Application of Directly Coupled HPLC-NMR-MS/MS to the Identification of Metabolites of 5-Trifluoromethylpyridone (2-Hydroxy-5-trifluoromethylpyridine) in Hydroponically Grown Plants

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Directly coupled HPLC-NMR-MS was used to characterize two major metabolites of 5-trifluoromethylpyridone (2-hydroxy-5-trifluoromethylpyridine), a model compound for herbicides, after it had been dosed into hydroponically grown maize plants. The combination of NMR and MS data allowed the identification of both of these metabolites, namely, the *N*-glucoside and *O*-malonylglucoside conjugates of the parent pyridone. This work demonstrates the efficiency and the potential application of HPLC-NMR-MS to the investigation of the metabolism of agrochemicals. The work also indicates that combination of the use of hydroponically grown plants and directly coupled HPLC-NMR-MS allows rapid identification of metabolites with little sample preparation.

Keywords: *HPLC; NMR; MS; 2-pyridone; hydroponics; metabolism; xenobiotic*

INTRODUCTION

The hyphenated technique of HPLC-NMR is now well established as a powerful technique for the structure elucidation of unknown species in a variety of applications (Lindon et al., 1996). Recently, the hyphenation has been extended with the direct connection of HPLC-NMR-MS (Pullen et al., 1995; Scarfe et al., 1997; Shockcor et al., 1996). HPLC-NMR-MS has several advantages over both "stand alone" HPLC-NMR and HPLC-MS systems. A potential problem overcome by HPLC-NMR-MS is that of correlating spectroscopic data if the chromatography alters between HPLC-NMR and HPLC-MS separations. It is also possible to use either MS or NMR as a direct detection trigger to implement data collection by the other technique. By using a triple-quadrupole mass spectrometer, it is possible to obtain additional information from secondary fragmentation of selected ions (MS/MS).

HPLC-NMR-MS/MS has been used to study the metabolism of a trifluoromethylpyridone in hydroponically grown maize plants. Hydroponically grown plants offer several advantages over either whole plant systems or tissue culture (Miller et al., 1989). The conditions are more easily controllable than plants grown in soil and allow the introduction of the xenobiotic directly into the nutrient solution. Unlike tissue culture, the hydroponically grown plant is more akin to the whole plants and may give results that are more easily translated into what is likely to occur in whole plant systems.

5-Trifluoromethylpyridone (5-TFMP) was chosen for study as it serves as a simple substrate to examine the possible occurrence of *N*- versus *O*-glycosylation in plants.

EXPERIMENTAL PROCEDURES

Chemicals. 5-TFMP (2-hydroxy-5-trifluoromethylpyridine) was obtained from Fluorochem (U.K.).

Growth of Maize Plants. The plants (maize, seeds obtained from Zeneca Agrochemicals, U.K.) were germinated for 2 days at nightime temperatures of 20−25 °C, daytime temperatures of 23−30 °C, with 16 h day length.

Germinated seeds were then placed into 3 in. pots with washed grit and silver sand (at a ratio of 1:1), ∼2 cm under the surface. The plants were then placed in a closed frame and grown at nightime temperatures of 19−23 °C, daytime temperatures of 22−27 °C, and a 16 h day length. The plants were watered with a solution of half-strength Hoagland's solution (standard nutrient solution) once a day. After 2 weeks, the plants were removed from the pots, and the roots were washed to remove sand and grit. The plants were then placed in hydroponic units (supplied by Zeneca Agrochemicals, U.K.) and secured with sponge bungs (three plants in each pot). The units contained full-strength Hoagland's solution, the level of which was maintained throughout the experiment. The plants were then left at ambient temperature, with a day length of 16 h. The plants were left in the pots to acclimatize for 1 week before dosing.

Dosing of 5-TFMP. A single dose was given to each plant, by direct addition into the nutrient (Hoagland's solution). 5-TFMP was prepared as a fine powder in a pestle and mortar to aid solution. Approximately 100 mg (in water) of 5-TFMP was added to each hydroponic pot (400 mL total volume).

