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Review

Hyphenation and hypernation
The practice and prospects of multiple hyphenation

I.D. Wilson[a,*], U.A.Th. Brinkman[b]

[a]*Department of Drug Metabolism and Pharmacokinetics, AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK*
[b]*Department of Analytical Chemistry and Applied Spectroscopy, Free University, de Boelelaan 1083, 1081 HV Amsterdam, Netherlands*

Abstract

In the past two decades, combining a chromatographic separation system on-line with a spectroscopic detector in order to obtain structural information on the analytes present in a sample has become the most important approach for the identification and/or confirmation of the identity of target and unknown chemical compounds. In most instances, such hyphenation can be accomplished by using commercially available equipment. For most (trace-level) analytical problems encountered today, the combination of column liquid chromatography or capillary gas chromatography with a mass spectrometer (LC–MS and GC–MS, respectively) is the preferred approach. However, it is also true that additional and/or complementary information is, in quite a number of cases, urgently required. This can be provided by, for example, atomic emission, Fourier-transform infrared, diode-array UV–vis absorbance or fluorescence emission, or nuclear magnetic resonance spectrometry. In the present review, the various options are briefly discussed and a few relevant applications are quoted for each combination. Special attention is devoted to systems in which multiple hyphenation, or hypernation, is an integral part of the setup. As regards this topic, the relative merits of various combinations—which turn out to include a mass spectrometer as one of the detectors in essentially all cases—are discussed and the fundamental differences between GC- and LC-based systems are outlined. Finally, the practicability of more extensive hypernation in LC, viz. with up to four spectrometers, is discussed. It is demonstrated that, technically, such multiple hyphenation is possible and that, from a practical point of view, rewarding results can be obtained. In other words, further research in this area is certainly indicated. However, in the foreseeable future, using several separate conventional hyphenated systems will be the commonly implemented solution in most instances.

Keywords: Reviews; Hyphenated techniques; Hypernation; Gas chromatography; Liquid chromatography; Mass spectrometry; Nuclear magnetic resonance spectroscopy; Atomic emission detection; Diode-array UV absorbance detection; Fourier-transform infrared spectroscopy

Contents

*Corresponding author. Tel.: +44-1625-582828; fax: +44-1625-516962.
E-mail address: ian.wilson@astrazeneca.com (I.D. Wilson).

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1. Introduction

In the past decade, *hyphenation*, a term first proposed by Hirschfeld [1], has become a catchword in separation science. As it is used most frequently, hyphenation refers to the on-line combination of a separation technique—primarily column liquid chromatography (LC) or capillary gas chromatography (GC), the only techniques to be considered in this paper—and a spectroscopic detection method which provides structural information for the analytes concerned [2]. Somewhat surprisingly, more often than is generally appreciated, these analytes of interest are target compounds, and a search for real unknowns is something of an exception. With mass spectrometry (MS) as the preferred detection method and single- and triple-quadrupole, ion-trap and time-of-flight (ToF) mass spectrometers as the instruments most frequently used, LC–MS and GC–MS are the most popular hyphenated techniques in use today. Especially in LC, the use of LC–MS–MS is rapidly increasing. If trace-level analysis is required and analyte enrichment is, consequently, an important issue, on-line combination with, primarily, solid-phase extraction (SPE), solid-phase micro-extraction (SPME) or large-volume injection (LVI) can be used to set up powerful integrated systems such as, for example, SPE–LC–MS–MS or LVI-GC–MS, which enable analyte detection, identification and/or quantification down to, at least, the low ng/g concentration level.

As regards detection in hyphenated systems, there are of course also other options, such as diode-array UV absorbance (DAD UV) or intensified linear diode array fluorescence (ILDA FLU) detection and nuclear magnetic resonance (NMR) for LC, Fourier-transform infrared (FTIR) for LC and GC, and atomic emission detection (AED) for GC. The use of these detectors is one alternative to further increase the potential of a hyphenated system. Recently, there has also been renewed attention for the application of coupled-column techniques to enhance resolution. Typical examples are (normal-phase) LC–GC and, increasingly, GC×GC and LC×LC, two so-called *comprehensive* techniques which start to replace the older, heart-cut-type techniques, GC–GC and LC–LC, respectively. On the other hand, the distinctly enhanced potential of modern analytical systems also makes analysts aware of the fact that such systems can, in many instances, be simplified on the front end without compromising the aim of the study. Reduction of sample mass/volume is one advantage that

can be gained, with, for example, the use of much less chemicals or the substitution of more straightforward LVI-LC for SPE–LC as an advantage. Sample throughput can be considerably enhanced by using a single short (1–2 cm length) column (SSC) instead of the SPE–LC part of a conventional setup (see Section 3.1.4). Resolution is, of course, adversely affected, but the use of MS, tandem MS or MS^n generally maintains the desired selectivity and analyte detectability. In GC, similar goals are pursued by means of fast or flash GC techniques.

The above clearly shows that an abundance of instrumental setups and, consequently, analytical strategies are available to address those problems in which identification and/or confirmation of the identity of analytes plays a predominant role. Generally speaking, the rewarding result of such work is more reliable and detailed knowledge of sample composition. Simultaneously, this elicits the question: ''can even more information be obtained by a better or extended combination of techniques?'' To come back to the comprehensive approach mentioned above, one area where this is an issue is GC×GC–ToF MS [3]. Here, it is today clear that much more information can be obtained quite easily, and the real problem turns out to be not how to improve the analytical performance, but how to handle the plethora of data generated per run. More to the point for the present paper, combining different hyphenated techniques is an obvious choice to tackle challenging problems. The next question then is whether to use two (or more) parallel systems or to introduce multiple hyphenation in a single setup. The main advantage of the former approach is that each individual system can be optimised separately and used to its best advantage. To quote an example, the most demanding technique, in LC generally LC–NMR [4], will now not compromise the other techniques with regard to, for example, eluent composition, sample loading, time constraints or D–H exchange. A drawback is that peak correlation between the various systems can easily cause problems for minor sample constituents. The latter difficulty self-evidently does not occur when the second approach, usually called *hypernation*—a term indicating that one is now ''one higher than'', or *hyper*, hyphenation—is preferred. A relevant example is GC with AED and MS detection [5]. Attention can now be focused on improving the peak correlation because the other problems mentioned above are typical for LC, not GC. For the rest, it has to be admitted that hypernation is, technically, more difficult than ''double hyphenation'' and that most laboratories in which the various spectroscopic detectors are available for analytical studies do not provide the opportunity to combine them into a single hypernated unit. This is not too surprising if one calculates the cost of what is, probably, the most desirable hypernated system, LC–DAD UV–NMR/MS–MS!

In the present paper, we will not discuss all instrumental options available today for those who want to design suitable hyphenated or hypernated systems. For the former category, a large number of research papers, reviews and books can be consulted, and only a few illustrative examples will be given, primarily for areas less well known to the general reader, i.e. for LC–NMR rather than GC–MS. Attention will be on the state-of-the-art in the hypernation category, with GC-based systems primarily serving as a reference point for LC-based systems. After all, it is in the latter group that the technical problems loom large and that there is considerable doubt concerning the practicability of the approach, specifically if the setup is extended to comprise not two, but three or four spectroscopic detectors. A more or less ''closed set of studies'' of one of the present authors (I.D.W.) will be used to highlight the possibilities and to indicate the, serious, problems.

2. Gas chromatography

2.1. Hyphenation

2.1.1. MS

A brief look at the literature suffices to show that, from among the many separation–identification setups that can be constructed, GC–MS was the earliest one to become useful for research and development purposes. Actually, dedicated instrumentation became commercially available many years before the term ''hyphenation'' was coined and, in the context of the present paper, there is no need to review the copious literature [6,7]. Quoting a

limited number of examples should suffice. As regards volatiles, headspace-GC–MS and SPME–GC–MS [8] are indispensable tools for the trace-level determination of, for example, flavours and fragrances [9], and volatile organic compounds (VOCs) [10]. The same is true for a wide variety of halogenated industrial chemicals and related compound classes, such as the polychlorinated—and, today, also the polybrominated—biphenyls and biphenyl ethers (PCBs, PBBs; PBDEs) and the polychlorinated dibenzodioxins and dibenzofuranes (PCDDs and PCDFs). Especially because of the high toxicity of the 17 priority CCDs and CDFs, and also the non- and mono-*ortho*-substituted CBs, this is an area in which a variety of GC–MS techniques are being used routinely whilst, at the same time, much attention is devoted to further study of the relative merits of high- and low-resolution MS, and the complementarity of the EI and NCI (electron ionisation; negative chemical ionisation) modes of operation. Moreover, the recent introduction of GC×GC–ToF MS, already referred to above, has led to markedly successful separations of, for example, essentially all CB, CDD and CDF congeners of interest [11]. An additional advantage is that the comprehensive separations display a so-called ordered structure in the GC×GC plane; the ordering is based on both the number of chlorine substituents per molecule and—in the case of the CBs—on the number of *ortho* positions that are occupied. Many illustrative examples are given in another review included in the present volume [3]. Other examples are the ubiquitous use of GC–MS in the petroleum industry [12]—another area where the considerable merits of GC×GC are rapidly becoming recognised [13,14] (see Fig. 1)—and for the determination of

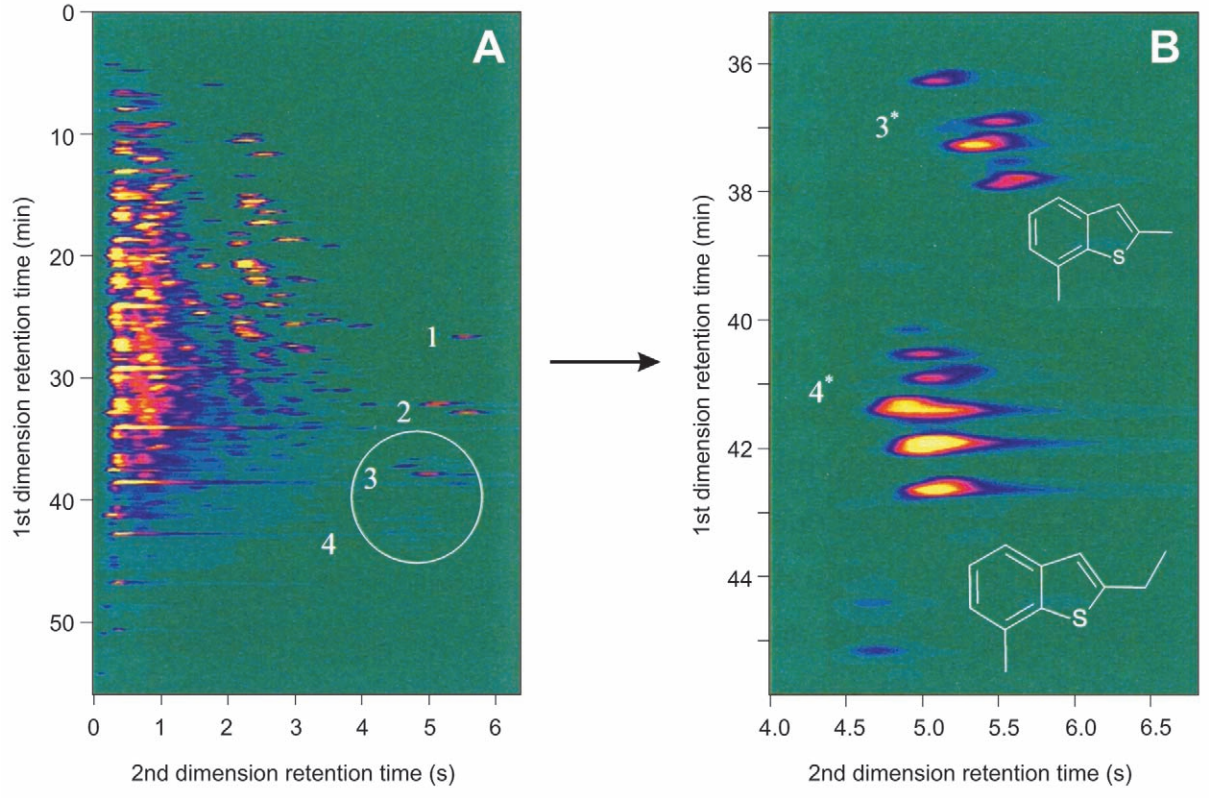


Fig. 1. (Left) TIC trace of GC×GC–ToF MS of petroleum fraction with high sulphur concentration. No sulphur-containing compounds are visible (see circle); key: 1, naphthalene; 2, methyl-naphthalenes; 3, dimethyl-naphthalenes; 4, trimethyl-naphthalenes. (Right) Selected ion traces (m/z 161+176), enlarged from left-hand-side chromatogram. Key: (3*) dimethyl-benzothiophenes and (4*) methylethyl-benzothiophenes, which co-elute with substituted naphthalenes, now clearly visible [14].

pesticides in water, soil and sediment, biota and food. As regards the latter class of analytes, it may be good to add that, contrary to what is frequently stated, some 85% of all pesticides presently in use can be determined without prior derivatization.

As for analytical performance data, quantification usually is straightforward (although one should always be aware of the possibility of matrix effects) and the three or four diagnostic ions typically required today can often be selected without too much loss of sensitivity (which is a distinct advantage over what is frequently observed in LC–MS; see Section 3.1.4). No generally valid statement on detection limits (LODs) can be made, because too much depends on the analyte(s) selected, the sample preparation effected and the mode of MS operation used. However, one can confidently say that, in a large majority of all cases such as are mentioned above, low nanogram to low picogram amounts injected can be detected. If one assumes that, when the more modern sample preparation and/or injection techniques are used, the equivalent of 1 g of a solid, or 10 ml of a liquid, sample can be introduced into the GC column, LODs in concentration units are seen to be in the low ng/g down to the sub pg/g level—which is what is, indeed, found in the literature. To quote two illustrative examples. In a study on on-line SPE–GC–MS^2 of surface water, 10-ml samples sufficed to obtain LODs of 0.1–1 ng/l for a series of pesticides [15]. In studies on chlorinated micro-contaminants, GC–NCI-MS in the selected ion monitoring mode was used to quantify as little as 1–10 ng of total toxaphene/g of wet weight in several types of fish, fish products and milk [16], while the LODs of the three planar CBs 77, 126 and 169 in similar samples were found to be at a level of about 0.1 pg with both GC–NCI-MS and GC–MS^2 [17].

