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Rapid Analysis of Tetracycline Antibiotics by Combined Solid Phase Microextraction/High Performance Liquid Chromatography/Mass Spectrometry

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The technique of solid phase microextraction (SPME) combined on-line with high performance liquid chromatography/mass spectrometry (HPLC/MS) has been applied to the analysis of seven tetracycline analogues. Rapid baseline separation was achieved in under 5 min using a short 3 μm RP-18e cartridge column. Optimisation of the SPME procedure is described including choice of extracting fibre and modification of the sample by heating or salting out of the analytes. Detection limits of 4–40 ng/mL were obtained for the various analogues from extracted aqueous samples and absolute amounts of analyte extracted by the method determined using external calibration. To demonstrate the applicability of the technique for real samples the extraction of tetracycline from milk is described. Copyright © 1999 John Wiley & Sons, Ltd.

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The tetracycline group of antibacterial agents has become widely used in veterinary practice and animal rearing for both therapeutic and prophylactic purposes. They have been licensed for use in a variety of food-producing animals including cattle, pigs, sheep, poultry and fish,[1] in which they function as broad-spectrum bacteriostatic agents by inhibiting protein synthesis. Their basic structure consists of a hydronaphthacene backbone containing four fused rings. The various analogues differ primarily by substitutions of the fifth, sixth or seventh position on the backbone (Fig. 1).

The use of tetracycline and its analogues as animal feed additives has led to concerns about their contamination of animal products destined for human consumption. Tetracycline residues have been widely identified in meat products collected from slaughtered animals[2,3] and in commercial milk supplies,[4–7] resulting in the implementation of maximum residue limits. Thus, their determination at low levels is an important analytical problem.

A variety of methods has been proposed for the analysis of tetracyclines in biological matrices. Although microbiological assays have been most commonly used, they are time consuming and lack specificity. High performance liquid chromatography (HPLC) has been increasingly successfully applied,[8,9] but the biological nature of the sample matrix necessitates sample pre-treatment. Oka *et al.*[10] recently reviewed the current extraction, clean-up, and analysis techniques for the determination of tetracycline contaminants in such matrices. Solid phase extraction with C_{18} cartridges is a common choice for sample preparation.[11] However, poor recoveries often necessitate cartridge or sample pre-treatment with ethylenediaminetetraacetic acid (EDTA).[12]

Tetracycline and its analogues are known to combine with metal ions in aqueous media.[13] This property has been utilised in the development of metal chelate affinity chromatography (MCAC) as a selective clean-up technique prior to the HPLC analysis of tetracyclines.[5,14–16] However, the eluant from the MCAC still contains components detrimental to the performance of the HPLC column, and C_{18} solid phase extraction or ultrafiltration have been described as further de-proteinisation steps.[5]

The application of HPLC/MS confers a significant advantage in the confirmation of residual tetracyclines. However, most previously reported LC conditions use involatile additives in the mobile phase to improve HPLC peak resolution, which are incompatible with the operation of a typical LC/MS interface due to the effects of solid deposition. Oka *et al.*[17] have recently described the electrospray HPLC/MS/MS analysis of tetracyclines using a volatile (acetonitrile/water) mobile and an alkyl-bonded silica gel column synthesised from a 99.99% pure silica column. Other approaches to mass spectral analysis of tetracyclines, such as fast-atom bombardment (FAB), thermospray (TSP), atmospheric pressure chemical ionisation (APCI), etc., are described in Oka's recent review.[10] The ability of tetracyclines to bind metal ions, and the relative efficiencies of ionisation by metal ion attachment or protonation, were recently probed using a quadrupole ion trap by Vartanian *et al.*,[18] with protonation proving to be the more efficient method.

The purpose of the present study was to develop a rapid, simple, extraction technique utilising solid phase microextraction (SPME) for tetracyclines from aqueous media, in combination with fast analysis by LC/MS. The advantages of SPME include simplicity, speed of operation, and the

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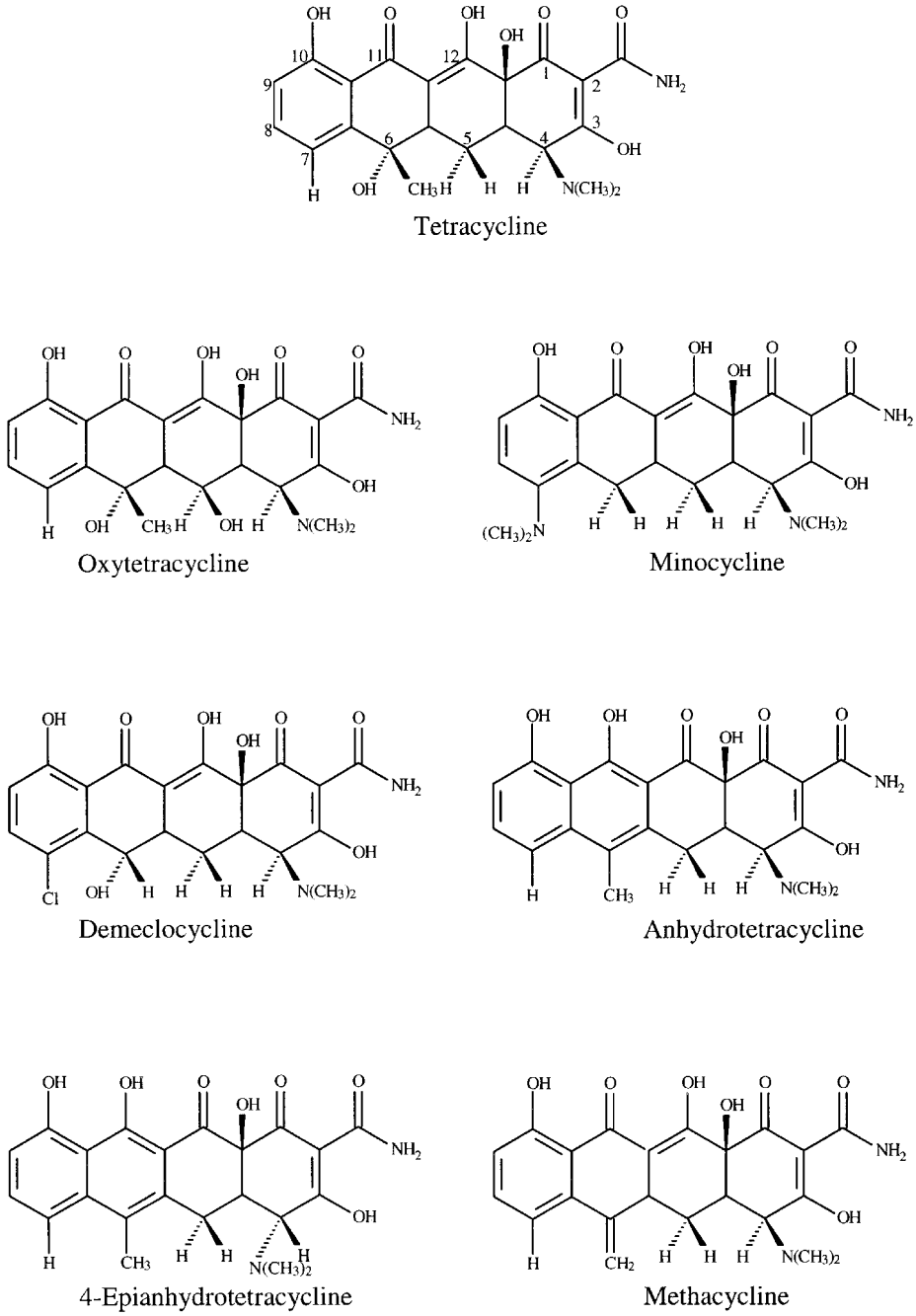

Figure 1. Structures of the tetracycline analogues investigated.

reduction in solvent requirements. The SPME technique was first described by Pawliszyn *et al.*[19,20] and has been routinely used in tandem with gas chromatography (GC) for a number of years. More recently SPME in combination with HPLC was introduced,[21–23] utilising solvent mobilisation as opposed to thermal desorption of the analytes from the SPME fibre. SPME/HPLC has gained increasing interest as an analytical technique, and its coupling to MS has been extensively investigated by Volmer *et al.*[24–26]

EXPERIMENTAL

Chemicals

Oxytetracycline, minocycline, methacycline, demeclocycline, anhydrotetracycline and 4-epi-anhydrotetracycline were obtained as their hydrochloride salts, in addition to tetracycline, potassium chloride and formic acid, from Sigma-Aldrich (Mississauga, ON, Canada). Acetonitrile (Caledon, Georgetown, ON, Canada) and Milli-Q organic free water (Millipore, Bedford, MA, USA) were used as solvents.

