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Review

Advantages and disadvantages of nuclear magnetic resonance spectroscopy as a hyphenated technique

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Abstract

A general overview of the advancements and applications of nuclear magnetic resonance (NMR) hyphenated with other analytical techniques is given from a practical point of view. Details on the advantages and disadvantages of the hyphenation of NMR with liquid chromatography as LC–NMR and also with mass spectrometry as LC–MS–NMR are demonstrated with two examples. Current developments of NMR with other analytical separation techniques, especially with capillary liquid chromatography (capLC) are discussed.

Keywords: Hyphenated analytical techniques; Structure elucidation of organic compounds; Review; NMR; LC–NMR; LC–MS–NMR; Flavonoids; Metabolites

1. Introduction

During the last decade hyphenated analytical techniques have grown rapidly and have been applied successfully to many complex analytical problems. The combination of separation technologies with spectroscopic techniques is extremely powerful in carrying out qualitative and quantitative analysis of unknown compounds in complex matrices. High-performance liquid chromatography (HPLC) is the most widely used analytical separation technique for the qualitative and quantitative determination of compounds in solution. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are the primary analytical techniques that provide structural information on the analytes. The physical connection of HPLC (or LC) and MS (LC–MS) or NMR (LC–NMR) increases the capability of solving structural problems of mixtures of unknown compounds. LC–MS has been the more extensively applied hyphenated technique because MS has higher sensitivity than NMR [1–3]. Recent advances in NMR, LC–NMR and even LC–MS–NMR have enabled these techniques to become routine analytical tools in many laboratories. The present article provides an overview of the LC–NMR and LC–MS–NMR techniques with a description of their limitations together with an example of LC–NMR and another for LC–MS–NMR to illustrate the data generated by these hyphenated techniques. This article is not meant to imply that LC–MS–NMR will replace LC–MS, LC–NMR or NMR techniques for structural elucidation of compounds. LC–MS–NMR together with LC–MS, LC–NMR and NMR are techniques that should be available and applied in appropriate cases based on their advantages and limitations.

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1.1. Historical background in NMR

In 1945, NMR signals in condensed phases were detected by physicists, Bloch et al. [4] at Stanford and Purcell et al. [5] at Harvard; Bloch and Purcell received the first Nobel Prize in NMR. Work on solids dominated the early years of NMR because of the limitations of the instruments and the incomplete development of theory. Work in liquids was confined to relaxation studies. A later development was the discovery of the chemical shift and the spin–spin coupling constant. In 1951, the proton spectrum of ethanol with three distinct resonances showed the potential of NMR for structure elucidation of organic compounds [6]. Scalar coupling provides information on spins that are connected by bonds. Spin decoupling or double resonance, which removes the spin–spin splitting by a second radiofrequency field, was developed to obtain information about the scalar couplings in molecules by simplifying the NMR spectrum [7]. Initial manipulation of the nuclear spin carried out by Hahn [8] was essential for further development of experiments such as insensitive nuclei enhanced by polarization transfer (INEPT) [9], which is the basis of many modern pulse sequence experiments. During the 1960s and 1970s the development of superconducting magnets and computers improved the sensitivity and broadened the applications of the NMR spectrometers. The Fourier transform (FT) technique was implemented in the instruments by Ernst and Anderson [10] in the 1960s, but it took time to become the standard method of acquiring spectra. Another milestone which increased the signal-to-noise (S/N) ratio was the discovery of the nuclear Overhauser effect by Overhauser [11], which improves the S/N in less sensitive nuclei by polarization transfer. The three-fold enhancement generally observed for the weak carbon-13 (^{13}C) signals was a major factor in stimulating research on this important nuclide. Several years later, the proton–proton Overhauser effect was applied to identify protons that are within 5 Å of each other. In the 1970s, Aue et al. [12] implemented the idea of acquiring a two-dimensional (2D) spectrum by applying two separate radiofrequency pulses with different increments between the pulses, and after two Fourier transformations the 2D spectrum was created. Two-dimensional experiments opened up a new direction for the development of NMR and Ernst obtained the second Nobel Prize in NMR in 1991. 2D correlation experiments are of special value because they connect signals through bonds. Examples of these correlation experiments are correlation spectroscopy (COSY) [12], total correlation spectroscopy (TOCSY) [13], heteronuclear correlation spectroscopy (HETCOR) [14] and variations. Other 2D experiments such as nuclear Overhauser effect spectroscopy (NOESY) [15] and rotating frame Overhauser effect spectroscopy (ROESY) [16] provide information on protons that are connected through space to establish molecular conformations. In 1979, Müller [17] developed a novel 2D experiment that correlates the chemical shift of two spins, one with a strong and the other with weak magnetic moment. Initially the experiment was applied to detect the weak ^{15}N nuclei in proteins, but was later modified to detect the chemical shift of ^{13}C nuclei through the detection of the protons attached directly to the carbons [18]. The heteronuclear multiple quantum correlation (HMQC) experiment gives the same data as the HETCOR but with greater sensitivity. Heteronuclear single quantum correlation (HSQC) [19] is another experiment widely used that provides the same information as the HMQC and uses two successive INEPT sequences to transfer the polarization from protons to ^{13}C or ^{15}N. Heteronuclear multiple bond correlation (HMBC) [20] experiment gives correlations through long-range couplings which allows two and three ^{1}H–^{13}C connectivities to be observed for organic compounds. In 1981, 2D incredible natural abundance double quantum transfer experiment (INADEQUATE) [21] was developed and defines all the carbon–carbon bonds thus establishing the complete carbon skeleton in a single experiment. However, due to the low natural abundance of adjacent ^{13}C nuclei, this experiment is not very practical. All of these experiments became available with the development of computers in the 1980s. With the accelerated improvements in electronics, computers and software in the 1990s, the use of the pulsed field gradients as part of the pulse sequences was developed [22] and applied to improve solvent suppression and to decrease the time required to acquire 2D experimental data.

This brief historical introduction is intended to give a simplified overview of some of the critical milestones of NMR mainly in chemical applications, excluding the innovations in the field of proteins, solid

state and magnetic resonance in clinical medicine. To find out more details see the articles written by Emsley and Feeney [23], Shoolery [24], and Freeman [25] and the references cited therein.

1.2. Last decade advancements in NMR

As mentioned at the end of Section 1.1, the development of the pulse field gradients extended the applications of NMR. One of the areas not mentioned is the hyphenated techniques. NMR is one of the most powerful techniques for elucidating the structure of organic compounds. Before undertaking NMR analysis of a complex mixture, separation of the individual components by chromatography is required. LC–MS is routinely used to analyze mixtures without prior isolation of its components. In many cases, however, NMR is needed for an unambiguous identification. Even though hyphenated LC–NMR has been known since the late 1970s [26–33], it has not been widely implemented until the last decade [34–39].

The first paper on LC–NMR was published in 1978 [26] using stop-flow to analyze a mixture of two or three known compounds. At that time, the limitations in the NMR side, e.g. sensitivity, available NMR solvents, software and hardware, and resolution achieved only with sample-spinning, made direct coupling to the HPLC difficult. Watanabe and Niki [26] modified the NMR probe to make it more sensitive, introducing a thin-wall teflon tube of 1.4 mm (inner diameter) transforming it into a flow-through structure. The effective length and volume of this probe were about 1 cm and 15 μl, respectively. Two three-way valves connected this probe to the HPLC detector. This connection needed to be short to minimize broadening of the chromatographic peaks. During the stop-flow mode, the time to acquire an NMR spectrum on each peak was limited to 2 h to avoid excess broadening of the remaining chromatographic peaks. The authors also mentioned that use of tetrachloroethylene or carbon tetrachloride as solvents and ETH-silica as normal-phase column limited the applications for this technique. Because solvent suppression techniques were not available at that time, the authors [26] recognized that more development was required in the software and hardware of the NMR side to include the use of reverse phase columns and their solvents which in turn would broaden the range of applications. A year later, Bayer et al. [27] carried out on-flow and stop-flow experiments with a different flow-probe design on standard compounds. They used normal-phase columns and carbon tetrachloride as solvent. One of their observations was that the resolution of the NMR spectra in the LC–NMR system was poorer than for the uncoupled NMR system, which made the measurement of small coupling constants difficult. The first application of on-flow LC–NMR was carried out in 1980 to analyze mixtures of several jet fuel samples [28]. Deuterated chloroform and Freon 113 and normal phase columns were the common conditions used for LC–NMR [29–33], limiting the application of this technique.

The use of reverse phase columns in LC–NMR complicates the NMR analysis because of (1) the use of more than one protonated solvent, which will very likely interfere with the sample, (2) the change in solvent resonances during the course of the chromatographic run when using solvent gradients, and (3) small analyte signals relative to those of the solvent. In 1995, Smallcombe et al. [40] overcame these problems by developing the solvent suppression technique which greatly improved the quality of the spectra obtained by on-flow or stop-flow experiments. The optimization of the WET (water suppression enhanced through T1 effects) solvent suppression technique generates high-quality spectra and effectively obtains 1D on-flow and stop-flow spectra and 2D spectra for the stop-flow mode, such as WET-TOCSY, WET-COSY, WET-NOESY and others [40].

During the last few years, more progress has been achieved by hyphenating LC–NMR to MS. The LC–NMR–MS (or named in this article as LC–MS–NMR) has expanded the structure-solving capabilities by obtaining simultaneously MS and NMR data from the same chromatographic peak. There are some compromises that have to be taken into account because of the differences between MS and NMR, such as sensitivity, solvent compatibility, destructive versus non-destructive technique, discussed below. LC–MS has been used for many years as a preferred analytical technique; however, with the development of electrospray ionization techniques, LC–MS has been routinely used for the analysis of complex mixtures. LC–MS–NMR is a combination of LC–MS with electrospray and LC–NMR.

2. LC–NMR

2.1. Introduction

The decision to use either NMR or LC–NMR for the analysis of mixtures depends on factors related to their chromatographic separation and the ability of NMR to elucidate the structure whether hyphenated or not. The major technical considerations of LC–NMR are NMR sensitivity, NMR and chromatographically compatible solvents, solvent suppression, NMR flow-probe design, and volume of the chromatographic peak versus the volume of the NMR flow cell or LC–NMR sensitivity. Fig. 1 shows the schematic setup of the LC–NMR connected to other devices, such as radioactivity detector and MS (see Section 3).

2.1.1. NMR sensitivity

NMR is a less sensitive technique compared to MS and hence requires much larger samples for analysis. MS analysis is routinely carried out in the picogram range. Modern high field NMR spectrometers (400 MHz and higher) can detect proton signals from pure demonstration samples well into the nanogram range (MW 300). With the cryoprobes that are currently available, the sensitivity of NMR markedly improves. The samples in the low nanogram range can be detected. In the high nanogram range, structural analysis can be carried out. For real world samples, however, purity problems become more intrusive with diminishing sample size and can be overwhelming in the sub-microgram domain. This places a current practical lower limit for most structural elucidation by NMR which is estimated by the writer to be close to 500 ng (MW 300).