Sample Preparation. At time periods of 1, 3, 7, 14, 28, and 38 days after dosing, plants were harvested. Roots and

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shoots were separated (below the first leaf) and the roots rinsed to remove excess nutrient solution. Samples were then placed in a freezer (−20 °C) until use. To prepare samples, plants were snap-frozen in liquid nitrogen and homogenized using a pestle and mortar while still frozen. The resulting sample was placed in a centrifuge tube with distilled water (volume dependent on sample size) and was centrifuged for 30−45 min (10 °C) at ∼3000 rpm, after which the supernatant was drawn off. The extract supernatants were then used for HPLC and HPLC-NMR studies. Typically, 10 μL injections for HPLC-UV and 300 μL injections for HPLC-NMR-MS were used.

Analytical Methods. The HPLC system comprised a Bruker (Coventry, U.K.) LC22C pump using an LC33 variable-wavelength detector with a Hypersil BDS C_{18} column (250 × 4.6 mm). The mobile phase was changed from 100% D_2O going to 98:2 D_2O/acetonitrile over 20 min and then to 80:20 D_2O/acetonitrile from 20 to 60 min (flow rate of 1 mL/min); 0.01 M ammonium formate (pH 7) was present in the aqueous mobile phase. Chromatographic conditions and stopped-flow NMR "peak parking" were controlled by Bruker Chromstar software.

Detection was by UV (254 nm), NMR spectroscopy, and positive ion electrospray MS. Stopped-flow NMR data were acquired for selected chromatographic peaks.

Following on-line UV detection of the chromatographic peaks, the eluent was split in the ratio 95:5 to the NMR probe and MS inlet, respectively. Transfer to the NMR and MS was via polyether (ether) ketone (PEEK) tubing of 3.6 m length and 0.18 mm i.d.

^{19}F continuous flow HPLC-NMR spectra were acquired at 470.5 MHz using a Bruker DRX 500 spectrometer fitted with a ^{1}H/^{19}F 4 mm HPLC probe, having a cell volume of 120 μL. Spectra were acquired using 32K data points (F2) with 200 time increments (8 scans per increment). Acquisition time was 0.58 s, with a 1 s delay between pulses. Spectra were referenced to trifluoroethanol at δ −77.0.

Stopped-flow ^{1}H HPLC-NMR spectra were measured at 499.87 MHz using a Bruker DRX 500 spectrometer. Spectra were typically acquired with each spectrum comprising the summation of several thousand free induction decays (FIDs; the decay of nuclear magnetization with time). FIDs were collected into 16K data points, with an acquisition time of 0.98 s. Acetonitrile and residual water resonances were suppressed using a preirradiation pulse of 2 s. FIDs were zero-filled by a factor of 2 and, prior to Fourier transformation, these data were resolution enhanced using a Lorentz−Gaussian transformation. Chemical shifts were referenced internally to the residual acetonitrile signal at δ 2.00 for ^{1}H.

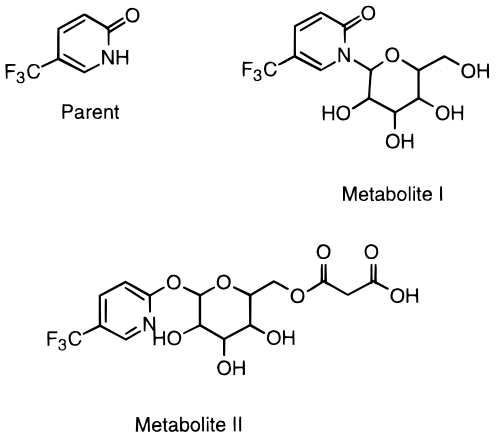
A minor proportion (5%) of the eluent was transferred to the inlet of a Micromass QuattroLC benchtop triple-quadrupole mass spectrometer. Ionization was by positive ion electrospray, and mass spectra were acquired up to mass *m*/*z* 500.

