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Size-exclusion chromatography with on-line ultraviolet, proton nuclear magnetic resonance and mass spectrometric detection and on-line collection for off-line Fourier transform infrared spectroscopy

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Abstract

The coupling of HPLC with UV detection and on-line NMR spectroscopy and mass spectrometry combined with a dedicated interface for the collection of the chromatographic eluent for subsequent Fourier transform (FT) IR has been investigated using a number of polymer additives as model compounds. Size-exclusion chromatography was performed using deuterated chloroform as eluent with the separation monitored on-line by UV detection at 254 nm and on-flow ^{1}H-NMR and MS. The effluent from the NMR probe was directed to a dedicated HPLC interface where it was deposited on a germanium plate for subsequent FT-IR. NMR and MS spectra were successfully obtained for 2,6-di-*tert*.-butyl-4-methylphenol, octadecyl-3-(3,5-di-*tert*.-butyl-4-hydroxyphenyl) propionate (Irganox 1076) and diisooctyl phthalate on-line and FT-IR spectra for all three compounds were obtained off-line. Practical problems encountered with this multiple hyphenation are described.

Keywords: Hyphenated techniques; Interfaces, LC–NMR–MS–IR; Detection, LC; Dibutylmethylphenol; Octadecyl(dibutylhydroxyphenyl) propionate; Diisooctyl phthalate

1. Introduction

High-performance liquid chromatography (HPLC) is now routinely coupled to both nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) enabling spectra of organic molecules to be obtained directly on chromatographic peaks (reviewed in Refs. [1–5]). Normally HPLC–NMR and HPLC–MS have been performed separately but more recently examples where both NMR and MS coupled to the same separation (HPLC–NMR–MS) have been shown [6–10]. Clearly such systems enable a much more complete characterisation of samples by providing both types of spectroscopic data on the same separation. The success of this combination has led us to examine the multiple hyphenation of HPLC–NMR with other spectro-

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scopic detectors and we have recently investigated the coupling size-exclusion chromatography (SEC)–NMR with on-line collection of peaks via a dedicated interface for subsequent Fourier transform (FT) IR spectroscopy [11]. This was performed using a dedicated interface to collect the eluent from flow probe of the NMR spectrometer for later off-line FT-IR of peaks of interest. The success of these preliminary experiments has further encouraged us to attempt to construct a chromatographic system capable of on-line MS in addition to NMR and FT-IR with the potential to provide a very complete method of peak characterisation.

Here we describe preliminary results for the separation and spectroscopic characterisation of a number of polymer additives, which have previously been successfully identified using hyphenated techniques such as HPLC–MS and HPLC–FT-IR [12,13], following SEC with a deuterochloroform-based solvent system as the eluent.

2. Experimental

2.1. Reagents

2,6-Di-*tert*.-butyl-4-methoxyphenol (BHT), octadecyl-3-(3,5-di-*tert*.-butyl-4-hydroxyphenyl) propionate (Irganox 1076) and diisooctyl phtalate (DIOP) were obtained from EVC (Runcorn, UK). Deuterochloroform (C^2HCl_3) 99.9 atom% was from Fluorochem (Glossop, UK). Solutions of the test analytes BHT, Irganox 1076 and DIOP, of approximately 100 mg/ml in C^2HCl_3 were used.

2.2. Chromatographic conditions

The HPLC–NMR system (Fig. 1) consisted of a Bruker LC22 pump (Bruker, Coventry, UK) which delivered C^2HCl_3 or C^2HCl_3 containing 10 g/l of ammonium acetate (in ^2H_2O) and 5% (v/v) $C^2H_3O^2H$ at 1.0 ml/min to two mixed-E SEC columns (3 μm, 30 cm×7.5 mm I.D.; Polymer Labs., Shropshire, UK) connected in series. Typically 10 μl of sample was introduced onto the column via a Model 7125 Rheodyne injector (Rheodyne, Cotati, CA, USA) fitted with a 100-μl sample loop.

