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RECENT ADVANCES IN DOPING ANALYSIS (7)

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J.-C. MATHURIN, J. DE CEAURRIZ, M.-T. SICART, B. MARION, M. AUDRAN, M.-P. KRAFFT:
Development of an Analytical Strategy for Detection of Perfluorocarbons (PFCs) for Anti-Doping Control: Analysis of Breath and Blood Samples after i.v. Injection of a PFC's Emulsion in Rats
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Development of an analytical strategy for detection of perfluorocarbons (PFCs) for anti-doping control : analysis of breath and blood samples after *i.v.* injection of a PFC's emulsion in rats

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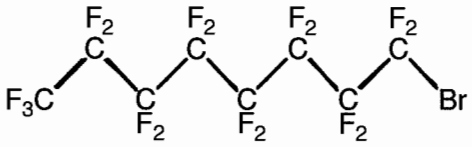
Introduction :

The first PFCs have been developed during the second world war with the Manhattan Project as chemical inert solvents for handling highly reactive uranium compounds. These PFCs, which can be produced in relative high purity by electrochemistry or telomerization techniques, are highly hydrophobic and lipophobic.[1] Thus, the medical use requires an emulsion form of these PFCs (with water, stabilisant and surfactant) for perfusion or *i.v.* administration.[2] The electronic shield created by fluoride atoms prevent the chemical and biochemical attacks of these molecules. Consequentely, PFCs are considered as totally inert materials. The fluoride atoms also create weak intermolecular forces facilitating the insertion of gas molecules, and enhancing the capacity of dissolution of oxygen which is about twenty times higher in PFC than in plasma. Moreover, these dissolved molecules are easily removed from the PFC as they do not bind oxygen as hemoglobin but only dissolve it.[3] So, PFCs have a great interest in order to support tissues oxygenation (under the condition of a high inspired concentration of O_2). Numerous clinical applications have been developed or are currently in clinical trials in different pharmaceutical companies. PFCs emulsions are investigated as blood substitutes during surgery and neat PFCs are tested in the treatment of the respiratory distress syndrome during liquid ventilation.[4,5] So, due to these particular physicochemical properties, the perfluorocarbons are included in the IOC list of prohibited compounds as

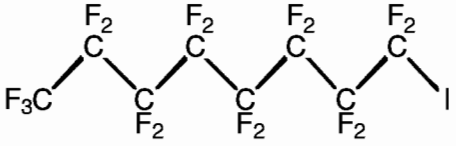
"artificial oxygen carrier". The use of PFCs in sport for doping purpose has not been established but such practice is suspected in cycling. We propose here different methods of detection of an administration of these PFCs using GC/MS. PFCs are mainly excreted by lung and they have not been detected in urine.[6] In fact, it seems that blood and breath may be considered as adequate media for the detection of PFCs in anti doping analyses. In order to develop an analytical procedure, animal experiments have been carried out and blood and breath samples have been examined. The extractions of the PFCs from these samples have been performed by a liquid-liquid extraction method (LLE) and a new tool in the field of anti-doping analysis, the solid phase micro-extraction (SPME).

Experimental :

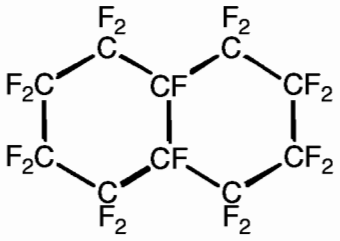
The PFCs used in these experiment were provided by Atochem (Pierre Benite, France) excepted the heptadecafluoro-1-iodo octane (Fluka) used as an internal standard for SPME experiments.