2.1.2. FTIR and AED

As regards the other two spectroscopic detectors that were mentioned in the Introduction, neither GC–FTIR nor GC–AED has ever achieved the same popularity as has GC–MS. With the former approach, one main disadvantage is that FTIR is eminently suitable for functional-group recognition, but much less so for true analyte identification. In addition, from among the three detector designs that are available, the lightpipe detector is user-friendly but does not provide very high sensitivity, while the matrix-isolation and cryotrapping detectors do meet trace-level requirements, but are rather expensive and somewhat difficult to operate. Nevertheless, detection limits (LODs) of about 5–10 ng injected for the former, and 0.1–1 ng injected for the latter two, detectors indicate that the potential of the technique is somewhat underestimated [18]. This also becomes clear from a study on on-line SPE–GC–cryotrapping IR, in which LODs of about 15 ng/l were obtained for a series of organic microcontaminants in only 20 ml of tap water, when using appropriate functional-group chromatograms [19]. In other words, in our opinion, more dedicated attention should be devoted to this technique, as will also become apparent from the examples included in Section 2.2 below.

In the case of GC–AED, which started to gain popularity after the launch of the HP 5921 in 1989, one main advantage is that essentially all major hetero-atoms, the halogens and metals such as Pb, As, Sn and Hg can be detected with high sensitivity, i.e. with LODs of 0.1–30 pg/s, as well as with an excellent selectivity over carbon (Table 1) [20]. Combined with the universal—that is, non-com-

Table 1
Analytical characteristics of AED detection for selected elements

Element	Wavelength (nm)	Set[a]	LOD (pg/s)	Selectivity over carbon ($\times 10^{-3}$)
N	174.2	1	15–50	2–5
S	180.7	1	1–2	5–20
C	193.1	1	0.2–1	–
P[b]	178.1	2	1–3	5–8
C	495.8	3	15	–
H	486.1	3	1–4	–
Cl	479.5	3	25–40	3–10
Br	478.6	3	30–60	2–6
F	685.6	4	60–80	20–50
O	777.2	5	50–120	10–30
Si	251.6	6	1–7	30
Hg	253.7	6	0.1–0.5	250
Pb	261	6	0.2–1	300
Sn	271	6	1	300

Data collected from various sources.
[a] Arbitrary order.
[b] In wavelength range of first set; different make-up flow conditions.

pound-dependent—calibration that has been amply demonstrated, and the possibility to register complete ''snapshot'' spectra for elements such as Sn and P, this provides the opportunity to calculate partial elemental formulae and perform, at the same time, quantification. The initially suspected vulnerability of the detector has turned out to be a hoax and the analysis of large sample volumes, as in LVI-GC, does not cause any problems. The main disadvantages are the fairly high price of the instrument and the limitation to recording four element channels per GC run. Today, several hundreds of papers have been published on GC–AED. These include many speciation studies of Hg, Sn and Pb [21], the determination of (invariably hetero-atom-containing!) pesticides in food and water [22], and N-containing compounds in gas oils [23].

2.1.3. Conclusion

The above, very brief overview can be summarised by stating that hyphenation in GC can be performed via various routes, which each bring their rewards in terms of confirmation/identification, and functional-group or element recognition, and have their own technical or operational problems. In all instances, trace-level studies can be carried out and, generally speaking, quantification does not meet with any major problems. It is difficult to compare analyte detectability for the three techniques, because too much depends on the experimental and instrumental conditions selected—which is specifically true for MS—and the analyte/sample type combination being studied. Even so, it is true to say that, in most instances, detectability decreases in the order MS>AED>FTIR.

2.2. Hypernation

2.2.1. FTIR/MS

In the previous section, it was shown that three powerful alternatives are available for hyphenation in GC. However, this does not imply that all problems can be solved adequately. One well-known example is isomer identification, where the use of MS detection serves little purpose because the spectra of the isomers are indistinguishable. A rewarding solution is to maintain the MS (for all other analytes of interest in the sample, and for additional information on the molecular structure of the isomers) and add an FTIR instrument to the system. It has, indeed, been shown in the past decade that the use of such hypernated GC–FTIR/MS systems dramatically increases capabilities for complex mixture analysis—with the additional option to enhance resolution by introducing multi-dimensional GC. A few relevant examples are discussed below.

The utility of GC–FTIR/MS has repeatedly been demonstrated in the analysis of natural products such as flavours and fragrances, and essential oils. In one study, with matrix-isolation (but not with a ''cruder'' lightpipe) detection, a pair of compounds as closely related as geraniol and nerol—isomers differing only in the *trans* vs. *cis* configuration of a double bond—could be properly identified as well as quantified [24]. In another study, and with a lightpipe detector, out of 58 peaks observed in the GC chromatogram of eucalyptus oil, 33 peaks could be identified after extensive library searches [25]. One practical problem was the much smaller size of IR compared with MS libraries and another, quite different, one the occasional loss of resolution (in the MS) caused by the rather large dead volume of the lightpipe detector which was positioned between the GC outlet and the MS. A detailed study on the successful identification and quantification of 50 targeted phenolic compounds may serve as a further illustration of the practicability of the hypernated approach [26]. In the studies quoted so far, the effluent from a single GC column was split to the two detectors. The alternative of splitting the sample, after injection, to two identical columns, one being connected with the MS, and the other with the IR detector, has also been reported [27]. With this configuration, which was used to study a sample prepared by steam distillation/extraction of coal, larger samples can be loaded—but keeping the columns ''identical'' may become a problem after prolonged use. Detailed discussions of these and related studies can be found in two recent reviews by Wilkins and co-workers [18,28].

It was also Wilkins and his group who realized that, for the complex samples typically subjected to GC–FTIR/MS, more sophisticated GC analysis would be highly welcome. To this end, they used a GC–GC setup with a cryogenic trap between the two GC columns. This provided a qualitative analysis of the total sample but, unfortunately, at the expense of

a great increase in analysis time. Their much more complicated, final design [29] therefore was a system featuring five parallel cryogenic traps, two or even three GC columns and, of course, the two detectors—with the 9:1 split ratio reflecting the general difference in analyte detectability for the two instruments (Fig. 2). The loss per recycle is on the order of 20% (10% due to the MS and 10% due to the valves). In a series of papers, a variety of real-life samples were studied, e.g. extracts of contaminated water, clay and soil samples, gasoline, essential oils and soap (detection of irritants), and most rewarding results were obtained in each case. As an illustration, some of the results obtained for an adulterated oil sample [30] are shown in Fig. 3. The explanation can be found in the legend.

As a somewhat different example, a GC equipped with a flame ionisation (FID), a lightpipe FTIR and an MS detector was used for the definitive structure identification of all mononitro- and dinitro-isomers formed during the nitration of *N*,*N*-dimethyldiphenylacetamide. The additional use of LC–DAD UV (see Section 3.1.3) confirmed that polynitration products did not form under the conditions used [31].

2.2.2. AED/MS

Isomers are not the only problem area in GC–MS.

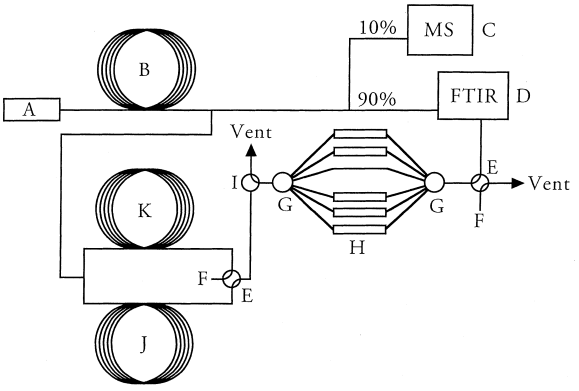


Fig. 2. Schematic of parallel cryogenic trap multi-GC–FTIR/MS system. A, splitless injection port; B, intermediate-polarity first-stage column; C, quadrupole MS; D, FTIR detector; E, four-port, two-way valve; F, external auxiliary carrier gas; G, six-port selection valve; H, stainless-steel cryogenic traps; I, three-port, two-way valve; J, polar higher-stage column; K, non-polar higher-stage column [29].

Difficulties also arise when unknowns having a common structural element have to be identified across the complete GC chromatogram. A well-known question is: ‘‘how many Cl-, Br- or Sn-containing compounds are present in the sample, and what is their nature?’’ In such cases, starting with a full-scan MS screening is not a good strategy—it is much too time-consuming, and peaks of minor constituents partly co-eluting with a much larger peak will often go undetected. Instead, the screening should be done by recording the traces of the pertinent AED channels (with their high element selectivity), with subsequent confirmation of identity by means of MS. Performing two subsequent runs—one with AED, and one with MS detection—already provides interesting results, but precision can be improved significantly, viz. from retention-time differences of up to 9 to 0.5 s or less, by setting up a hypernated GC–AED/MS system [5,32]. Because of the fairly similar LODs of many key elements with both detectors (cf. above), the GC effluent is usually split in a ratio of about 1:1. It is, of course, essential to keep the composition of the carrier gas mixture—which has somewhat different optima for different elements in AED detection—constant throughout the run. That is, a compromise usually has to be made here, but adverse effects on analyte detectability are generally less than two- to three-fold.

Typical examples deal with the analysis of surface and wastewater [5,32], effluents from a sewage-treatment plant [33] and vegetable extracts [5]. Because of the aqueous nature of the samples, trace enrichment was done by an on-line combination with SPE on a copolymer sorbent, i.e. an SPE–GC–AED/MS system was used. Since this ensures that, instead of a (minor) aliquot, the total sample is subjected to the final chromatographic analysis, 10–30 ml samples typically sufficed to obtain LODs of 20–50 ng/l for most of the target and non-target pesticides and industrial chemicals of interest in these studies. An example of the good agreement of the AED and MS data for a surface water sample—also as regards quantification—is presented in Table 2. The potential of the hypernated approach for complex samples is demonstrated in Fig. 4, which shows parts of several elemental traces, the total ion chromatogram (TIC) and relevant mass traces for a sewage-treatment-plant sample [33]. The spiked chlorpyrifos (peak 9;

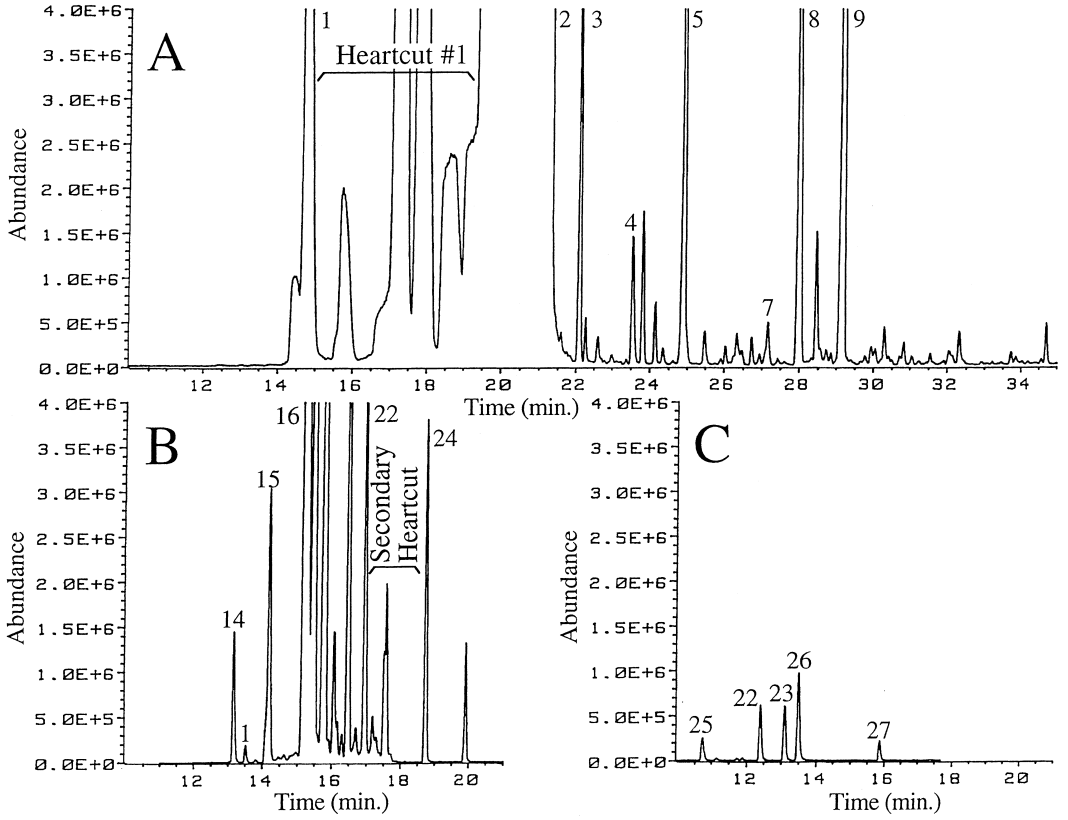


Fig. 3. Use of the system shown in Fig. 2 for the analysis of an adulterated oil sample using full-scan MS. (A) ''Precolumn'' chromatogram. (B) Chromatogram from secondary separation of heart-cut #1. (C) Chromatogram from tertiary separation of 17.0–18.5 min heart-cut from secondary separation. Key: 22, α-terpinene; 23, limonene; 25, carvomenthene; 26, β-phellandrene; 27, *p*-cymene [30].