The eluent from the columns was monitored at 254 nm via a Bischoff Lambda 1000 UV detector (Brucker). On leaving the UV detector flow cell the eluent was split 95:5 with 5% of the flow being directed to the mass spectrometer. Immediately prior to the flow probe of the NMR spectrometer the flow was again split 50:50 with one half of the flow sent to the NMR flow probe and the other to the LC-Transform interface for the collection of the peaks for subsequent off-line FT-IR. The details of this system are shown in Fig. 1.

2.3. Spectroscopy

NMR spectra were acquired using a Bruker DRX-500 NMR spectrometer. On-flow ^{1}H-NMR detection was carried out in the pseudo-two dimensional (pseudo-2D) mode at 500.13 MHz using a flow-through probe of 4 mm I.D. with a cell volume of 120 μl. Free induction decays (FIDs) were collected into 4000 data points with a spectral width of 8278 Hz, 90° pulses were used with an acquisition time of 0.25 s and each row in the pseudo-2D plot was acquired from 24 scans. As described above, immediately prior to the NMR probe 50% of the eluent was taken via 2 m of polyether ether ketone (PEEK) tubing from an in-line splitter to an LC-Transform Series 400 interface (Viscotek, Basingstoke, UK). The major portion of this stream of eluent (70%) was taken through a heated nebulizer nozzle (90°C), which rapidly evaporates the eluent solvent while depositing a focused track of solute onto a rotating (10°/min) IR reflective disk, whilst the remainder was diverted to waste. This disk was subsequently transferred to an optics module connected to an FT-IR system for infra-red analysis of the deposited sample track. FT-IR spectra of the individual components were acquired using a Nicolet 5DXC IR spectrometer (Nicolet Instruments, Warwick, UK) equipped with a DTGS room temperature detector over a spectrum width of 4000 to 650 cm^{-1} with a spectral resolution of 8 cm^{-1}.

Mass spectra were acquired on a Micromass LC-Quattro triple quadrupole mass spectrometer (Micromass, Altrincham, UK) using electrospray ionisation (ESI) with a Z spray source. Four functions were acquired simultaneously, two in positive ion and two in negative ion, with cone voltages of 25 and 50 V. The source temperature was set at 150°C