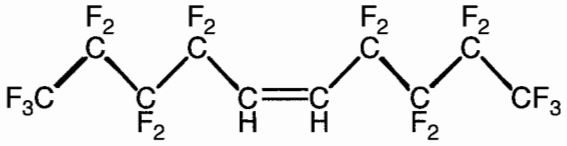
F- octylbromide
Perflubron : PFOB (M=498)



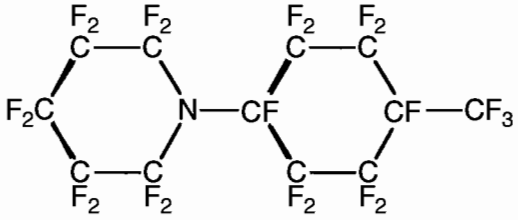
F- octyliodide : I.S.
Heptadecafluoroiodooctane : HDFIO (M=546)



F-decaline
Perfluorodecaline : FDC (M=462)



bis (F-butyl)ethene
F-44E (M=464)



F-methylcyclohexylpiperidine
Perfluoromethylcyclohexylpiperidine : FMCP (M=595)

Two emulsions of PFCs prepared and characterized at the Institut Charles Sadron (UPR 22, Strasbourg, France) were administred to Wistar strain male rats. After *i.v.* injection of the PFC's emulsion, the rats were leaved in a hermetic glass cage (9L). Two types of emulsion have been used. Emulsion A contains 90 % (w/w) of PFOB and emulsion B contains 6.5 % (w/w) of FMCP and 13 % (w/w) of FDC. The compounds were emulsified by egg yolk phospholipids and the emulsion was stabilized by a semifluorinated alkane $C_6F_{13}C_{10}H_{21}$ for emulsion A and the pluronic F68 (a copolymer of polyethylene and polypropylene glycol) in the case of emulsion B. After *i.v.* injection of these emulsions to rats (1.8 g/kg of PFOB for emulsion A; 0.6 g/kg of FMCP and 1.3 g/kg of FDC for emulsion B) breath and blood samples were simultaneously sampled. About 100 µl of blood were obtained from retro-orbitary sinus or intra-cardiac puncture and collected in glass tubes coated with sodium heparinate as the anticoagulant.

Air samples were transfered from the cage with a 100 ml syringe into a hermetic gas sampling bag (Tedlar bag, Supelco). Static head-space and solid phase micro extraction were therefore carried out from these samples.

Blood samples :

-LLE combined with GC/MS

Liquid-liquid extractions were performed from 100 µl of blood and 1 ml of 1,1,2-trichlorotrifluoroethane (4°C) containing the internal standard (F44-E). The mixture was vortexed for 1 min, then 100 µl of ethanol (4°C) were added and the samples were mixed (15 min) in an ultrasonic bath and vortexed, then the tubes were centrifuged and thawed at –30°C before analysis. One µl of the solvent was injected into the column of a HP 5890 chromatograph (CP-Select 624 CB : 30m, 0.32 mm, 1.8 µm) combined with a HP 5989A mass spectrometer. The initial oven temperature was maintained for 1 min at 40°C and then increased by 11°C/min up to 65°C. The injector was set to 200°C in the split mode (1/40).

-SPME combined with GC/MS

Automation of the SPME adsorption and injection was performed with a Varian 8200 Cx Autosampler combined with a 3800 Varian GC. Twenty µl of blood and the internal standard (HDFIO) were introduced in a 2 ml auto-sampler vial

The adsorption time was fixed at 10 min at room temperature with a 100 μm Polydimethylsiloxane fiber (Supelco). The fiber was let 2 min in the GC injector in order to assure a total desorption of the analyte and to avoid cross contamination.
Oven temperature program (DB-5 : 30m, 0.25 mm, 0.25 μm) was fixed at 40°C during 2.5 min and increased at 50°C/min up to 150°C in splitless mode with a helium flow regulated at 1 ml/min. Mass spectra were recorded in full scan mode or MS/MS mode using an ion trap instrument (Saturn 2000, Varian).

Air samples :

-SPME combined with GC/MS :

The hermetics sampling bags were equipped with septa which were manually pierced with a manual SPME syringe (Supleco). After an adsorption period of 20 min the syringe was introduced into the GC injector. SPME, GC and mass spectrometric detection conditions were similar as those described above.