SPME/LC procedure

An SPME/HPLC interface from Supelco (Bellefonte, PA, USA) was used during this investigation. Four SPME fibres were initially evaluated: 60 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB), 85 μm polyacrylate (PA), 50 μm Carbowax/templated resin (CW/TPR) and 65 μm Carbowax/divinylbenzene (CW/DVB). 3.5 mL of each aqueous sample for extraction were placed in a 4 mL glass

vial and rapidly stirred with a magnetic stirring bar. The solution was then heated to 65 °C in a hot water bath to aid extraction of the analytes. The solution was saturated with KCl and the entire SPME fibre immersed in the liquid. Subsequently the fibre was transferred to the desorption chamber previously filled with acetonitrile/water, 15:85 (v/v). The chamber was heated to 40 °C to aid desorption of the analytes which occurred in static mode for a period of 5 min, before exposing the chamber to the initial mobile phase flow of the HPLC. The fibre remained exposed to the solvent gradient for the entire length of the analysis, after which the mobile phase gradient was repeated to eliminate any carryover from the fibre into the next analysis. The fibre was then removed and the chamber flushed with 100% acetonitrile. The extracted fibre was first washed under a stream of distilled water and subsequently immersed in a rapidly stirred vial of water to remove any residual organic solvent. The fibre remained immersed until required for the next extraction. This procedure was found to significantly improve the reproducibility of the extraction efficiency, compared with conventional drying of the fibre.

LC procedure

The HPLC system used was a Model 1090 liquid chromatograph (Hewlett Packard, Palo Alto, CA, USA). A PuroSphere cartridge column (4.0 × 50 mm), packed with 3 μm RP-18e stationary phase from Merck (Darmstadt, Germany) was used at a flow rate of 1 mL/min. The solvents were acetonitrile + 0.2% formic acid (A) and water + 0.2% formic acid (B). The separations were achieved with the following mobile phase gradient program: at 0 min A/B = 16:84, 1.5 min A/B = 24:76, 2 min A/B = 30:70, 7 min A/B = 50:50. The initial mobile phase composition was slightly higher in organic modifier than the SPME desorption mixture to reduce the effects of chromatographic peak broadening.

Ionspray mass spectrometry and MS/MS

Ionspray data were acquired using an API 300 (Sciex, Concord, On, Canada) triple quadrupole mass spectrometer, ($Q_1q_2Q_3$). A spray voltage of 4.5 kV, a ring voltage of 300 V and an orifice skimmer potential of 25 V were used. Ultrapure nitrogen was used as the nebuliser and curtain gas at a flow rate of 1.0 and 1.1 L/min, respectively. The mobile phase was split post column, delivering ~50 μL/min to the mass spectrometer. Q_1 was scanned over the range *m/z* 350–470 for conventional full scan experiments. Tandem mass spectrometry experiments (MS/MS) were performed using nitrogen collision gas at a pressure of 2.2 mTorr in q_2 and a laboratory collision energy of 35 eV.

RESULTS AND DISCUSSION

LC separation and electrospray MS/MS

The LC method used a short, base-deactivated column similar to that recently described for the analysis of antibiotic polyether ionophores,[27] to enable rapid baseline separation of all seven tetracyclines studied in only 4 min, using a binary solvent system. The more polar tetracyclines (minocycline, oxytetracycline, demeclocycline, tetracycline) proved to possess very rapid elution times, necessitating the use of a high initial aqueous percentage in the mobile phase to obtain good chromatographic peak resolution. SPME desorption is normally carried out at high levels of organic modifier (typically 50% methanol). However, the large volume of the desorption chamber, ~200 μL, resulted in the composition of the desorption solvent exerting a significant influence on the chromatography of the more polar tetracyclines. A high percentage of organic modifier (acetonitrile in this case) initially resulted in the co-elution of these analytes, necessitating a reduction in the percentage of organic modifier used for desorption down to 15%, as described above.

The highly efficient separation of a mixture of seven tetracyclines achieved by the short C_{18} column used throughout this investigation is demonstrated in Fig. 2. A 20 μL aliquot of an aqueous mixture of the tetracyclines, with each analyte at 1 ppm, was injected via the HPLC instrument onto the column. Separation was carried out using the solvent gradient described above using a full Q_1 scan of *m/z* 350–470. The elution order along with retention times are summarised in Table 1.

All peaks are less than 7 seconds in width, requiring rapid scanning of the mass spectrometer to obtain an adequate number of data points across the peak from each analyte. The predominant ion observed for all analytes, under the acidic mobile phase conditions used (pH 2–3), was $[M + H]^+$. Low energy fragmentation (5 eV) indicated that, for those tetracyclines possessing a hydroxyl group at the C_6 position (tetracycline, oxytetracycline and demeclocycline), the $[M + H - H_2O]^+$ and $[M + H - H_2O - NH_3]^+$ ions were the most abundant fragments. $[M + H - NH_3]^+$ was also observed to a lesser extent with the loss of ammonia occurring from the common carboxyamide group. For the remaining tetracyclines analysed, which lack this hydroxyl group, the $[M + H - NH_3]^+$ fragment ion dominated with no evidence for the formation of $[M + H - H_2O]^+$ or $[M + H - H_2O - NH_3]^+$. In order to produce more diagnostic ions a higher collisional energy of 35 eV was adopted. This generated an abundance of structurally informative fragment ions, the most significant of which are summarised in Table 1. Under this higher energy regime, for those analytes with the C_6 hydroxyl group, the formation of $[M - 35]^+$ dominated, with a lesser abundance of $[M - 17]^+$; $[M - 18]^+$ was no longer observed suggesting that it is formed by a lower energy process. The remaining analytes all produced dominant $[M - 17]^+$ fragment ions, with some evidence of $[M - 35]^+$ at this higher energy. Several fragment ions were identified as being characteristic of these tetracyclines and were common to the fragmentation spectra of those studied, namely *m/z* 154, 126, 98. The commonality of these fragments would suggest them as excellent diagnostic ions for the identification of tetracycline analogues in complex sample mixtures. Possible structures for these fragments were proposed recently by Vartanian *et al.*[18] The fragmentation patterns of protonated tetracyclines have been reported previously,[10,18,28–30] and therefore are not presented in detail here.

Log P and log D predictions

It has been shown that, for liquid chromatography,[25,31] the elution for a series of analytes can be predicted by determining their apparent octanol-water partition coefficients (log D) under the pH conditions of the mobile phase. The partition coefficient of the neutral species (log P), on the other hand, might be useful in predicting relative extraction efficiencies obtained by the SPME procedure.