Although several other important nuclides can be detected by NMR, proton (^{1}H) remains the most widely used because of its high sensitivity, high isotopic natural abundance (99.985%) and its ubiquitous presence in organic compounds. Of comparable importance is carbon (^{13}C), 1.108% abundance, which, because of substantial improvements in instrument sensitivity, is now utilized as routinely as proton. Fluorine (^{19}F), 100% abundance, is less used since it is present in only about 10% of pharmaceutical compounds. Another consequence of the intrinsic low sensitivity of NMR is that virtually all samples require signal averaging to reach an acceptable signal-to-noise level. Depending on sample size, signal averaging may range anywhere from several minutes to several days. For metabolites in the 1–10 μg

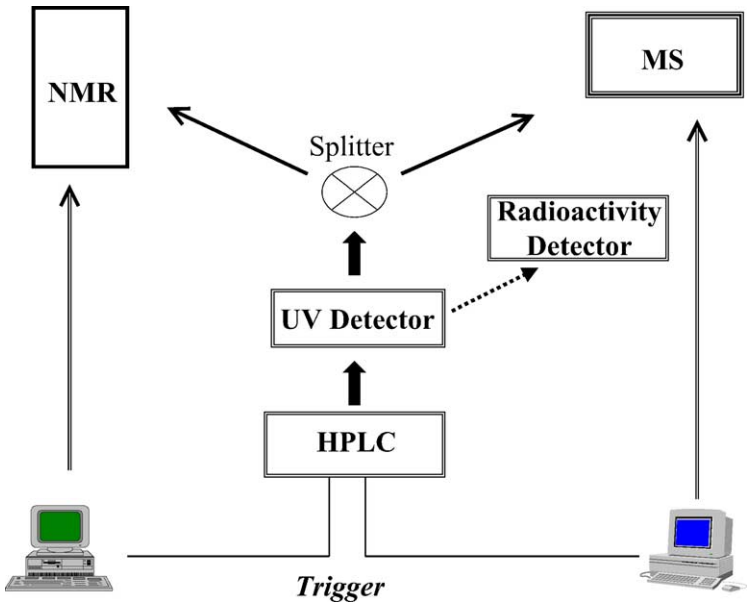

Fig. 1. Schematic setup for the LC–MS–NMR system.

range, for example, overnight experiments are generally necessary.

2.1.2. NMR and chromatographically compatible solvents

Liquid NMR requires the use of deuterated solvents. Conventionally the sample is analyzed as a solution using a 5- or 3-mm NMR tube depending on the NMR probe, which requires ca. 500 or 150 μl, respectively, of deuterated solvents. The increased solvent requirements for LC–NMR make this technique highly expensive. Deuterium oxide (D_2O) is the most readily available, reasonably priced solvent (over US$ 300/l). The cost of deuterated acetonitrile (CD_3CN) is decreasing and varies depending on the percentage of included D_2O, but is still over US$ 1000/l. Deuterated methanol (CD_3OD) is even more expensive. Deuterated solvents for normal-phase columns are not readily available but those that are have even more prohibitive prices. This necessitates the use of reverse-phase columns. Another factor to be concerned with is compatibility of the HPLC gradient–solvent system with the NMR operations. An HPLC gradient–solvent system greater than 2–3%/min causes problems in optimizing the magnetic field homogeneity (shimming) due to solvent mixing in the flow cell.

2.1.3. Solvent suppression

During the LC–NMR run, the solvent signal in the chromatographic peak is much larger than those of the sample and needs to be suppressed. This applies even with deuterated solvents. In the case of acetonitrile, the two ^{13}C satellite peaks of either the protonated or residual protonated methyl group for CH_3CN or CD_3CN also require suppression because they are typically much larger than signals from the sample. With the optimization of the WET solvent suppression technique by Smallcombe et al. [40] in 1995, the quality of spectra generated during LC–NMR has been greatly improved and is routine. The WET solvent suppression technique is the standard technique for LC–NMR because it has the capability of suppressing several solvent lines without minimum base line distortions, compared with others such as presaturation or watergate. One disadvantage of suppressing the solvent lines is that any nearby analyte signal will also be suppressed, resulting in loss of structural information.

2.1.4. NMR flow-probe design

Conventional NMR flow cells have an active volume of 60 μl (i.e. corresponds to the length of the receiver coil), and a total volume of 120 μl. This means that NMR will only "see" 60 μl of the chromatographic peak. If the flow rate in the HPLC is 1 ml/min, when 4.6 mm columns are used, only 3.6 s of the chromatographic peak will be "seen" by NMR. Chromatographic peaks are generally much wider than 4 s indicating that less than half of the chromatographic peak will be detected. This is one of the disadvantages of LC–NMR compared with conventional 3-mm NMR probes where the amount of sample "seen" by the NMR receiver coil is independent of the width of the chromatographic peak.

2.1.5. LC–NMR sensitivity

Because NMR is a low sensitivity technique which requires samples in the order of several micrograms, analytical HPLC columns have to be saturated when injecting samples in that range. This will affect the chromatographic resolution and separation since resolution often degrades when sample injection is scaled-up to that level. Another factor that can affect chromatographic performance is the use of deuterated solvents. In many cases analytes show chromatographic peak broadening and occasional different retention times from non-deuterated solvents. When this occurs more chromatographic development is required in order to obtain reasonable resolution. One way to increase the LC–NMR sensitivity is by decreasing the flow rate to less than 1 ml/min. At this flow rate, a greater portion of the chromatographic peak will be "seen" by NMR. However, this is only possible if the pump of the LC system is accurate at rates lower than 1 ml/min.

2.2. Modes of operation for LC–NMR

The HPLC is connected by red polyether ether ketone (PEEK) tubing to the NMR flow cell which is inside the magnet. With shielded cryomagnets the HPLC can be as close as 30–50 cm to the magnet versus 1.5–2 m for conventional magnets. Normally a UV detector is used in the HPLC system to monitor the chromatographic run. Radioactivity or fluorescent detectors can also be used to trigger the chromatographic peak of interest.

There are four general modes of operation for LC–NMR: on-flow, stop-flow, time-sliced, and loop collection.

2.2.1. On-flow

On-flow or continuous-flow experiments require more sample to analyze "on the fly" because the resident time in the NMR flow cell is very short (3.6 s at 1 ml/min) during the chromatographic run, which limits this approach to 1D NMR spectra acquisition only. This mode can be used to analyze the major components of the mixture.

2.2.2. Stop-flow

Stop-flow requires the calibration of the delay time which is the time required for the sample to travel from the UV detector to the NMR flow cell, which depends in turn on the flow rate and the length of the tubing connecting the HPLC with the NMR. Because the chromatographic run is automatically stopped when the chromatographic peak of interest is in the flow cell, the amount of sample required for the analysis can be reduced compared to the on-flow mode and 2D NMR experiments, such as WET-COSY, WET-TOCSY and others [40], can be obtained since the sample can remain inside the flow cell for days. It is possible to obtain NMR data on a number of chromatographic peaks in a series of stops during the chromatographic run without on-column diffusion that causes loss of resolution, but only if the NMR data for each chromatographic peak can be acquired in a short time (30 min or less if more than four peaks have to be analyzed, and less than 2 h for the analysis of no more than three peaks). The use of commercially available cryoprobes will improve the sensitivity of the stop-flow mode (see Section 2.1.1).

In our laboratories, stop-flow is the preferred mode for the analysis of metabolites when the chromatography is reasonable or the metabolite is unstable. One example is the analysis of the major metabolites of compound **I** (Fig. 2), a *ras* farnesyl transferase inhibitor in rats and dogs [41]. Preliminary studies by LC–NMR using a linear solvent gradient [5–75% B 0–25 min, 75–95% B 25–35 min, A: D_2O, B: ACN (acetonitrile), 1 ml/min, 235 nm, BDS Hypersil C_{18} column 15 cm × 4.6 cm, 5 μm] indicated that even with the use of protonated acetonitrile in the solvent mixture, all the resonances were visible (Fig. 3).

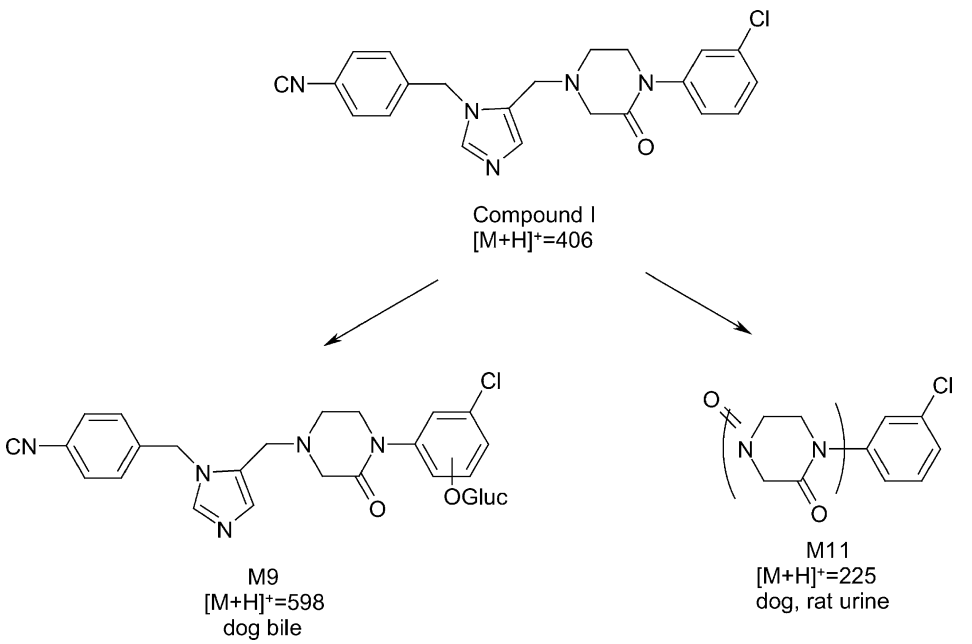

Fig. 2. Structure of compound **I**, a *ras* farnesyl transferase inhibitor in rats and dogs, and proposed structures by MS of its major metabolites in dog bile (**M9**) and dog and rat urine (**M11**).