-Static head-space combined with GC/MS :

500 μl of air was collected from the hermetic bag with a gas syringe and injected into the GC under similar conditions as those described above.

Results and discussion :

Mass Spectra :

Mass spectra of pure PFCs have been recorded in order to create a new library in MS and MS/MS mode with the ion trap mass spectrometer. EI/MS and EI/MS/MS mass spectra are reported in figure 1 and 2. In almost all cases the ion with the higher mass corresponds to the loss of a fluorine atom. Thus, successive loss of CF_2 or F_2 are observed. So $(M\text{-}C_nF_{2n+1}\text{-}mF_2)^+$ are characteristic ions of a PFC, with M the molecular weight of the molecule, m and n are two integer. In the case of PFOB the loss of the bromine atom substituted the loss of a fluorine atom. When hydrogen atoms are present, losses of HF can also be observed.

Comparison between static head-space and SPME/GC/MS

500 μl of the vapor phase of the mixture of three PFCs have been introduced into the hermetic bag. The bag was stored 24 hours at room temperature, a static head-space experiment and a SPME experiment were performed. The static head-space was supposed to create a negligible

impoverishment in gas so that the signal obtained by the two experiments can be compared. The results are reported in figure 3. We can observe that SPME experiments result in a five fold increase of the signal for PFOB and FMCP, due to the pre-concentration step on the fiber prior to the analysis. In the case of FDC, no significant improvement is observed. An explanation may be that the SPME process is dependent on the physicochemical properties of the analyte (partition coefficients in particular).

Excretion studies in breath by SPME/GC/MS:

We report in Figure 4 the signal obtained for each PFC at different times after the *i.v.* injection (the signal was recorded by focusing on the specific ions m/z 331 for PFOB, m/z 293 for FDC and m/z 576 for FMCP). If we compare the evolution of the signal as a function of time for the three PFCs, we observe similar results for PFOB and FDC but not for FMCP. Indeed, the signal is decreased by a factor of 2 within a 20 days period after the injection for FMCP whereas this signal is divided by a factor of 30 for the two other PFCs. This result can be explained by the structure of the PFC. Indeed, even if excretion and elimination processes of PFC is not still well-known[7], half life time in organs is related to the weight of the PFC and in a smaller way to the presence of heteroatoms. PFOB and FDC have half life times of about 4 and 7 days with a molecular weight of 498 and 462 g/mol, respectively. In the opposite FMCP has a half life time of about 100 days with a molecular weight of 595. So these values can be correlated with our prolonged observation of FMCP in breath. Under the present conditions we still observed the presence of PFOB in full scan mode up to 2 month after the injection of the emulsion.

Excretion studies in blood by LLE/GC/MS and SPME/GC/MS:

In the case of blood analysis by LLE/GC/MS the decrease in the signal within the first few hours seems to be faster in comparison with breath samples (Figure 5). The differences between blood and breath have not been interpreted yet. However, we observed that the PFOB molecule can still be detected in SIM mode by liquid-liquid extraction up to one month after the injection of perflubron. Only few samples of blood have been analysed by SPME and comparison of the two methods can not be done. The chromatogram for a sample obtained 6 hours after injection of the FDC/FMCP emulsion injection is represented in Figure 6. We can observe the two isomers (cis and trans) of the FDC and the different isomers of FMCP which can correspond to four stereoisomers (two couples of conformer). One can note the presence

of a very low chemical background due to the high selectivity of the fiber for volatile or semi-volatile compounds which are present in the blood sample.

Conclusion

SPME is an extraction/concentration method that preserved the use of solvent, reduced sampled handling and increased the sensitivity of the analysis. Due to the pre-concentration step of the SPME, this technique improved sensitivity in comparison with static classical head-space on breath samples. Regarding these preliminary results it is expected that PFCs could be detected by SPME in air samples several weeks after their injection.