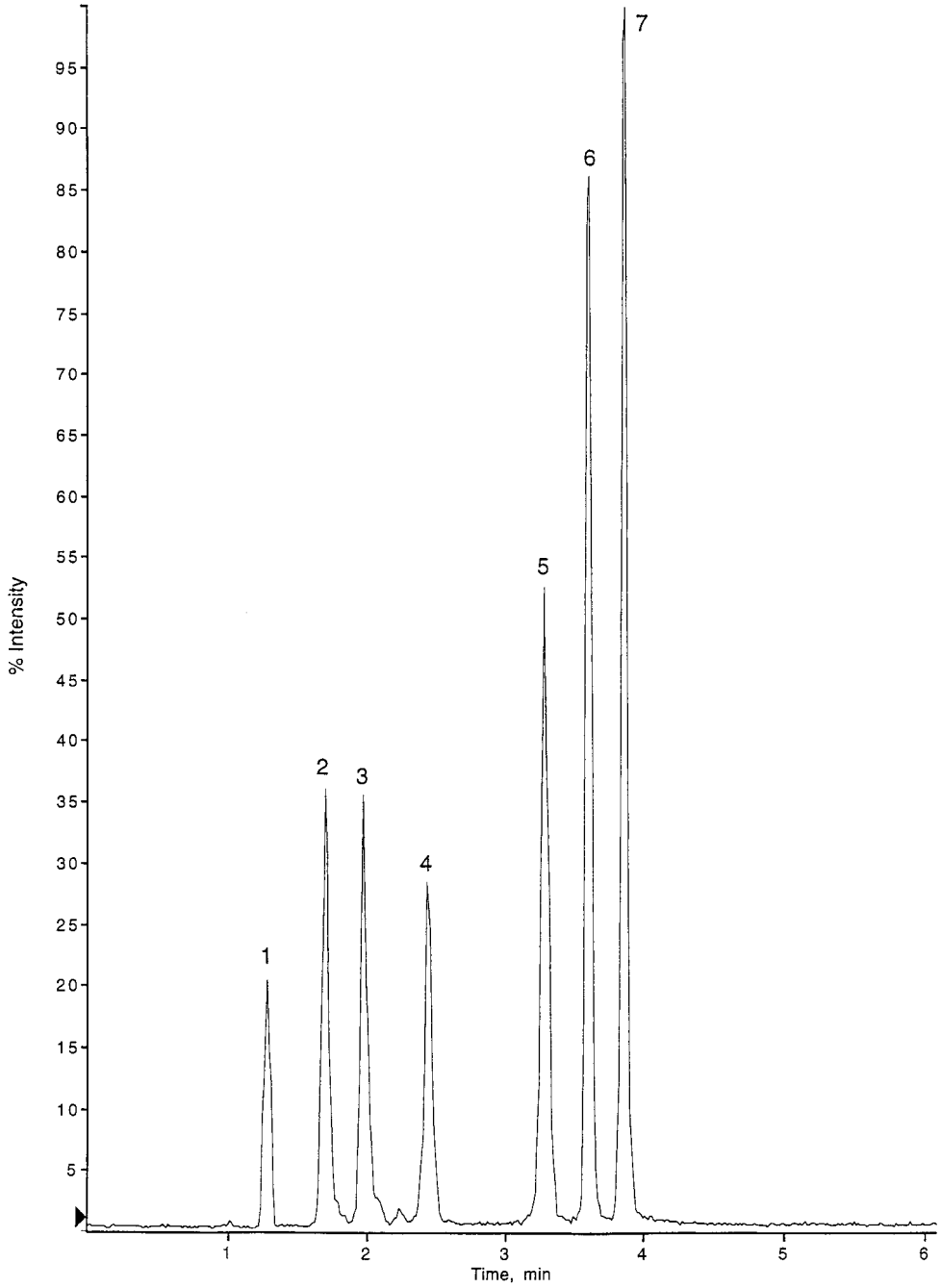

Figure 2. Total ion chromatogram (Q_1 scan *m/z* 350–470) obtained by a short column separation of a 20 μL aqueous aliquot spiked at 1 ppm with the tetracyclines under investigation.

Table 1. Retention and MS/MS data for the tetracycline compounds investigated

No.	Compound	Calculated[a] log P	Calculated[a] log D	t_r (min)	$[M+H]^+$	MS/MS (35 V, 2.75 mTorr N_2) major product ions (*m/z*)
1	Minocycline	−1.01	−5.10	1.29	458	441, 352, 283, 154
2	Oxytetracycline	−3.34	−5.35	1.70	461	426, 365, 337, 226, 201, 154
3	Tetracycline	−1.94	−4.26	1.90	445	410, 337, 269, 241, 154
4	Demeclocycline	−1.39	−3.83	2.45	465	430, 289, 154
5	Methacycline	−2.06	−4.86	3.29	443	426, 381, 226, 201, 154
6	4-epi-Anhydrotetracycline[b]	0.05	−2.68	3.61	427	410, 321, 269, 154
7	Anhydrotetracycline[b]	0.05	−2.68	3.86	427	410, 321, 269, 154

[a] Theoretical calculations performed using the PrologD 2.0 and PrologP 5.1 modules of Pallas 2.0 software (Compudrug).
[b] Structures are described in 2D for the calculations, therefore no distinction can be made between conformers by the Pallas software.

Theoretical calculations of log P and log D values for the tetracyclines under analysis were performed using the PrologD 2.0 module of the Pallas-for-Windows software (Compudrug, Chemistry/VCH Software, Weinheim, Germany), and are included in Table 1. A comparison of the order of elution with that predicted by the log D values shows fairly good agreement, with the notable exception of methacycline and, to a lesser extent, minocycline. While a log D value of −5.10 for minocycline is only slightly out of order, a log D value of −4.86 for methacycline differs significantly from that expected from its relative retention time. This discrepancy between experimental observation and theory may stem from the fact that the Pallas software does not account for any interaction between functional groups which may occur as a result of the particular conformation that the molecule adopts. For example, no distinction can be made between anhydrotetracycline and its epimer 4-epi-anhydrotetracyline. It is known from both quantum mechanical[32] and circular dichroism[33] investigations that the conformations of tetracyclines have a pH and solvent dependence as a result of the changes in the charge state of the molecule. Tetracyclines can adopt two distinct families of conformations, one adopted when the molecule exists as a zwitterion or charged species formed in aqueous phases, and the other a non-ionised form in more hydrophobic conditions. The zwitterion possesses a positive charge on the C_4-dimethylamino group and a delocalised negative charge involving the C_3-enolic proton. The two conformations exist in a dynamic equilibrium, each possessing a distinct structure with interconversion requiring rotation about the C_4–C_{12} bond.

In addition, the first conformer, primarily adopted in aqueous solution, can be subdivided into two groups. Under neutral and acidic conditions (in which the molecule exists as a zwitterion or protonated species), a twisted conformer predominates, characterised by the occurrence of an N_4–O_3 hydrogen bond and a twisting of the A-ring to relieve the steric crowding between the C_4-dimethylamino and C_{12}-hydroxyl groups (Fig. 1). In basic conditions deprotonation of the C_4-dimethylamino group enables the extended form to exist, stabilised by an N_4–O_{12} hydrogen bond.