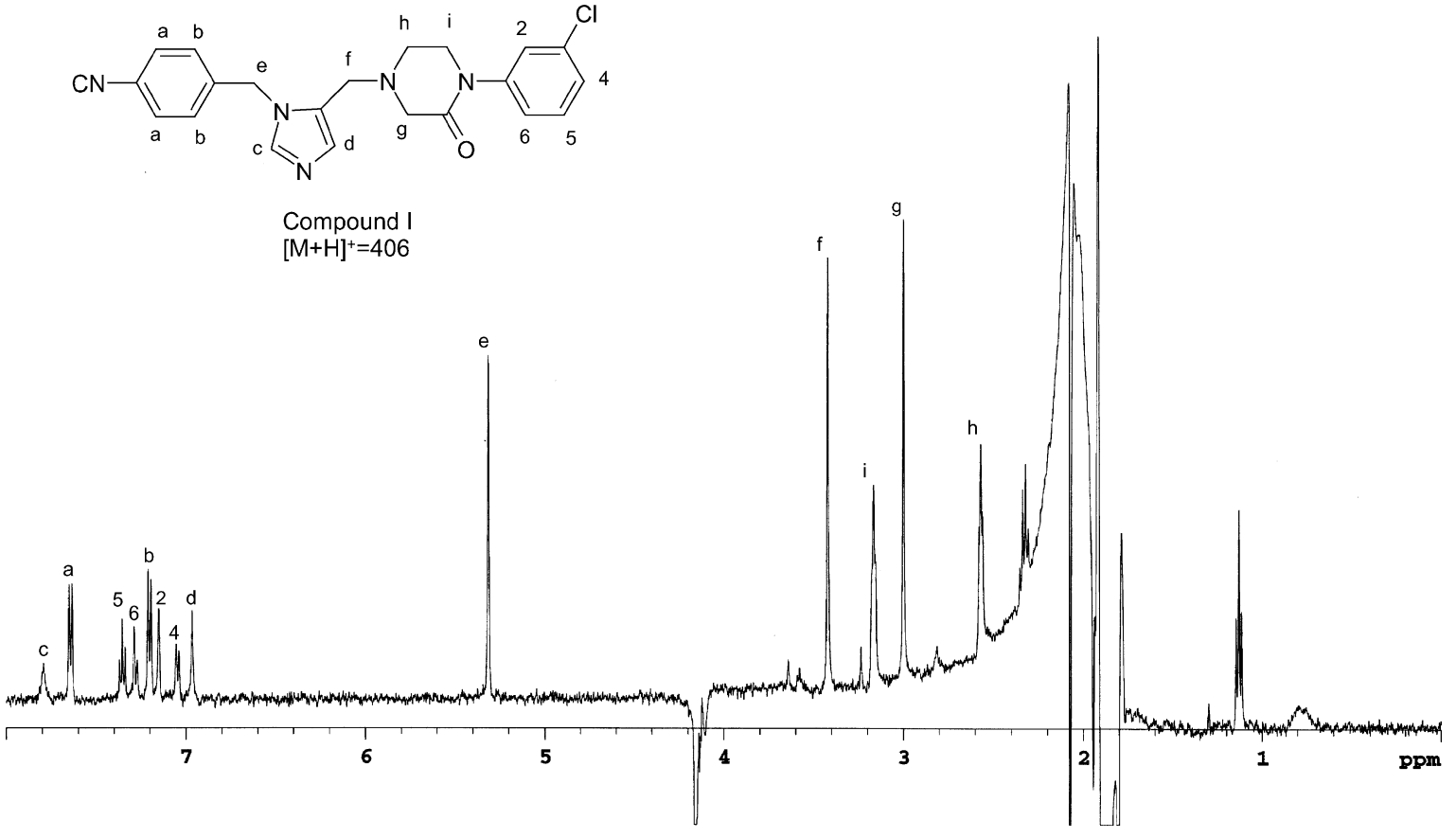

Fig. 3. ^{1}H NMR spectrum of compound **I** in stop-flow.

Fig. 4A and B is the UV chromatograms from a small injection of dog bile and dog urine for metabolites **M9** (retention time 10 min) and **M11** (retention time 21 min), respectively. Metabolite **M11** was also found in rat urine. To analyze the structures of **M9** and **M11** by NMR, larger injections of dog bile, dog urine and rat urine were carried out for the stop-flow experiments. The ^{1}H NMR spectrum on the

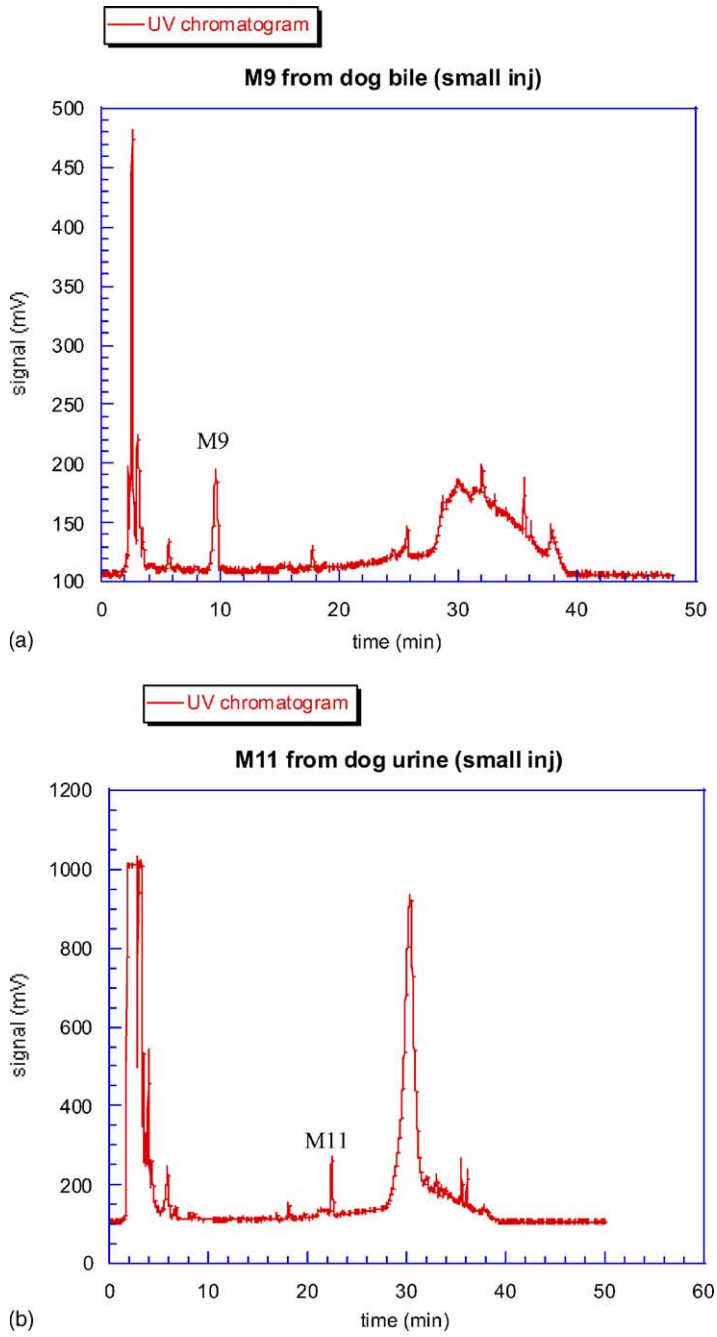

Fig. 4. UV chromatograms from small injections of the dog bile containing metabolite **M9** (a) and dog urine containing metabolite **M11** (b).

LC–NMR system (Varian Inova 500 MHz equipped with a ^{1}H–^{13}C pulse field gradient indirect detection microflow NMR probe with a 60 μl flow cell, Palo Alto, CA, USA) of **M9** (Fig. 5) revealed the presence of a 1,2,4-trisubstituted aromatic ring in the 3-chlorophenyl ring and the glucuronide moiety. Neither of the two possibilities for the position of the glucuronide moiety ring, positions 4 or 6, could be distinguished. NOE experiments on the LC–NMR were not successful because of problems with the solvent suppression. The sample was collected and the NOE was performed (Varian Unity 400 MHz, equipped with a 3 mm ^{1}H–^{13}C pulse field gradient indirect detection Nalorac probe, Palo Alto, CA, USA) over a weekend (Fig. 6). Even though the collected sample contained more impurities, the NOE experiment showed that the glucuronide moiety was attached at C-4 by irradiating the methylene at i which elicited NOE signals from H-2 and H-6 thus eliminating the C-6 possibility (Fig. 6). LC–MS on **M11** indicated it to be only the 1-(3-chlorophenyl)piperazinone moiety with an additional oxidation on the piperazinone ring. The ^{1}H NMR spectrum on the LC–NMR system of **M11** lacked the isolated methylene signal on the piperazine ring (Fig. 7) indicating it to be the (1-(3-chlorophenyl)piperazine-2,3-dione).

Recently, we studied a radioactive volatile metabolite with a small molecular weight by LC–NMR [42]. To be able to identify the UV chromatographic peak corresponding to the radioactive metabolite, a radioactivity detector equipped with a liquid cell (Radiomatic C150TR, Packard) was connected on-line to the LC–UV system of the LC–NMR. Small injections were carried out initially to identify the metabolite UV chromatographic peak with the radioactive peak prior to the stop-flow experiments. Stop-flow experiments were triggered by UV because the transfer delay from the UV to the NMR was shorter than from the radioactive detector to the NMR. Fig. 1 shows the schematic diagram for this setup.

2.2.3. Time-slice

"Time-sliced" involves a series of stops during the elution of the chromatographic peak of interest. Time-sliced is used when two analytes elute together or with close retention times, or when the separation is poor.

2.2.4. Loop collection

Loop collection can be used when there is more than one chromatographic peak of interest in the same run. The chromatographic peaks are stored in loops for later off-line NMR study. In this case the analytes must be stable inside the loops during the extended period of analysis. Capillary tubing should be used to avoid peak broadening with concomitant loss of analyte "seen" by the NMR spectrometer. The stored chromatographic peaks can be analyzed in different order from the chromatographic run.

