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Aerobic biotransformation of 4-fluorocinnamic acid to 4-fluorobenzoic acid

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Abstract

The biotransformation of 4-fluorocinnamic acid (FCA) using non-acclimated industrial activated sludge was investigated. FCA is a common intermediate in organic synthesis, and it is often present in aqueous waste streams. Hence, the biotransformation reactions this compound undergoes when exposed to activated sludge micro-organisms should be understood before waste streams are sent to biological wastewater treatment plants (WWTPs). FCA biotransformation was monitored using a wide range of analytical techniques. These techniques were used to monitor not only FCA disappearance, but also the formation of degradation products, in order to propose the metabolic pathway. FCA was biotransformed to 4-fluorobenzoic acid via the formation of 4-fluoroacetophenone. The removal of FCA up to 200 mg L^{-1} followed first order kinetics. The half-lives for removal of FCA from the test solutions supplied with 200 mg L^{-1}, 100 mg L^{-1}, and 50 mg L^{-1} were 53, 18, and 5 hours respectively.

Introduction

Pharmaceutical synthesis uses a range of halogenated compounds (either as reagents, solvents, or intermediates) that pose particular challenges to microbial degradation. Biodegradation of chlorinated aromatics has been extensively studied, but there is relatively limited information on the biodegradation of fluorinated aromatics. A few examples are given in (Harper and Blakley 1971; Horvath and Flathman 1976; Oltmanns et al. 1989; Ramanand et al. 1995; Haeggblom et al. 1996; Kobayashi et al. 1997). However a significant number of pharmaceutical and fine chemical syntheses use fluorinated aromatics (such as fluorocinnamic acid and fluorobenzoic acids), and these are often carried into the waste streams. Since there is little or no information about the biodegradation of these compounds, a large fraction of these waste streams is currently incinerated. However an increase in understanding of their biodegradation would allow these aqueous waste streams to be treated in a biological waste water treatment plant (WWTP). In this context, the degradation of 4-fluorocinnamic acid (FCA) as the sole source of carbon and energy was investigated using non-acclimated industrial activated sludge. The metabolic pathway was investigated by identifying some of the key intermediates in the biotransformation. A range of analytical techniques – High Performance Liquid Chromatography (HPLC), Gas Chromatography/Mass Spectrometry (GC/MS), Ion Chromatography (IC), Liquid Chromatography/Mass Spectrometry (LC/MS) and Fluoride-Nuclear Magnetic Resonance (F-NMR) – were employed in this study.

Materials and methods

Chemicals

All fluorinated aromatics were supplied from Aldrich (UK). All other chemicals were supplied from Merck

(UK) and Acros (UK). All compounds were over 99% pure.

Micro-organisms and culture conditions

Non-acclimated sludge samples from a SmithKline Beecham (SB) biological WWTP were employed in this study. They were mixed with mineral salts media (MSM) to provide buffering and trace elements. This MSM contained per dm^3: 5.5×10^{-4} g $ZnSO_4.7H_2O$, 2.25×10^{-3} g $FeSO_4.7H_2O$, 8×10^{-5} g $MnSO_4.H_2O$, 2×10^{-5} g $CuSO_4.H_2O$, 0.1×10^{-3} g $CoCl_2.6H_2O$, 0.1×10^{-3} g $Na_2B_4O_7.10H_2O$, 0.06 g H_3BO_3, 0.6 g $MgSO_4.7H_2O$, 0.07 g $CaCl_2.H_2O$, 2×10^{-3} g $Na_2MoO_4.2H_2O$, 1.3 g KH_2PO_4, l g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$.

Eight 250-mL conical flasks were placed in an incubator containing 100 mL of FCA in MSM solution and 100 mL of activated sludge. Three solutions were prepared so as to contain 200 (flasks 1 and 2), 100 (flasks 3 and 4), and 50 (flasks 5 and 6) mg L^{-1} of FCA respectively at the beginning of the experiment. Two flasks of each concentration were set up and monitored. Two control flasks (flasks 7 and 8) were also set up containing 100 mg L^{-1} of FCA and no activated sludge.