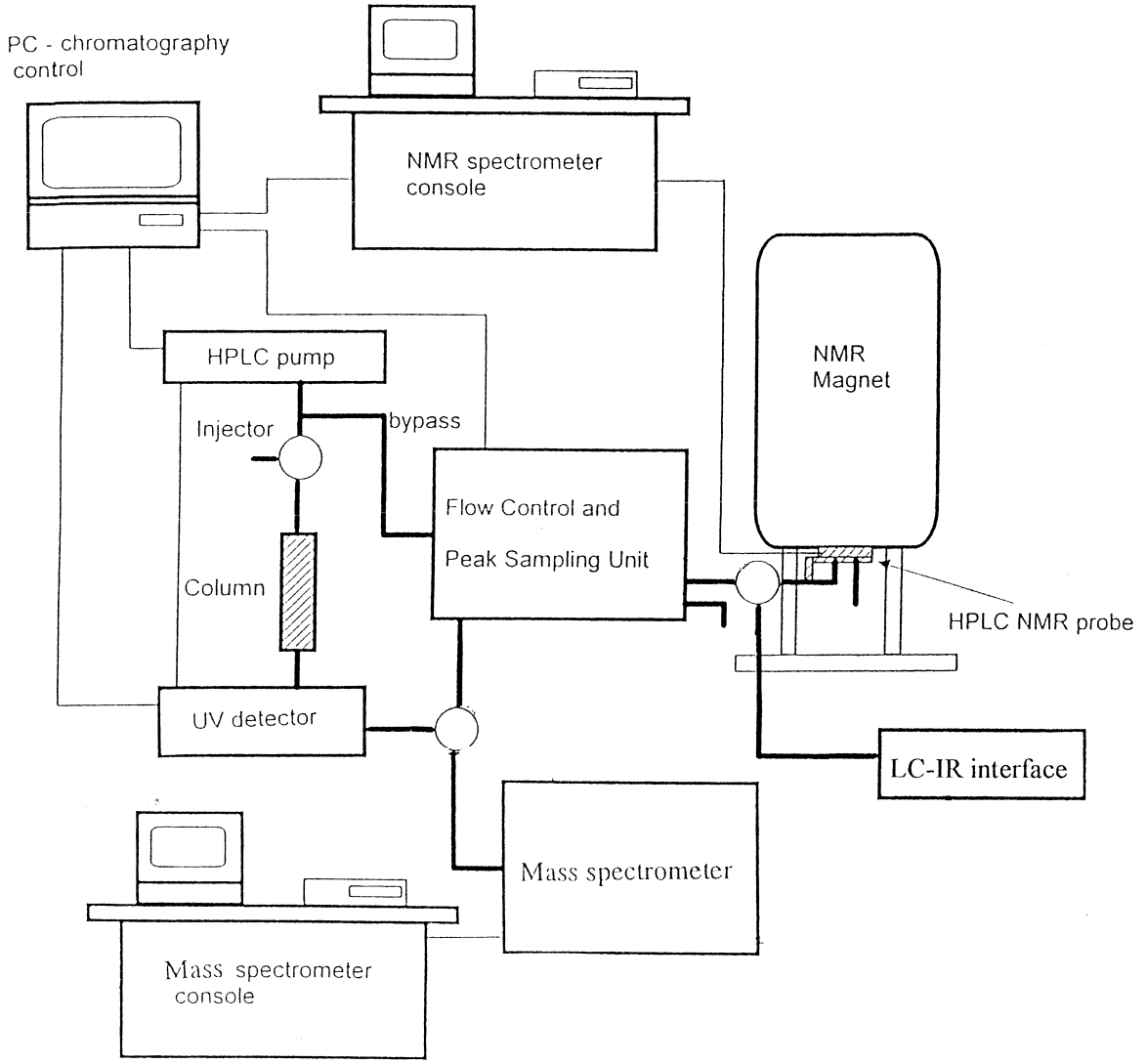

Fig. 1. A schematic diagram of the SEC–UV–NMR–MS–IR system used in these studies.

and the desolvation temperature at 70°C. The high temperatures were employed to minimise the contamination of the source with the samples and they did not appear to introduce any significant amounts of fragmentation.

3. Results and discussion

The experimental set-up used here (Fig. 1) is similar to that employed by us in previous HPLC–NMR–MS studies [9,10], with 95% of the effluent from the UV flow cell directed towards the flow probe of the NMR spectrometer and 5% to the mass spectrometer interface. The reasons for placing these instruments in parallel, rather than in-line, have been discussed in detail elsewhere [9,10]. However, the primary reason for the use of this layout is the fact that placing the MS in-line, and after, the NMR results in a small but significant back pressure on the NMR flow-probe which results in leaks. That said, depending upon the relative delays for peaks entering

the MS and NMR detectors certain advantages accrue from connecting the two detectors in parallel. Thus, in our previous studies it has often proved to be possible to use information gained in real time via monitoring the output from the MS to make decisions as to which peaks deserve a more detailed, stopped flow, analysis by NMR etc.

In our original work on coupling SEC with NMR and FT-IR [11] we used an LC-Transform Series 300 interface that employed an ultrasonic nebulizer under vacuum to remove the solvent. In contrast to the HPLC–NMR–MS set-up this type of interface did not cause a build up of pressure in the NMR flow probe and could therefore be used in-line [11]. The Series 400 LC-Transform interface used in the present study has been developed for use with aqueous eluents and the heated nebuliser around which it is based does cause a back pressure. Thus, when we attempted to place it in-line with the NMR spectrometer as we had done previously with the Series 300 interface leakage of the eluent from the flow probe was observed. We therefore also placed this interface in parallel with the NMR spectrometer by inserting an additional split in the line to divert 50% of the total flow being sent to the NMR to the LC-Transform (30% of this flow was diverted to waste in the interface itself).