2.3. Other analytical separation techniques hyphenated with NMR

Recently, other chromatographic techniques have been coupled on-line to NMR for additional applications, such as size-exclusion chromatography (SEC) as SEC–NMR for the characterization of polymer additives [43], solid-phase extraction (SPE) as SPE–NMR for trace analysis [44], capillary electrophoresis (CE) as CE–NMR for small volume samples [45,46], and capillary electrochromatography (CEC) as CEC–NMR for on-flow identification of metabolites with small volume samples [46,47] as examples. CE–NMR and CEC–NMR are techniques that work with very small-volume NMR probes with capillary separations. Lately, more developments have been carried out to hyphenate capillary-based HPLC (capLC) with NMR (capLC–NMR) and the use of commercial microcoil NMR probes [46,48]. With microcoil NMR probes, the range of sample used in capLC–NMR could reach the nanogram level (low nanogram level only for detection limit but not for structural analysis) [46,48]. With this technique, the volume of the chromatographic peak is comparable to the volume of the microcoil NMR flow cell. The volume observed for a commercial microcoil NMR flow cell is approximately 1.5 μl and there is a wider range of solvent gradient variation than in the standard LC–NMR. capLC–NMR can be used without a column for analysis of small concentrated pure compounds, such as 1 μg, or with the column to study mixtures of compounds. One of the requirements for capLC–NMR is that the sample has to be soluble in a volume of approximately 5 μl or less which is not always possible. The delay time between the UV detector of the capLC and the NMR flow cell has to

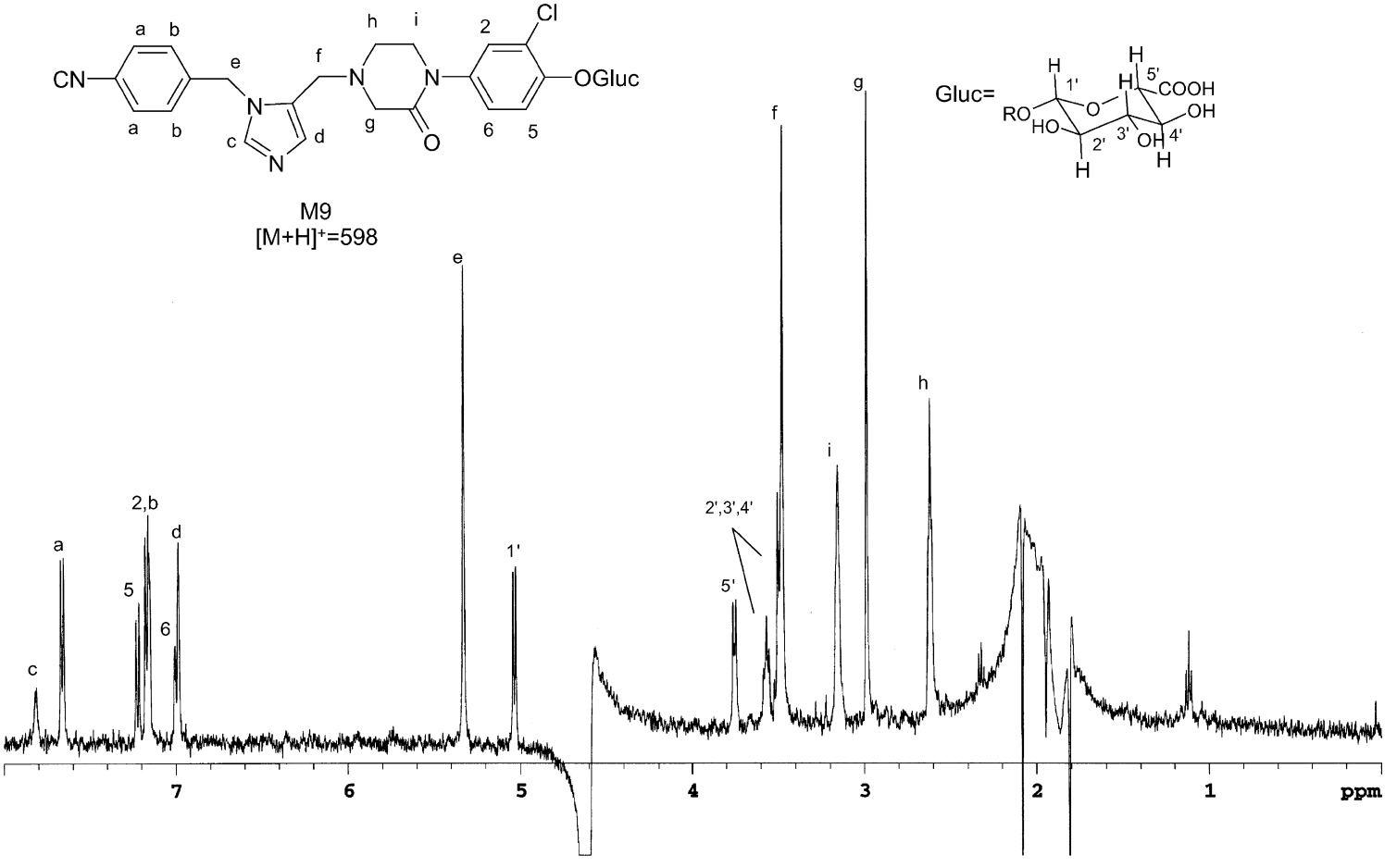

Fig. 5. ^{1}H NMR spectrum of metabolite **M9** from dog bile in stop-flow mode.

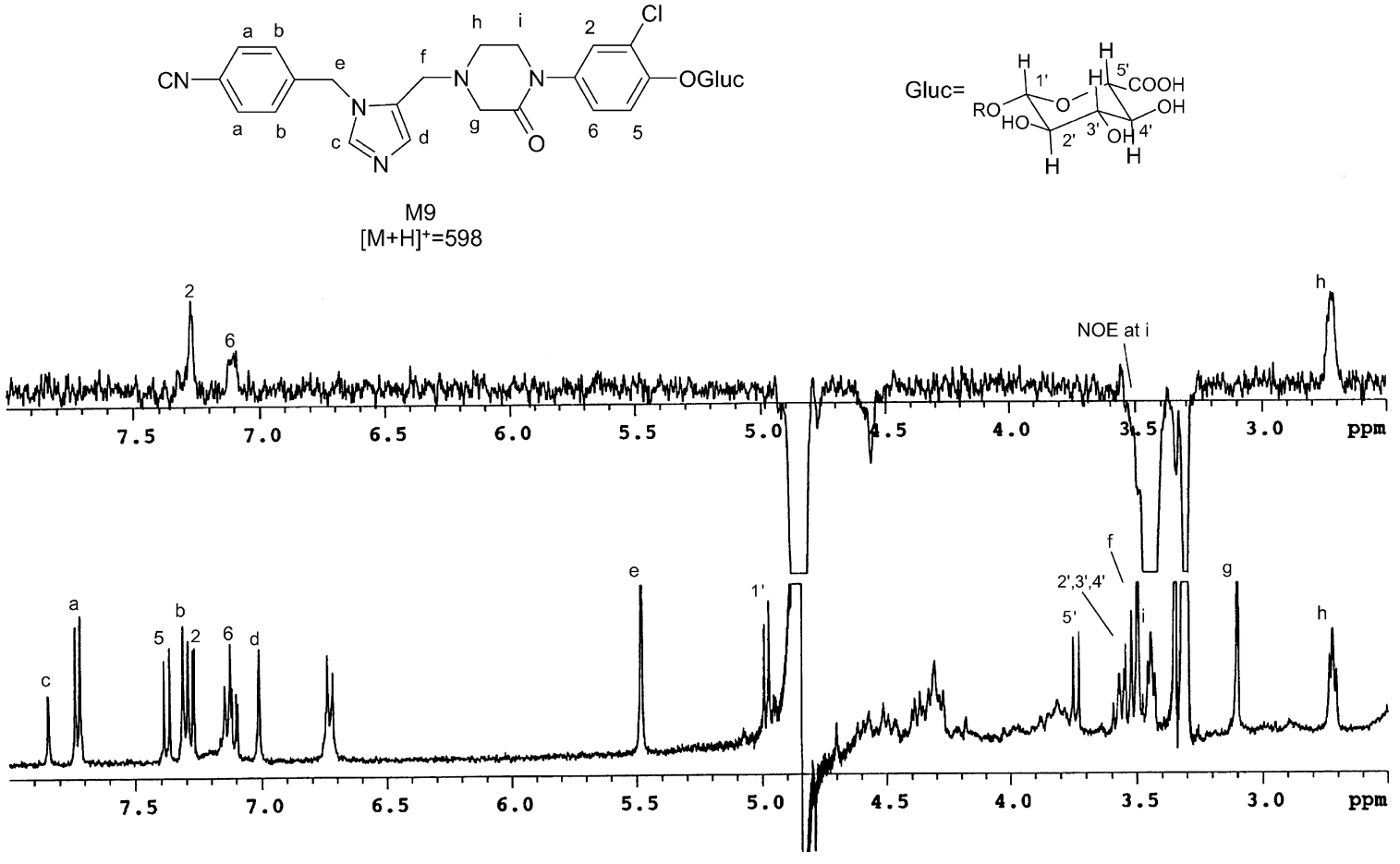

Fig. 6. ^{1}H NMR (bottom) and 1D NOE spectra at i (top) of **M9** from dog bile recovered from LC–NMR.

