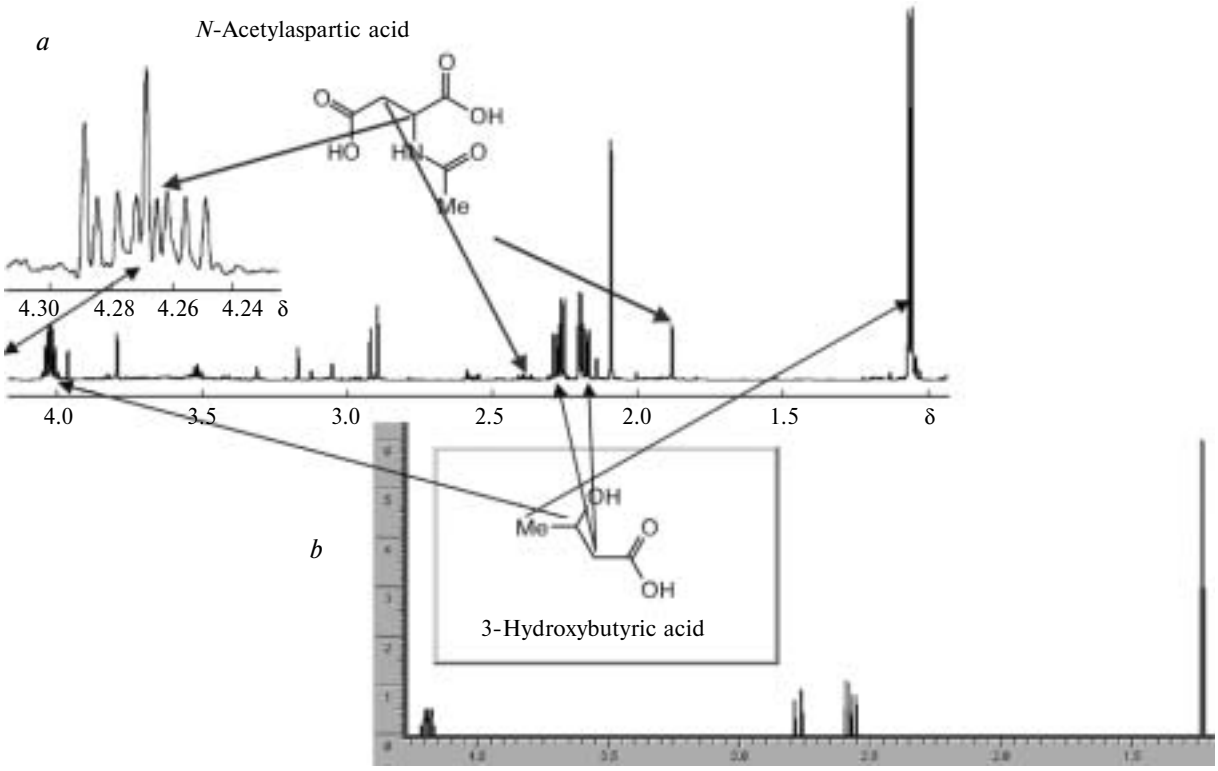

Fig. 15. Metabolite identification by comparison of the measured 1D spectrum and the library spectrum: *a*, urine spectrum of a two-year-old boy with Canavan syndrome (aspartoacylase deficiency); *b*, predicted spectrum.

nique for clinical practice should be easy to use by non-specialists in NMR and provide clear "yes/no" answers.

Measured spectra of urine are converted to so-called bucket spectra by the replacement of the original spectrum by integral signal values over a pre-defined small spectral region (Fig. 13). All values from the bucket spectrum are put into a bucket table containing the complete spectra of all measured samples. Then the data are subjected to a mathematical procedure called "Principal Component Analysis" (PCA). The computer determines the spectral features, which exhibit the biggest differences between the individual spectra, and these principal components are plotted against each other. Principal component analysis of identical spectra gives one point in the center. Two hundreds of urine samples group as a center cluster around the zero point but five samples show clear deviations (Fig. 14). This means that only these five spectra with the suspected metabolic errors (*3*, *4*, *16*, *55*, and *73*) have to be inspected. For the Canavan syndrome, an often lethal disease leading to the motoric and mental retardation we found an unusual amount of *N*-acetyl-aspartic acid and 3-hydroxybutyric acid. This indicates a deficiency of the aspartoacylase enzyme (Fig. 15). For a more detailed metabolite identification, 2D spectra were measured and compared with the library spectra.[20] In the case of mevalonic acidurea, all four mevalonic acid peaks were identified with a hit quality of 0.968 against the next alternative hit with 0.156. The conversion of mevalonic acid to 5-phosphomevalonic acid is blocked due to a deficiency of the corresponding enzyme (Fig. 16).