In the case of blood samples, analyses by SPME combined with GC/MS can be easily performed and moreover automation SPME is available. About 50 samples can be analysed in one day without any pre-preparation step. Liquid-liquid extraction can also be used and performed in every anti-doping laboratory with a "classical" material but this procedure is relatively time consuming in comparison with SPME.

In all cases, MS/MS mass spectra can be easily recorded with an ion trap in order to confirm the nature of the molecule.

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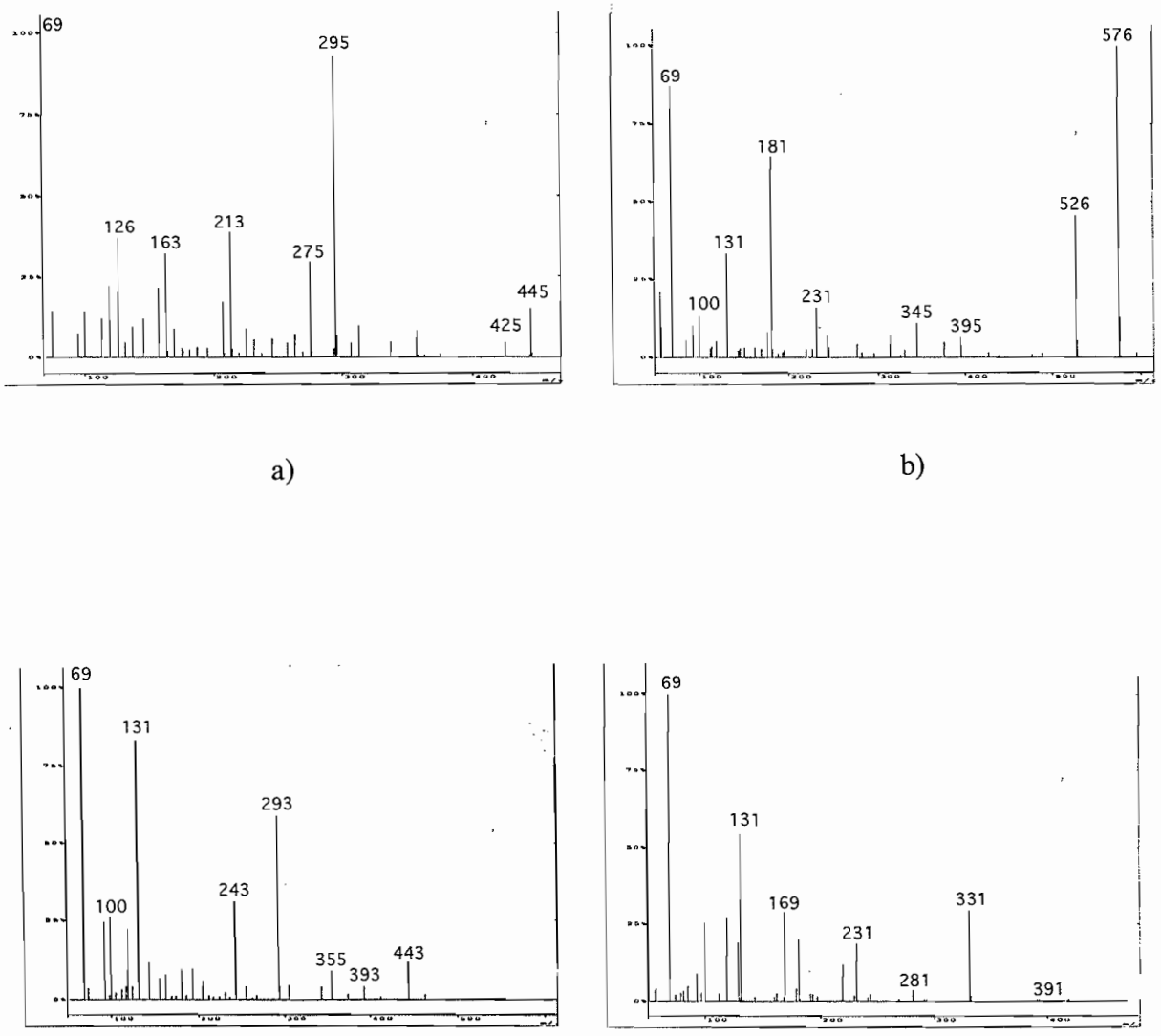
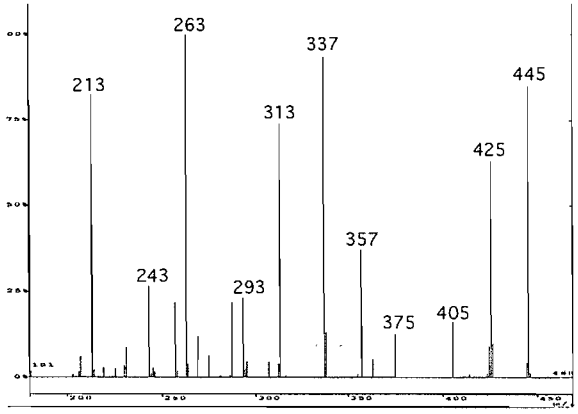
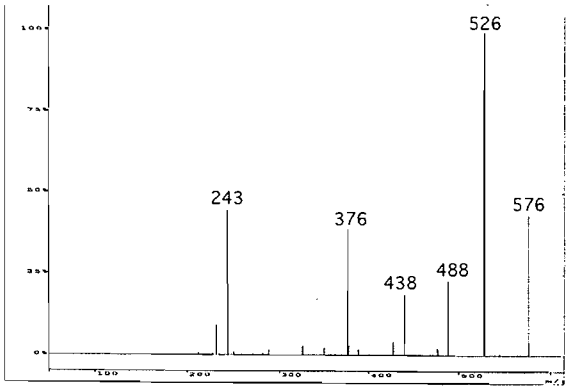