1 μg/l) gives a distinct peak in the Cl and N traces, but the small TIC-trace peak would go unnoticed during non-target analysis; alachlor (peak 8) produces an even smaller shoulder. Non-target compounds that were identified included the disinfectant triclosan (peak 11) and tris(2-chloroethyl) phosphate (peak 5). In both instances, no peak was visible in the TIC trace.

Table 2
Comparison of MS and AED detection in quantitative GC-based surface-water analysis [5]

Analyte	True value (μg/l)	Experimental concentration (μg/l)						
		MS[a]		AED				
		m/z 1	*m/z* 2	Br	Cl	N	P	S
Trifluralin	0.82	0.84	0.83			0.77		
Atrazine	0.97	0.96	0.99		0.83	0.84		
Diazinon	0.73	0.69	0.68			0.64	0.83	0.72
Alachlor	1.03	1.02	1.02		0.94	1.12		
Terbutryn	1.05	1.04	1.07			0.88		0.94
Bromophos-ethyl	0.70	0.77	0.75	0.63	0.71		0.60	0.69
Azinphos-ethyl	1.30	1.52	1.42			1.08	1.33	1.13

[a] Specific ions for each analyte.

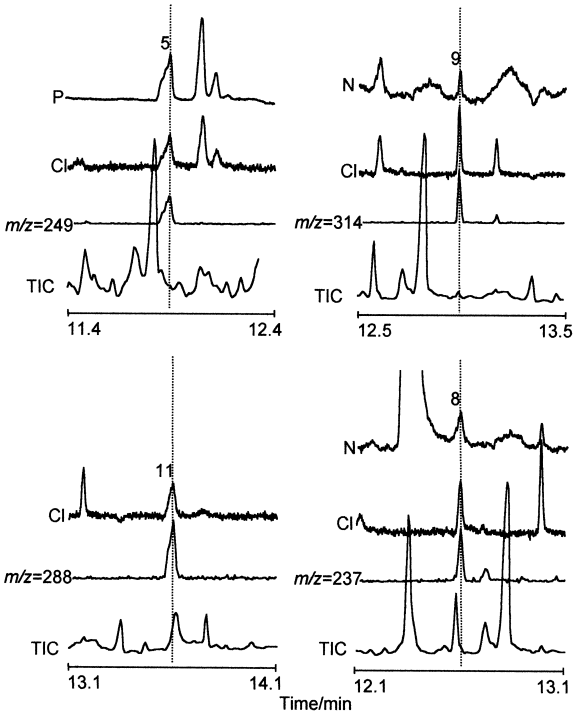


Fig. 4. Blow-ups of parts of GC–AED/MS chromatograms showing obscured peaks of chlorinated compounds in the TIC trace, and the corresponding extracted-ion chromatograms and AED elemental traces [33].

2.2.3. Conclusion

To summarise the above discussions, even though a single system featuring multiple hyphenation is, of course, more complex than two parallel hyphenated systems, hypernation in GC does not appear to create major technical problems. Neither does it cause dramatic losses in detection performance, mainly because there is little need to compromise the experimental conditions which are optimal for each separate detector. The combination of MS and FTIR has the advantage that a number of well-documented isomer problems can easily be addressed. The use of the MS/AED combination provides the opportunity to exhaustively screen on an element-selective basis, and for several elements simultaneously—an approach which is especially rewarding when unknowns rather than target compounds are a major aim of a monitoring programme or the analytical study at hand. To the best of our knowledge, GC with AED/FTIR hypernation has never been reported. Indeed, it is difficult to envision problems with which sacrificing the extremely valuable MS-type information would not adversely affect the final outcome. In addition, the very different nature of the main application areas for the two combinations in use today, FTIR/MS and AED/MS, makes it rather unlikely that there will be much interest in the near future to make things more complicated by introducing a GC–AED/FTIR/MS system.

Finally, one may have, at this stage, two further questions: (i) why does hypernation not cause too many real problems in GC; and (ii) what is an interesting option for further study. As regards the former aspect, one main cause is that—contrary to what is true in LC (see below)—the ‘‘mobile phase’’ in GC is, in essence, a carrier stream only: the selectivity of the technique resides in the stationary phase. In addition, analyte detectabilities in matrix-isolation or cryotrapping FTIR, AED and full-scan MS (often the preferred mode when non-target compounds are one’s aim) are fairly close together. That is, with some adaptation of the experimental conditions, each part of the hypernated system will perform equally well. As regards further developments, apart from more wide-ranging applications, which are required to convince newcomers to the field of the practicability of the techniques, it lies at hand to devote more attention to the separation part of the system. As was mentioned in Section 2.1 with regard to the potential of GC×GC–ToF MS, and as could already be observed in the systems proposed in Wilkins’s studies on multi-column-multi-trap-FTIR/MS systems, improving the overall resolution in a two-dimensional plane is what now should have priority. The gain in selectivity that can thereby be obtained will be highly rewarding by itself and will, besides, trigger the required further innovations in the field of hyphenation/hypernation.

3. Column liquid chromatography

3.1. Hyphenation

When going from GC- to LC-based separation systems, there is one main difference: compared to GC, the role of the stationary and mobile phases is, in LC, essentially reversed. This is especially true for

reversed-phase LC (RPLC), which is the separation technique used in a large majority of all cases today. The selectivity mainly resides, not in the stationary phase (which usually is an alkyl-bonded silica), but in the mobile phase. Although most RPLC eluents are, at first glance, mutually rather similar water–organic modifier mixtures, the nature and proportion of that modifier—usually either methanol or acetonitrile, the nature of the buffer salts and the pH value used for the separation, and the presence of any other added reagents, often seriously influence the performance of the detection system. Obscuring analyte peaks, causing a high absorption or emission background, and affecting the ionisation efficiency of analytes of interest are well-known or, rather, notorious phenomena. Since instantaneous cryotrapping of the analytes or, alternatively, evaporation of the usually water-rich LC eluents is not a practical solution (and heat treatment should, anyway, be avoided with a technique in which the study of thermolabile compounds plays an important role), it will be clear that hyphenation in LC will often require that compromises are made with regard to the optimisation of the separation and detection parts of the procedure. This aspect should always receive due attention.

3.1.1. Fluorescence

From among the five types of spectroscopic detectors that should be compared and/or combined with each other, fluorescence (FLU) detection has, probably, gained least popularity. One cause is that, if used for identification purposes, the approach is self-evidently limited to compounds displaying native fluorescence. This seriously limits the applicability range of the technique: it is much too selective for almost all screening procedures, and the need to use fairly expensive ILDA detectors or charge-coupled devices does not add to its attractiveness. It will not come as a surprise that many published applications deal with polynuclear aromatic hydrocarbons (PAHs); these analytes are generally highly fluorescent and their spectra are very rich [34]. In one of the first papers, an in-house assembled full-spectrum fluorescence detector was built with a diode-array-based emission monitor. A variety of PAH standards were studied, but the analysis of a carbon black extract was only partly successful because of a lack of reference compounds. Nevertheless, the high-molecular-mass PAHs rubrene and decacyclene were provisionally identified [35]. In another early study, the LC eluent obtained during PAH analysis was deposited on a moving thin-layer (TL) plate. After a second-dimension separation on that plate (not a prerequisite but an extra option), the emission spectra of the analytes were recorded [36]. The procedure, which was used for sediment analysis, admittedly is at-line rather than on-line and would require an on-line matrix-isolation or similar trapping step to become truly hyphenated. Recently, another group used laser-induced excitation and a charge-coupled device for the emission detection of a series of PAHs. The output was an excitation–emission matrix for each LC peak of interest. LODs were in the range of ng/ml of injected sample [37] (as quoted in Ref. [34]).

One may add here that, if the use of cryo techniques is taken into consideration, there is another option open, viz. LC (or CE)-TL-FLN, where the final acronym stands for fluorescence line narrowing, a low-temperature (<20 K) high-resolution laser-based technique. FLN is extremely powerful and allows the vibrational states of analyte molecules to be recorded. The—again, at-line—technique has been used to distinguish several closely related PAH–DNA adducts (CE) and, also, a number of stereoisomeric benzo[*a*]pyrene tetrols (μLC) [38].

As a more interesting—because really hyphenated—example, a series of amino- and hydroxy-substituted naphthalene sulphonates was separated by means of capillary electrophoresis (CE) and subjected in-line to wavelength-resolved CE–LIF (LIF: laser-induced fluorescence) for identification in river water down to the low μg/l level [39]. The results, shown in Fig. 5, can be considered self-explanatory. In a subsequent study by another group, the same principle was used, but now with ion-pair LC as the method of separation [40]. Another appealing example is the analysis of peptides containing tyrosine (em. max., 304 nm) and/or tryptophan (em. max., 358 nm). Because of the widely different emission maxima, peptides containing either tyrosine or tryptophan and those containing both amino acids could be distinguished as three separate groups [41].

3.1.2. FTIR

Over the years, quite some attention has been devoted to the design of LC–FTIR systems, which

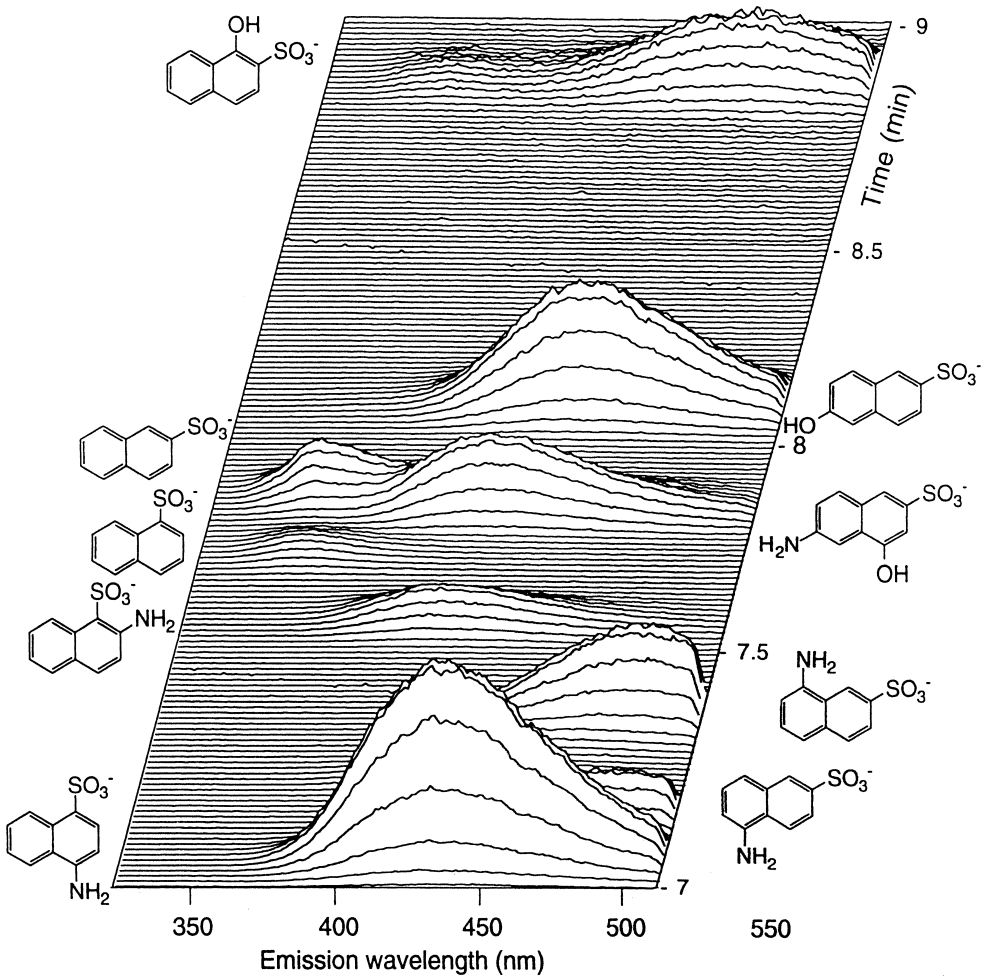


Fig. 5. 3D CE–FLU electropherogram (SDS-containing pH 8.75 borate buffer) of naphthalene monosulphonates (structures shown on *Y*-axis) [39].

are either of the flow-cell or the solvent-elimination type [42]. The major obstacle to the use of the former approach is the IR absorption of the LC eluent. Consequently, LODs generally are rather poor. Nevertheless, experience has shown that LC–flow-cell-FTIR can be useful for the specific detection of major constituents of mixtures, typically at the 0.1–10 g/l level. The identification of carbohydrates in soft drinks and wine is one interesting example. Other recent studies use size-exclusion chromatography (SEC) or flow-injection analysis (FIA) rather than conventional LC: with both approaches, the type of solvent selected often is not essential for the separation process or carrier-solvent function, respectively, and a solvent that does not spectrally interfere with the marker bands of the analytes of interest can be selected.