RESULTS

HPLC-NMR analysis using on-flow ^{19}F NMR detection is shown in Figure 1a. This allowed the retention times of the three most abundant compounds to be determined, and the peaks were identified by correlation with UV detected HPLC (Figure 1b), giving retention times of approximately 33 and 43 min (two compounds coeluted at ∼43 min).

HPLC-MS results supported the above data. Precursor ion scanning functions were employed to determine which peaks in the HPLC chromatogram contained the pyridone substructure (looking for *m*/*z* 166 in the positive ionization mode and *m*/*z* 162 in the negative ionization mode). The resulting ion chromatogram indicated the presence of three major compounds at retention times corresponding to those mentioned above, as well as an additional minor metabolite (data not shown).

Stopped-flow ^{1}H NMR (Figure 2a) of the peak at 33 min indicates a compound containing the pyridone moiety (singlet at δ 8.1, doublets at δ 7.7 and 6.6), a conjugate containing an anomeric resonance at δ 5.9, and resonances consistent with a sugar between δ 3.4 and 3.8. Integration of the δ 3.4−3.8 region indicates the presence of six protons, consistent with a glucosyl moiety. The $^3J_{H1'-H2'} = 8.8$ Hz coupling is consistent with a β-isomer. Overall, this spectrum is consistent with an *N*- or *O*-glucoside. MS (Figure 2b) supports this



hypothesis. In HPLC-NMR-MS, the deuterated solvents result in exchange between the hydroxyl protons and the deuterated water. This means that the molecular ion will be correspondingly higher. The product ion spectrum of the *m*/*z* 331 species (positive ion) showed the characteristic loss of the glucose moiety to leave the deuterated pyridone at *m*/*z* 166 (the pyridone fragment has an *m*/*z* 163; exchanging the NH for ND results in *m*/*z* 164, and adding D^+ instead of H^+ gives an *m*/*z* of 166). Repeating the MS work using nondeuterated solvents indicated an ion with *m*/*z* 326, corresponding to the glucoside conjugate with *H*-hydroxyl rather than *D*-hydroxyl functional groups (data not shown).

By stopped-flow ^{1}H NMR (Figure 3a) it was seen that there are two compounds present in the chromatographic peak at 43 min. The major component is readily identifiable as the parent (labeled P in Figure 3a), with resonances at δ 7.9, 7.8, and 6.6. The remaining resonances are again suggestive of a sugar conjugate, with an anomeric proton at δ 5.6 and other resonances between δ 3.0 and 4.4. The difference between this NMR spectrum and the one for metabolite I is an extra singlet at δ 3.1. This is consistent with an isolated methylene group such as in a malonyl structure, metabolite II. Further confirmation of this structure is provided by the downfield shift (of ∼ +0.7 ppm) of the glucose methylene signal, H6′, indicative of ester formation on the primary alcohol group.

Full-scan MS data showed the major ions to be *m*/*z* 166 (positive ion mode) and *m*/*z* 162 (negative ion mode), indicating the presence of the pyridone fragment (data not shown). However, the precursor ion experiment indicated the presence of a further component of MW 415 (Figure 3b), consistent with the fully deuterated O- or N-linked malonylglucoside conjugate of the parent pyridone. The product ion spectra of *m*/*z* 417 (positive) and *m*/*z* 413 (negative, not shown) typically show that the only major fragmentation is the loss of the malonylglucose unit. The nondeuterated MS data (not shown) confirm this hypothesis, with an *m*/*z* of 412.