Figure 1 : Full scan mass spectra (ion trap) of a) F44-E (M=464), b) FMCP (M=595) c) FDC (M=462), d) PFOB (M=498)



a)



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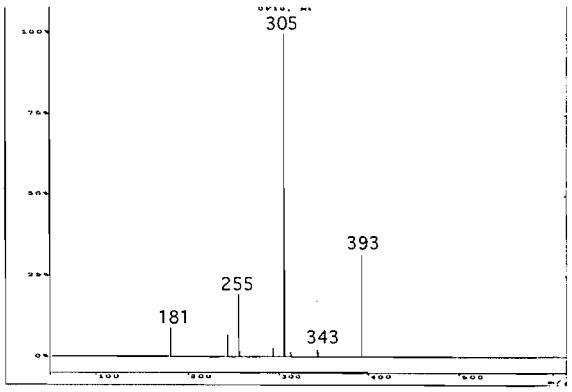
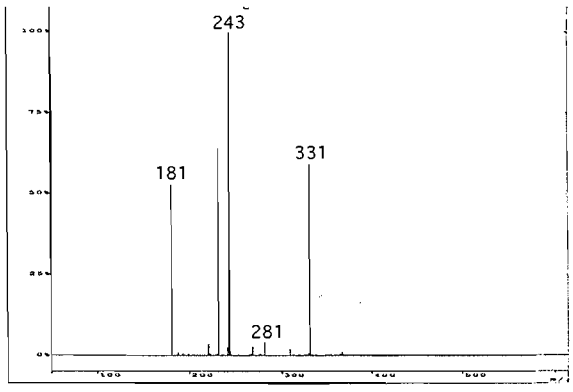


c)



d)

Figure 2 : MS/MS mass spectra (ion trap) of a) F44-E (m/z 445, 90V: q_z=0.35), b) FMCP (m/z 576, 85V: q_z=0.2), c) FDC (m/z 393, 80V: q_z=0.3) d) PFOB (m/z 331, 80V: q_z=0.4)

