LC-NMR : AN ALTERNATIVE TO LC-MS AND HPLC-DAD FOR THE ANALYSIS OF COMPLEX MIXTURES.

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Introduction

NMR (Nuclear magnetic resonance) spectroscopy has become an essential technique for the organic chemist for its interpretative power. However analytical chemists have exploited this technique less largely because of problems of analysing mixtures on a routine basis. Within the past decade LC-NMR (liquid chromatography NMR) has become increasingly available, although many of the earlier reports involve fairly slow analyses using quite high concentrations which often are readily separable. Recently it has become easier to couple HPLC (High Performance Liquid Chromatography) to NMR allowing cheaper and more routine applications.

In parallel to improved instrumentation, advances in data analysis such as Fourier transformation, digital signal processing and maximum entropy played a major role in the 1980s by improving the quality of NMR spectra. With the ability to obtain LC-NMR, chemometrics methods for deconvolution and enhancement of spectral quality also have an important role. Many LC-NMR experiments are performed using high concentrations, slow analysis times and often stopped-flow conditions. Although these experiments overcome many of the problems of sensitivity and distortion in the chromatography due to high concentrations necessary to obtain acceptable spectral signal to noise ratio, the inherent expense and difficulty means that LC-NMR is not yet a routine technique in the same way as HPLC-DAD and LC-MS (liquid chromatography mass spectrometry). Despite this LC-NMR is now possible using mid range NMR and LC instrumentation. In addition to interpretative power proton NMR has a major advantage over many other forms of quantitative coupled chromatography in that the intensities of the signals are proportional to the number of protons and concentrations under suitable pulse conditions.

In this article we report the use of LC-NMR for the on-flow resolution of a seven component mixture. Using rapid flow rates, the LC-NMR signals coelute. It would not be easy to determine how many compounds are in the mixture without chemometrics.

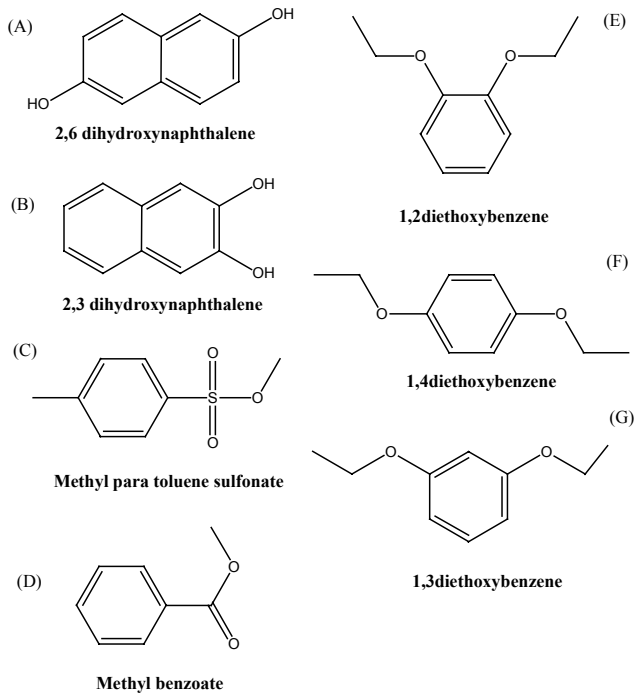
Keywords: HPLC, NMR, Chemometrics, Mixture Analysis

Experimental

A mixture of the seven compounds A to G as illustrated below (50 mM each) were prepared as a solution in 80% acetonitrile : 20% deuterated water, to be injected on the column.

A Waters HPLC system was used and consisted of a 600S controller, a model 616 pump and a model 717 autosampler running with the Millenium Session Manager software. The system was run in isocratic mode at 0.5 $mL.min^{-1}$ using 80% acetonitrile : 20% deuterated water, the

latter added for a lock. A few drops of tetramethylsilane (TMS) were added to the mobile phase as it was used as reference to zero ppm for the later NMR spectra. The mixture of the seven compounds (50 µL) was injected on a C_{18} reversed phase column (Waters Symmetry, 100 × 4.6 mm, 3.5 µm) at room temperature. A photo diode array detector (Waters model 996) was used on-line to detect the elution of the compounds from the column and trigger the NMR acquisition.



A 4 m PEEK tubing of width 0.005" was used to connect the eluent from the HPLC to a custom-made flow cell (300 µl) into the NMR probe. The system used was a 500 MHz Jeol Alpha spectrometer (Jeol, Tokyo, Japan). The spectra were acquired in quadrature mode with a spectral width of 7002.8 Hz and a digital resolution of 0.855 Hz, the pulse width was 16 µs (90^o). Solvent suppression was applied on the acetonitrile singlet by pre-saturation using a DANTE sequence. The acquisition time was 1.1698 s with a pulse delay of 2 s. A stack of 256 spectra was recorded and 84 datapoints in time starting at 1.5 min, containing the elution of the seven compounds, were retained for further processing.