The ability to adopt the twisted structure explains the significant difference observed in retention time, and hence hydrophobicity, between the anhydrotetracycline epimers. Under the acidic conditions of the mobile phase anhydrotetracycline will adopt the twisted conformation described above. However, the structure of 4-epi-anhydrotetracycline is such that the C_4-dimethylamino group is now in a *trans* position relative to the C_{12}-hydroxyl group, and therefore can no longer form the required hydrogen bond to generate the twisted conformer. Thus, the important conformational changes that the tetracycline compounds can undergo reasonably explains the discrepancy observed between retention times predicted by log D values and those obtained experimentally. It would be expected, however, that the SPME extraction efficiency should closely parallel the relative retention times of the analytes. The longer eluting compounds possess higher hydrophobic character, and therefore would be expected to partition more favourably into the SPME fibre from the aqueous solution than the earlier eluting, more hydrophilic, analytes.

SPME fibre evaluation

Volmer *et al.*[24–26] recently detailed the evaluation of SPME fibres for SPME/HPLC analysis of a variety of different analytes including steroids and carbamate pesticides. Four fibres were selected for evaluation to perform the extraction of the tetracyclines from aqueous solution, namely 60 μm PDMS/DVB, 85 μm PA, 65 μm CW/DVB and 50 μm CW/TPR. The standard procedure for conditioning new fibres is to expose them to the mobile phase gradient flow for 30 min (acetonitrile/water in this study). However, the CW/TPR, CW/DVB and PDMS/DVB fibres were found to be highly susceptible to damage to the resin by this method. Extensive pitting of the polymer coating was frequently observed both visually and with the aid of an optical microscope. The loss of solid phase material was extensive enough that the non-adsorbing fibre core beneath the solid phase was often revealed. Conditioning the fibre by three 10 min periods of exposure to the initial mobile phase, with drying of the fibre in between, appeared to resolve this problem. Thereafter, the resin became much more stable with no loss of material observed. Unfortunately the CW/DVB fibre had to be removed from the study as the solid phase proved to be readily stripped from the fibre under the experimental conditions used.

A comparison of the relative extraction efficiencies, under identical conditions, was made for the three remaining fibres. Each fibre was immersed for 15 min in a rapidly stirred aqueous mixture of all seven analytes, each spiked at 5 ppm. The analytes were then desorbed from the fibre as described previously and analysed by HPLC/MS. The procedure was carried out in triplicate for each fibre to evaluate reproducibility. Figure 3 shows a typical LC/MS chromatogram obtained from each fibre, using a Q_1 scan of *m/z* 350–470. As expected, and in agreement with the observations of Volmer *et al.*,[24–26] the CW/TPR fibre exhibited the best performance closely followed by the PDMS fibre. The polyacrylate fibre proved to be particularly poor in its extraction of these analytes. The CW/TPR fibre was therefore selected for further use in this study.

Optimisation of the SPME procedure

Quantitative SPME relies on establishing equilibrium of the sample analyte between the aqueous solution and the solid phase. Therefore, a number of factors can be manipulated in order to push the equilibrium point further towards the extracting medium or to increase the speed at which it is attained. In order to investigate this aspect a series of experiments were conducted with the CW/TPR fibre. Rapid stirring of the aqueous sample is a common characteristic of liquid phase SPME extractions. This is thought to enhance recovery by constantly exposing the fibre to fresh sample and reducing the layer of 'static' liquid next to the fibre surface through which any analyte must diffuse before absorption.

The efficiency of SPME has been shown to be enhanced by increasing the ionic strength of the aqueous sample solution,[22,23] an effect analogous to the salting out technique frequently used to remove proteins from biological matrices. In agreement with these earlier investigations, it was found that increasing the ionic strength by the addition of NaCl or KCl had a marked improvement on the extraction efficiency from an aqueous solution of all seven tetracyclines investigated.

The use of elevated temperatures enables the use of higher ionic strength solutions due to the increased solubility of the salts. The extraction efficiency obtained

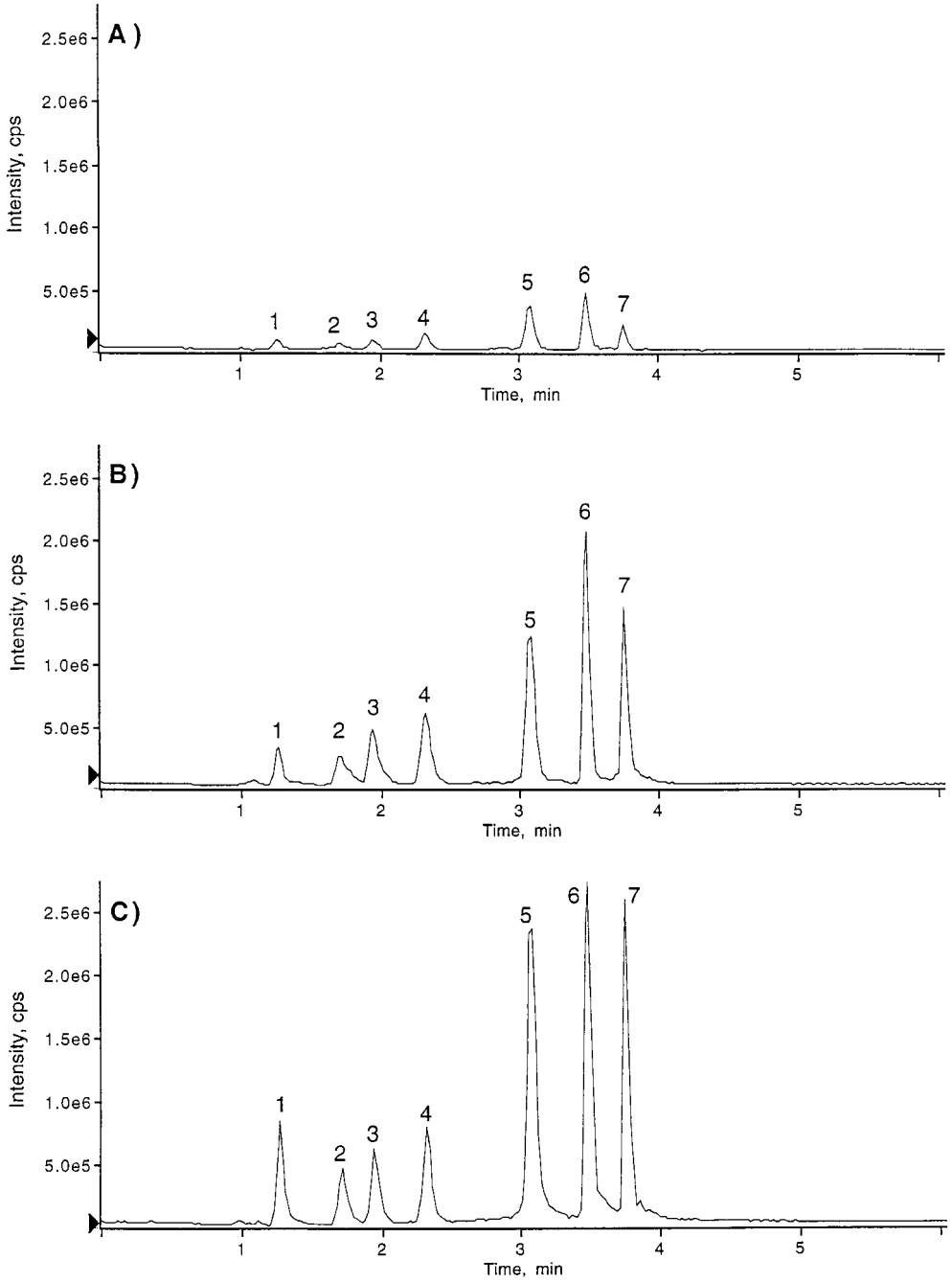

Figure 3. Comparison of the relative extraction efficiency of the (a) 85 μm PA, (b) 60 μm PDMS/DVB and (c) 50 μm CW/TPR SPME fibres under identical conditions from an aqueous mixture of the tetracyclines investigated.