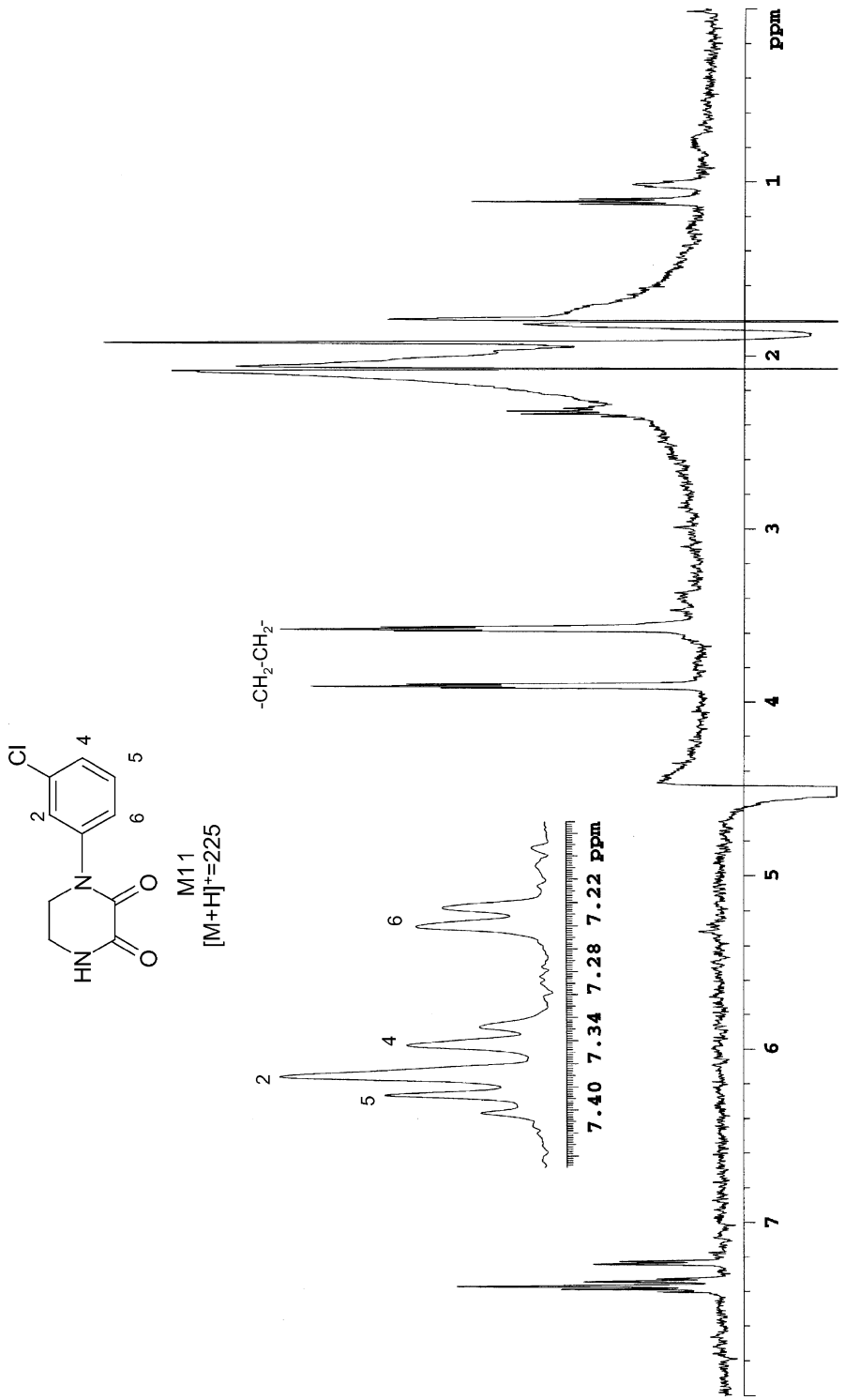

Fig. 7. ^{1}H NMR spectrum of metabolite **M11** from dog urine in stop-flow mode.

be calibrated for all chromatographic conditions due to the changes of viscosity of the different solvent compositions which has an effect on the pump of the capLC. More recently, the development of multiple coils connected in parallel may be applicable to acquire NMR data of several samples at the same time [39,49]. So far, four samples can be run at the same time, but future developments may be going towards analysis of 96-well plates emulating techniques such as LC–MS [39].

2.4. Applications of LC–NMR

There are many examples in the literature of applications of LC–NMR. In the area of natural products, LC–NMR has been applied to screen plant constituents from crude extracts [50,51], to analyze plant and marine alkaloids [52–54], flavonoids [55], sesquiterpene lactones [56,57], saponins [58], Vitamin E homologues [59] and antifungal constituents [60] as examples. In the field of drug metabolism, LC–NMR has been extensively applied for the identification of metabolites [41,61–69] and even polar [70] or unstable metabolites [42]. And finally, LC–NMR has been used for areas such degradation products [71–73], drug impurities [74,75], drug discovery [76] and food analysis [77–79].

3. LC–MS–NMR

3.1. Introduction

NMR and MS data on the same analyte are crucial for structural elucidation. When different isolates such as metabolites are analyzed by NMR and MS, one cannot always be certain that the NMR and the MS data apply to the same analyte. To avoid this ambiguity, LC–MS and LC–NMR are combined. MS data should be obtained initially since with NMR, data collection in the stop-flow mode can take hours or days depending on the complexity of the structure and the amount of sample. This is why it is preferable to designate this operation as LC–MS–NMR rather than LC–NMR–MS.

Since MS is considerably more sensitive than NMR, a splitter is incorporated after the HPLC to direct the sample to the MS and NMR units separately. In our laboratories the MS used in these studies is a Classic LCQ instrument (ThermoFinnigan, CA, USA). A custom-made splitter was used with a splitting ratio of 1/100 (Acurate, LC Packings, CA, USA). It was designed to deliver 1% of the sample initially to the MS and the balance 20 s later to the NMR. With a flow rate of 1 ml/min, the final flow rate going to the NMR will be 0.990 ml/min and to the MS will be 0.010 ml/min. Electrospray is the only source of ionization that will work with such low flow rate (10 μl/min) in LCQ. Fig. 1 depicts the scheme of our LC–MS–NMR system. The technical considerations of LC–MS–NMR are the same as LC–NMR (see Section 2) plus the effect of using deuterated solvents for the MS of the LC–MS–NMR.

3.1.1. The use of deuterated solvents

Another consideration for the LC–MS–NMR is the use of deuterated solvents needed for NMR. Analytes with exchangeable or “active” hydrogens can exchange (i.e. equilibrate) with deuterium (^{2}H) at different rates. The analyst should be alert to this possibility since it could result in the appearance of several closely spaced molecular ions. When buffers or other compatible solvents for MS are needed, it is recommendable to use deuterated buffers to avoid the suppression of additional solvent lines in the NMR spectra (see Section 2.1.3).

3.2. Modes of operation for LC–MS–NMR

As mentioned in Section 2.2, with the use of shielded cryomagnets, the location of the MS instrument will follow the same rule as for the HPLC. The most common modes of operation for LC–MS–NMR are on-flow and stop-flow. With stop-flow, the MS instrument can also be used to stop the flow on the chromatographic peak of interest that is to be analyzed by NMR.

In the last 3 years, there have been relatively few examples in the literature dealing with the application of LC–MS–NMR. We have been interested in evaluating this technology in our laboratory to determine the *pros* and *cons*, and to decide which cases are suitable for this application. To illustrate these modes of operation, a group of flavonoids was chosen. These compounds have simple structures composed primarily of aromatic protons; some have low field aliphatic protons which

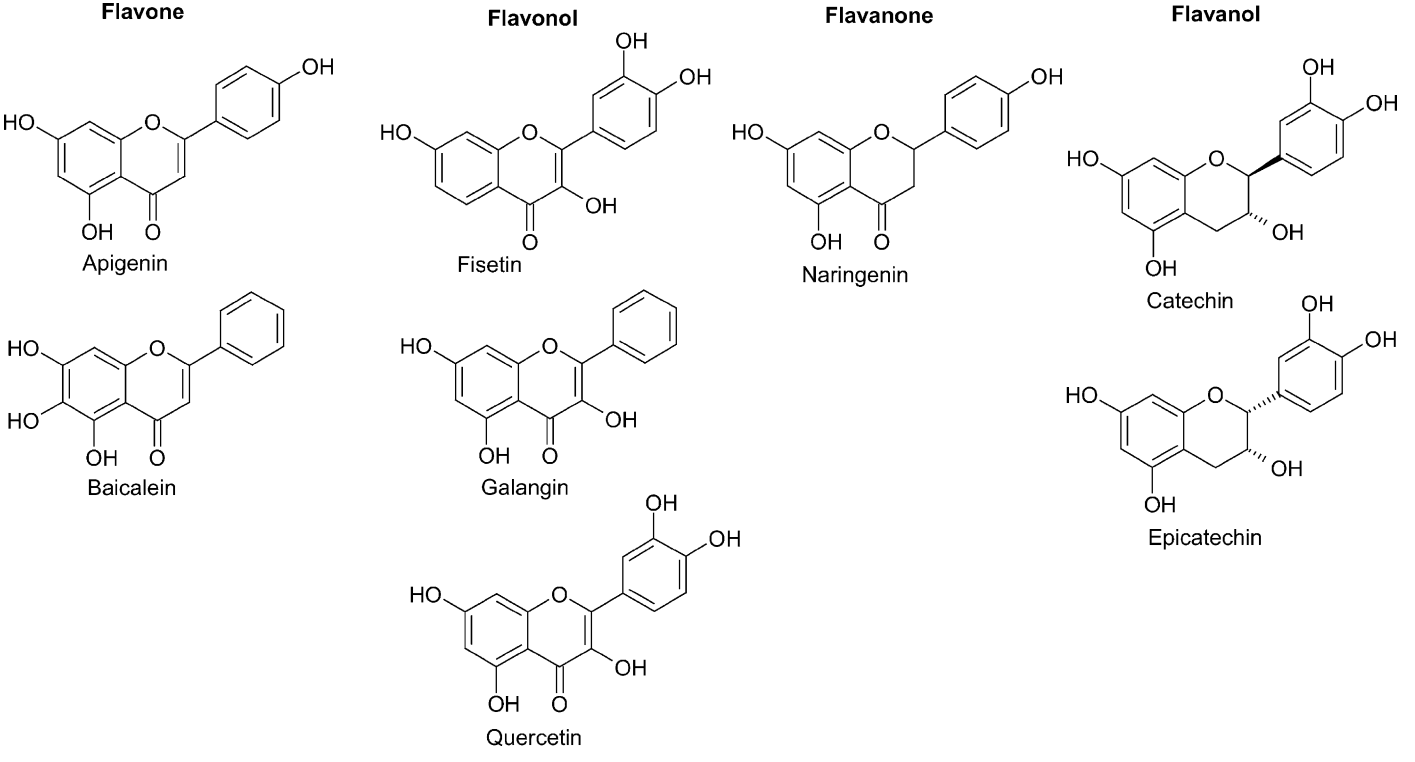

Fig. 8. Structures of eight flavonoids used for the LC–MS–NMR technology development studies.

would not be hidden under the NMR solvent peaks. Phenolic protons exchange rapidly with D_2O, so that each compound will only show one molecular ion. Flavonoids are natural products with important biological functions acting as antioxidants, free radical scavengers and metal chelators, and are important to the food industry. Fig. 8 shows the eight flavonoids (Aldrich).

The chromatographic conditions are as follows: 35–50% B 0–10 min, 50–80% B 10–15 min, A: D_2O, B: ACN, 1 ml/min, 287 nm, Discovery C_{18} column 15 cm × 4.6 cm, 5 μm. Stock solutions of each compound were prepared at 1 μg/μl in ACN:MeOH 1:1.

A Varian Unity Inova 600 MHz NMR instrument equipped with a $^1H\{^{13}C/^{15}N\}$ pulse field gradient triple resonance microflow NMR probe (flow cell 60 μl; 3 mm o.d.) was used. Reversed-phase HPLC of the samples was carried out on a Varian modular HPLC system (a 9012 pump and a 9065 photodiode array UV detector). The Varian HPLC software was also equipped with the capability for programmable stop-flow experiments based on UV peak detection. An LCQ Classic MS instrument, mentioned in the previous section, was connected on-line to the HPLC–UV system of the LC–NMR by contact closure. The ^2H resonance of the D_2O was used for field-frequency lock and the spectra were centered on the ACN methyl resonance. Suppression of resonances from HOD and methyl of ACN and its two ^{13}C satellites was accomplished using a train of four selective WET pulses, each followed by a B_o gradient pulse and a composite 90° read pulse [40].