The flasks were placed in an Innova 4000 incubated orbital shaker (New Brunswick Scientific Company, New Brunswick, USA) set to a temperature of 27 °C and a speed of 150 rpm. Samples were taken from the flasks at regular intervals over a 5 day period. The solutions were filtered using 0.2 μm Minisart® filters (Sartorius Göttingen, Germany), in order to remove the bacteria and debris from the samples and hence stop further reaction prior to analysis. Samples were refrigerated between analyses at 0–4 °C in polyethylene plastic vials.

Analytical methods

High Performance Liquid Chromatography (HPLC)
HPLC analysis were carried out using a Hewlett Packard HP 1100 (Hewlett Packard, Palo Alto, CA, USA) instrument equipped with a Novapak C18 column 3.9 $\times$ 150 mm 5 μm particle size (Waters, Watford, UK). An injection volume of 20 μL was used. The flow rate was set to 0.8 mL min^{-1}, the UV detector wavelength was 230 nm, and the column oven temperature was set to 40 °C. The eluent consisted of 70/30 (v/v) 0.02 M ammonium acetate adjusted to pH 4.5 with glacial acetic acid/methanol. The run was isocratic for 10 minutes.

Ion Chromatography (IC)
Ion chromatography analysis was carried out using a Dionex DX 500 Ion Chromatograph (Dionex, Sunnyvale, CA, USA). This was equipped with an AS11 analytical column (250 $\times$ 4 mm) and a AG11 guard column (50 $\times$ 4 mm), also supplied by Dionex. An injection volume of 10 μL was used. The column temperature was set to 30 °C and flow rate 1.0 mL min^{-1}. Detection was by suppressed conductivity. An hydroxide gradient was produced electrically using a Dionex EG4O eluent generator. This gradient was at time = 0; 5 mM hydroxide, at time = 5 minutes; 5 mM hydroxide, at time = 7 minutes; 45 mM hydroxide, at time =10 minutes; 45 mM hydroxide, at time =12 minutes; 5 mM hydroxide, and at time =15 minutes; 5 mM hydroxide.

Gas chromatography/Mass Spectrometry (GC/MS)
GC/MS analysis were carried out using a Hewlett Packard MSD 6870 (Hewlett Packard, Palo Alto CA). This instrument was equipped with an SGE BP1 25 m $\times$ 0.22-mm internal diameter, 0.25 μm film thickness. The temperature program was as follows 50 °C for 2 minutes, then 10 °C min^{-1} to 150 °C, and then 30 °C min^{-1} to 210 °C. A post-run time of 1.0 minutes gave a total run time of 15 minutes. The inlet mode was pulsed split, with a pulse pressure of 5.0 psi for 1.0 minute and a split ratio of 20:1 and a split flow of 23.1 mL min^{-1}. Helium was used as a carrier gas with a flow rate of 1.2 mL min^{-1} in constant flow mode. The nominal initial pressure was 13.64 psi and the average velocity was 43 cm sec^{-1}. Injection volume was 1 μL. The MSD transfer line temperature was set to 280 °C. The mass spectrometer source temperature was set to 230 °C and the quadrupole temperature to 150 °C. The ionisation mode was electron ionisation (EI) and the quadrupole was set to scan from m/z 25–550. The electron multiplier voltage was set to 1188 volts.

Liquid Chromatography/Mass Spectrometry (LC/MS)
LC/MS analysis were carried out using a HP 1090 (Hewlett Packard, Palo Alto, CA, USA) coupled to a Micromass Q-ToF™ (Micromass, Manchester, UK) quadrupole orthogonal time-of-flight mass spectrometer equipped with a Z-Spray™ electrospray ion source. The HPLC method was as above except that a 50 μL injection volume was used. The mass spectrometer was used in negative ionisation mode with a cone voltage of 20 V, source block temperature of 100

°C, desolvation temperature of 300 °C, mass range of 60–1 000 u, and a scan cycle time of 1 second.

