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Measurement of the free concentration of octylphenol in biological samples with negligible depletion-solid phase microextraction (nd-SPME): Analysis of matrix effects

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Abstract

A negligible depletion-solid phase microextraction (nd-SPME) method is presented to measure free concentrations of octylphenol in biological samples. Potential confounding factors, such as matrix effects, are studied as well. Fouling of the fibre appears to occur, but it does not seem to reduce or enhance the measured uptake of octylphenol. In the setup applied here, without any agitation, it has also been found that there is a large effect of protein presence on the kinetics of octylphenol uptake. In addition, an apparent affinity constant of octylphenol for bovine serum albumin was determined.

Keywords: nd-SPME; Octylphenol; Matrix effects; Albumin; Fouling; Kinetics; GC–MS; Affinity

1. Introduction

Octylphenol is a degradation product of the surfactant octylphenol ethoxylates. It is found in the environment [1,2] and has shown to possess estrogenic potency [3–5]. Estrogenic effects are often studied in in vitro tests, where free concentrations represent a more intrinsic dose parameter than the often used nominal or total concentration [6]. Therefore, methods are necessary to enable the measurement of free concentrations of xenoestrogens, such as octylphenol, in in vitro assays.

The free concentration of a compound is the concentration of compound molecules that are dissolved in the aqueous phase without being bound to something. There are many methods with which free concentrations can be determined, such as equilibrium dialysis, ultrafiltration and fluorescence quenching [7,8]. For small biological samples, however, negligible depletion-solid phase microextraction (nd-SPME) seems most promising [9]. SPME is a sample preparation technique introduced by Arthur and Pawliszyn [10], which applies the polymer coating of optical fibres as a very small, solid extraction phase. The solid state of the polymer does not necessarily mean that compounds can only adsorb to the surface of the polymer. Some widely-used fibre coatings, such as polyacrylate and polydimethylsiloxane (PDMS), behave as liquids, allowing compounds to be absorbed into the coating. SPME is used to determine total concentrations of chemicals, as is done with solid phase extraction (SPE), with the advantage that smaller samples can be analysed and that the solid phase can directly be desorbed in a GC or HPLC.

The miniature size of this extraction phase has been used by Vaes et al. [11] to perform extractions where the free concentrations were negligibly depleted (nd-SPME), leaving the freely dissolved concentration virtually constant. This way, all binding equilibria remain undisturbed. The concentration of compound in the fibre coating is then directly related to the free concentration in the sample through the partition coefficient. Measuring the fibre concentration after an extraction can thus provide the free concentration of a compound in a sample. The key difference between SPME and nd-SPME is the difference in extracted fraction of the compound of interest: with nd-SPME this has to be negligible, enabling the determination of the free concentration of the compound. With normal SPME there is no such condition. Further details on nd-SPME can be found in a review on this method [9].

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For correct application of nd-SPME in samples containing a binding matrix, such as proteins, the occurrence of matrix effects needs to be verified, as has been explained extensively in Heringa et al. [9]. Matrix effects are defined here as differences in measurement outcomes in samples with and samples without a binding matrix. A binding matrix can theoretically adsorb to the fibre surface (fouling), possibly blocking the uptake of analyte into the fibre coating. Fouling could also lead to an overestimation of the concentration in the fibre coating, as the matrix-bound analyte in the fouling layer is measured along with the analyte in the fibre coating. Another possible matrix effect is the net desorption of analyte from the binding matrix in the aqueous diffusion layer around the fibre coating. In this diffusion layer, the analyte is transported from the bulk solution to the surface of the fibre by diffusion. If the diffusion inside the fibre coating is faster than the diffusion in this aqueous layer, the free concentration in the layer will become temporarily depleted. This will lead to a net desorption of compound from the matrix in the layer, speeding up the uptake of compound in the fibre in samples with binding matrix, in comparison with samples without binding matrix.