3.2.1. On-flow

The on-flow experiment was carried out on a mixture of eight flavonoids (Fig. 8) (20 μg each). MS and NMR data were obtained during this on-flow experiment. The UV chromatogram is depicted in Fig. 9. Table 1 and Fig. 11 show the pseudo-molecular ion information $[M - {}^2H]^-$, where M is the molecular weight with all the hydroxyl protons deuterated, in negative mode for the eight flavonoids obtained in this on-flow experiment. Fig. 10 is the 2D data set (time versus chemical shift) where each ^1H NMR spectrum

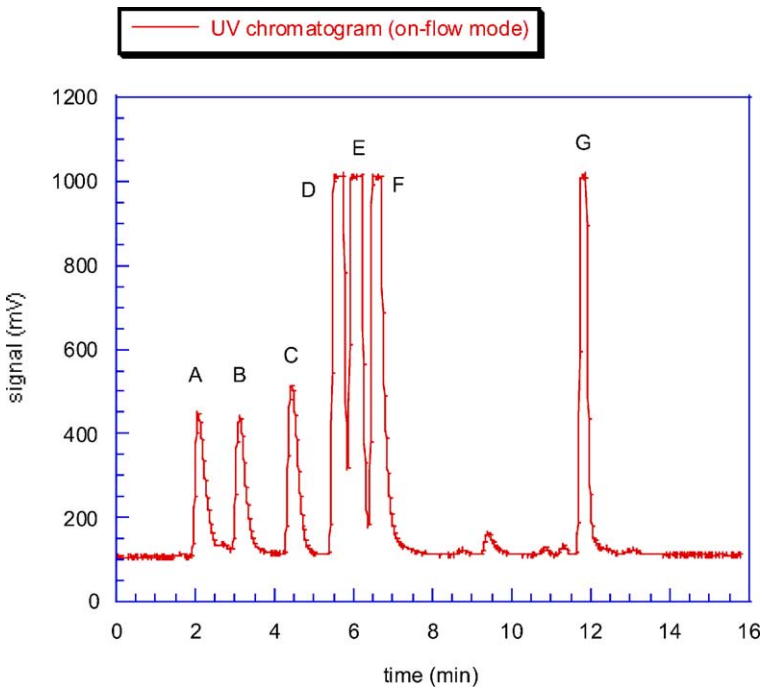

Fig. 9. UV chromatogram of the on-flow experiment injecting a mixture of eight flavonoids (A: catechin + epicatechin; B: fisetin; C: quercetin; D: apigenin; E: naringenin; F: baicalein; G: galangin).

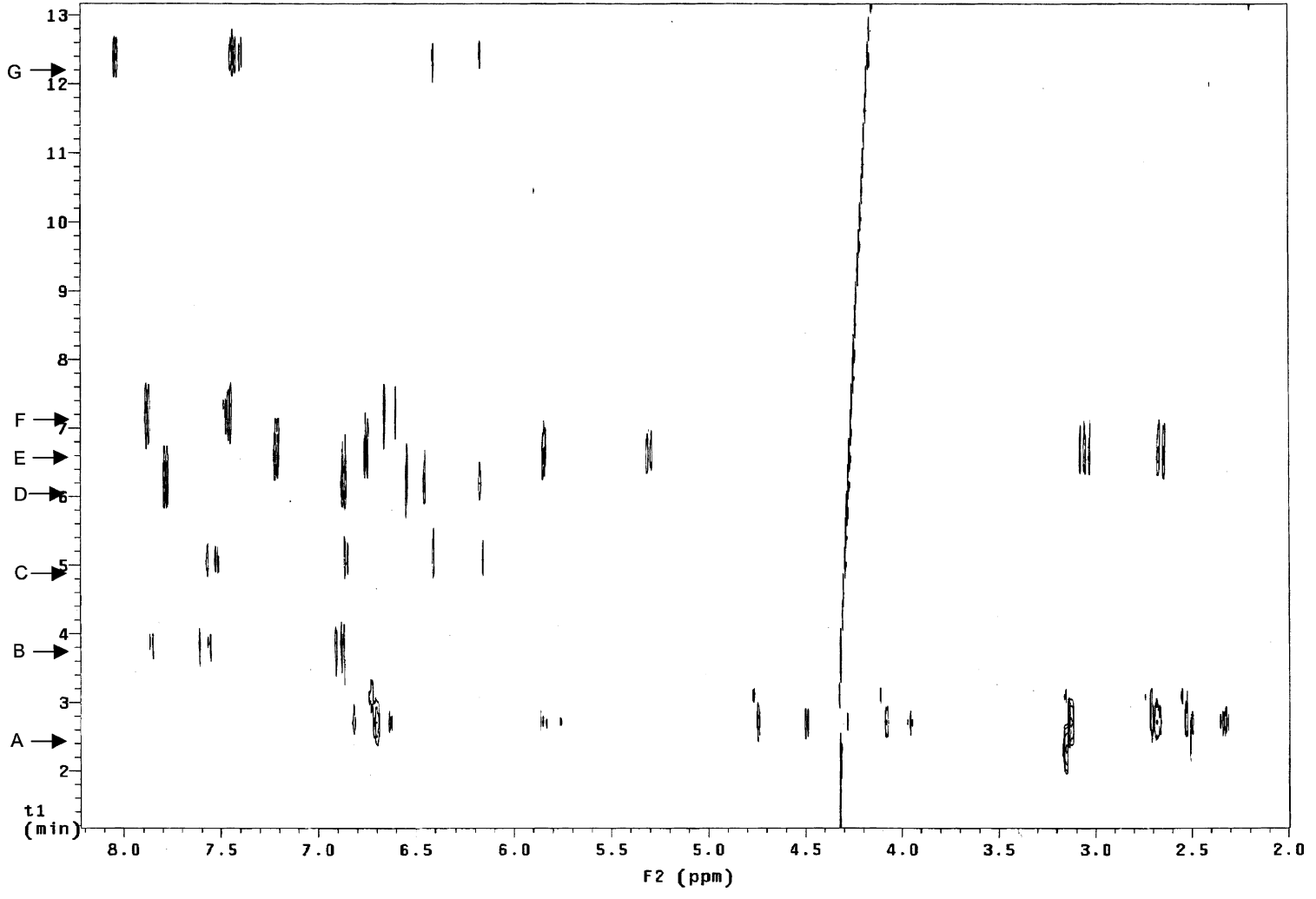

Fig. 10. 2D data set (time/min vs. chemical shift/ppm) for the on-flow experiment injecting a mixture of eight flavonoids (A: catechin + epichatechin; B: fisetin; C: quercetin; D: apigenin; E: naringenin; F: baicalein; G: galangin).

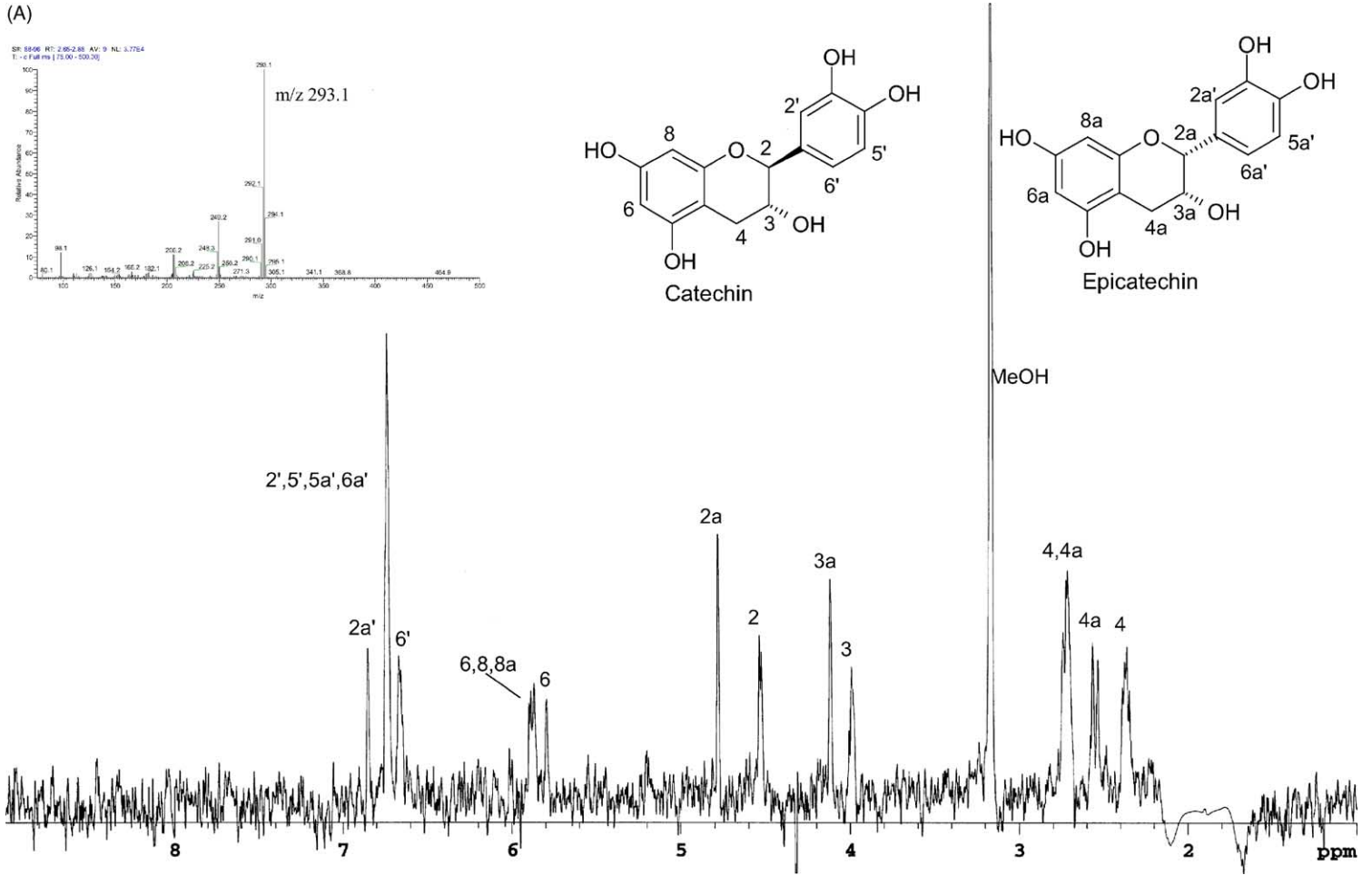

Fig. 11. (A) MS and ^{1}H NMR spectra from the 2D data set of the on-flow experiment of catechin and epicatechin. (B) MS and ^{1}H NMR spectra from the 2D data set of the on-flow experiment of fisetin (bottom) and quercetin (top). (C) MS and ^{1}H NMR spectra from the 2D data set of the on-flow experiment of apigenin (bottom) and naringenin (top). (D) MS and ^{1}H NMR spectra from the 2D data set of the on-flow experiment of baicalein (bottom) and galangin (top).