The major problem that had to be overcome in this work was that of finding a chromatographic eluent that was compatible with the NMR and mass spectrometers and the IR interface. The SEC solvent system that was used in our previous work was based simply on deuterochloroform [11]. This system gave good separation and was well suited to both on-flow NMR (being essentially free of spectral interferences) and also, because of its volatility (which facilitated the easy removal of the solvent) for the LC-Transform interface. As such it was possible to obtain good on flow NMR data and off-line IR spectra without the need for any compromises around the eluent used with the SEC system itself [11]. We therefore attempted to obtain mass spectra of the test analytes as well using C^2HCl_3 as eluent. However, this proved to be unsuccessful using electrospray ionisation, and in order to obtain spectra the addition of water–methanol to the chloroform was necessary. Our first approach to solving this problem was to add water–methanol, via a syringe pump, to the 5% of the eluent that had been directed to the MS system, following the splitting of the flow. However, in practice the combined ''back pressure'' from the mass spectrometer interface and syringe pump was sufficient to cause the ''makeup'' flow of methanol–water to flow back into the splitter, rather than into the MS system, and then to the NMR system. This caused additional signals to be present in the resulting NMR spectra without actually resolving the problem of non-ionisation of the analytes.

In order to promote ionisation we therefore included a small proportion of ammonium acetate and methanol in the deuterochlorofom used as the SEC solvent. The resulting UV chromatogram is shown in Fig. 2. The inclusion of these additives did not significantly change the separation of the analytes obtained using C^2HCl_3 alone. This expedient enabled SEC–MS to be performed on all three compounds as shown in Figs. 3 and 4. As seen in Fig. 4A–C, spectra with all of the exchangeable protons replaced with deuterium, were obtained for each the three compounds. An $[M-{}^2H]^-$ was observed for BHT and Irganox and a $[M+{}^2H]^+$ for DIOP. In addition an $[M+N^2H_4]^+$ was observed for DIOP. Little fragmentation was observed as the spectra were acquired using a low cone voltage.

However, whilst the addition of $C^2H_3O^2H$, ^2H_2O and ammonium acetate did allow mass spectra to be acquired, and did not affect the separation process there were effects on the NMR spectra of the analytes. Thus, whilst these mobile phase additives did not prevent us from obtaining interpretable results for each of the analytes they did contribute a number of additional signals to the spectrum that might, in the case of different compound types, have caused relevant signals to have been obscured. In addition the signal for the phenolic OH that we had observed in our previous study for both BHT and Irganox 1076 was lost due to exchange.

These effects are illustrated in the on-flow spectra for BHT shown in Fig. 5A and B. In Fig. 5A the spectrum for the straight C^2HCl_3 eluent is provided (showing an additional signal due to a small amount of residual acetonitrile resulting from previous experiments) whilst Fig. 5B shows the result obtained for the additive-containing solvent system. The resonances for all protons of BHT, with the exception of the phenolic OH, are present in both spectra. Similar

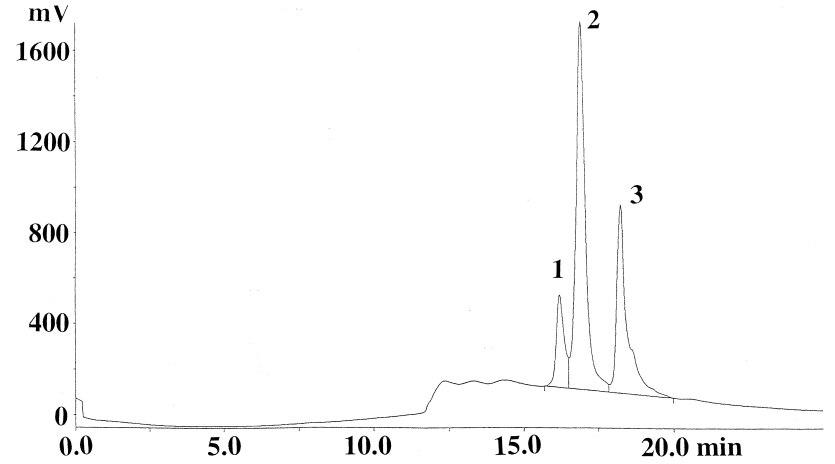

Fig. 2. The SEC–UV chromatogram of the mixture of Irganox 1076 (1), DIOP (2) and BHT (3).