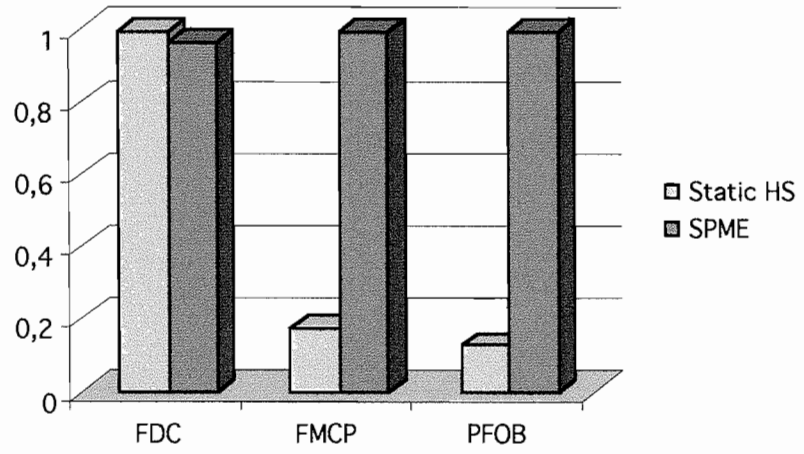


Figure 3 : Comparison between Static Head-Space/GC/MS (500 µl injected) and SPME/GC/MS for three PFCs in air samples

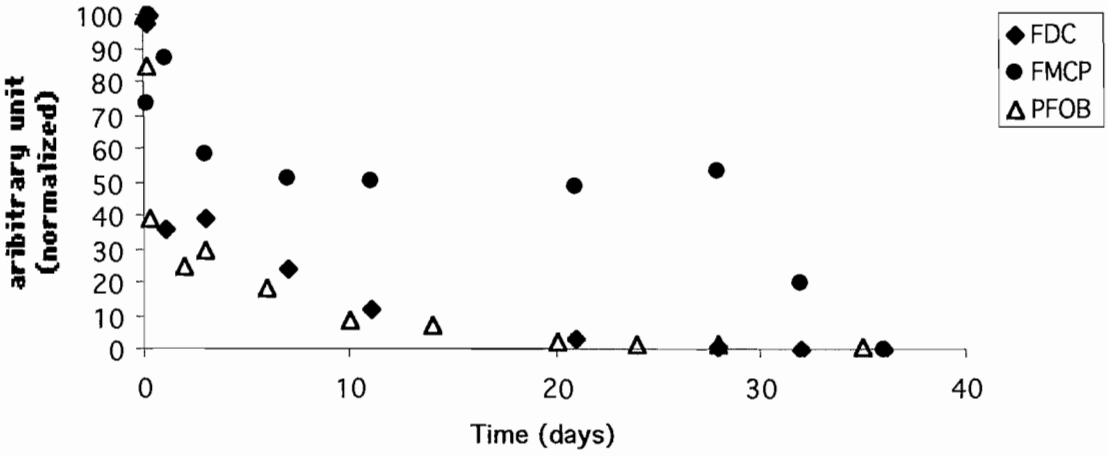


Figure 4 : Measurements of three PFCs in air samples as a function of time, after *i.v.* injection