Fluorine Nuclear Magnetic Resonance (F-NMR)
For each time point sampled, 500 μL was taken for NMR analysis, and 100 μL D_2O added to act as a ^2H lock. The samples were analysed by ^{19}F magnetic resonance spectroscopy, using proton decoupling during the acquisition period and a relaxation delay of 2 seconds. Spectra were recorded on a Bruker DPX400 NMR spectrometer operating at a frequency of 376.49 MHz for ^{19}F observation. For samples at a 100 mg L^{-1} nominal concentration, spectra were acquired with 1024 scans.

Experimental strategy

The active flasks containing FCA were inoculated with non-acclimated industrial activated sludge, and samples were taken at 0 (starting point), 3, 6, 21, 29, 50 and 99 hours. These samples were analysed using the range of complimentary analytical techniques described above. The techniques were used to monitor the disappearance of the compound and the formation of degradation products, in order to propose a biotransformation pathway for FCA.

Results and discussion

The amount of FCA in each of the samples taken from the active and control flasks over time was estimated using HPLC, and the results are shown in Figure 1. The data are presented as averages of duplicate samples for each concentration tested. For the highest concentration, after 50 hours the amount of FCA was reduced to 100 mg L^{-1} and after 99 hours was totally removed. Figure 1 also shows that the amount of FCA in the control flasks remained constant throughout the experiment, proving that there was no chemical degradation.

Plotting the logarithm of FCA concentration divided by the FCA initial concentration versus time $[\ln([FCA]/[FCA]_0) = -kt]$ gave a good fit to first-order kinetics. Figure 2 shows typical data obtained for flasks 3 and 4. The rate of FCA disappearance was 0.036 h^{-1} and the half-life was 18 hours. For starting concentrations of 200 mg L^{-1}, the half-life was 53 hours, and for starting concentrations of 50 mg L^{-1}, it was 5 hours.

The HPLC data also showed the appearance of an unknown peak, which increased during the course of the experiment following zero-order kinetics. This compound is expected to be a metabolite due to FCA biotransformation, since it only appeared in the active flasks and not in the controls. The peak areas of FCA and the unknown compound for flask 3 are plotted against time in Figure 3. This compound was identified using GC/MS and LC/MS (described below).

Ion chromatography was used to investigate the possible production of fluoride ion and attempt to correlate the production of fluoride with the disappearance of FCA. The production of fluoride ion also gives some indication of the transformation pathway. The fluoride ion theoretically expected in the different flasks, if complete mineralisation had occurred, was calculated as 23 mg L^{-1} for flasks 1 and 2, 11.5 mg L^{-1} for flasks 3 and 4, and 5.7 mg L^{-1} for flasks 5 and 6. However the IC analyses showed that there was no significant increase in the fluoride ion concentration after 99 hours. A small increase was observed for the active flasks (approximately 1 mg L^{-1} in the flasks with starting concentration of 200 mg L^{-1} and less then 0.5 mg L^{-1} in the other active flasks) in comparison to the controls (negligible increase), which suggests that some fluorinated intermediates may have been slowly produced, releasing fluoride ion into solution.

In order to attempt to identify the degradation product observed by HPLC, LC/MS analysis was initially carried out. The major degradation product observed in the chromatograms was identified as 4-fluorobenzoic acid (FBA). The accurate mass measurement of the $[M\text{-}H]^-$ ion of FBA, i.e. 139.0190, agrees with the theoretical value (139.0195) to within 4 ppm. FBA was mass measured against residual trifluoroacetic acid TFA ($[M\text{-}H]^- = 112.9850$).

An additional GC/MS investigation was carried out to attempt to identify further metabolites. Two of the peaks in the chromatogram are aromatic fluorine containing compounds identified as FBA (Peak A) and 4-fluoroacetophenone (Peak B) by a library search (NIST98 library within the HP MSD software). The mass spectra for these compounds are shown in Figures 4(a) and 4(b). Using this information, a pathway for the biotransformation of FCA is proposed and shown in Figure 5.