results were obtained for both Irganox 1076 and DIOP

The modification of the mobile phase did not result in problems with evaporation in the LC-Transform interface except for the need for a slightly increased nebulizer temperature. As before FT-IR spectra were obtained off-line following collection of the peaks using the LC-Transform interface. The resulting spectra were essentially identical to those obtained in our previous study clearly showing signals for phenolic OH at ca. 3650 cm^{-1} for BHT and Irganox 1076, aliphatic CH absorptions at ca. 2900 cm^{-1} and carbonyl absorptions for Irganox 1076 and DIOP at ca. 1750 cm^{-1}. The example shown in Fig. 6 is for BHT. From this spectrum it is clear that the addition of a small amount of ammonium acetate to the solvent did not cause any extra signals to appear in the off-line IR spectrum. This presumably reflects the volatility of this buffer salt under the conditions employed to remove the eluent. The excellent quality of the IR spectra that can be obtained in this way obviously opens up the opportunity of using library searching to obtain spectral matches for unknowns.

Using the system described above it did indeed prove possible to obtain NMR and MS spectra on-

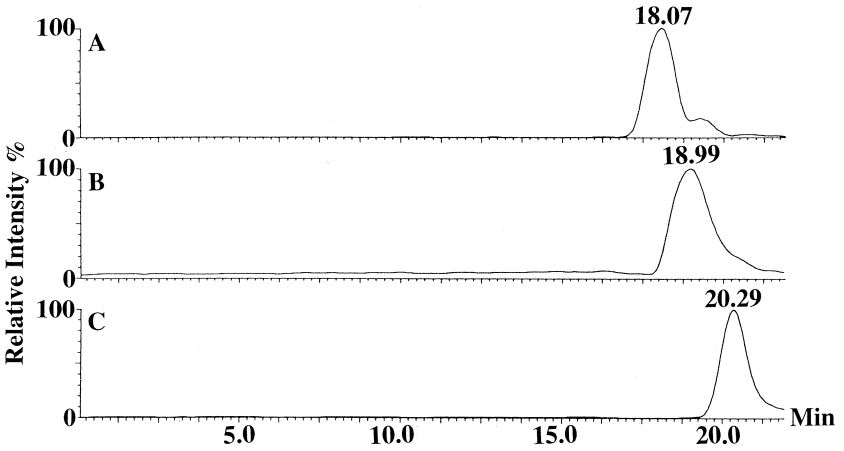

Fig. 3. SEC–MS traces for (A) Irganox 1076, (B) DIOP and (C) BHT.

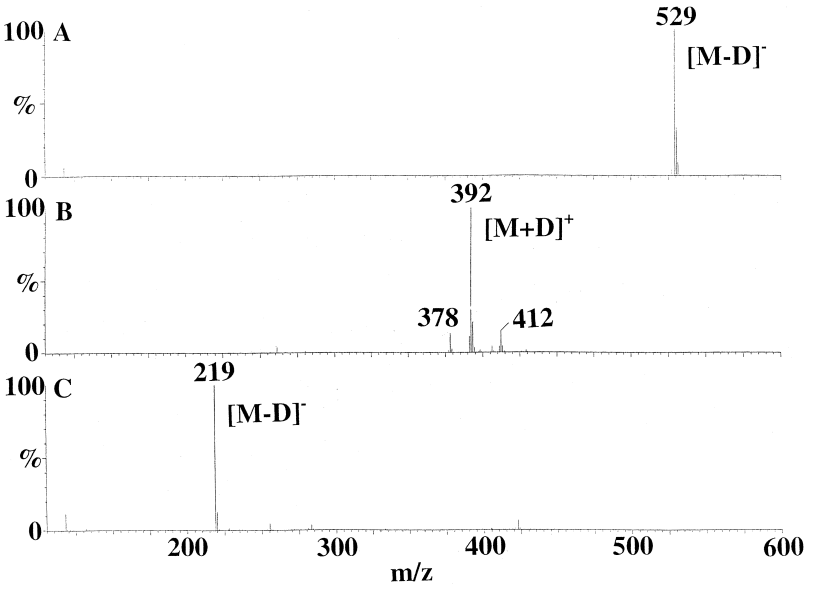

Fig. 4. The ESI-MS spectra of (A) Irganox 1076, (B) DIOP and (C) BHT obtained following the separation shown in Fig. 1.