LC–solvent elimination-FTIR is, undoubtedly, a more powerful alternative. From among the various designs for direct or indirect solvent elimination that have been proposed, pneumatic nebulizers appear to be best [42]. They combine rapid solvent elimination with a relatively narrow spray. This allows analytes to be deposited in narrow spots, so that FTIR transmission spectroscopy can be applied to achieve mass sensitivities in the low-to-sub-ng range. The main limitation is that flow-rates are restricted to 2–50 μl/min, with lower flow-rates being required

for LC eluents containing higher proportions of water; actually, up to about 5 μl/min, even pure water can be handled. Buffered eluents should be avoided because even volatile buffer salts are not completely eliminated by a pneumatic nebulizer and may therefore cause interfering absorbances in the analyte spectra. Thermal degradation of analytes is usually not observed [43,44]. Probably, even though the nebulizer gas itself is rather hot, the spray droplets are cooled considerably due to the rapid evaporation of the solvent. Applications have been reported for a variety of compounds such as quinones, steroids, polymer additives and herbicides. Identification LODs typically are in the low-mg/l range, which is certainly useful for some applications but cannot be called satisfactory for environmental and bioanalysis. Considerably improved detectability requires the use of micro precolumns [45] or on-line SPE procedures [43], while another option is in-line liquid–liquid extraction (LLE) to effect a water-to-organic solvent phase switch which will facilitate the subsequent evaporation step. Under optimum conditions, i.e. with SPE–LC–LLE-FTIR, identification LODs of about 2 μg/l have been obtained for herbicides in river water (up to 100 ml sample) [43]. Admittedly, this is what is required for trace analysis, but the rather complicated setup cannot really be called user-friendly. Probably the best way in which to illustrate the still rather weak position of what can certainly become a powerful hyphenated technique is that, until now, most LC–FTIR interfaces have been used only by their designers.

3.1.3. DAD UV

Today, it is easy to forget that, some 15–20 years ago—that is, when LC–MS still was very much in its infancy—it was the introduction and rapid further development of DAD UV instrumentation which considerably promoted the wide-ranging application of LC–DAD UV for detection (plus peak-purity control) as well as (provisional) identification purposes [46]. Since the application of on-line SPE–LC procedures reached maturity at about the same time, SPE–LC–DAD UV easily became the workhorse for many pharmaceutical, biomedical, food and environmental analyses of aqueous samples or sample extracts. Going from on-line to semi- and fully automated setups was a logical next step. To quote one example only, this considerably facilitated the installation of automated early-warning/monitoring stations along the banks of several major European rivers. Typically, loading 100 ml of river water at a speed of about 5 ml/min on a copolymer SPE cartridge can be used to detect—and provisionally identify—up to 100–150 organic microcontaminants using a 50–60 min LC run, with LODs in the 0.1–1 ng/ml range [47–49]. If the early-warning procedure indicates the possible presence of a contaminant at too high a level, a subsequent LC–MS run is used for confirmation.

There is no doubt that, also today, LC–DAD UV plays an important role in many research and development studies, and for a wide variety of routine analyses—specifically in application areas such as mentioned above. Screening for the presence of specific classes of compounds with their, usually, mutually closely similar UV spectra is a typical area of interest. At the same time, it is also true to say that (RP)LC–DAD UV, which is a robust and user-friendly technique, has gradually become a tool rather than the subject of scientific studies. Routine procedures are available to obtain, store and interpret the data; or, in other words, no further attention is required in the context of this review. However, it may be good to emphasise one aspect of interest, also in view of what will be stated below regarding LC–MS techniques.

The very advantage of the similarity of the UV absorption spectra of a related set of analytes is, at the same time, a distinct drawback when, instead of general screening, identification of individual compounds is the main aim of a study. If, in addition, rather high and structureless backgrounds show up due to the presence of matrix compounds, the limitations caused by the insufficient selectivity of the detection technique become clear. An illustrative example [50] is the trace-level detection of highly polar environmental contaminants such as phenol, oxamyl and desethylhydroxyatrazine in river water. These analytes elute in the very early part of an RPLC run, i.e. right on top of the notorious humic-substances hump. It was found that, even with an on-line dual-stage SPE clean-up, the peaks of the analytes could hardly be recognised at the 2 μg/l level, and satisfactory UV spectra could not be recorded. When, instead, LC–MS/MS was used (see

Section 3.1.4), no problems were encountered even at the 0.1 μg/l level, and the LODs were 1–50 ng/l. A typical example is shown in Fig. 6.

The above example should be appreciated in the context of the increasing interest in (highly) polar analytes in general. These include many modern human and veterinary drugs, recently introduced pesticides and, maybe even more importantly, (bio)degradation products of all these and many more analytes. As a class, they all elute very rapidly in RPLC systems and are all easily more or less obscured by the matrix background, irrespective of the sample type studied. Combined with the rapidly growing demands made by customers, governmental bodies, etc., for reliable and unambiguous information, this causes UV detection/DAD UV ''identification'' gradually to become less popular: its power as a partner in hyphenation is increasingly being considered to be below par. As will be discussed in Section 3.2, this slowly changes the role of DAD UV detection in systems with multiple hyphenation.

3.1.4. MS

The past decade has witnessed the coming of age of LC–MS, a combination once considered the mismatch of the twentieth century [51,52]. In earlier years, most attention had to be devoted to solving interfacing problems and building new technology. However, after the introduction of the electrospray ionisation (ESI) and atmospheric-pressure chemical ionisation (APCI) interfaces—which are both atmospheric-pressure ionisation (API) devices—most technical problems can be considered solved. As a consequence, there has been a major change of attention in the recent literature: most workers now use commercially available instrumentation to perform application-orientated studies. At the same time, next to ''simple'' LC–single quadrupole MS, more advanced techniques such as, specifically, LC coupled with tandem MS or ion-trap MS, i.e. LC–MS–MS and LC–MS^n, respectively, are rapidly gaining in popularity—with the use of time-of-flight mass spectrometry, LC–ToF MS, with its highly improved mass accuracy of 5–10 ppm rather than the conventional mass unit resolution, being a promising recent addition to the list [53–55].

Actually, what is the most striking observation today is that there is an avalanche of papers, reviews and books being published on the principles and instrumentation, modes of operation and, above all, the application of the technique(s) to solve a wide variety of problems. These are frequently found in the fields of environmental, pharmaceutical, biomedical, biological and food analysis. Classes of compounds that are often the goal of such work include naturally occurring bioactive substances [56,57], pesticides [58,59] and related organic micro-contaminants such as endocrine disruptors [60] and detergents [61], veterinary and human drugs [62] and, in many instances, also their (bio)degradation products. In some cases, for example the early-warning and/or monitoring of micro-contaminants in water, sediment and soil, or the residue analysis of veterinary drugs, target analysis dominates the field. On the other hand, however, with the increased attention for transformation products of parent compounds and, more so, the characterisation of natural products, ''semi-unknowns'' and real unknowns are gradually coming to the fore. Combined with an ever-increasing demand for the unambiguous identification of analytes whenever environmental concern, health care, food safety or governmental directives require this, this readily explains why advanced techniques such as MS–MS, MS^n and Q-ToF MS (Q, quadrupole) have become so popular so rapidly. It also explains why, especially for the more complicated types of study, the use of multiple hyphenation is considered as a worthwhile option.

In view of the abundant information on LC–MS techniques provided in the recent literature (cf. above) and, also, in several papers included in the present volume (e.g., Refs. [60,63–66]), this review will be limited to reporting a few selected examples to illustrate the potential of MS detection, and discussing one topic that needs careful consideration to prevent undue errors, i.e. ion suppression.

One example [50] was already presented in Section 3.1.3 to demonstrate the dramatically enhanced sensitivity (largely caused by the much improved selectivity!) of MS–MS compared with DAD UV detection (cf. Fig. 6). Another interesting development is the use of 1–2 cm analytical-quality single short LC, or SSC, columns which were already briefly mentioned in the Introduction. They are used for both sample loading and separation and are, preferably, combined with tandem MS detection

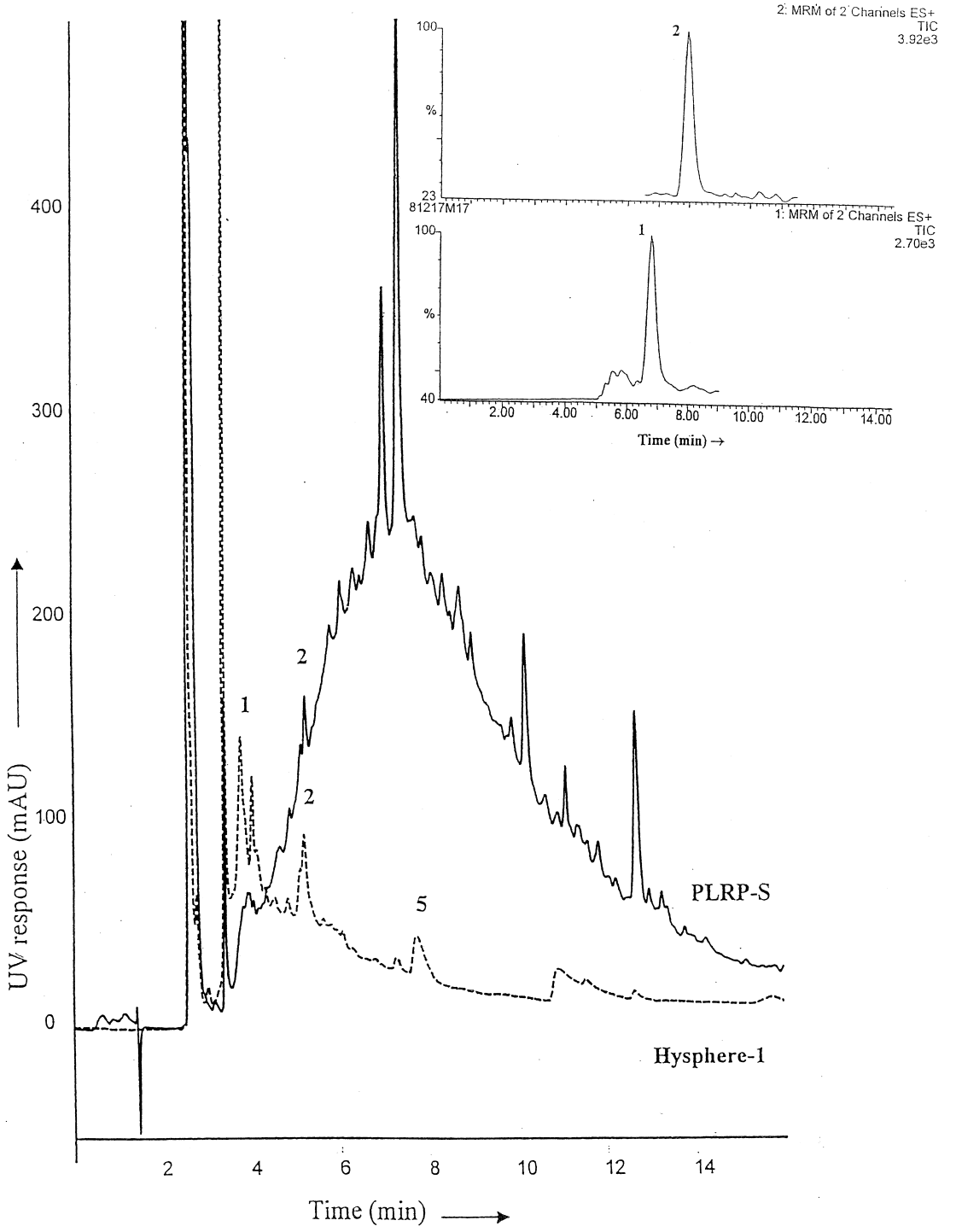


Fig. 6. On-line dual-SPE–LC–UV (215 nm) of 50 ml of surface water spiked at the 2 μg/l level. Inset: on-line dual-SPE–LC–ESI-MS–MS SRM chromatograms of the same sample spiked at the 0.05 μg/l level. Key: 1, desethylhydroxyatrazine (SRM, *m/z* 170→128, 86); 2, oxamyl (SRM, *m/z* 220→90, 72) [50].

[67,68]. Experience shows that 1–5 ml samples suffice for the identification of target analytes at the low-ng/ml level, that run times are on the order of only 3–5 min and that up to 10 compounds can easily be included in one analysis. The approach is especially useful for real-time degradation studies of a parent compound and its several degradation products [68]. Another innovation is the use of LC–ToF MS for the accurate mass analysis of parent xenobiotics and their degradation products. Applications illustrating the potential and limitations of the approach deal with ethanesulphonic acid degradates of acetochlor and alachlor [69], the screening of surface water for unknowns by combining ToF MS and data-dependent MS-to-MS/MS switching [54], and a detailed study of alachlor breakdown [70]. Another emerging topic is the ultra-trace-level determination of pharmaceuticals in surface and wastewater [71]. A combination of LC- and GC-based procedures is required to cover the whole range of analytes of interest, and adequate clean-up is needed to prevent suppression of ionisation with highly contaminated samples. LC–ESI-MS–MS is the only alternative for beta-blockers such as atenolol and sotalol, and the preferred procedure for antibiotics such as chlortetracycline, nafcillin and sulphametoxazole, and X-ray contrast media such as iopamidol and iopromide. With the procedures proposed in the literature—which are not always as integrated as one would like them to be—quantification limits of 5–50 ng/l can be obtained for all compound(s) classes quoted. A quite different example deals with the determination of sulphonamides in milk, a notoriously difficult matrix [72]. Because LC–MS–MS is used for analysis, sample pre-treatment can be limited to ultrafiltration. This is, admittedly, an off-line procedure but, since 24 samples can be treated simultaneously, the real preparation time is only 2–3 min. With a sample volume of 1 ml and a 1% aliquot being subjected to the final analysis, LODs are, typically, 5–10 μg/kg. Another area of much current interest is the measurement of flavonoids in, specifically, food and plants [57,73]. There are over 4000 known flavonoids comprising 12 sub-classes such as flavones, flavonols and flavanones, and LC methods have been developed for analysis across up to three sub-classes (which implies that there is still quite a challenge that has to be met). Results from one recent study, in which LC–ESI(+)-MS was used to analyse flowers and leaves of red clover for the presence of flavonoids and their glycoside malonates, show that some 50 compounds were identified; six of these were malonates not previously reported for this plant, and three of them were new compounds [74]. In another study, 14 isoflavone glycoside malonates and six acetyl glycosides were added to the list [75].