by spiking with NaCl appeared to maximise and eventually decrease with increasing ionic strength, but spiking with KCl continued to improve the extraction yield up to saturation of a solution at 65 °C. KCl therefore became the preferred choice due to its increased performance relative to NaCl at elevated temperatures. As a consequence all future samples for extraction were spiked with KCl beyond the point of saturation prior to analysis so that, even when heated to 65 °C, some undissolved KCl remained. Increasing the temperature of the sample solution might at first seem counter-productive as it results in a corresponding increase in the aqueous solubility of the contained analytes. However, this is more than compensated for by the significant increase in the diffusion rate of the analytes enabling the equilibrium to be much more rapidly obtained. This results in a much faster extraction rate over the initial time period of exposure of the fibre. All extractions were therefore carried out with the sample vial immersed in a water bath at 65 °C. The relative extraction efficiencies of the CW/TPR fibre under various conditions, when exposed to an aqueous mixture of the tetracyclines at 1 ppm for 15 min, are summarised in Fig. 4. It is evident that the saturation of the solution with KCl results in a substantial increase in recovery for all analytes. The increase in extraction efficiency attained by heating and salting the sample with KCl, relative to extraction from a mixture prepared solely in de-ionised water at room temperature, is indicated in Table 2. Increasing hydrophobicity seems to

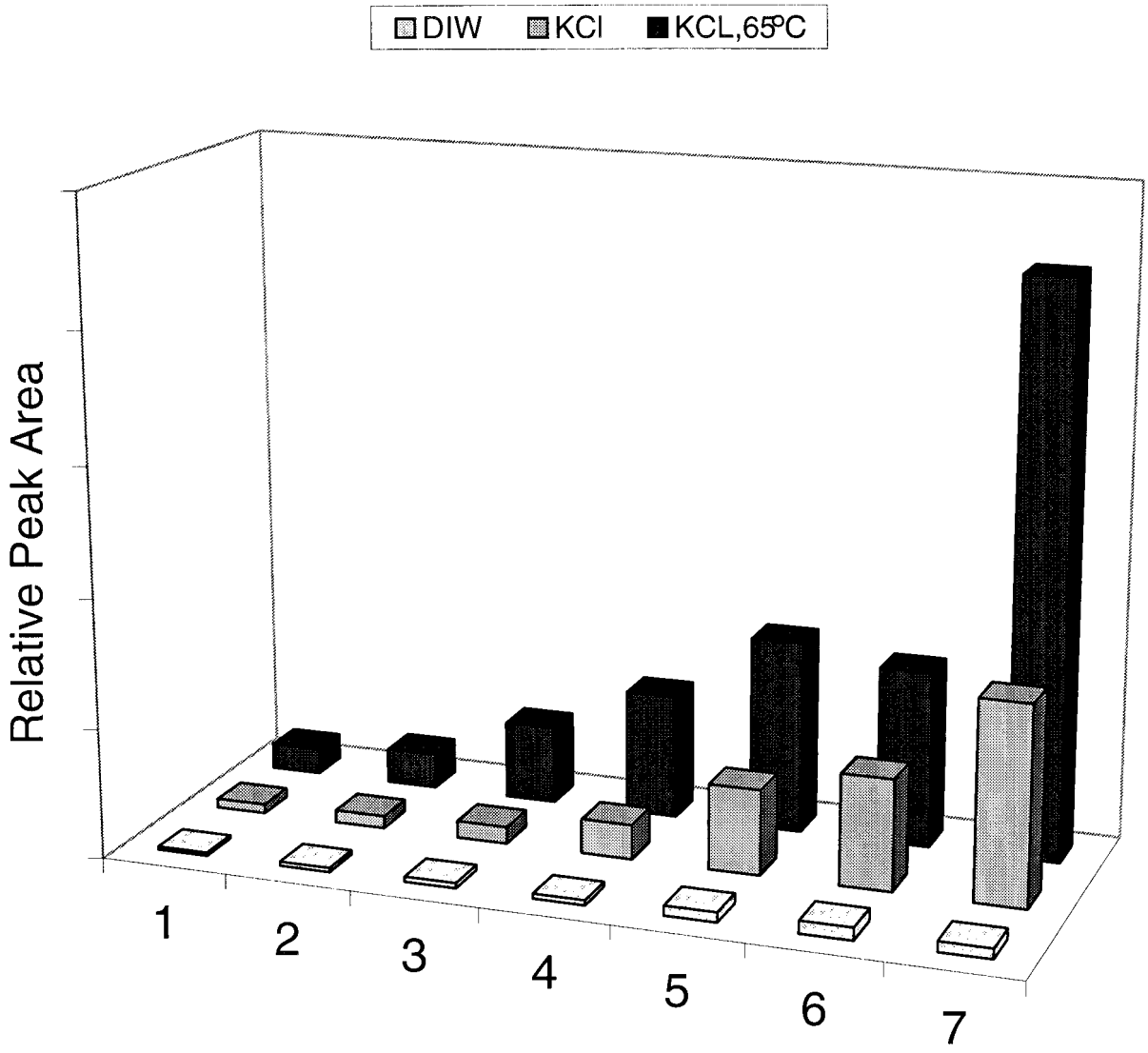

Figure 4. Comparison of the extraction efficiency of the 50 μm CW/TPR fibre from an aqueous solution of the tetracyclines (DIW) an aqueous solution saturated with KCl (KCl) and a heated aqueous solution at 65 °C saturated with KCl (KCL, 65 °C).

coincide with increasing extraction efficiency, as expected. A determination of the extraction efficiencies of anhydrotetracycline and it's 4-epimer was made difficult by the known re-epimerisation reaction that occurs between them. It is clear that a significant conversion of 4-epi-anhydrotetracycline to anhydrotetracycline occurs during the heated extraction procedure. Evidence for this conversion is also seen to a lesser extent in the unheated extracted sample. Such re-epimerisation reactions have been observed previously for other tetracycline analogues, for example, the conversion of 4-epi-oxytetracycline to oxytetracycline at elevated temperatures by Prewo *et al.*[34] No evidence was

Table 2. Improvement in extraction efficiency obtained by addition of KCl and heating of the sample in addition to the ultimate limit of detection using the procedure developed for each analyte from an aqueous solution

No.	Compound	E_s/E_w[a]	E_{sh}/E_w[a]	Mass extracted[c] (μg)	Limit of detection[d] (ng/mL)
1	Minocycline	2.9	8.3	0.17	40
2	Oxytetracycline	3.0	8.4	0.20	30
3	Tetracycline	3.3	15.0	0.25	14
4	Demeclocycline	7.1	25.0	0.57	9
5	Methacycline	8.5	19.1	0.60	6
6	4-epi-Anhydrotetracycline[b]	8.8	13.9	0.31	<4
7	Anhydrotetracycline[b]	19.0	55.0	0.92	<2

[a] Ratio of peak areas obtained for extraction from an aqueous sample, E_w a sample saturated with KCl, E_s and a heated sample saturated with KCl, E_{sh}.
[b] A direct measurement of extraction efficiency for these two epimers is difficult to due the re-epimerisation reaction described previously resulting in the conversion of 4-epi-anhydrotetracycline to anhydrotetracycline.
[c] Absolute amount of analyte adsorbed by the fibre (μg) at a solution concentration of 1 μg/mL.
[d] A signal-to-noise ratio of 3:1 is assumed.

observed for significant epimerisation of any of the remaining tetracycline analytes investigated here. The use of elevated extraction temperatures clearly produces an increased recovery for all analytes over the time frame of analysis.