For clinical screening the total procedure has to be completely automated. In the first step a 1D NMR spectrum is measured. In the second step the principal component analysis including a database of normal spectra detects deviation from normal. In the third step the 2D spectra identify the metabolite signals indicative for the metabolic error. In the fourth step the structures of all compounds are verified using simulation routine spectra and spectral databases. A relationship to the corresponding biochemical pathway is established in the final step. All these steps can be performed in a single automatic procedure.

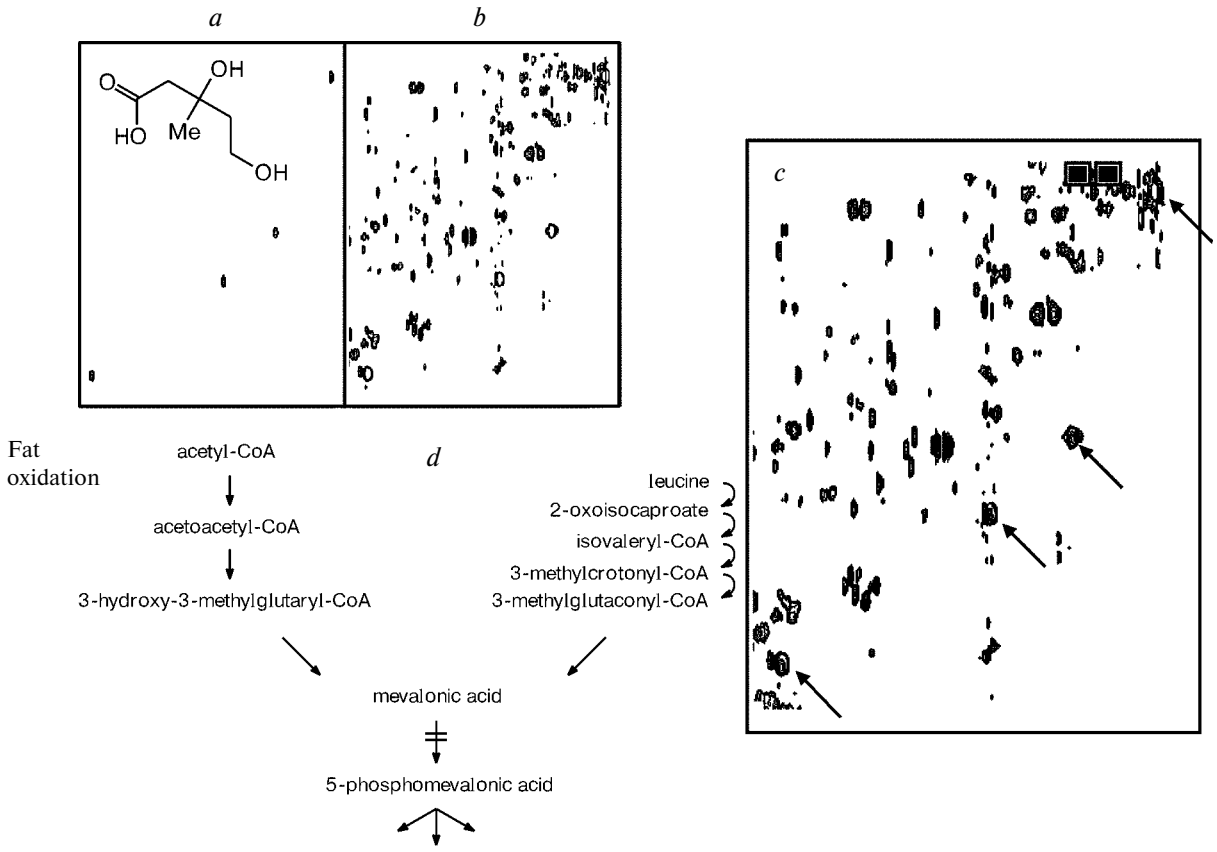

Fig. 16. Identification of mevalonic acid peaks in the 2D spectrum and metabolic pathway: *a*, structure and 2D spectrum of mevalonic acid; *b*, 2D urine spectrum; *c*, identification of all four peaks of mevalonic acid in the 2D urine spectrum; *d*, metabolic defect site in the biochemical cycle of fat oxidation; mevalonic acid phosphorylation to 5-phosphomevalonic acid was interrupted because of the mevalonate kinase deficiency. Mavalonic aciduria (mevalonate kinase deficiency) results in the mental and motor retardation (ataxia).

Thus, the LC—NMR—MS system can act as the complete analytical laboratory appropriate for extensive studies in the fields of metabolism, toxicology, or pharmacology.

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