Ion suppression is one of the most serious problems in LC–MS. Analyte responses can be considerably affected by the addition of (buffer) salts to the LC eluent and/or by altering the pH. Changing the nature of the buffer salt or the organic modifier can also have dramatic effects. In the latter case, even a complete loss of signal has been reported. A further cause of signal reduction are compounds present in the matrix: during ionisation, they compete with the analytes of interest and suppress their ionisation. Such suppression can occur even for sample components that do not show up in the mass chromatogram at all! A number of relevant examples is summarized in Ref. [58]. Humic acids and co-extracted food constituents are two notorious classes of interfering compounds. Keeping the (gradient) LC conditions rigidly constant and using matrix-matched standards are ways to overcome most such problems. For the rest, contrary to what was sometimes stated in early papers, quantification itself is straightforward in LC–(ESI or APCI)-MS for both standards and samples: calibration plots are, typically, linear over several orders of magnitude.

It is good to issue a note of warning regarding the choice of interface. While there is little doubt when the analytes of interest are of a really polar (ESI) or distinctly less-polar (APCI) nature, errors are easily made with ‘‘in-between’’ compounds such as, for example, flavonoids and many phenolic compounds. For the flavonoids this is illustrated by data on preferred MS conditions taken from recently published studies and summarised in Table 3 [73]. Actually, differences in signal intensity may be up to two orders of magnitude when ESI (+ or −) and APCI (+ or −) are compared, with the choice of the LC eluent creating further differences. Consequently, using two sets of conditions in order not to lose specific analytes is often required. It will also be clear that, especially in such situations, it is the MS detection rather than the LC separation (or UV

Table 3
Selected papers on the LC–API-MS(–MS) of flavonoids [72]

Sample	LC eluent	API mode	Type of MS
Fresh herbs	MeOH–water 1% FA	APCI−	Q
Soy foods	ACN–water 0.1% TFA or 0.1% AA	APCI± and IS±	QqQ
Red clover	ACN–water 0.25% AA	ESI±	Q
Soy, onions	ACN–water 10% FA	ESI−	Q
Olives	MeOH–water 1% AA	ESI±	QqQ
Not applied	ACN–water 0.5% AA	APCI± and ESI±	IT and Q-TQF
Wood pulp, waste water	MeOH–water 0.5% AA	ESI±	QqQ
Urine	MeOH–ACN–water 0.5% FA	APCI−	Q
Not applied	MeOH–water 0.1% FA	ESI−	IT
Onion, blossom and St. John's wort	ACN–water 20 m*M* TFA	ESI+	IT
Not applied	MeOH–water or ACN–water 0.1–0.4% FA or 10 m*M* AAc or 0.1% AH, 0.05% TFA	IS± APPI± and APCI±	QqQ
Red clover	ACN–water 0.2% AA	ESI+	Q

AA, acetic acid; AAc, ammonium acetate; ACN, acetonitrile; AH, ammonium hydroxide; APPI, atmospheric pressure photo-ionisation; FA, formic acid; IS, ion spray; IT, ion trap; MeOH, methanol; TFA, trifluoroacetic acid; TOF, time of flight; Q, single quadrupole; QqQ, triple quadrupole.

detection) which determines the composition of the eluent. Finally, one should be aware that despite the impressive progress in LC–MS made in recent years, there are still major differences with GC–MS when identification is considered. In the latter case, large libraries are available, and meeting properly defined criteria is an essentially routine operation. In LC–MS, however, analyte spectra are often influenced by the type of MS instrument used and the instrument settings and, as was briefly outlined above, also by LC conditions and matrix influences. Thirdly, and this is often a serious problem, the structural information that can be derived from API-MS spectra is much more limited than the information that can be obtained, in GC, from EI-MS spectra. Formulating suitable identification and confirmation criteria is, therefore, urgently required and the recent EU-wide initiative to introduce and apply so-called identification points (IPs) is to be applauded [76].

3.1.5. NMR

It is well known that NMR is a spectroscopic technique with a very high information content and the first attempts to add LC–NMR to the other options open for hyphenation in LC date from some 30 years ago (see, e.g., Refs. [77–79]). However, analysts will also be aware of the very high price of NMR instrumentation (especially now that 400 and 500 MHz machines are rapidly being replaced by 600 and, even, 750 MHz instruments) and, fundamentally more important, of the poor sensitivity of the technique—specifically when compared with the

other spectrometric techniques which are available. For obvious reasons, the present discussion will be limited to highlighting aspects which are of special importance when considering (multiple) hyphenation.

Since, in LC–NMR, sensitivity is such a crucial point, many authors stress that the LC separation should be properly optimised to have as concentrated analyte zones as possible, and some authors argue (Chapter 1 of Ref. [4]) that stationary phases such as the newly developed C_{30}-bonded silicas should be used, which tolerate extensive overloading. Somewhat surprisingly, the use of routine on-line SPE–LC procedures to create such effects has, as yet, not received much attention [80,81]. Another highly important point is the suppression of solvent signals. This can be achieved by so-called NOESY presaturation (reduction of one particular signal), soft-pulse multiple irradiation (reduction of multiple solvent signals) and WET presaturation (removal of ^{13}C satellites), where one special area of application is added in brackets for each technique. Signal-intensity reductions of up to 1000:1 can be effected. However, compound signals lying under the solvent signals are also suppressed; in other words, multiple-solvent suppression is only useful to a limited extent. Gradient LC–NMR has its own specific problems because the changing dielectric constant causes severe chemical shift alterations. Solvent-signal suppression now requires a prior scout scan to detect the effective shift of these signals if on-flow spectra are to be obtained (see below). Efficient suppression has reduced the need for deuterated solvents to prepare LC eluents. Still, (inexpensive) D_2O is frequently used rather than H_2O because this makes multiple solvent suppression easier. In most applications, D_2O–non-deuterated acetonitrile mixtures are the eluent. However, in the pharmaceutical industry, acetonitrile-d_3 is increasingly used because the cost of this solvent is negligible in relation to the other operating costs and, again, a substantial gain in quality of the results can be obtained.

There are four main types of data acquisition in LC–NMR (see, e.g., Ref. [4]): (i) stopped-flow acquisition; (ii) time-sliced acquisition, where a peak is moved incrementally through the probe with stopped-flow spectra taken at each step; (iii) on-line collection of peaks in storage loops; and (iv) continuous-flow acquisition. With the first two alternatives, peak selection requires the use of a monitoring device, typically an LC detector. Stopped-flow acquisition is the simpler solution; however, the repeated stop/go disturbances of the LC part of the system, the possibility of undue peak diffusion during long-lasting experiments and memory effects if a small peak is preceded by a large one, are disadvantages. However, generally speaking, using properly optimised and robust LC conditions will prevent such problems. With intermediate storage in loops, these problems virtually disappear, but decomposition/isomerisation of labile compounds can still create problems when storage is prolonged. In daily practice, measurement times per peak are often on the order of 20 min. This implies that, even with a limited number of four or five peaks of interest per run, total analysis times tend to become much longer than is usual in conventional LC. In other words, one has to be very careful in selecting analytical problems for which the technique should be used. With the continuous- or on-flow acquisition mode, this problem does not exist because measurements occur under dynamic conditions; standard LC equipment can now be used, no monitor detector is needed (although a UV detector remains useful for monitoring the on-going LC separation) and the entire chromatogram is ''covered'' by the NMR spectra. However, the very short period of time available for data acquisition and the reduced stability under flowing conditions limit the operation to acquiring spectra for the major sample constituents. Calculations show that LODs are in the range of 5–10 μg injected. One way to improve the situation is to run an initial experiment overnight at a much reduced flow-rate of about 0.05 ml/min. Experience shows that, with conventional LC–NMR systems, up to 128 scans can then be recorded per spectrum. Such an, admittedly, very expensive run is especially useful to obtain a first, straightforward overview of mixtures of closely related (e.g., natural) products.

Because of the inherent lack of sensitivity, LC–NMR is, typically, not used for the trace analysis of drugs in biofluids—for which LC–MS, anyway, is a better alternative. However, it is applied successfully for the characterisation of drug impurities and synthetic chemistry in the form of combinatorial peptide libraries, and the identification of drug metabolites (as reviewed in, e.g., Chapters 3 and 4 of Ref. [4]).

To quote a few examples, a study using chiral LC–UV–NMR [and also separate LC–circular dichroism (CD) detection] was devoted to an investigation of the 10 isomers of atracurium besylate; the role of each part of the system to characterise these compounds is explained in Fig. 7 [82]. Of more general importance, in a study on another drug, fluticasone propionate, it was demonstrated that LC–NMR can be used to characterise impurities below the 0.1% level, which is the relevant limit for regulatory submissions. However, the authors also remarked that, for a level of 0.2% or less, recording acceptable NMR spectra required considerable and time-consuming data acquisition. In other words, because of the high cost in NMR analysis time, such an approach is justified only in cases where there are few analytical alternatives [83].

As regards the identification of drug metabolites, there have been studies on, for example, ibuprofen [84], antipyrine [85], naproxen [86], flurbiprofen [87] and iphosfamide [88]. With the latter two compounds, ^{19}F- and ^{31}P-NMR, respectively, were used next to ^{1}H-NMR. Studies in animals included applications to paracetamol [89] and phenacetin in rat [90] and iloperidone in rat, dog and man [91].

Next to pharmaceutical analysis and metabolism research, natural product analysis is, today, one of the main application areas of LC–NMR. However, whereas in the first two fields the parent compound is known and, consequently, some knowledge of the expected structures is available, the composition of a natural-product extract is often completely unknown beforehand, and non-target analysis is the catchword. While NMR detection turns out to be particularly powerful for the differentiation of isomers, different sugar configurations and differences in substitution patterns on, for example, aromatic ring systems, MS and MS–MS techniques are needed to obtain information on, for example, molecular mass and functional groups. At this point it should be noted that for a comprehensive structural elucidation of a novel natural product, a preparative isolation still has to be performed because (i) in LC–NMR part of the spectral region is lost (cf. above) and (ii) even if the available sample amount is generally not a limiting factor, LC–NMR does not provide the indispensable ^{13}C-NMR data. This intriguing and rewarding field of study has specifically been explored by Hostettmann and Wolfender and their group, who have published several reviews [92–94] on the application of LC–NMR in phytochemical analysis. Therefore, and also because a contribution by these authors is included in the present volume [65], no detailed discussion is presented here. However, for the convenience of the reader, Table 4 summarises a number of applications.

LC–NMR has also been applied in environmental chemistry: elegant studies on, for example, hazardous waste sites, landfill leachates and the simulation of environmental processes such as phototransformation or hydrolysis reveal that valuable structural information can be obtained for large numbers of organic microcontaminants ([95], Chapter 6 of Ref. [4]). Again, combination with information derived from LC–MS is often indispensable. From the point of view of selectivity, LC–NMR is especially suited for many classes of aromatic compounds, for example nitro-aromatics, aromatic amines, PAHs and PCBs, but not for aliphatic compounds with long alkyl chains, such as surfactants. A relevant example of determining the position of substituents on an aromatic ring is the unambiguous identification of hydroxy-PCBs, most of which are not commercially available. The stop-flow NMR spectra of four such compounds, produced by the chlorination of 2-hydroxybiphenyl, are shown in Fig. 8.

Other promising areas of application of LC–NMR are the study of *Z*/*E* stereo isomers of carotenes such as lycopene (Chapter 5.2 of Ref. [4])—where MS techniques provide no, and DAD UV only a partial, solution—and the characterisation of polymers, specifically copolymers, which differ in chemical composition or stereo-regularity (Chapter 7 of Ref. [4]).

3.1.6. Conclusion

From among the five types of spectroscopic detection discussed above, fluorescence has the fundamental problem that the number of compounds displaying native fluorescence is limited—much more so than is commonly thought by workers thinking in terms of fluorescent labelling for detection, not identification, purposes. Therefore, even though the information that is provided, specifically at low temperatures, can be impressive, LC–ILDA FLU is not a generally useful hyphenated system. With FTIR detection, there is a quite different