Extraction time

The most rapid absorption by the fibre occurs in the initial period of exposure, due to the overwhelming imbalance in the relative concentrations of the analytes between the aqueous and solid phase. As equilibrium is approached the relative rate of absorption is expected to decrease. This is clearly evident in Fig. 5, which shows a characteristic extraction profile obtained using the CW/TPR fibre for four of the tetracyclines under analysis. In each case the fibre was exposed to a rapidly stirred aqueous mixture at room temperature (saturated with KCl) of all four analytes for the specified time, and the extraction yield determined by LC/MS. As a comparison the profile obtained for methacycline extracted from an identical solution at 65 °C has been included. Not only does the extraction show an increased initial rate compared with that when carried out at ambient temperature, but also the absolute amount of analyte extracted has been increased significantly. Similar plots were obtained for the other tetracyclines under analysis.

SPME/LC mass spectrometric analysis of tetracyclines from aqueous and milk samples

Using the optimised procedures described above, the extraction, separation and analysis of the seven selected tetracyclines were conducted using single ion monitoring (SIM) for the precursor ions of interest. A typical chromatogram obtained from SPME extraction of 3.5 mL of a 1 ppm mixture of the analytes is shown in Fig. 6(a). The sensitivity of the method was determined by directly injecting 200 μL of the sample solution into the desorption chamber (which contained an SPME fibre stripped of its solid phase coating), and proceeding with the analysis by LC/MS as before, shown in Fig. 6(b). It can clearly be seen that the two methods give comparable sensitivity for the more hydrophilic analytes minocycline and oxytetracycline, while the later eluting tetracyclines are detected with increasing sensitivity by the SPME method. A 200 μL injection of a 1 ppm mixture represents 0.2 μg of each analyte injected onto the column. Since both analyses were carried out under identical conditions the extent of recovery of each analyte by the SPME method can be determined from the respective chromatographic peak areas obtained. The yields of recovery determined from a theoretical maximum of 3.5 μg per analyte are included in Table 2. The limits of detection (LOD) attained are also shown (Table 2), and represent analysis using the present multi-residue method with SIM of the respective known precursor ions for each analyte. If identification of only a single compound is required the LOD will naturally improve accordingly, since Q_1 can be set to exclusively monitor the particular ion of interest.

When considering real samples such as milk, which may contain both inorganic particulates and/or biological matrix components, direct injection of a sample onto the column is extremely unwise. The limit of detection of the SPME/LC/MS method was explored by examining the analysis of tetracycline spiked at the 100 ppb level into each of water and milk. It was found, however, that the salting out procedure using KCl caused precipitation of the protein component of the milk sample. This caused the viscosity of the sample to rapidly increase, preventing an efficient SPME extraction from the sample. Thus, while the present

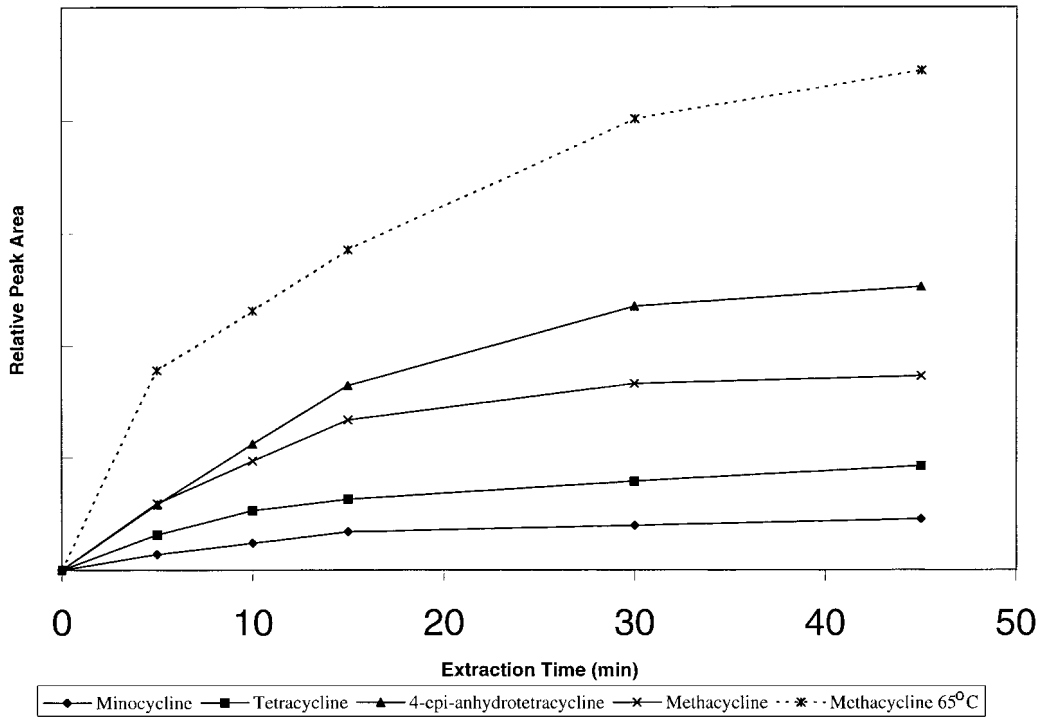

Figure 5. The variation in total analyte extracted with time from aqueous solutions saturated with KCl of the four tetracyclines indicated at 5 ppm. The extraction profile obtained for methacycline at 65 °C is shown, demonstrating the enhanced initial extraction rate and increase in overall yield.