The formation of benzoic acids from non halogenated cinnamic acids has previously been shown, for example the conversion of ferrulic to vanillic acid (Fewson et al. 1968; Ruzzi et al. 1997). In addition according to our own studies (data not shown)

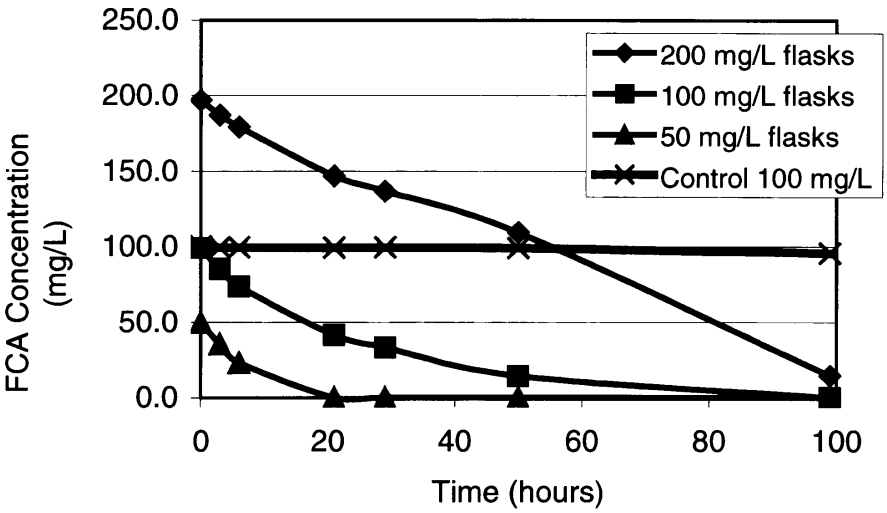

Figure 1. FCA concentration over time for the 200 (flasks 1 and 2), 100 (flasks 2 and 3), 50 (flasks 5 and 6) mg L^{-1} active flasks and control flasks (flasks 7 and 8). Data shown as average of duplicate samples.