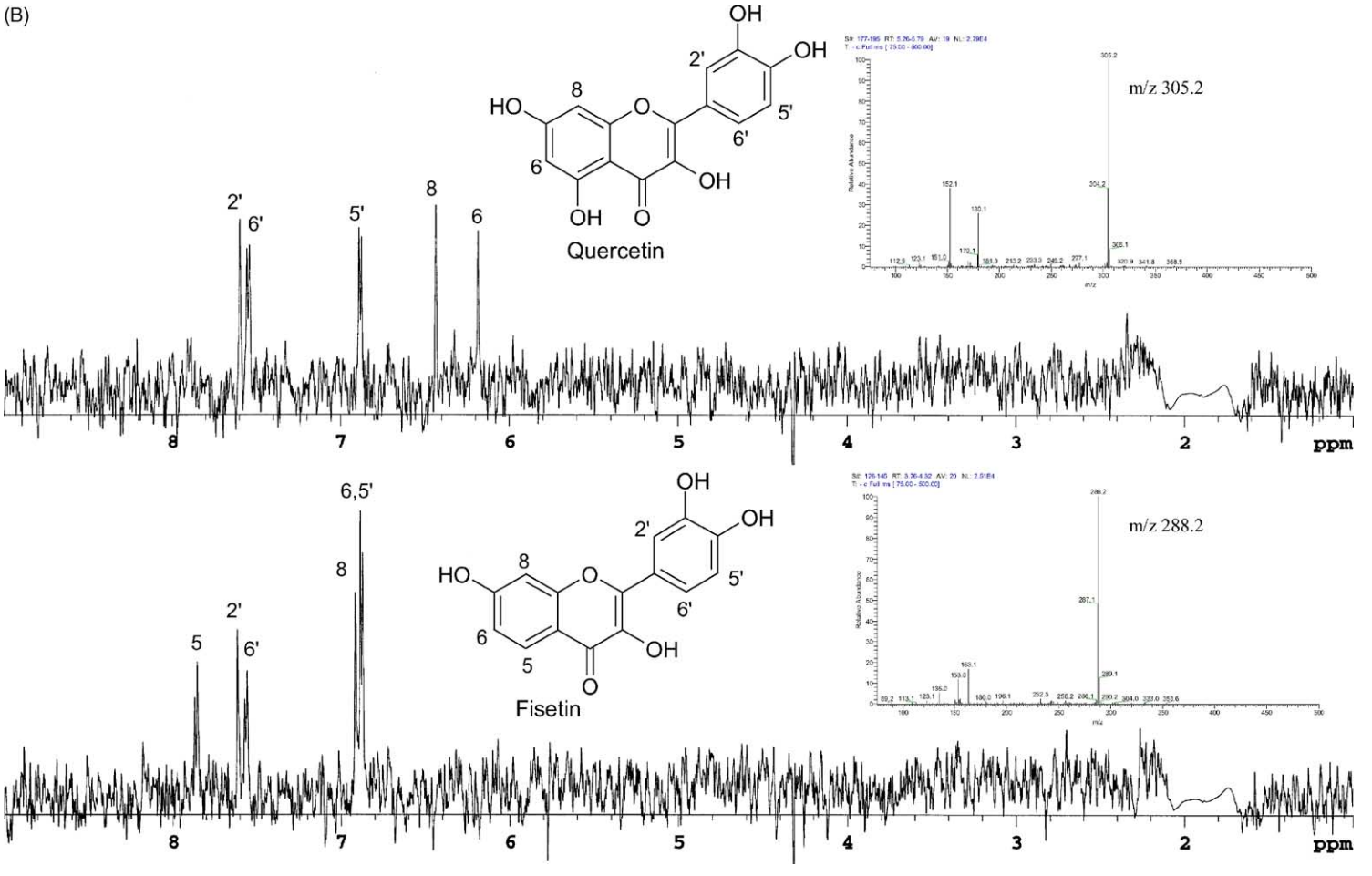

Fig. 11. (*Continued*).

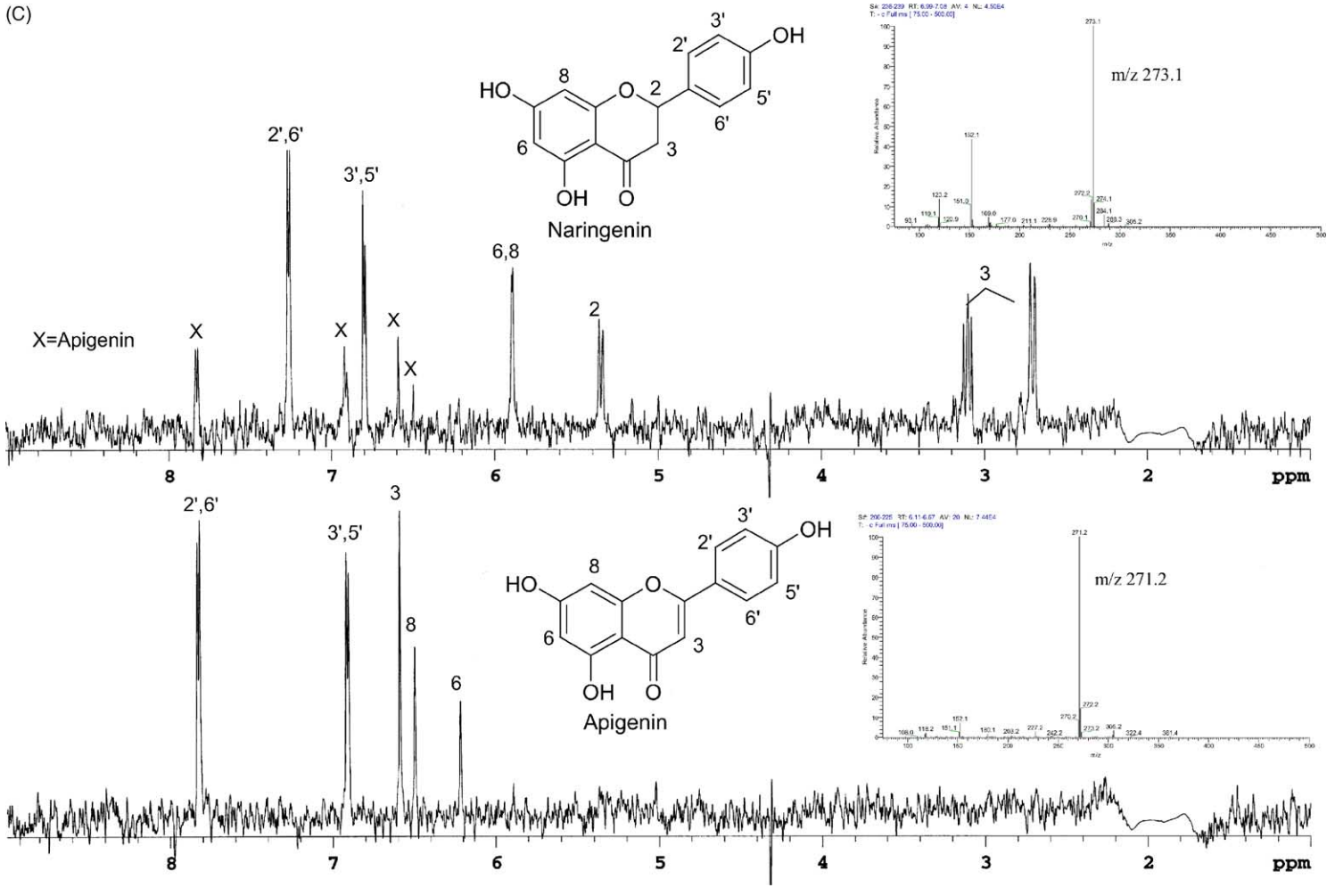

Fig. 11. (*Continued*).

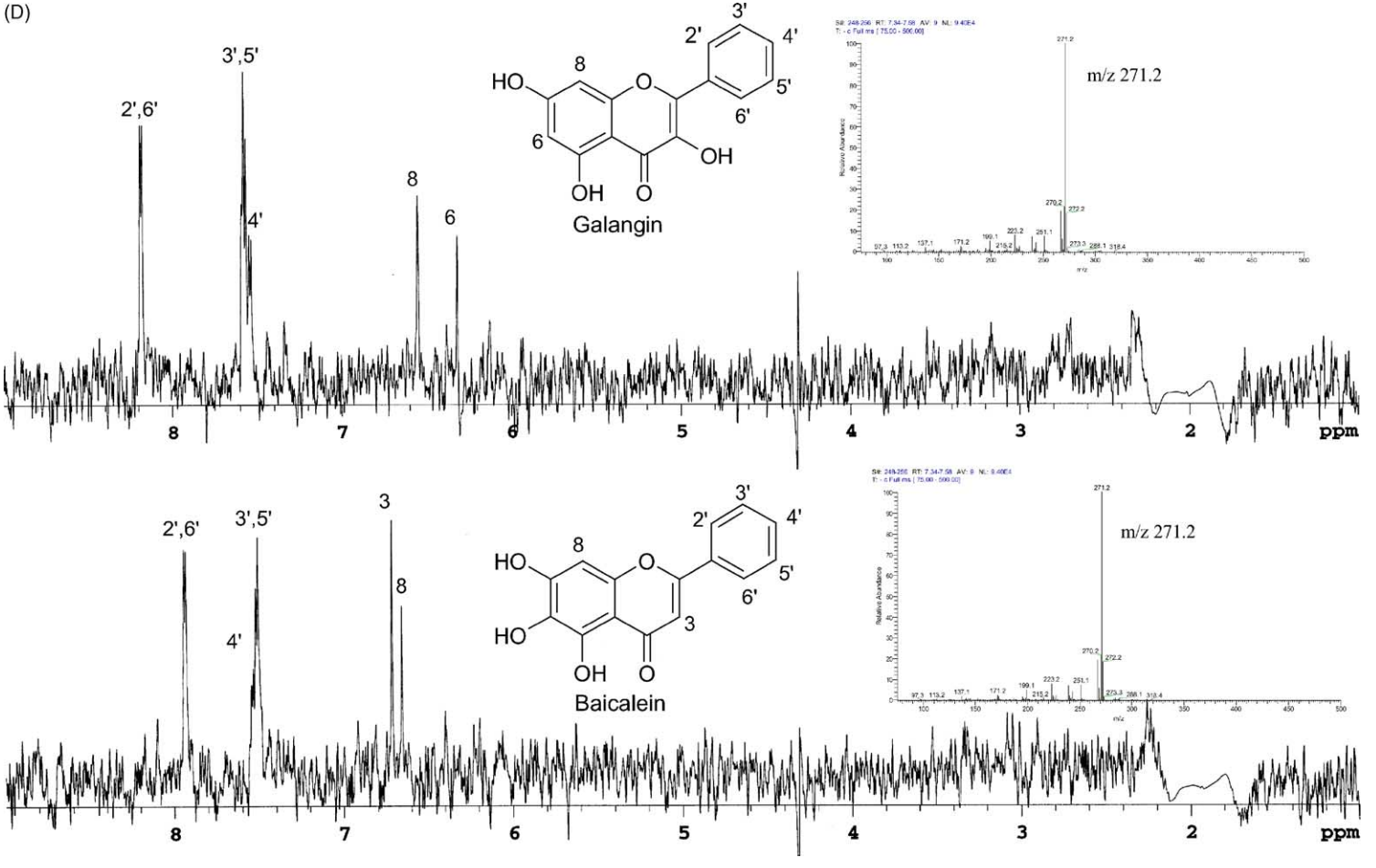

Fig. 11. (*Continued*).