Data Analysis

The 2-dimensional stack dataset was transferred to Matlab version 6 on a 600 MHz PC with 128 Mbyte memory using software developed in Bristol. The first steps involve apodisation, Fourier transformation, phasing, chemical shift alignment to 0 ppm for TMS, baseline correction and removal of the region (1.57 - 2.74 ppm) where the solvent elutes and replacing by zeros. Because of quadrature detection the chromatogram appears to oscillate in the raw data but this is solved by baseline correction. This gives a matrix of 85 × 8192 datapoints. The matrix is reduced slightly in later calculations to remove high field frequencies where no protons resonate. In order to retain chromatographic digital resolution of 3.1698 s but to smooth the chromatogram a 4 point moving average of the spectra is computed, so that the first smoothed spectrum consists of the average of spectra 1 to 4, and the second of the average of spectra 2 to 5 and so on.

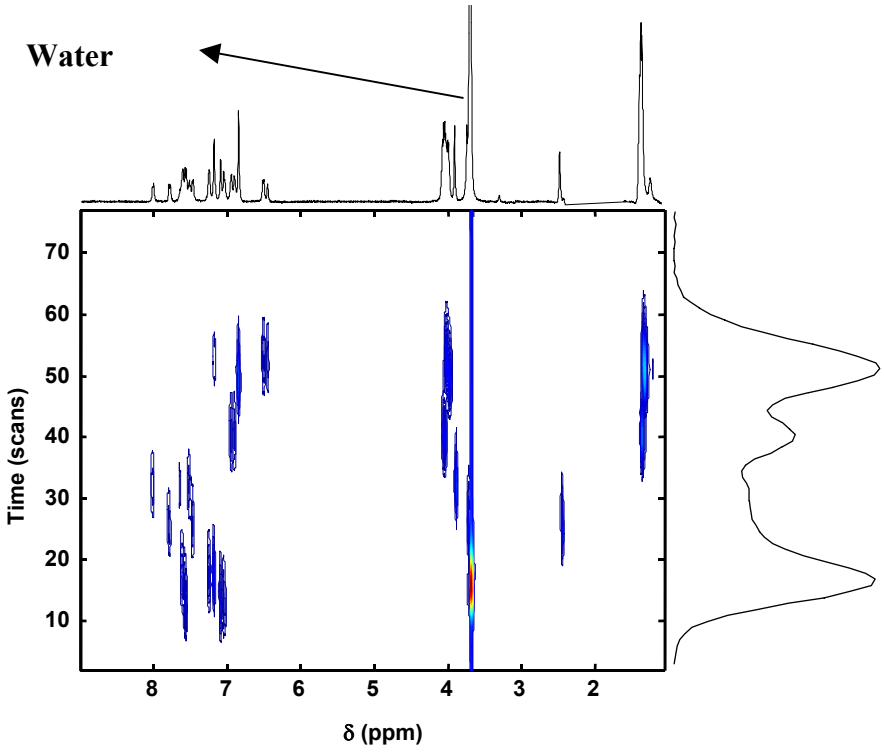
The next step was to use PCA (principal components analysis) [1-3] to explore the complex chromatograms. This method can be employed to simplify the very complex dataset; scores relate to elution times and can help pinpoint where the compounds in the mixture elute, and loadings to resonances. Finally PCA is performed and the three most discriminatory PCs are retained, the scores are then presented graphically as a 3-D plot. The number of changes in direction of the scores plot provides information on the number and elution times of the compounds in the mixture. This provides graphical information about the chromatogram.

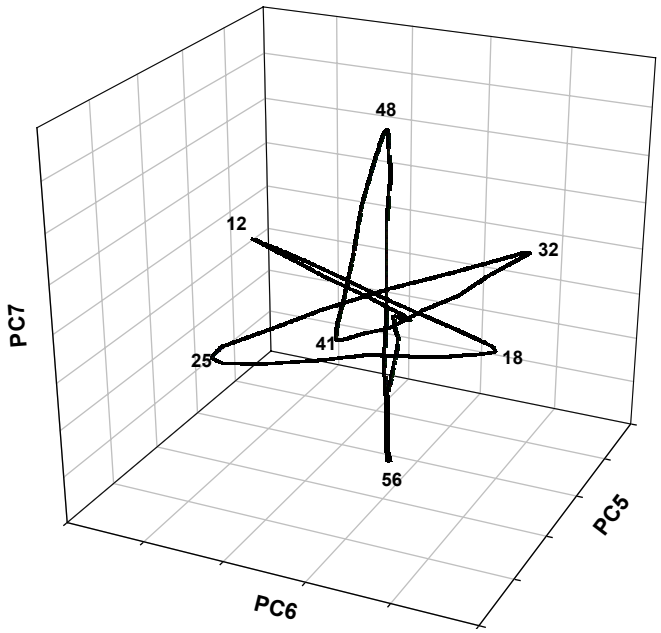
The next step is to use a method called the simplified Borgen method [4,5], to identify seven key variables. This is a multivariate method based on PCA which looks for extreme behaviour in the spectroscopic variables. It starts by identifying a pure variable most diagnostic of one compound. The variable least correlated to this will correspond to a different compound. By examining the correlations between all the frequencies each key variable is selected stepwise. Ideally all the key variables should be unimodal but sometimes because of spectral overlap there is an element of bimodality, under such circumstances, regression is used to assign a minor contribution to one of the other main variables, resulting in seven unimodal key variables. These represent the first estimate of the seven chromatographic profiles.