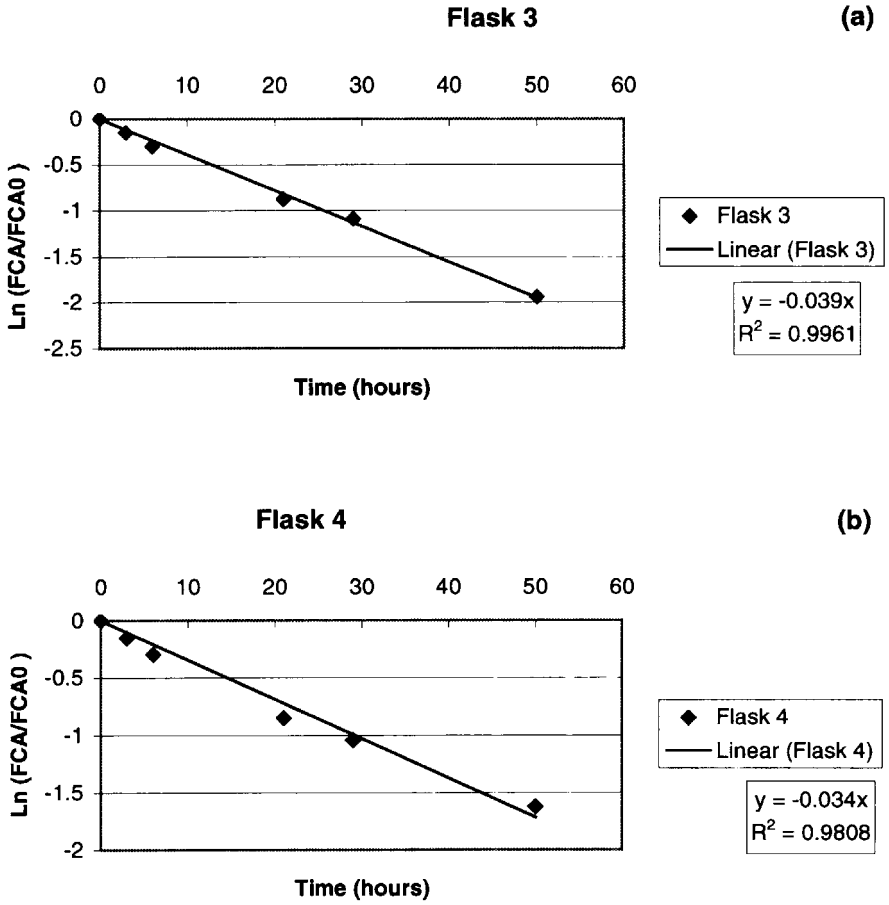

Figure 2. ln(FCA/FCA_0) vs. time for flasks 3 (a) and 4 (b).

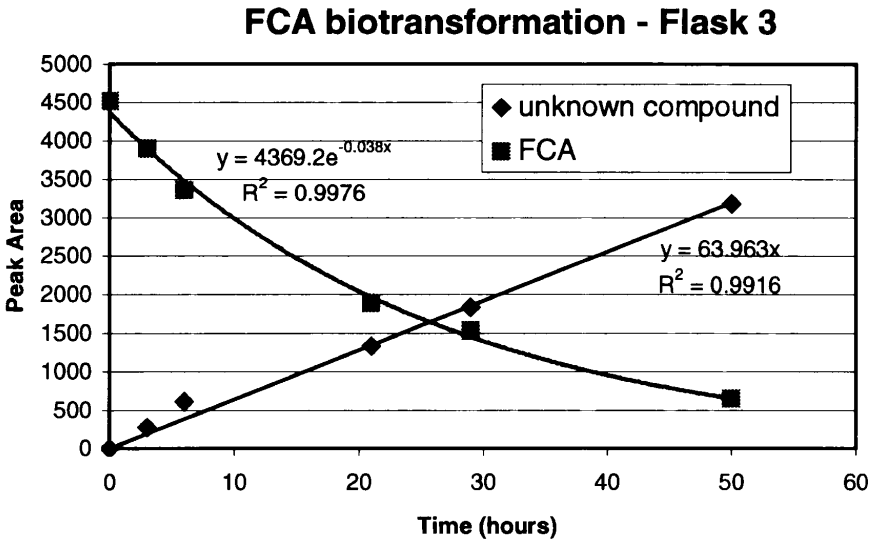

Figure 3. FCA disappearance and unknown compound appearance over time for flask 3 (first 50 hours).