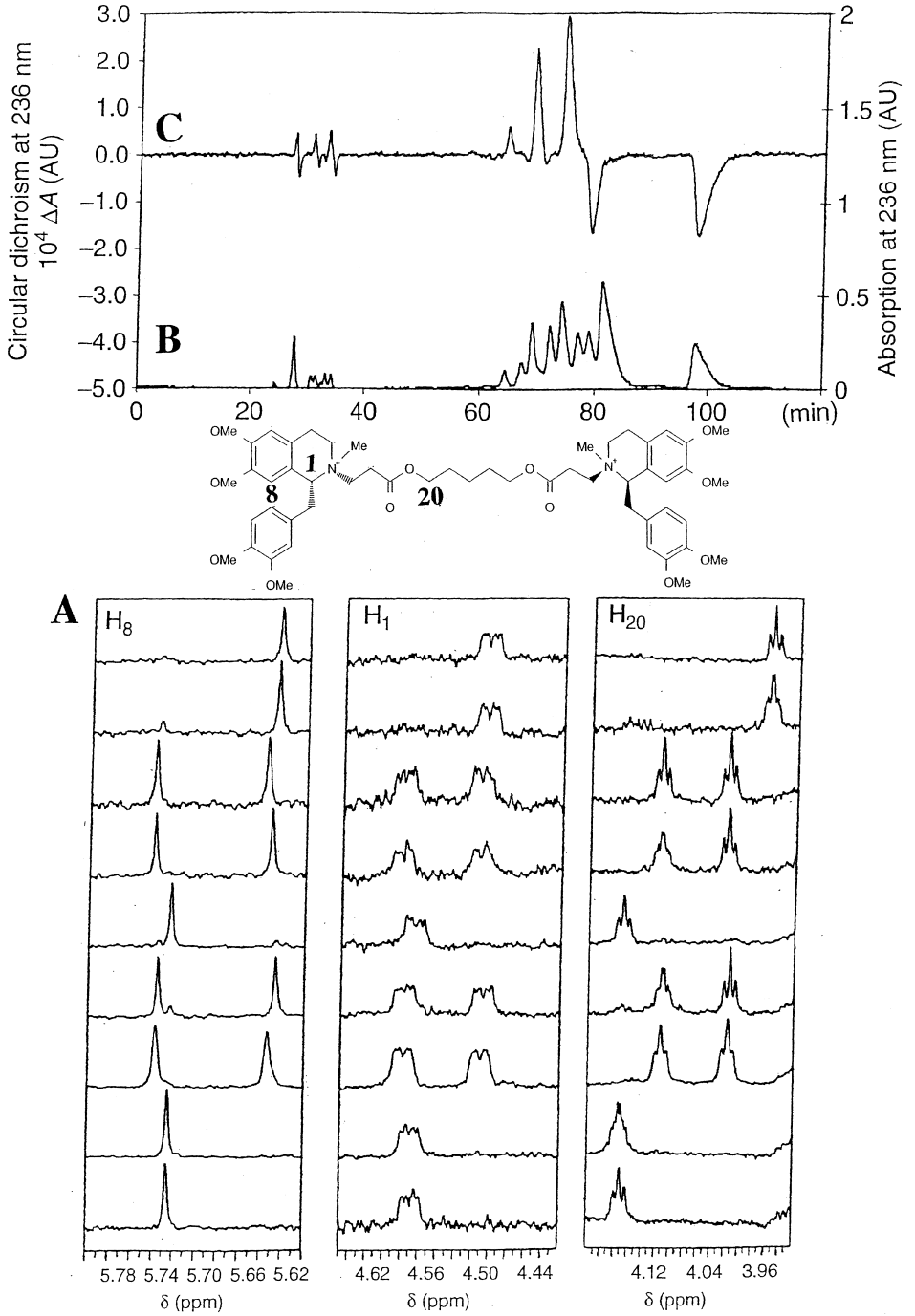


Fig. 7. LC–NMR and LC–UV–CD for identification of the various isomers of atracurium besylate (structure, see inset) following separation on a chiral LC column. (A) Expansions of parts of stopped-flow 750 MHz ^{1}H-NMR spectra (protons indicated on structure), (B) LC–UV trace and (C) corresponding LC–CD trace. The NMR data allowed the isomeric configuration at the C1–N2 bond to be determined, revealed the enantiomeric pairs and allowed them to be distinguished from the *meso* forms. The corresponding CD data on each peak provided the absolute stereochemistry, e.g. *S-trans*/*S-trans*, *R-trans*/*S-trans*, etc. Adapted from Ref. [82].

Table 4
Selected applications of LC–NMR in plant natural product analysis

Compound type	Compound type
Prenylated flavones	Taxanes
Secoiridoids	Lignanes
Naphthoquinones	Phenylphenalenones
Pyrrolidizidine alkaloids	Ecdysteroids
Benzophenones	Fasciculol triterpenes
Naphthylisoquinoline alkaloids	Tocopherols and tocotrienols
Sesquiterpene lactones	Carotenoid isomers
Azadiractin	Flavonoids
Triterpene saponins	Hop bitter acids

Often combined with LC–MS and LC–UV information. Data collected from various sources.

problem, viz. (i) the limited compatibility of optimised separation and detection conditions, and (ii) the rather complicated interfacing that is required. As a consequence, even though FTIR information is highly desirable in the case of, specifically, isomer identification, the state-of-the-art is that—as with GC–FTIR discussed above!—the bright future which the technique should have cannot be read from the somewhat indifferent usage typically encountered today.

As for the other three detection principles discussed above, all of these contribute significantly to the present-day success of hyphenation in LC. While DAD UV can often provide a rapid general impression of the complexity of a sample and is frequently useful for a provisional analyte identification or a confirmation of identity, the technique is slowly becoming less essential. Today, a multi-wavelength UV monitor detector is, in many instances, all that is required, because more detailed information is provided either by one of the many MS techniques that are available—which combine versatility and analyte detectability at the trace level—or by the extremely powerful but, unfortunately, not really sensitive NMR, a combination of characteristics which frequently requires that operational compromises have to be made. If one, moreover, keeps in mind that it is with MS and NMR instruments and techniques that most progress has been made in the recent past, it will come as no surprise that, from among the many combinations of spectroscopic detectors in use in hyphenated LC, only two have gained widespread acceptance, DAD UV–MS and (UV)–NMR/MS, where ''MS'' is used as an umbrella for all techniques of interest; that is, MS–MS and MS^n and the use of time-of-flight instruments are included.

3.2. Hypernation

3.2.1. DAD UV–MS

This combination became available much earlier than NMR–MS, i.e. at a time when the MS part of such systems was much less powerful than today. Early work in, for example, environmental analysis really did rely on the combined information derived from the two types of detector. Also today, monitoring stations on river banks often do not have MS facilities, and provisional identification on the basis of retention time and UV spectral match is used to reach a decision regarding sample status, with suspected samples being sent to a central laboratory for a re-run involving the use of LC–MS. However, in much laboratory-based work one notices that the, frequently still called ''DAD UV'', detection actually merely serves as a selective type of monitoring—as is also true for much LC–NMR work (cf. above). That is, while there certainly still are quite a number of cases where DAD UV is most useful (e.g., with flavonoids, for which several sub-classes can be distinguished [57]) or, even, indispensable (characterisation of unknown natural products, when every additional bit of information is most welcome [65,92–94]), there is a tendency to limit the technique to excluding false positives or to reduce its role to that of a sophisticated selected-wavelength detector. Since DAD UV–MS interfacing does not create any real problems and it is, moreover, the more powerful MS detector with its much greater sensitivity to changes in LC conditions (cf. above) which almost invariably causes the experimental conditions to be selected, there is no need to discuss DAD UV–MS hypernation in any detail here. Many relevant examples can be found in virtually all reviews on modern LC–MS-based trace-level studies and, implicitly, also in some of the applications discussed in the present review.

3.2.2. NMR/MS

In recent years, a number of studies on the use of NMR and MS detection in a single setup have been

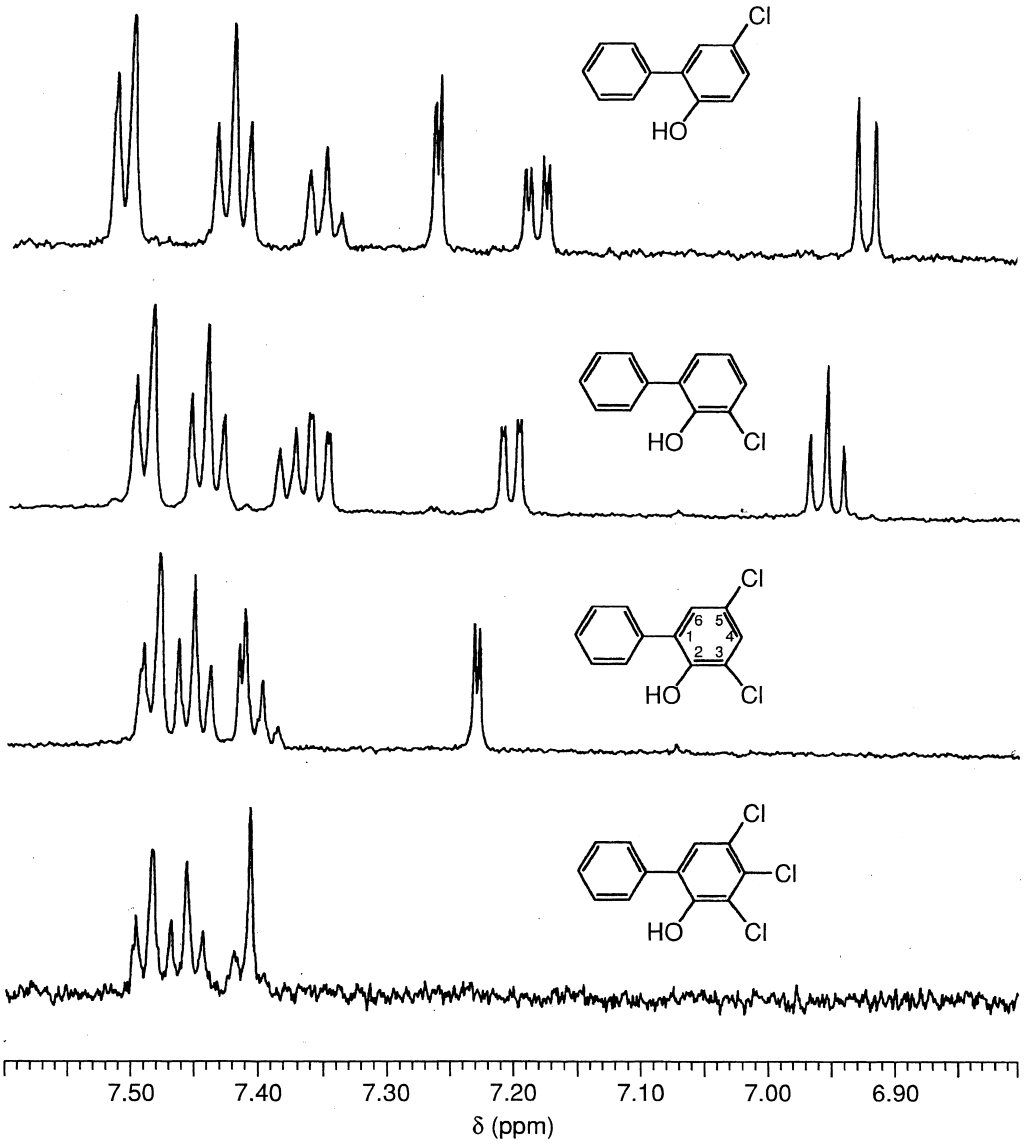


Fig. 8. Stopped-flow ^{1}H-NMR spectra obtained for the chlorination products of 2-hydroxybiphenyl using RPLC with acetonitrile–D_2O. Adapted from Chapter 6 of Ref. [4].

reported. In an early paper on LC–NMR/MS of a mixture of fluconazole and two related triazole-type compounds, the LC eluent was split 6:4 for on-flow NMR and CI-PB-MS detection (PB, particle beam), respectively. Subsequent studies dealt with metabolites of paracetamol isolated from human urine and metabolites of ibuprofen. To illustrate the merits of the hypernated setup, with the latter study [96] both detectors easily found the major glucuronide metabolites, but the much greater sensitivity of MS detection for several minor ibuprofen-related compounds helped to direct the NMR analysis to obtain spectra of these peaks that would have gone undetected by NMR alone. On the other hand, information obtained by ^{1}H-NMR that would not have been found by MS concerned the ratio of the

diastereo-isomers of the glucuronide conjugates of various metabolites of ibuprofen which was used as a racemate. In a study [97] on a [^{14}C]-labelled β-blocker (with an on-line radioactivity monitor to direct the spectroscopic analyses), the combined information enabled the parent drug, its ring-hydroxylated metabolite and the ring hydroxy-glucuronide to be identified. In addition, the NMR spectrum clearly showed that enantioselective metabolism/excretion had occurred. In another study, an on-line NMR–MS rather than a parallel NMR/MS configuration was used to study a novel non-nucleoside reverse transcriptase inhibitor, and several hydroxylated and glucuronidated metabolites were identified [98].

LC–NMR/MS has also been used to characterise the major metabolites of the herbicide 5-trifluoromethylpyridone (which can also exist as the tautomer, 2-hydroxy-5-trifluoromethylpyridine) in maize plants (Fig. 9A) [99]. A combination of ^{19}F-NMR spectroscopy, on-line LC–NMR and stopped-flow LC–NMR/MS was used to identify the parent compound and two metabolites. Subsequently, the metabolism of 5-nitropyridone (no ^{19}F handle!) was studied [100]. This application required first running the on-line MS experiments and, next, selecting the peaks of interest and subjecting these to LC–NMR; three metabolites could be characterised. For comparison, it is good to quote a study (Chapter 4 of Ref. [4]) of another fluorinated compound, efavirenz, which also has a CF_3 group (Fig. 9B). In this case, as there were no endogenous fluorinated compounds in the control urine, responses in the initially conducted LC–^{19}F-NMR/MS run must arise from efavirenz metabolites. That is, this first—now an NMR!—experiment located all metabolites of sufficient concentration to be detected. Without going into details regarding the further procedure, what is important to note is that the NMR-*cum*-MS approach should start with either one or the other technique, depending on the analyte structure and the goal of the study—which is closely analogous to what was earlier observed for the AED-*cum*-MS approach in Section 2.2.

In view of the demanding nature of the non-target approach generally prevalent in natural-product analysis, one might well expect this to be a preferred area for LC with NMR/MS hypernation. However, the actual situation turns out to be somewhat different: there apparently are two ‘‘schools’’ today. Some workers, notably Wolfender and Hostettmann (also see above), prefer to use the two spectroscopic techniques separately. In their opinion, LC–NMR, which generally is the most demanding technique, may well compromise the use of (the) other hyphenated technique(s). To quote a typical example [101], they acquire the data from LC–DAD UV and LC–MS–MS at the same time, while LC–NMR and LC–micro-fractionation are performed in a separate, second analysis. Loading of the sample can now be optimised for each technique and D–H exchange problems during MS detection do not occur. In addition, 10% of each micro-fraction (used for a bioassay) is kept for post-run UV/MS analysis in case of doubt regarding the attribution of LC peaks. The latter precautionary measure indicates the weak point of the approach, the problem of peak correlation between the two runs—especially if minor peaks have to be considered, and this will invariably be part of any analytically challenging problem. Such less than ideal peak correlation may be caused by differences in sample loading, differences in LC behaviour of D_2O and H_2O and, simply, by very small differences in LC flow-rates and/or LC column quality. Irrespective of the pros and cons of the approach, impressive results can be achieved, as is clearly illustrated in a paper included in the present volume [65].