By comparing the chemical shifts of the aromatic protons, it can be determined that the glucoside conju-

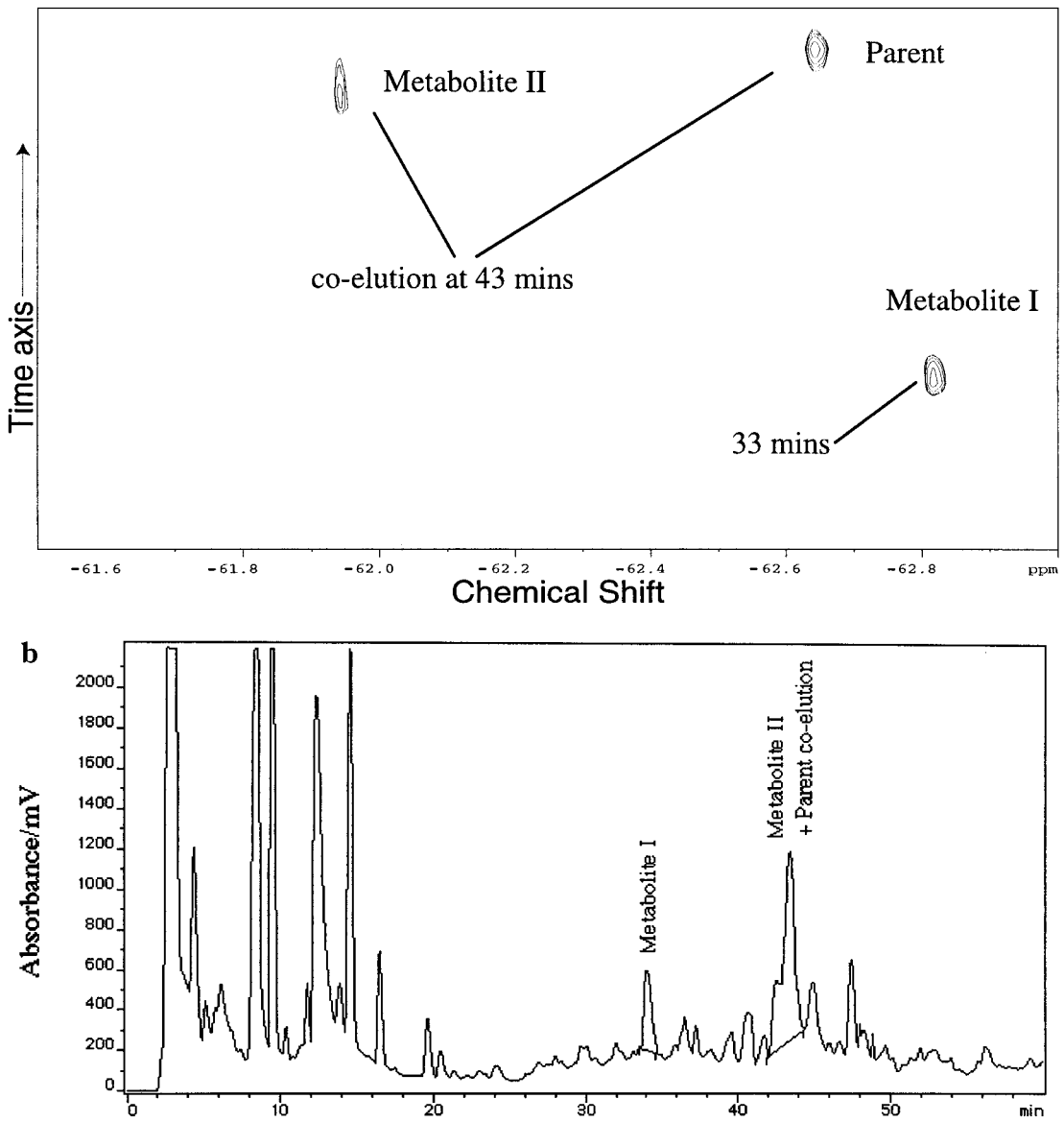


Figure 1. (a) On-flow 470.5 MHz ^{19}F NMR detected HPLC chromatogram; (b) UV detected HPLC chromatogram. Metabolite peaks are indicated at 33.9 and 43.3 min (two coeluting peaks).