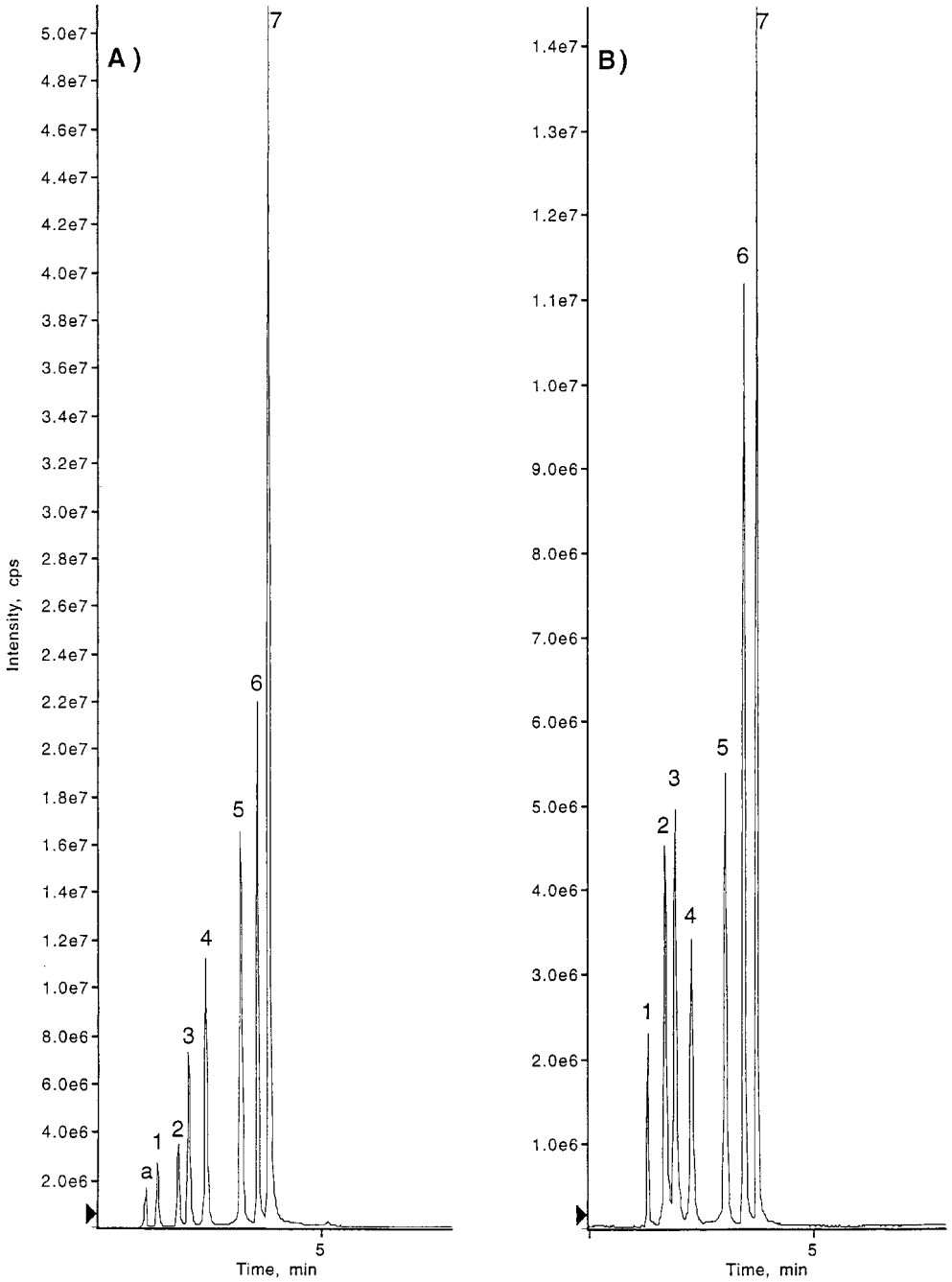

Figure 6. Comparison of the sensitivity of the SPME method (using the protocol developed) (a) for a 1 ppm aqueous solution of the tetracycline mixture with (b) direct injection of a 200 μL (SPME desorption chamber volume) aliquot of the mixture. Peak 'a' indicates the initial salt peak at the solvent front.

SPME method developed proved excellent in its recovery of analyte from aqueous samples, it could not be directly applied to extraction from a biological matrix such as milk with a high protein and lipid content. No such problems would be envisaged from a more SPME friendly sample such as urine. It would be feasible to filter the sample to remove the denatured protein before proceeding with the SPME extraction. However, the purpose of this investigation was to develop a rapid method avoiding such purification procedures. It was therefore decided to omit the spiking with KCl from the extraction procedure, and to rely on only heating the milk sample to increase the extraction efficiency.

After confirming the absence of tetracycline residues in the milk, obtained by performing the extraction of a blank sample, 3.5 mL of milk spiked at 100 ppb with tetracycline were extracted using the present method but omitting the KCl. SIM analysis for the protonated tetracycline ion at *m/z* 444.8 was used to increase sensitivity (see Fig. 7(a)). The LOD obtained by this method, after extracting for 30 min, was determined to be ~100 ppb from 2% homogenised milk, assuming a 3:1 signal-to-noise ratio. The LOD could be improved further by increasing the time period over which the extraction takes place. The LOD is thus in accordance with the European Economic Community (EEC) regulatory limit for tetracycline contamination in milk set at 100 μg/kg or 100 ppb and slightly lower than the 80 ppb limit imposed by the US Food and Drug

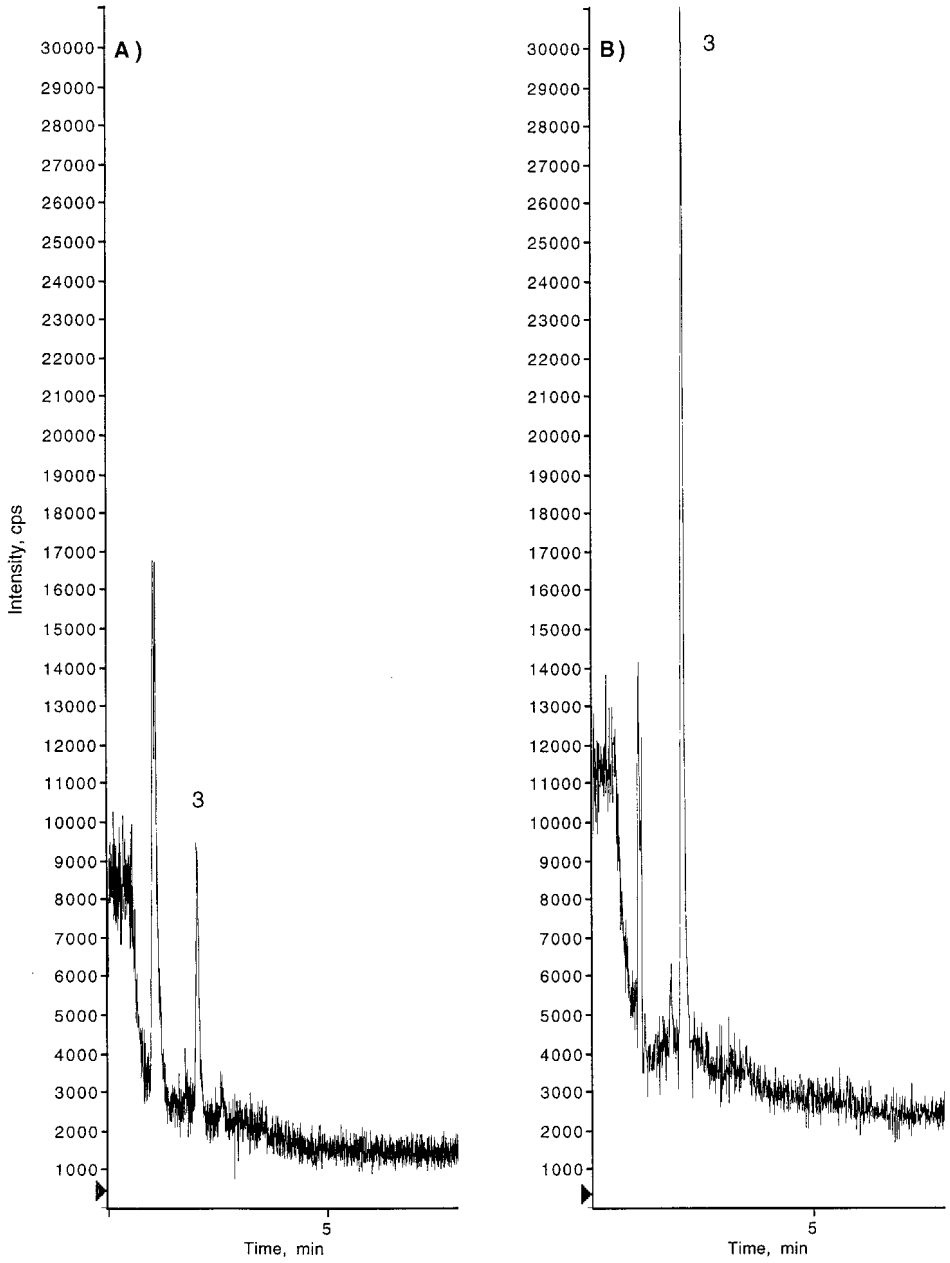

Figure 7. SPME analysis of (a) tetracycline from a milk sample spiked at 100 ppb and (b) tetracycline from an aqueous sample spiked at 100 ppb.