flow and IR spectra off-line and the inclusion of a UV diode array detector in such a system would enable an essentially ''complete'' spectral characterisation of peaks eluting from a chromatographic system. The system that we have described here was constructed as a ''proof of concept'' and was by no means optimised. However, the problems encountered in this study do serve to highlight the difficulties in trying to hyphenate spectroscopic detectors with different requirements. Thus the NMR flow probe was not designed to work with back pressures, whilst the MS had different mobile phase require-

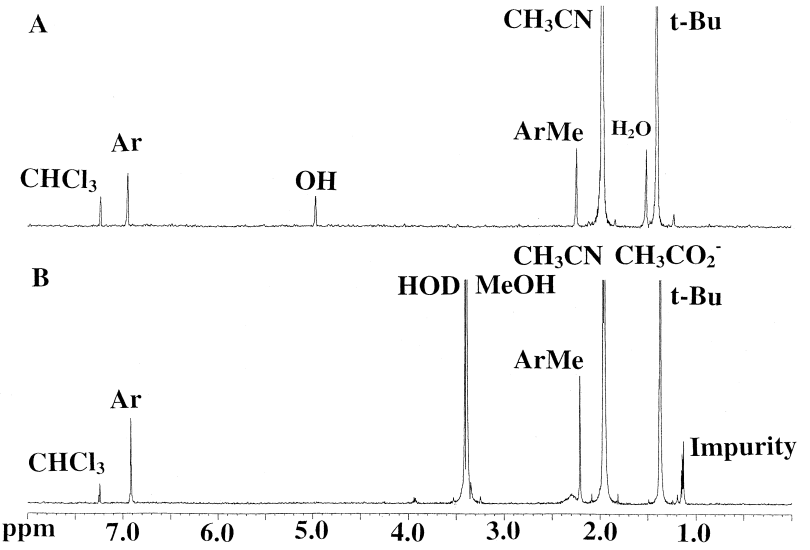

Fig. 5. The on-flow ^{1}H-NMR spectra of BHT (A) obtained in our previous SEC–NMR–IR study [10] with $C^{2}HDCl_3$ alone as mobile phase and (B) with the mobile phase used in this study to show the effect on the NMR spectrum of the mobile phase additives needed for MS.