Table 1. Chemical Shifts of the Aromatic Protons in the Parent, Metabolite I, and Metabolite II

compound	H3	H4	H6
parent	6.6	7.7	7.9
metabolite I	6.6	7.7	8.1
metabolite II	7.0	8.0	8.4

gate is in the *N*-form, whereas the malonylglucoside is in the *O*-form. By comparing the chemical shifts of the parent, metabolite I, and metabolite II (Table 1), it can be seen that the chemical shifts of metabolite I are more similar to those of the parent. It is well established that in aqueous solution state, the lactam/lactim equilibrium lies well over to the lactam side (Albert and Phillips, 1956). It follows that metabolite I is therefore in the lactam form, whereas metabolite II is the lactim. The two major metabolites of 5-TFMP are thus proposed as the *N*-glucoside and *O*-malonylglucoside conjugates.

DISCUSSION

This paper demonstrates the advantages of HPLC-NMR-MS/MS over the more conventional discrete techniques of HPLC-NMR and HPLC-MS. By correlating on-flow ^{19}F detected HPLC-NMR and MS with stopped-flow ^{1}H HPLC-NMR, it was possible to identify the peak of interest from a complicated chromatogram, after minimal sample preparation and cleanup. In addition, the use of MS/MS to search for fragments relating to the parent ion means that future work using nonfluorinated compounds has an additional tool from which to identify peaks of interest. Both NMR and MS identified that two peaks were coeluting, something that the UV detected chromatogram alone would have failed to observe. Taking this one stage further, the technique, utilizing the NMR and MS data, has the ability to identify compounds containing no chromophore at all, further enhancing the power of the technique.

The combined use of NMR and MS data derived from the same experiment is clearly demonstrated in distinguishing between the glucoside and malonylglucoside conjugates. With the NMR spectra for both compounds being very similar, a conclusive answer would have been difficult. With the aid of the molecular weights of each compound, however, as well as MS/MS data, structural elucidation was possible.