Administration. The extraction profiles obtained for the remaining tetracycline analogues indicate that the more hydrophobic analytes will possess improved LOD than tetracycline itself. As a comparison, to determine the influence on the extraction efficiency of the hydrophobic components present in the milk, an identical extraction from an aqueous sample was carried out (Fig. 7(b)). The recovery was improved by a factor of three to less than 30 ppb LOD, indicating either that tetracycline has an increased solubility in milk relative to a purely aqueous medium or that components within the milk physically interfere with the extraction into the fibre. Increased solubility is a plausible explanation since the extraction procedure itself relies on the analyte diffusing into a more hydrophobic environment. This suggests that any means of removing the hydrophobic content of the milk, in order to permit addition of KCl to improve the extraction efficiency, may result in a loss of analyte if the removal is a purely physical rather than a chemical process. This will be especially true if the tetracycline analogues become bound to proteins within the biological matrix impeding their adsorption by the SPME fibre. Krogh[35] demonstrated this effect with the SPME extraction of the benzodiazepine, diazepam, from plasma. Diazepam exhibits high binding affinity with plasma proteins. Krogh used the addition of 10% methanol to the sample prior to extraction to lower the binding affinity and enable removal of the precipitated proteins. However, this technique would severely impact on the extraction efficiency of the relatively polar tetracycline compounds.

If the tetracycline analogues can be efficiently extracted by the SPME fibre, which represents a more hydrophobic environment than the aqueous medium, then it is probable that the tetracyclines will partition themselves between the aqueous and more hydrophobic components in the milk, i.e. the protein and lipid components. Tetracyclines are known to move freely within the body between such highly

hydrophobic and aqueous environments. In fact the antimicrobial action of tetracycline is highest in the pH range 5.5–6.0, where the maximum lipid solubility also occurs,[36] consistent with a direct correlation between the two. Further studies to develop improvements in the extraction efficiency from biological samples are currently being explored in addition to the development of the technique as a fully quantitative method for tetracycline analysis.

CONCLUSIONS

The present SPME/LC/MS multi-residue method, developed for the detection of tetracycline antibiotics, has proven to be a rapid and sensitive procedure. The SPME technique eliminates the need for lengthy sample clean-up and is extremely economic in comparison with the wet chemistry procedures typically required. A fast LC/MS short column analytical method enabled rapid analysis of the extracted fibres, with all analytes eluting in less than 5 min. The LC procedure developed is completely MS compatible, requiring no involatile buffers that are frequently used for the analysis of tetracyclines. Modification of the extraction medium by spiking with KCl and/or heating was shown to significantly enhance the recovery of all analytes. The application of the salting out procedure to contaminated milk samples was prevented due to the effects of the precipitation of components in the biological matrix interfering with the SPME efficiency. However, a LOD of 100 ppb for tetracycline (the EEC regulatory limit) was attainable using the SPME technique and heating of the sample. SPME may therefore prove to be a useful screening technique for the identification of tetracycline contamination prior to performing a more sensitive but equally more costly and lengthy analysis by metal chelate affinity chromatography followed by high performance chromatography.[7,15] The Carbowax/templated resin fibre proved the most efficient at extracting the analytes, closely followed by the Carbowax/divinylbenzene fibre. The restricted variety of fibres initially available for the study is an obvious limitation to a more widespread development of the SPME/LC/MS method. It is hoped that with the development of new solid phases compatible with LC, that the sensitivity and versatility of this technique will continue to improve.

REFERENCES

1. Debuf Y (ed). *The Vetinary Formulary*, Pharmaceutical Press: London, 1988; 97.
2. Oka H, Ikai Y, Kawamura N, Hayakawa J. *J. Assoc. Off. Anal. Chem.* 1991; **74**: 894.
3. Oka H, Ikai Y, Kawamura N, Hayakawa J, Ishikawa N, Shibata S, Saito F, Ohmi S, Ando K, Kawada M, Nakanishi K. *J. Food Hyg. Soc. Jpn.* 1995; **36**: 759.
4. Brady MS, Katz SE. *J. Food Prot.* 1988; **51**: 8.
5. Carson MC. *J. AOAC Int.* 1993; **76**: 329.
6. *Food Chemical News*, 1997; **39**: 1.
7. Carson MC. *FDA Veterinarian – Food and Drug Administration*, 1997; **12**: 5.
8. Bryan PD, Stewart JT. *J. Pharm. Biomed. Anal.*, 1993; **11**: 971.
9. Moats WA, Harik-Khan R. *J. Agric. Food Chem.* 1995; **43**: 931.
10. Oka H, Ito Y, Ikai Y, Kagami T, Harada K. *J. Chrom. A* 1998; **812**: 309.
11. Sokol J, Maisova E. *J. Chromatogr. A* 1994; **669**: 75.
12. Walsh JR, Walker LV, Webber JJ. *J. Chromatogr.* 1992; **596**: 211.
13. Day ST, Crouthamel WG, Martinelli LC, Ma J K H. *J. Pharm. Sci.* 1978; **67**: 1518.
14. Farrington WHH, Tarbin JA, Bygrave J, Shearer G. *Food Addit. Contam.* 1991; **8**: 55.
15. Cooper AD, Stubbings GWF, Kelly M, Tarbin JA, Farrington WHH, Shearer G. *J. Chromatogr. A* 1998; **812**: 321.
16. Degroodt JM, Wyhowski de Bukanski B, Srebnik S. *J. Liq. Chromatogr.* 1993; **16**: 3515.
17. Oka H, Yoshitomo I, Ito Y, Hayakawa J, Harada K, Suzuki M, Odani H, Maeda K. *J. Chromatogr. B* 1997; **693**: 337.
18. Vartanian VH, Goolsby B, Brodbelt JS. *J. Am. Soc. Mass Spectom.* 1998; **9**: 1089.
19. Arthur CL, Pawliszyn JB. *Anal. Chem.* 1990; **62**: 2145.
20. Zhang Z, Yang M, Pawliszyn JB. *Anal. Chem.* 1994; **66**: 844A.
21. Chen J, Pawliszyn JB. *Anal. Chem.* 1995; **67**: 2530.
22. Boyd-Boland AA, Pawliszyn JB. *Anal. Chem.* 1996; **68**: 1521.
23. Kiyokatsu J, Muramatsu T, Saito Y, Kiso Y. Magdic S, Pawliszyn J. *J. Chromatogr. A* 1996; **754**: 137.
24. Volmer DA, Hui JPM. *Rapid Commun. Mass Spectrom.* 1997; **11**: 1926.
25. Volmer DA, Hui JPM. *Arch. Enviro. Contam. Toxicol.* 1998; **35**: 1.
26. Volmer DA, Hui JPM. *Rapid Commun. Mass Spectrom.* 1998; **12**: 123.
27. Volmer DA, Lock CM. *Rapid Commun. Mass Spectrom.* 1998; **12**: 157.
28. Voyksner RD, Smith CS, Knox PC. *Biomed. Environ. Mass Spectrom.* 1990; **19**: 523.
29. Kijak PJ, Leadbetter MG, Thomas MH, Thompson EA. *Biological Mass Spectrom.* 1991; **20**: 789.
30. Coleman WM. *J. Chromatogr. Sci.* 1997; **35**: 245.
31. Volmer DA, Mansoori B, Locke SJ. *Anal. Chem. Columbus* 1997; **69**: 4143.
32. Dos Santos HF, De Almedia WB, Zerner MC. *J. Pharm. Sci.* 1998; **87**: 190.
33. Lambs L, Venturini M, Reverend BD, Kozlowski H, Berthon G. *J. Inorg. Biochem.* 1988; **33**: 193.
34. Prewo R, Stezowski JJ. *J. Am. Chem. Soc.* 1979; **101**: 7655.
35. Krogh M, Grefslie H, Rasmussen KE. *J. Chromatogr. B* 1997; **689**: 357.
36. Stezowski JJ. *J. Am. Chem. Soc.* 1976; **98**: 6012.