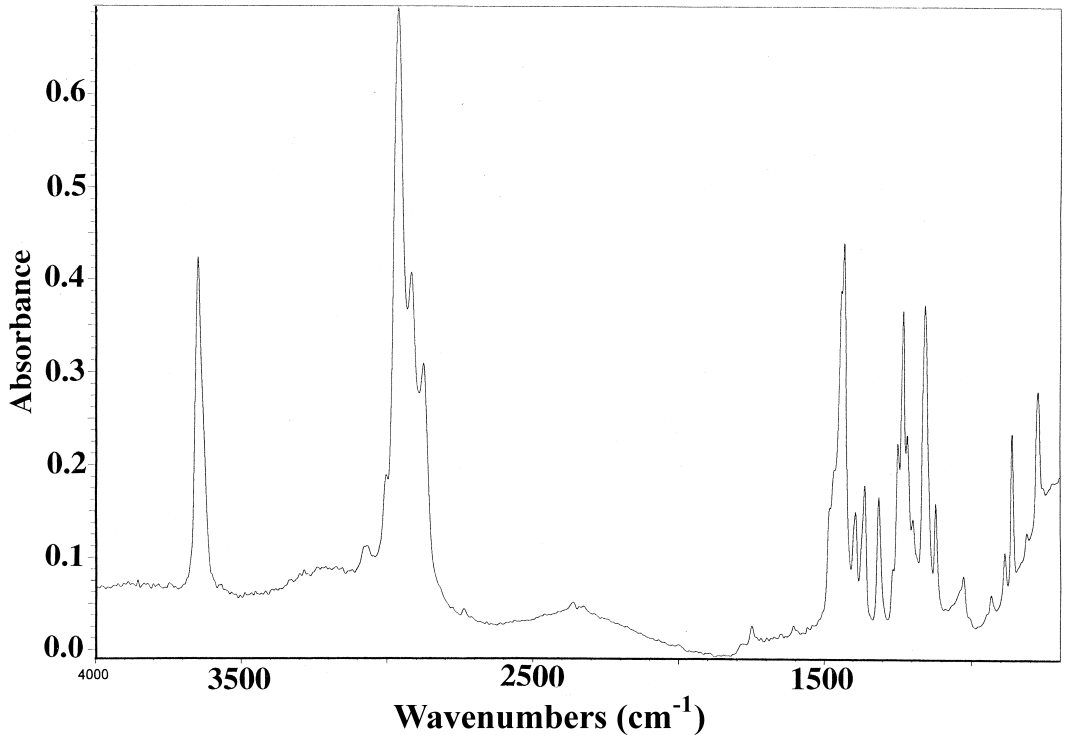

Fig. 6. The off-line FT-IR spectrum of BHT obtained from the separation shown in Fig. 1.

ments to the NMR and IR interface. Thus initial strategy to deal with the mobile phase requirements of the mass spectrometer, that involved adding the additives needed to obtain ionisation in the electrospray interface of the mass spectrometer, is undoubtedly the preferred solution and will be explored further in future work. However, the problem of obtaining ESI-MS data was due to the use of essentially ''dry'' C^2HCl_3 and is unlikely to be so severe with reversed-phase systems. There is also no doubt that a modified design for the NMR flow probe, that enabled a back pressure to be applied without resulting in leakage, would also benefit the construction of this type of multi-hyphenated system as it would put fewer constraints on the subsequent instrumental layout. Whilst it is clear that the use of on-flow IR would be of benefit in such a system, and flow through cells are available, the loss of large portions of the spectrum due to interference by the solvent, and the general incompatibility of these flow cells with aqueous solvent of the type that would be encountered in reversed-phase chromatography mitigates against their use. However, the use of a ''moving belt'' type of interface to the IR, where the solvent is evaporated prior to spectrometry would remove these objections to an on-flow acquisition of IR spectra. The use of this type of interface, which is commercially available, deserves evaluation.

We have made no attempt here to explore the limits of sensitivity of this system, but we have no reason to believe that it should be greatly inferior to that of the individual spectroscopic detectors themselves. In this case, for an on-flow analysis, the limiting factor will be the sensitivity of the NMR spectrometer (assuming that the mobile phase composition has been optimised for MS detection). In this case, with the NMR instrument, flow probe and flow-rates used here it should be possible to reliably obtain spectra on quantities of ca. 50 μg of an individual analyte (depending upon the structure) on column.

It is perhaps worth emphasising that the system described here was a prototype designed to explore the possibilities of multiple hyphenation for complex

mixture analysis, with a chromatographic step to separate the individual components for subsequent spectroscopy. It is also possible to speculate on the use of this type of system without the chromatographic separation for the flow injection analysis (FIA) of individual analytes in an automated structure determination laboratory. This would reduce some of the problems associated with solvents used as these would not need to be selected for their ability to achieve a separation but could be chosen for their compatibility with the spectrometers.

4. Conclusions

This preliminary investigation is the first practical demonstration of this combination of instruments and shows that it is possible to couple UV–NMR–MS–IR with an SEC separation to provide a single multiply hyphenated system for mixture analysis. Not surprisingly it proved to be more difficult to construct this system than less complex combinations (e.g., HPLC–NMR–MS, HPLC–NMR–IR) due to problems with ensuring compatibility of solvent systems. However, the complementary nature of the MS, NMR and IR data and the potential analytical power that results from the simultaneous coupling of all of these to a chromatographic separation (or indeed in the flow injection analysis mode) is self evident.

Future investigations will explore the potential of FIA and chromatographic separations to HPLC–UV–NMR–MS–IR systems with both normal and reversed-phase eluents.

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