The final step is called resolution, and involves using this information to provide estimates of the deconvoluted spectra. In this application, there are several embedded chromatographic peaks, so the spectra could not be obtained by stopped-flow or simply by taking a profile through the LC-NMR spectrum, and neighbouring compounds often share regions of resonance in the mixture spectra. This can be done by regression of the seven key variables onto the LC-NMR spectra. Using non-negative least squares [6,7] the quality of the spectra can then be improved.

Results

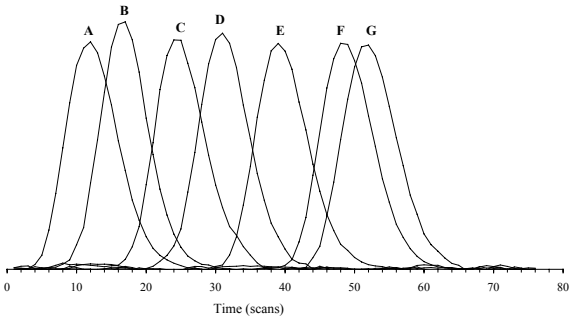
The 3-D chromatogram in the elution region together with the summed elution and spectral profiles is presented below. It is not obvious how many components are in the mixture and what their spectra are. There is obviously severe coelution. Note that solvent suppression has been used to reduce the acetonitrile peak in intensity, so about 1 ppm is missing from the spectrum.

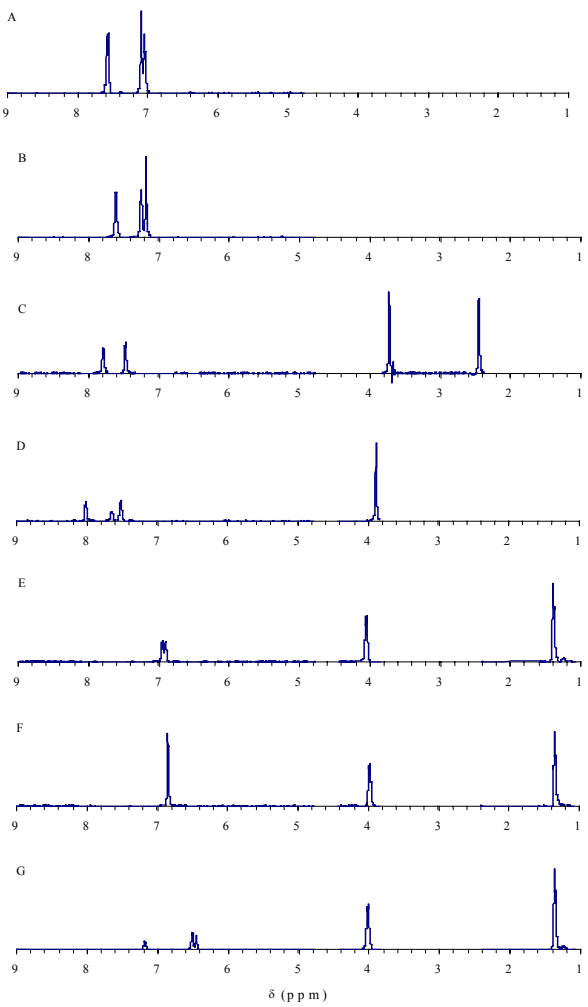




The PC scores plot shows seven directions, the elution times of the main compounds being indicated. Notice that the fifth, sixth and seventh PC have been selected. Hence the fastest eluting compound has an elution maximum at time=12, or around 38 s from the beginning of the cluster.

Using seven key variables results in a deconvoluted chromatogram as illustrated below, showing smooth underlying elution profiles for all components. The spectra of each compound can then be estimated and related to their structures.