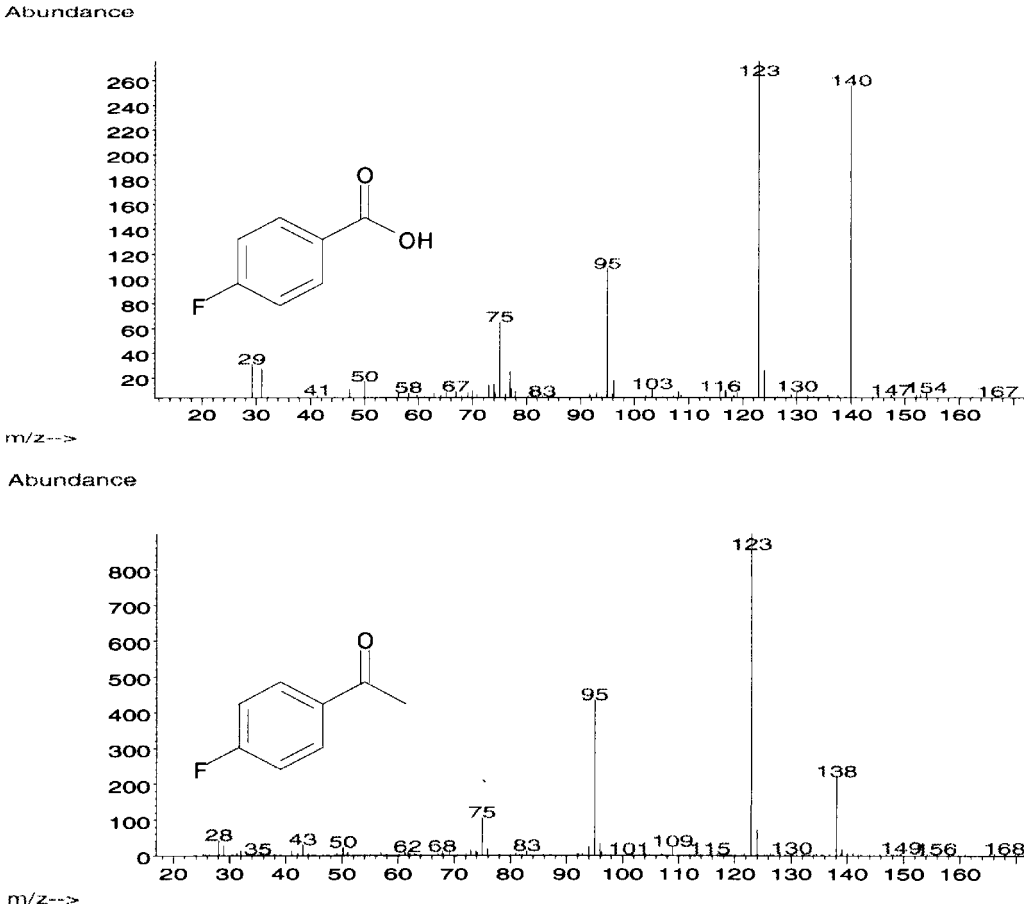

Figure 4. Mass spectra for Peaks A (4-fluorobenzoic acid (FBA)) and B (4-fluoroacetophenone).

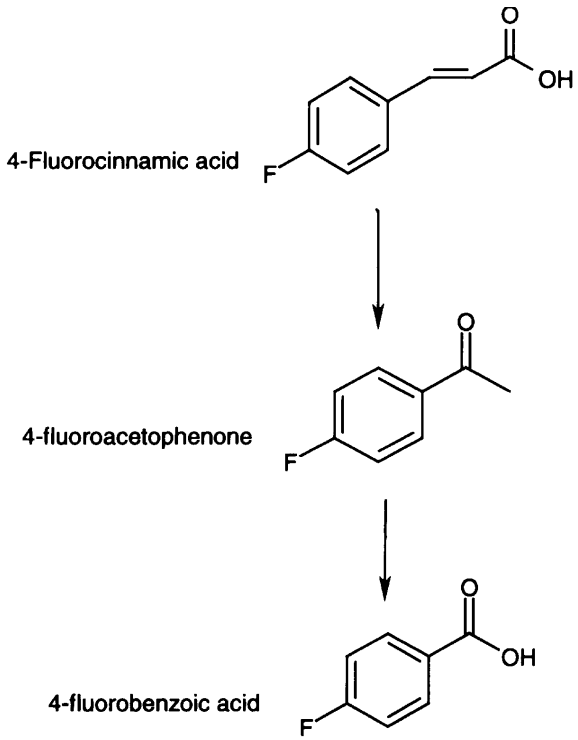

Figure 5. Proposed biotransformation pathway for FCA.

and literature published in the late eighties (van den Tweel et al. 1987; Oltmanns et al. 1989), FBA is fully mineralised with the formation of stoichiometric amounts of fluoride. The same authors have found that FBA degradation proceeds via the formation of 4-hydoxybenzoic acid (with loss of fluoride) or by 3,4-dihydroxybrenzoic acid followed by complete mineralisation.