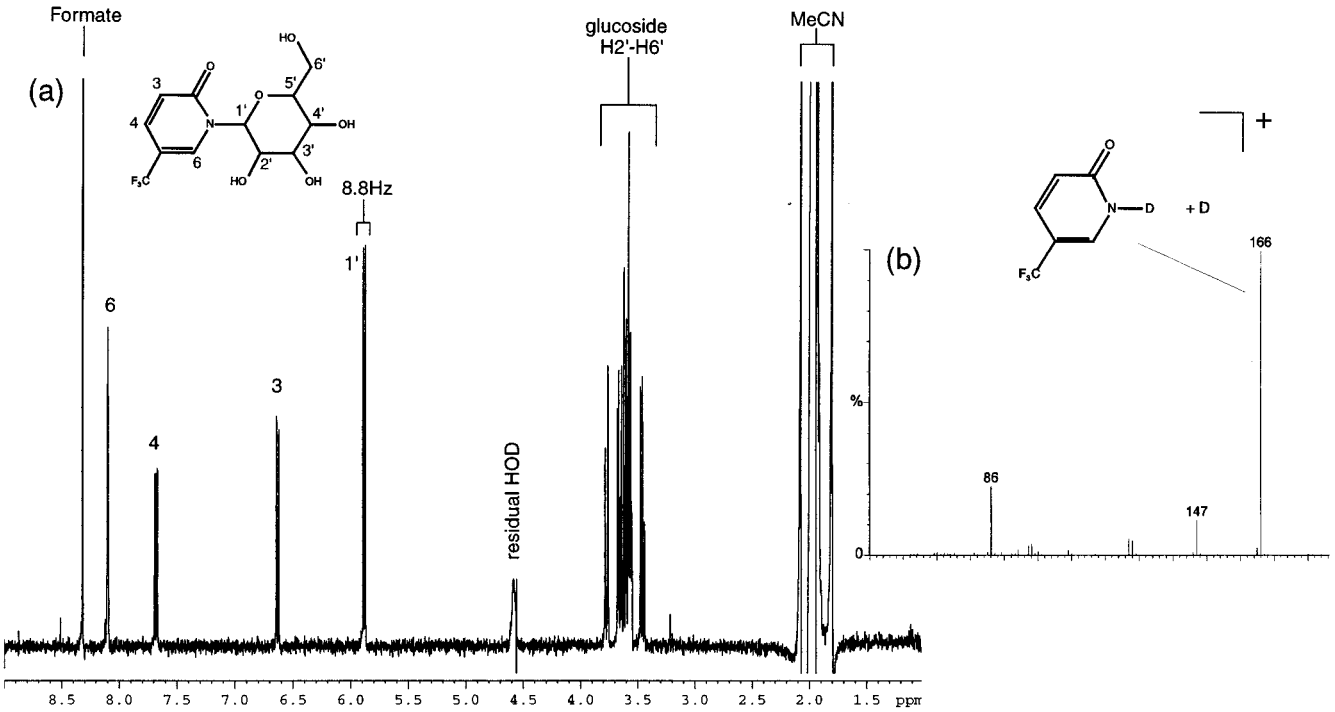


Figure 2. Stopped-flow 500 MHz HPLC-^{1}H NMR (a) and HPLC-MS (b) data for metabolite I.