A recent clear demonstration of the potential of having both NMR and MS available in one LC run was recently reported in a study on the Baltic starfish, *Asterias rubens* [102]. Previously unreported asterosaponins were identified from sub-fractions after rapid sample preparation by matrix solid-phase dispersion. Despite the high complexity and close analogy of their structures, the targeted isolation and off-line structural elucidation of seven new compounds was achieved and up to 17 individual constituents could be characterised in a single chromatogram of an asterosaponin fraction. Fig. 10 should serve as a partial illustration of the complicated nature of the problem at hand.

Recently, LC–NMR/MS was also used for the separation and characterisation of two sec-oisolariciresinol diglucoside isomers (structure below) in flaxseed [103].

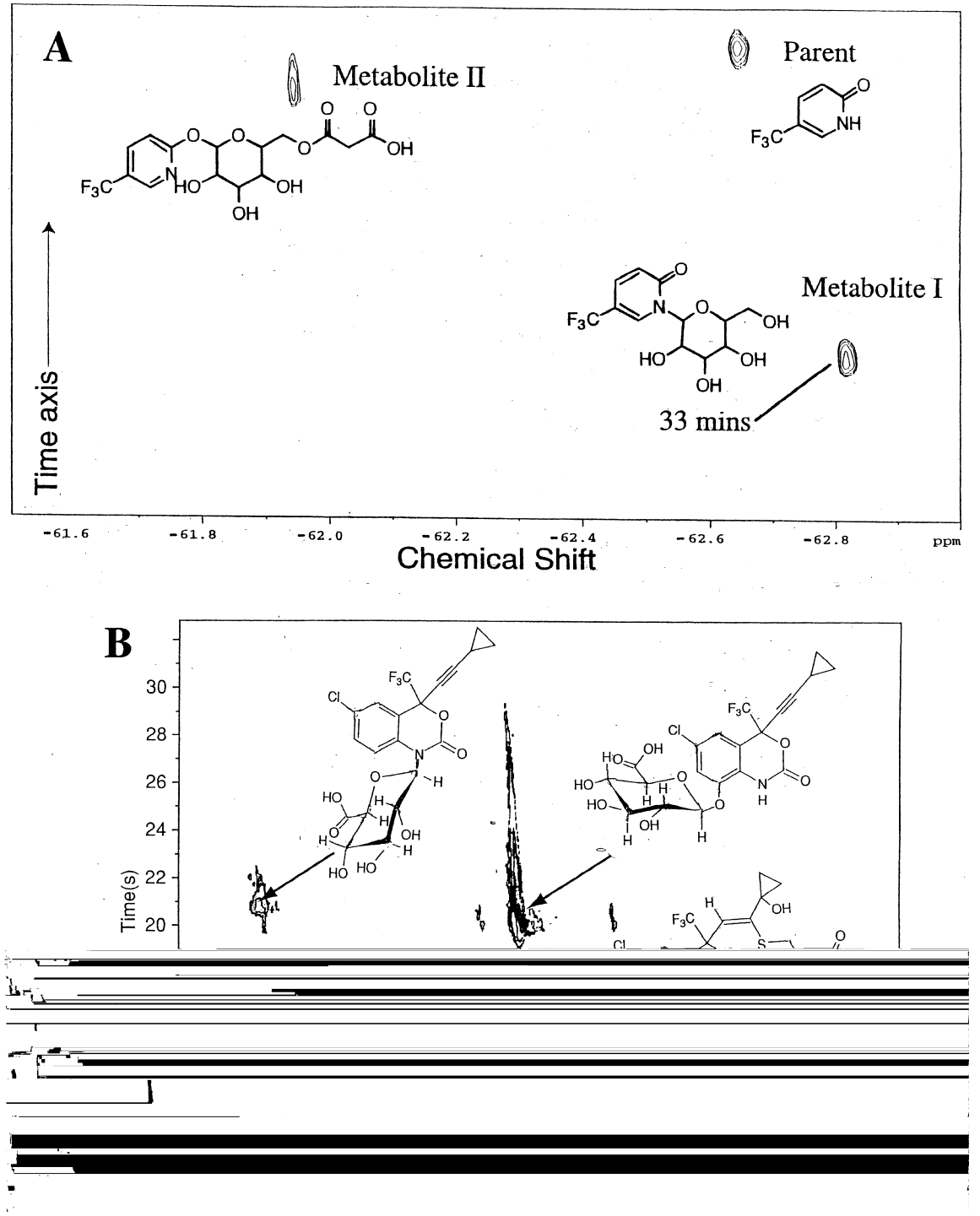
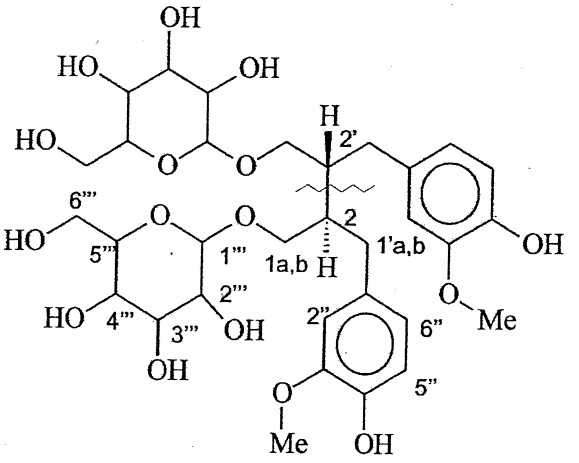


Fig. 9. On-flow ^{19}F-detected LC–NMR of (A) unchanged parent compound and *N*-glucoside and *O*-malonylglucoside metabolites of 5-trifluormethylpyridone (structures, see inset) produced by hydroponically grown maize (adapted from Ref. [99]), and (B) the *O*-sulphate-cysteinylglycine, *O*-glucuronide and *N*-glucuronide conjugates (structures, see inset) of efavirenz (adapted from Chapter 4 of Ref. [4]).



CD analysis confirmed the presence of two optically active compounds. Gradient LC at 1 ml/min with a D_2O–acetonitrile mixture on a conventional-size C_{18}-bonded silica column was used for separation, with a subsequent 20:1 split ratio to the 600 MHz NMR and ESI-MS instruments. A UV detector set at 280 nm was used for monitoring. This somewhat detailed, basic information is added to emphasise that, from a technical point of view, rather similar conditions are applied in most of the quoted studies.

3.2.3. Conclusion

The above discussions show that there is, in the present context, in essence only one type of hypernated system that deserves further attention, viz. the combined use of NMR and (tandem) MS detection in either a serial (LC–NMR–MS) or a parallel (LC–NMR/MS) setup. Inserting a DAD UV (or a UV) and, potentially, a CD detector on-line does not cause any real technical complications and may provide useful additional information. The complementarity of the, often, detailed structural information that can be obtained, has been shown to be especially rewarding in the ''bio'' sphere, for example with drug impurities, metabolites and bioactive substances. It is stimulating to note that what was, typically, a ''milligram operation'' and a, consequently, not very useful technique some 20 years ago, today is a low-, and, occasionally, sub-microgram technique. This helps to explain why most of the successful applications are in the field of impurity profiling (relatively high, 0.1–0.2%, levels) and the characterisation of unknowns (long measurement times acceptable), while environmental target analysis and conventional bioanalysis with their ng/g trace-level nature clearly are out of bounds.

It is also clear that, for reasons partly mentioned already in the Introduction, at the present time two stand-alone LC–MS and LC–NMR setups will probably be preferred by most workers over a single hypernated system. However, the very importance of the experimental information that can be obtained and the increasing need to have proper data correlation also for minor peaks will effect a general trend in the direction of the latter option. Finally, a most relevant observation is that almost all recent in-depth studies—for example, several chapters of Ref. [4] quoted earlier in this review—conclude by saying that the power of the hypernated NMR-*cum*-MS approach has been convincingly demonstrated, but that the utility of LC–NMR and LC–NMR/MS is still hampered by the inherently low sensitivity of NMR spectroscopy. Another item of interest is the further development of NMR in the micro-domain, discussed in another contribution to the present volume [104]. Several authors indicate that progress is expected when new capillary flow cells and suitably designed cryoprobes will become available. Hopefully, such developments will bring the ranges of analyte detectabilities of the two techniques closer together: partly closing the existing gap of several orders of magnitude which exists for most classes of compounds is a major challenge.

3.3. Extended hypernation

The previous discussions have shown LC–DAD UV–NMR/MS—with the parallel generally preferred to the serial NMR-plus-MS sequence—to be the hypernated system in pole position. It is not surprising, therefore, that the next attempt was to include FTIR by collecting the effluent from an NMR instrument on-line on a slowly rotating germanium disk with concomitant solvent evaporation and subsequent off-line FTIR detection. As was discussed above (cf. Section 3.1.2), such off- or at-line fraction collection ensures that chromatographic resolution is preserved. A normal-phase SEC–NMR[–FTIR]/MS setup was used for the characterisation of several model polymer additives [105]. From the point of

view of spectroscopy, the results were satisfactory. However, some problems were encountered regarding overall eluent compatibility: to ensure sufficient ionisation in ESI-MS, 1% ammonium acetate and 5% deuteromethanol had to be added to the dry $CDCl_3$ used in the earlier LC–NMR–FTIR study. Next, an LC–DAD UV–NMR–FTIR/ToF MS system was designed, with IR spectra obtained on flow using an attenuated total reflectance (ATR) flow cell, and used to study a fairly concentrated mixture of four non-steroidal anti-inflammatory drugs such as ibuprofen and naproxen, as model compounds (2 mg of each analyte injected) [106]. Good-quality spectroscopic data and accurate masses were obtained for all analytes. In other words, a ''proof of concept'' was provided. This was confirmed in a subsequent study on ecdysteroids in plant material, where two compounds, 20-hydroxyecdysone and polypodine B, were unequivocally identified. In both studies, stopped-flow ^{1}H-NMR was used [107].

In some recent studies, attention has been devoted to the use of superheated water (or, rather, D_2O) as the eluent in LC–NMR/MS. The attraction in terms of detection performance will be clear, although one should keep in mind that the approach will not be suitable for all analytes of interest, because the elevated temperatures may cause degradation of thermolabile compounds. An LC–FTIR–DAD UV–NMR/MS system (operated at 160 °C; Fig. 11) was used for the analysis of ecdysteroids present in crude extracts of several *Silene* species [108]. For all samples, UV, IR, NMR and MS data could be recorded that identified 20-hydroxyecdysone as the main ecdysteroid. A compound mass of ca. 100–150

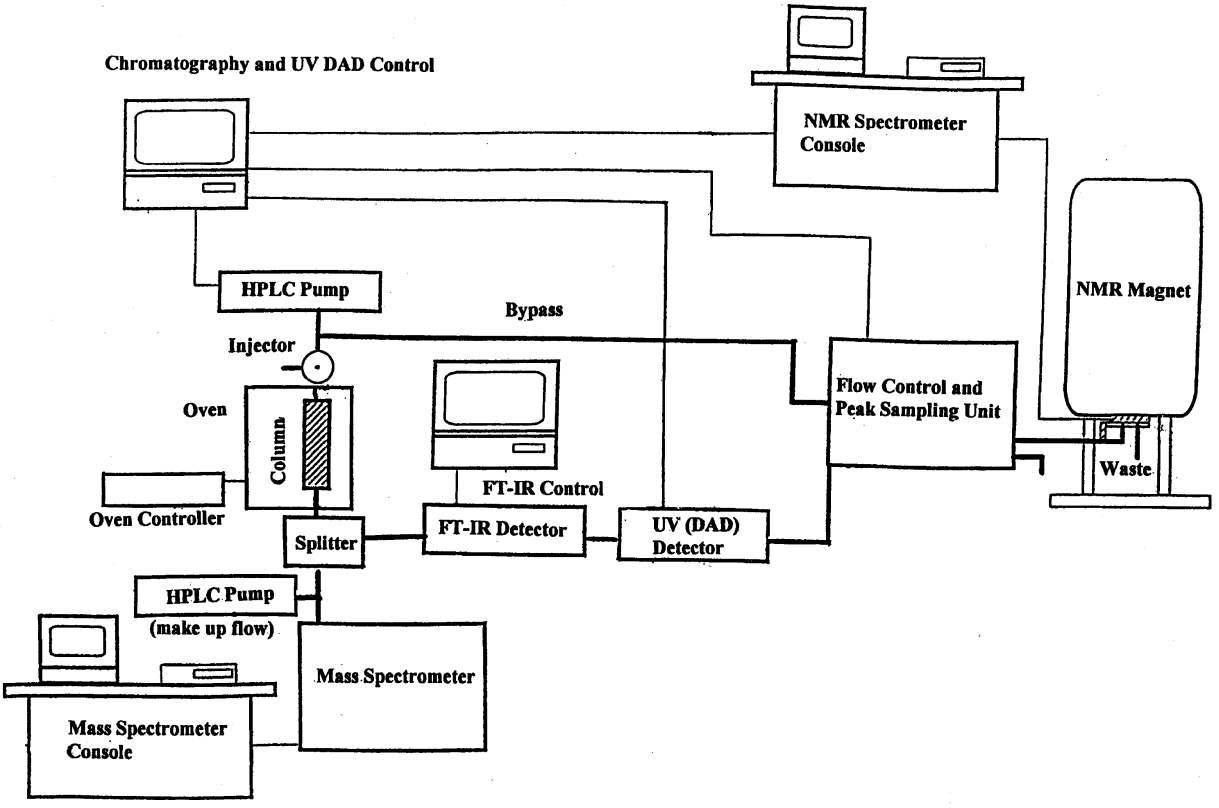


Fig. 11. Instrumental setup for LC–DAD UV[–FTIR]–NMR/MS used for the analysis of ecdysteroids from various *Silene* species with superheated D_2O as the eluent [109].