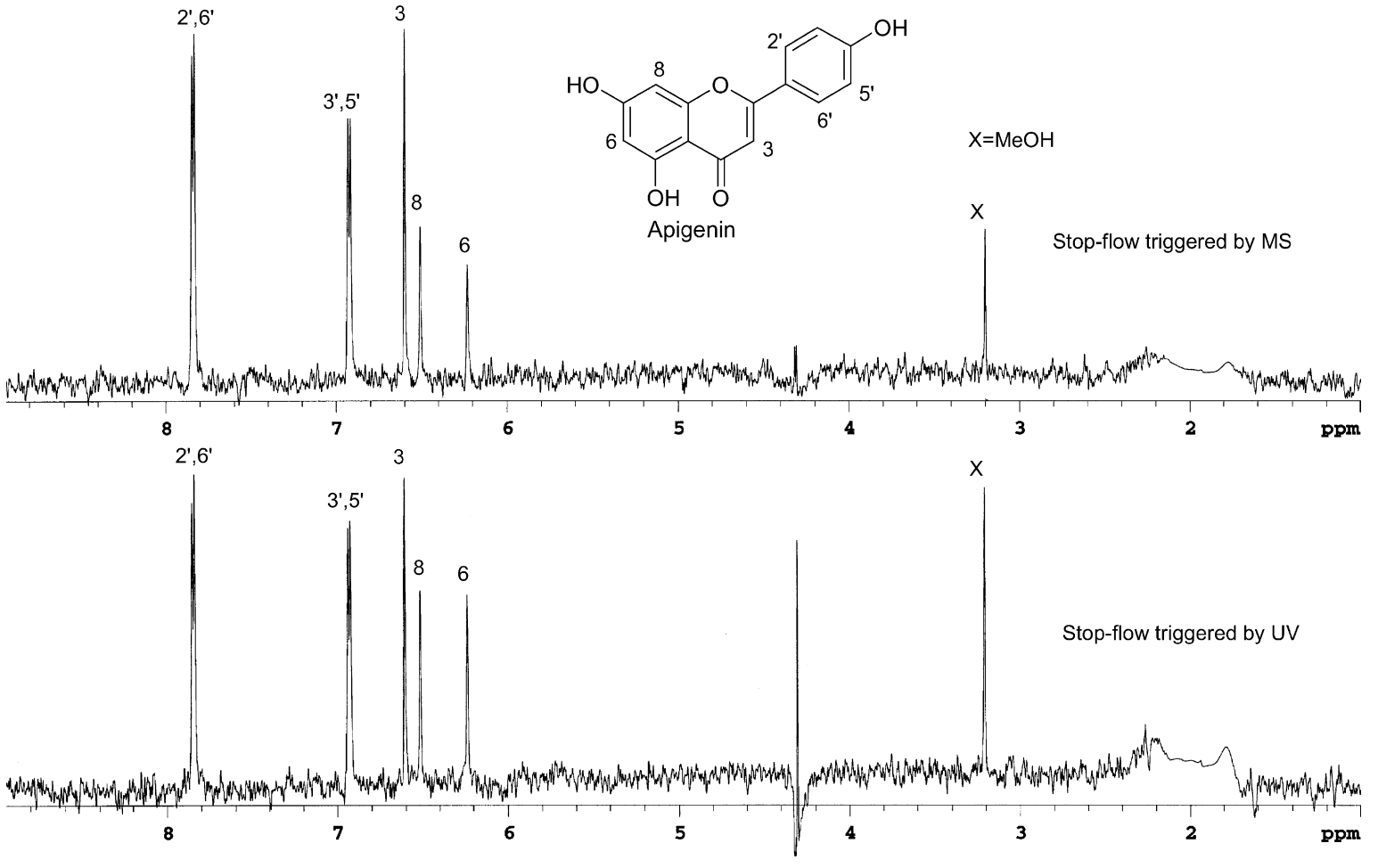

Fig. 12. ^{1}H NMR spectra of apigenin triggering the stop-flow by UV (bottom) and by MS (top).

Table 1
MS data of flavonoids in negative mode from the on-flow run in the LC–MS–NMR

Peak	Compound	MW[a]	M[b]	m/z, $[M - {}^2H]^-$
A	Catechin + epicatechin	290	295	293
B	Fisetin	286	290	288
C	Quercetin	302	307	305
D	Apigenin	270	273	271
E	Naringenin	272	275	273
F	Baicalein	270	273	271
G	Galangin	270	273	271

[a] Molecular weight.
[b] Molecular weight with all the hydroxyl protons deuterated.

was acquired for 16 scans and decreasing the delays (total time per spectrum of 20 s). Fig. 11 depicts the ^{1}H NMR traces of each flavonoid extracted from the 2D data set. Notice that catechin and epicatechin co-elute under these conditions (peak A of the UV chromatogram of Fig. 9). Distinguishing these diastereomers by MS alone is not feasible (Table 1 and Fig. 11A). Differences in the NMR spectra would be expected and are, in fact, observed (Fig. 11A). The ability of LC–MS–NMR to distinguish signals from the individual diastereomers is illustrated in Figs. 10 and 11A. The ^{1}H NMR spectrum of Naringenin in Fig. 11C shows the ability of NMR to analyze a mixture of two components in different ratio (X indicates the signals coming from Apigenin as the minor component of this chromatographic peak).

3.2.2. Stop-flow

Two stop-flow experiments were carried out on Apigenin (10 μg) (Fig. 8) using, independently, the UV peak maximum or the molecular ion chromatographic peak seen in the MS instrument to trigger the stop-flow. Since the Varian software automatically triggers the stop-flow with the UV peak, this mode was used as a reference point. When the MS was used to trigger the stop-flow, it was carried out manually with a chronometer while monitoring the molecular ion of Apigenin in negative mode (m/z 275). After peak detection in the UV or MS and a time delay of about 52 or 20 s, respectively, the HPLC pump was stopped, trapping the peak of interest in the LC–NMR microprobe. ^{1}H NMR stop-flow spectra were acquired using an acquisition time of 1.5 s, a delay between the successive pulses of 0.5 s, a spectral width of 9000 Hz, and 32 K time-domain data points. The methyl resonance of ACN was referenced to 1.94 ppm. These two experiments were carried out injecting 10 μg of Apigenin and acquiring ^{1}H NMR spectra for ca. 4.5 min (128 scans) giving rise the same quality of ^{1}H NMR spectra of Apigenin (Fig. 12).

These experiments indicated that for sample mixtures, the on-flow mode of LC–MS–NMR is useful for obtaining structural information on the major components. If more detailed analysis is required, or the amount of sample is small and the compound(s) cannot be isolated because of instability or volatility, stop-flow is the mode of choice. LC–MS and LC–NMR chromatographic conditions must be compatible; in addition, prior evaluation of the LC conditions on the LC–MS–NMR system is required to assure consistency with the chromatographic resolution needed in the LC–NMR part of the system. The sample must ionize well by electrospray to obtain MS data. When stop-flow mode is triggered by MS, prior MS information of the chromatographic peak(s) of interest is needed in deuterated solvent(s) to evaluate the suitability of the system to provide structural information.

3.3. Applications of LC–MS–NMR

There are examples in the literature for the application of LC–MS–NMR. In the area of natural products, this technique has been applied as a rapid screening method of searching unknown marine natural products in chromatographic fractions [80], and for the separation and characterization of natural products from plant origin [81,82]. Another application is in the area of combinatorial chemistry [83]. In the field of drug metabolism, LC–MS–NMR has been extensively applied for the identification of metabolites [84–90], and in pharmaceutical research [35,91].

4. Conclusions

The hyphenation of analytical and spectroscopic techniques has enhanced the ability to solve structural problems. LC–MS had been the only hyphenated technique for qualitative analysis of structures on mixtures until recent developments in NMR. Prior

to the last decade, NMR could be applied only to reasonably pure compounds. LC–NMR has expanded the capability to solve structural problems in complex mixtures. LC–NMR, however, is not comparable to LC–MS because of its lower sensitivity, the need of expensive deuterated solvents, the need of solvent suppression of the residual protonated solvents and the compatibility of the volume of the chromatographic peak with the volume of the NMR flow cell. To overcome some of these problems, more development has led to the hyphenation of capLC and NMR, where the amount of solvent used is minimal and the volume of the chromatographic peak is comparable to the volume of the NMR flow cell, but the suppression of the residual protonated solvents must still be carried out.

Within the last decade hyphenated LC–MS, LC–NMR and LC–MS–NMR have become available analytical techniques. Since MS is a destructive technique (in contrast to NMR) and requires far less sample than NMR, a splitter is incorporated on-line to direct the bulk of the sample to the less sensitive technique. In addition to the advantage of having MS and NMR information on the same chromatographic peak, the combination of these two techniques with different sensitivities must deal with other issues such as the effect of deuterated solvents on the MS, the limitation of source of ionization on the MS compatible with low flow rates, and the timing which depends on the slower NMR technique. There is still room for improvement for LC–MS–NMR and the next decade will define the areas where this hyphenated technique is best suited.

LC–MS–NMR cannot replace LC–MS, LC–NMR or even NMR techniques for the structural elucidation of compounds. There will always be cases where purification of the analyte(s) is required, when the structural problem is too complex or the separation of the chromatographic peak is not suitable. LC–MS–NMR. LC–MS, LC–NMR and NMR have to be available to the analyst to choose the appropriate technique for each structural problem. The success rate of problems will depend on choosing the right technique depending on the difficulty and nature of the problem. Each technique has its own advantages and limitations, and it is in the hands of the analyst to choose the one(s) that will help to solve the structural problems.

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