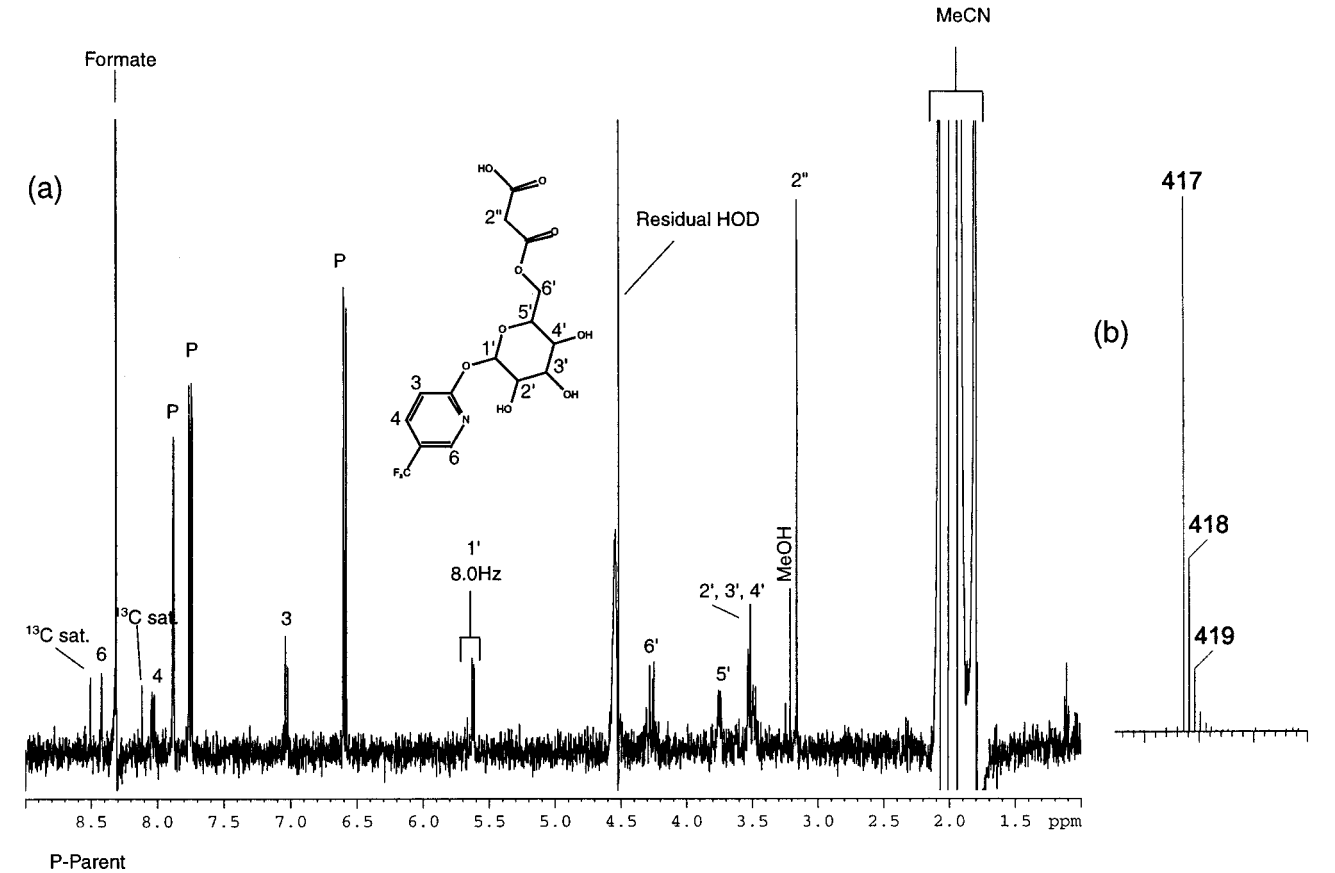


Figure 3. Stopped-flow 500 MHz HPLC-^{1}H NMR (a) and HPLC-MS (b) data for metabolite II.

Conversely, the mass spectral data, although indicating the presence of two different metabolites, was unable to conclusively identify the structures, particularly with respect to the site of conjugation.

Overall, the results indicate that metabolism of xenobiotics in plants can be followed by HPLC-NMR-MS/MS and that by using MS or NMR as appropriate for initial detection, compounds both with and without NMR active heteroatoms can be identified without the use of sample cleanup.

ABBREVIATIONS USED

5-TFMP, 5-trifluoromethylpyridone (2-hydroxy-5-trifluoromethylpyridine); HPLC, high-performance liquid

chromatography; NMR, nuclear magnetic resonance; MS, mass spectrometry; PEEK, polyether (ether) ketone; FID, free induction decay.

LITERATURE CITED

Albert, A.; Phillips, J. N. Ionization constants of heterocyclic substances. Part II. Hydroxy derivatives of nitrogenous six membered ring cpds. *J. Chem. Soc.* **1956**, 1294−1304

Lindon, J. C.; Nicholson, J. K.; Wilson, I. D. Direct Coupling of Chromatographic Separations to NMR Spectroscopy. *Prog. NMR Spectrosc.* **1996**, *29*, 1-49.

Miller, M. H.; Walker, G. K.; Tollenaar, M.; Alexander, K. G. Growth and yield of maize (*Zea mays* L.) grown outdoors hydroponically and in soil. *Can. J. Soil Sci.* **1989**, *69*, 295−302.

Pullen, F. S.; Swanson, A. G.; Newman, M. J.; Richards, D. S. On line LC/NMR/MS−A powerful spectroscopic tool for the analysis of mixtures of pharmaceutical interest. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1003−1006.

Scarfe, G. B.; Wilson, I. D.; Spraul, M.; Hofmann, M.; Braumann, U.; Lindon, J. C.; Nicholson, J. K. Application of directly coupled high performance liquid chromatography nuclear magnetic resonance mass spectrometry to the detection and characterization of the metabolites of 2-bromo-4-trifluoromethylaniline in rat urine. *Anal. Commun.* **1997**, *34*, 37−39.

Shockcor, J. P.; Unger, S. H.; Wilson, I. D.; Foxall, P. J. D.; Nicholson, J. K.; Lindon, J. C. Combined hyphenation of HPLC, NMR spectroscopy and ion trap mass spectrometry (LC−NMR−MS) with application to the detection and characterization of xenobiotic and endogenous metabolites in human urine. *Anal. Chem.* **1996**, *68*, 4431−4435.

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