We have developed an nd-SPME method for octylphenol in biological samples and verified if such matrix effects occur in the presence of protein. We checked for net desorption from protein in the diffusion layer by creating absorption profiles of octylphenol in presence of different concentrations of bovine serum albumin (BSA), a major serum binding protein. Further, the potential occurrence of fouling was studied by measuring the amount of human serum albumin (HSA) on exposed fibres with a competitive ELISA. Oomen et al. found no protein on fibres exposed to chyme, by using a Bradford assay [12]. However, this assay has a detection limit of 1 μg, which is much higher than what could be expected to be present on a fibre, considering we have never visually detected any fouling. The detection limit of the ELISA kit was 0.015 μg.

Lastly, the affinity constant (K_a) of octylphenol for albumin was determined to obtain an impression of how available octylphenol is in blood or serum-containing cell culture medium.

2. Experimental

2.1. Apparatus and chemicals

4-*n*-Octylphenol (99%) and 2,3,4-trichlorophenol (TCP; 99%) were purchased from Aldrich (Steinheim, Germany) and PCB #138 (2,2′,3,4,4′,5-hexachlorobiphenyl; 99%) from Riedel-deHaën (Seelze, Switzerland). Ethylacetate (99.8%) and the derivatisation agent MTBSTFA (*N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide; >97%) were purchased from Lab-Scan (Dublin, Ireland) and Fluka (Buchs, Germany), respectively. Polydimethylsiloxane (PDMS) fibre (7 μm) was purchased from Supelco (Bellefonte, PA, USA). BSA (98%, essentially fatty-acid free, essentially γ-globulin free) and HSA (fraction V, 96–99%) were supplied by Sigma (St. Louis, MO, USA).

2.2. Effect of BSA on absorption profile of octylphenol

To determine whether the presence of BSA has an effect on the uptake kinetics of octylphenol in PDMS fibre, absorption profiles were created at different BSA concentrations in the aqueous solution: 0, 1×10^{-8}, and 1×10^{-4} M BSA. For an absorption profile, 4.0-mL vials were filled with 4.00 mL of a solution of octylphenol and BSA in Tris buffer (50 mM, pH 7.4), which was allowed to equilibrate overnight. Pieces of ±3 cm of 7-μm PDMS fibre were pierced through the septa of the lids with the help of a syringe, and adjusted so that 5 mm of fibre stuck out under the septum. These lids with fibres were screwed on the sample-containing vials (in upright position). The fibres were exposed in the vials in horizontal position for a certain time, without agitation. The fibres were then pulled out of the septum and processed as described in paragraph 2.5. Water samples of 0.5 mL were taken from each vial after fibre exposure as well as from the remainder of original solution, and were processed as described in paragraph 2.5. Fibre concentrations were calculated with a calibration series and the known volume of the fibre (14.8 nL). These fibre concentrations (C_f) were divided by the vial-specific aqueous concentrations (i.e. free plus protein-bound concentrations; L_{total}) and plotted against the absorption time (t). Eq. (1) was fitted through the data points using Graphpad (Prism software):

$$\frac{C_f}{L_{total}} = \frac{k_1}{k_2} ff_L (1 - e^{-k_2 t}). \quad (1)$$

In this equation k_1 and k_2 are the uptake and release rate constants of ligand into and from the SPME fibre, respectively, and ff_L is the free fraction of ligand. The first absorption profile was prepared with a solution of 1.00×10^{-6} M octylphenol in milli-Q water without any BSA (pH 5.9). Absorption times for duplet fibres were 0.5, 1, 2, 4, 6, 8, 16, 20, 24 and 32 h. For this experiment, the extracted amounts in the fibre coating at equilibrium were also compared to the amount of octylphenol present in the original solution (known by measurement of the initial aqueous octylphenol concentration), to determine the extent of depletion.

For the second absorption profile, the sample contained 1.00×10^{-6} M octylphenol and 1.0×10^{-8} M BSA in Tris buffer. For this curve, absorption times were 5, 10, 30 and 45 s, 1, 2, 5, 10, 15 and 30 min and 1, 4, 6, 17, 20, 24 and 31 h. The last absorption profile was created with 1.00×10^{-4} M octylphenol and 1.0×10^{-4} M BSA in Tris buffer and absorption times of 5, 10 and 30 s, 1, 2, 5, 10 and 30 min and 1 and 2 h.