μg injected on-column was required to provide a reliable set of on-flow spectral data; that is, this quantity can be considered as a reasonable limit of identification for the present setup. Actually, in another study, a 50 μg limit was found for caffeine, but this was for a mixture of standard compounds and for a compound with a relatively simple ^{1}H-NMR spectrum [109]. As for other, less abundantly available sample constituents, DAD UV could repeatedly be used to demonstrate their ecdysteroid or non-ecdysteroid nature, while MS-based information enabled tentative identification—e.g. of integristerone A, 20,26-dihydroxyecdysone and 26-hydroxypolypodine B. However, these compounds were not always present in amounts sufficient for NMR or FTIR spectra to be obtained.

As was suggested above, although superheated water is an excellent eluent per se, the use of conventional reversed-phase eluents has its advantages. The latter approach was used for the characterisation of several polymer additives, Bisphenol A, BHA, Irganox 245, BHT and Topanol CA. Their rather non-polar nature required the use of an organic-rich eluent, acetonitrile–water (80:20, v/v), and fully deuterated solvents were used to simplify solvent suppression in NMR [110]. The separation was achieved for the latter three compounds, but Bisphenol A and BHA showed up as a single peak. Fortunately, Bisphenol A eluted in the leading, and BHA in the tailing, edge of that peak. This allowed diagnostic spectra to be obtained for both analytes. With compound quantities ranging from about 250 to 900 μg injected, UV, MS, ^{1}H-NMR and IR spectra were obtained for all additives except Topanol CA; for that compound, no IR spectrum could be recorded because of signal overlap with the solvent. Using this system, the four types of spectroscopic data were acquired for a suspected polymer additive; this enabled its conclusive identification as BHT (Fig. 12).

Finally, it is interesting to briefly introduce another recent example of extended hypernation to demonstrate the versatility and potential of state-of-the-art analytical chemistry. Today, the hyphenation of (RP)LC and inductively coupled mass spectrometry (LC–ICP MS) is a routine methodology in many laboratories that enables speciation studies of analytes on the basis of their elemental composition. Whilst most popular for the study of metal-containing compounds, the technique is also admirably suited for halogen-, P- and S-containing analytes. A recent trend is to combine ‘‘atomic’’ and ‘‘molecular’’ mass spectrometry in one setup to provide a system capable of speciation *and* identification. A hypernated LC–DAD UV–ICP MS/ToF MS system—with, typically, a 95:5 effluent split—was used for drug metabolite identification (ToF MS), and speciation and quantification (ICP MS). Examples in which Br-selective detection was used include the study of the metabolite 2-bromo-4-trifluoromethylacetanilide in rat urine [111], the metabolite profiles of bromobradykinin in rat and human plasma [112], and 4-bromoanaline metabolites in rat urine [113]. In another study, Cl- and S-selective detection were used for diclofenac metabolites in rat urine; again, metabolite identification was achieved by means of ToF MS [114].

Practical issues. As the above examples and, also, the technical information provided in the quoted papers show, setting up a hypernated system does not pose overwhelming technical problems. Rather, the practical difficulties centre around (i) finding compatible eluents, (ii) coping with large mutual differences in analyte detectability, and (iii) data handling. Experience shows that the eluent-compatibility problem is gradually being solved. Addressing the data handling-*cum*-reduction and interpretation situation will require dedicated and sustained attention in the near future—both here and with other more-dimensional systems such as, for example, GC×GC–ToF MS (cf. Introduction)—but the subject matter is beyond the scope of the present review. With respect to analyte detectability, it goes without saying that any hypernated system is only as sensitive as the least sensitive spectrometer used. For the present discussion this means that, in by far the most, though not all, cases, the MS—and, generally, also (depending upon the chromophore) the DAD UV—data can be obtained on a fraction of the sample amount needed for NMR, with FTIR in an intermediate position. However, this does not imply that hypernation is not suitable for problem solving. If, to take the extremes, both NMR and MS spectra are required to solve a specific structural problem, the amount of material that must be provided is the same irrespective of the degree of hyphenation. Using a

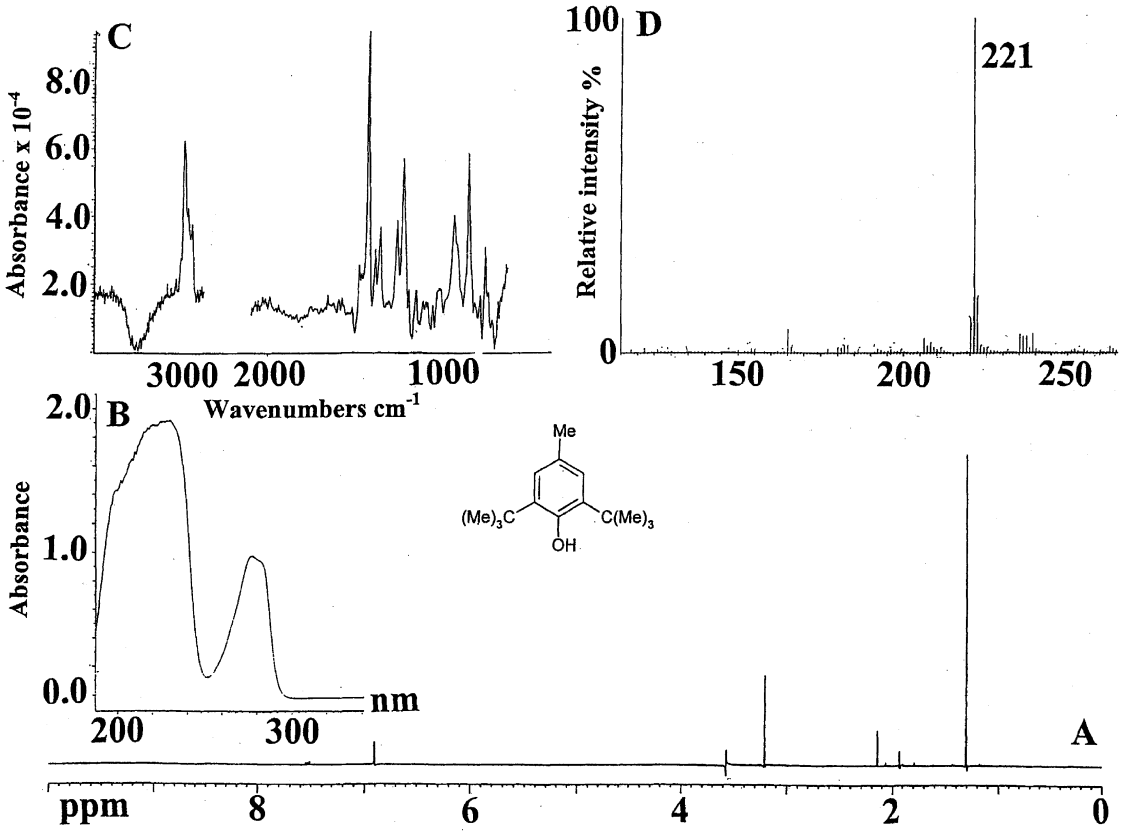


Fig. 12. Spectroscopic data obtained using LC–DAD UV[–FTIR]–NMR–MS for the identification of the polymer additive BHT (butylated hydroxytoluene, 2,6-di-*tert*.-butyl-4-methyl-phenol) (structure, see inset). Key: A, NMR; B, UV; C, IR; D, MS [110].

single setup then improves efficiency in terms of time and sample use, while dreaded inter-system interpretation problems (cf. Section 3.2.2) are avoided.

Finally, one should emphasise that extended hypernation requires capital investment, the co-location of several large spectrometers in a single laboratory, and additional technical and academic skills to operate the complex setup in an efficient way. In other words, to build such a system for a limited range of problems to which it might be applied will be hard to justify. On the other hand, there is little doubt that the number and range of problems which require this approach because of their inherent complexity will keep increasing. That is, preparing for a future in which (extended) hypernation will play a strategic role is indicated.

4. Conclusions

Hyphenated systems have been with us for quite some time already, and the wide-ranging and successful application of GC–MS, LC–DAD UV and a variety of LC–MS techniques in, for example, agricultural, biomedical, environmental, food and pharmaceutical analysis is generally recognised. Especially in the field of LC–MS, innovation is very rapid, with ion-trap MS^n or triple-quadrupole MS–MS frequently replacing single-quadrupole MS, and ToF MS and ion-cyclotron resonance (ICR) MS further enhancing the potential of the technique. Even so, it is good to add that the analyst should always be aware of the nature of the problem at hand: when conventional single-stage MS can do the job, this robust and relatively inexpensive technique

should be used and not exchanged for a more ''prestigious'' alternative.

Combining either GC or LC with FTIR-based detection or, rather, isomer or group-type recognition, still has its technical problems when the most sensitive, i.e. the analyte-deposition, techniques are used. Because of the valuable information that can be obtained, further research in this area should be stimulated. Such stimulation is also indicated for GC–AED. In this instance, it is unfamiliarity with the many benefits of multi-(element-specific) information rather than a technical problem that has, so far, prevented the ready acceptance of the technique. The increasing number of mutually rather divergent applications should help to remedy this problem in the near future.

For a completely different situation, one should go to the field of LC–NMR. What was considered, not too long ago, a less-than-useful technique is, today, a fully accepted and even indispensable tool in much sophisticated bio-related work. The complementarity of NMR- to MS-derived information is dramatically illustrated in a large number of recent papers on the identification of bioactive substances, metabolites and drug impurities—many of which have been quoted and discussed in the present review. On the other hand, while all this is true and LC–NMR has, without any doubt, a bright future, the repeated emphasis—by workers in the field—on the need to improve the sensitivity of NMR-based techniques should not go unnoticed. Fortunately, there is continual progress in this area and, according to one recent study [110], it is not unrealistic to predict that, in the not too distant future, low-microgram amounts of a single compound will be sufficient to obtain diagnostic NMR spectra.

All of the above ''simple'' hyphenated setups have by now been used as building blocks to design multiple-hyphenated, or hypernated, systems. However, the state-of-the-art in GC is rather different from that in LC. With the former separation technique, there are two distinct advantages that facilitate hypernation, viz. the carrier-stream nature (no chemical interaction!) of the mobile phase and the not too widely different ranges of analyte detectabilities of the three techniques of interest, full-scan MS, AED and FTIR. Consequently, there are no serious (additional) technical problems with regard to interfacing and effluent-stream splitting when combining two of these techniques, as is convincingly demonstrated by the applications of both GC–AED/MS and GC–FTIR/MS reviewed in the present paper. The distinct advantage of hypernation over double hyphenation in terms of improved detector-to-detector data correlation has specifically been shown for the former combination (cf. Section 2.2.2).

As regards LC-based separations, it has convincingly been argued in the present review that—since a UV detector can easily be inserted on-line in essentially all systems for either identification (DAD UV) or monitoring (multi-wavelength UV) purposes—LC–NMR/MS is the hypernated technique of primary interest. The main difference compared with hypernation in GC is that, firstly, the eluent composition has a marked influence on the LC separation that can be achieved and can, also, adversely affect detector performance (ion suppression; signal obscuring); secondly, analyte detectability is often several orders of magnitude better in MS as compared with NMR. In other words, compromises have to be made to arrive at the best possible result. Several groups of workers therefore prefer to use two parallel, i.e. separate, hyphenated systems: there is now no or, at least, less need to compromise the optimisation and the setup is less complex. It is, however, increasingly realised that, for more complicated problems, the data correlation referred to before is extremely important. In such cases, having to compromise somewhat more with respect to the experimental conditions would seem to be a lesser evil than losing vital information for minor peaks in the sample chromatogram(s). Since the amount of sample is—in both instances!—determined in the same way by the same, less sensitive, detector it is not too provocative to predict a gradual trend in the direction of the hypernated approach. That such combinations are now commercially available points in the same direction.

Stimulated by the proven potential of LC–NMR/MS, one group of workers [105–110] recently started to study the field of extended hypernation. Their prototype LC–DAD UV–FTIR–NMR/MS systems have meanwhile been used successfully for a variety of applications. The results satisfactorily demonstrate that the concatenation of LC to a wide range of spectroscopic detectors is, technically,

possible. They also show that diagnostic UV, IR, MS and ^{1}H-NMR spectra can be obtained on-flow, and in a single run, for quantities of material on the order of 50–100 μg. Actually, the lay-out of the instrumentation was not fully optimised and preliminary work indicates [109] that a five- to 10-fold improved performance can be achieved by paying attention to minimising band broadening. This is, no doubt, a promising development and one should not underestimate the considerable advantage of acquiring four sets of properly correlated data rather than data from four parallel separations. On the other hand, the obvious disadvantage of such extended hypernation is that, whilst it may be efficient in terms of run time, sample size and data correlation, it may be very inefficient in the utilisation of (expensive) spectrometers. In other words, one may well agree with the authors when they conclude [110] that it is not yet clear whether the benefits of hypernated systems of the type discussed here outweigh the costs of assembling such an array of instruments.

To the present authors' opinion, however, we may well end on a more positive note, specifically because of such highly rewarding recent developments as the introduction of another extended-hypernation technique which combines DAD UV, ICP MS and ToF MS for LC-based separations [111–114], and can be considered the mirror image of hypernated AED/MS detection for GC-based operations. If, in addition, one considers that (i) the whole area of LC-based (extended) hypernation is still in an early stage of development and major improvements can be expected, while (ii) the analytical problems that have to be solved tend to become increasingly complex, there is every reason to believe that, in the longer term, the benefits will certainly outweigh the costs—not for routine problem solving, but for whatever is new, challenging and complicated.

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