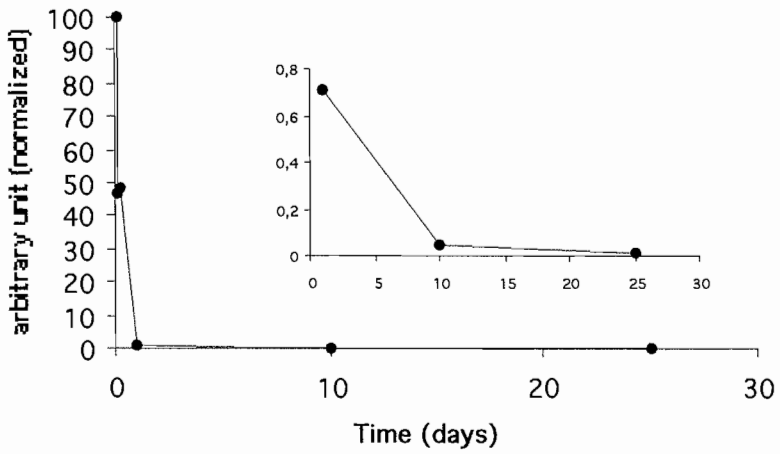


Figure 5 : Measurements of PFOB in blood samples as a function of time, after *i.v.* injection.

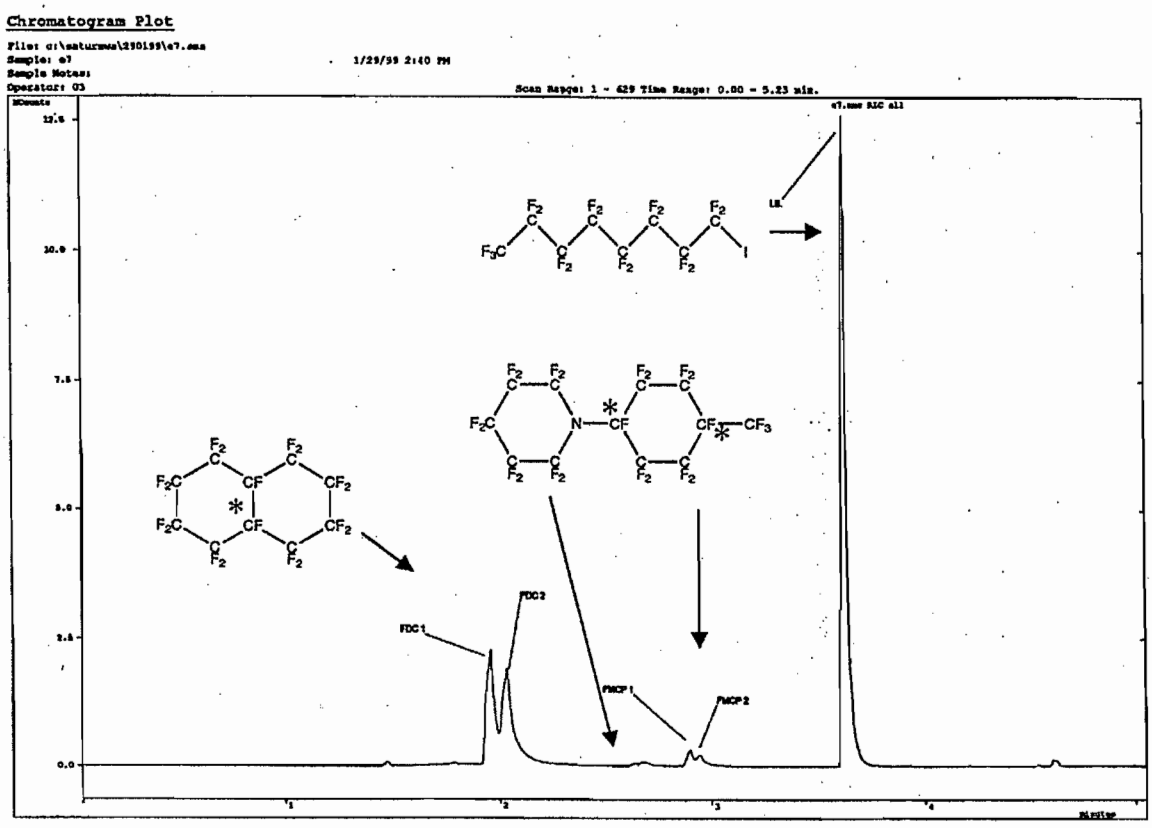


Figure 6 : Chromatogram of a blood sample by SPME

(6 hours after *i.v.* injection of emulsion B)