^{19}F-NMR was used to simultaneously monitor the disappearance of FCA and appearance of fluorinated metabolites. This was attempted in order to test the possibility of monitoring fluorinated compound biotransformations using a single analytical technique. The chemical shift of the ^{19}F nucleus in FCA acid under the conditions used is -112.4 ppm. After 6 hours, two fluorinated species were observed in addition to FCA at chemical shifts of -111 and -116.2 ppm. The resonance at -111 ppm has been confirmed as FBA by spiking. As time increased the FBA resonance continued to increase relative to the FCA resonance, which decreased. Additionally, after 21 hours, another resonance at -105.5 ppm was observed. Interestingly the resonances at -116.2 and -105.5 ppm appeared to have reached their maxima between 6 and 21 hours. Unfortunately, it was not possible from the current data to determine the structures of the minor biotransformation intermediates. Removal of FCA was complete after 99 hours, with only the resonance of FBA remaining, which agrees well with the results obtained with the other analytical techniques used in this study. Quantitative data were obtained by comparing resonance line heights of the ^{19}F spectra. The percentage figures for each species are plotted against time in Figure 6. This figure clearly shows the disappearance of FCA and the build-up of FBA, with the minor unknown species (marked uk1 & uk2) rising to a maxima between 6 and 20 hours. This preliminary investigation using F-NMR proved useful and in the future it will be carried out in the presence of an internal standard added at the time of NMR sample preparation. The internal standard will act as a reference and a constant reference for integration purposes In addition, it may be possible to sample a greater volume of solution at each time point, so that for those time points of interest, more detailed structural information may be gathered by concentrating the sample and possibly recording data in a solvent other than H_2O/D_2O.

Conclusions

These experiments have shown the biotransformation of FCA to FBA by non-acclimated industrial activated sludge. For the flasks supplied with 200 mg L^{-1}, FCA was totally removed in approximately 99 hours. FCA removal in all the active flasks could be approximated to first-order kinetics. In summary, the half-lives for FCA removal from the flasks supplied with 200 mg L^{-1}, 100 mg L^{-1} and 50 mg L^{-1} were calculated as 53, 18, and 5 hours respectively.

The ion-chromatography data did not show any significant increase in fluoride ion concentration after 99 hours. This suggests that in order to test if complete mineralisation would occur, the experiment should have been left running for a longer period of time.

A number of analytical techniques were used to investigate and propose the biotransformation pathway of FCA. FCA was biotransformed to FBA via the formation of 4-fluoroacetophenone. In addition, F-NMR could be used to simultaneously monitor fluorinated compounds removal and metabolite formation. This is an alternative to the combined use of HPLC, LC/MS, GC/MS, and IC.

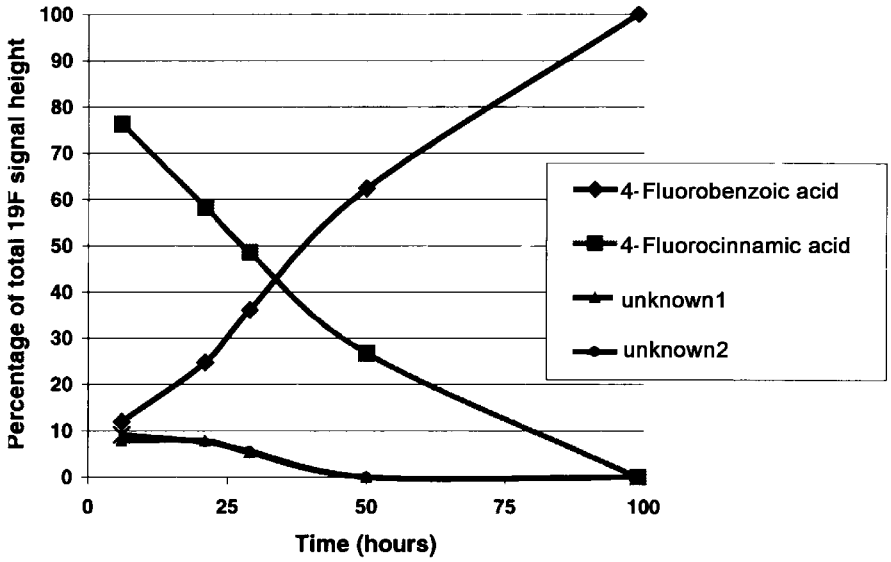

Figure 6. FNMR results showing the percentage of total ^{19}F signal height versus time.

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