It is also possible to obtain relative estimates of concentrations, providing a slow pulse rate is used, since the intensities are proportional to number of protons × concentration. However these depend on relaxation rates (T_1s) if pulsing is rapid. The results are tabulated. Within a group of isomers the relative peak areas are very quantitative and could be used as accurate indicators of relative concentrations. However, between groups of isomers there are differences. These could be solved by applying more than one pulse delay or pulse angle and recording several chromatograms. Note that long pulse delays will result in lower chromatographic resolution, however.

	Aromatic region						Aliphatic region			
	Number of protons			*Relative area after chemometrics*			*Number of protons*		*Relative area after chemometrics*	
	clust 1	clust 2	clust 3	clust 1	clust 2	clust 3	clust 1	clust 2	clust 1	clust 2
A	4	2	0	1.795	1.061	.	0	0	.	.
B	4	2	0	1.994	1.024	.	0	0	.	.
C	2	2	0	0.696	0.642	.	3	3	1.107	1.085
D	2	1	2	0.687	0.336	0.578	3	0	1.391	.
E	4	0	0	1.619	.	.	6	4	3.214	2.234
F	4	0	0	1.697	.	.	6	4	2.857	1.965
G	3	1	0	1.215	0.367	.	6	4	3.384	2.279

Comparison of methods

As yet HPLC-DAD and LC-MS are far more common in routine analytical chemistry. In the pharmaceutical industry, LC-NMR is slowly evolving from a specialised technique to one that is more widespread. A problem continues to be the rather specialist nature of NMR spectroscopy, and also that it is unlikely that bench-top technology will become available in the foreseeable future.

HPLC-DAD	**LC-MS**	**^{1}H LC-NMR**
Smooth peakshapes	Very noisy chromatographic peakshapes in electrospray mode in many cases due to irreproducible ionisation	Oscillations in chromatographic peakshapes due to quadrature detection, removed by baseline correction
Most spectroscopic channels useful in analysis but not very characteristic structurally	Discrete channels (masses) are very characteristic and must be selected in advance	Channels in NMR are clustered around spectral peaks
All compounds need chromophores and extinction coefficients can vary widely.	Intensities critically dependent on ionisation mechanism.	All compounds containing protons universally detected.
High detector sensitivity	Very high sensitivity but dependent on ionisation technique	Low sensitivity, problems with column overloading
In many cases symmetrical chromatographic peakahapes	Often severely tailing chromatographic peakshapes dependent on HPLC conditions	Normally tailing not too severe but overloading can influence peakshapes
Always aligned in spectral direction	Mass spectra are accurately aligned	Alignment in NMR dimension is relative to lock and there is often drift
Physical appearance of spectra are largely independent of instrument	Physical appearance can be altered using instrumental parameters	Physical appearance of spectra are largely independent of instrument under normal conditions providing adequate digital resolution.
Few restrictions on solvent	Few restrictions on solvent	Restrictions on solvent and buffers due to cost of deuterated compounds
Require calibration curves, proportional to extinction coefficients	Calibration depends critically on ionisation mechanism and must be repeated regularly	Relative peak areas proportional to concentration and number of protons providing pulse sequence is suitable and T1's comparable.

However, LC-NMR flow cells are now modest in cost, and it is not necessary to have a good HPLC detector. With solvent suppression it is only necessary to use deuterated water, which is quite cheap. Some modern spectrometers allow multi-channel solvent suppression but the apparatus is much more expensive. There are some problems which we have overcome in this study, and the sensitivity is lower than most other common chromatographic techniques. However, it is possible to achieve somewhat lower concentrations than discussed in this article, the signal to noise ratio being quite good. Higher field strengths coupled with stopped flow can achieve much greater sensitivity.

HPLC-DAD, LC-MS and LC-NMR are complementary techniques and the table above lists some of the pros and cons of each method. Ideally all three approaches should be used and there are now possibility for combining LC-MS-NMR.

Acknowledgements

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References

1. S.Wold, K.Esbensen, P.Geladi, *Chemom. Intell. Lab. Syst*., **2**, 37 (1987)

2. R.G.Brereton *Multivariate Pattern Recognition in Chemometrics, illustrated by case studies*, Elsevier, Amsterdam, 1992

3. C.Y.Airiau, H.Shen, R.G.Brereton, *Anal. Chim. Acta*, in press

4. B.V.Grande, R.Manne, *Chemom. Intell. Lab. Syst.* **50**:19 (2000).

5. H.Shen, C.Y.Airiau, R.G.Brereton, *J. Chemometrics,* in press

6. H.Shen, C.Y.Airiau, R.G.Brereton, *Chemom. Intell. Lab. Syst.,* submitted

7. M. Vives, R. Gargallo, R. Tauler, *Anal. Chem*., **71** 4328 (1999).