2.3. Competitive ELISA

To determine whether proteins adsorb to SPME fibres, causing fouling of the fibre, a competitive ELISA was performed with HSA-exposed fibres. Fifty pieces of 7-μm PA fibre were exposed for 5 mm overnight to a solution of 5.0×10^{-4} M HSA as described in paragraph 2.2. The competitive ELISA was performed using the Exocell Albuwell® kit (bioGnosis Ltd., Hailsham, UK), which provides 96-well plates pre-coated with HSA. The kit was used in conformity with the supplier's instructions. In short, a series of duplet HSA standard dilutions was prepared

and added to two columns of the plate. Five other wells were then filled with 100 uL of the supplier's diluent, but instead of adding 10 uL of urine sample, 10 HSA-exposed fibres were placed in each of the five wells (with the exposed side of the fibre down in the diluent). Then the antibody conjugate was added and left to incubate for 30–60 min, after which the fibres were removed and the wells were washed. Colour developer and colour stopper were added serially according to the instructions and absorbance was read at 450 nm on a EL_x800 plate reader of Bio-tek Instruments (Winooski, VT, USA). Absorbance of the fibre-wells was calibrated with the absorbances of the HSA standards to obtain the amount of HSA that had been adsorbed to the ten fibres.

2.4. Determination of K_a of octylphenol for BSA

For the determination of the K_a of octylphenol for BSA, duplet fibres were exposed to solutions with the same total octylphenol concentration but with varying BSA concentrations, in the same manner as for the absorption profiles. Seven BSA concentrations were tested, varying from 0 to 10^{-5} M. The nominal total octylphenol concentration was 1×10^{-6} M.

Fibres were exposed for 16 h. As detection problems were expected, 5 μL of the fibre extracts were injected on the GC–MS instead of 1 μL and the water extracts were concentrated as described in Section 2.5. The obtained fibre concentrations for all BSA-containing samples were plotted against the BSA concentration (P_{total}).

To fit the data and obtain K_a, a simple mathematical model was written using the software package Berkeley Madonna, assuming one binding site per protein molecule. The essence of this model is formed by Eqs. (2) and (3), of which the derivation can be found in Appendix A:

$$P_{free} = P_{total} - \frac{K_a L_{free} P_{total}}{1 + K_a L_{free}}, \quad (2)$$

$$L_{total} = L_{free} + \frac{K_a L_{free} P_{total}}{1 + K_a L_{free}}. \quad (3)$$

In these equations, P_{free}, L_{total} and L_{free} are the concentration of unoccupied protein, total and free concentration of ligand, respectively. The fibre measurements are incorporated in the model by Eq. (4), where $C_{f,0}$ is the fibre concentration at a protein concentration of 0:

$$ff_L = \frac{L_{free}}{L_{total}} = \frac{C_f}{C_{f,0}}. \quad (4)$$

The complete script of the model can be found in Appendix B. L_{free} was calculated first by Berkeley Madonna, using Eq. (3) in the GUESS. . .ROOTS function. With the obtained L_{free}, the software used Eq. (2) to calculate K_a. The value for L_{total} was obtained from the analysis of the water samples, while K_a and $C_{f,0}$ were fitted.

$C_{f,0}$ was fitted, although experimental values for it were available, because these experimental values cannot be used in Eq. (4) due to an experimental artefact. We have experienced that in samples without protein or other macromolecule, there is more loss of hydrophobic ligands than in samples containing protein. This loss is caused by non-specific binding to, for example, vial walls, and decreases the total aqueous concentration (L_{total}) of the ligand. The free concentration of ligand is therefore lower in samples without protein than it theoretically should be, based on the total concentrations in the samples with protein. The measured $C_{f,0}$ can thus not be used in series with the fibre concentrations in the samples with protein, and thus not in Eq. (4) or our model. The measured value of $C_{f,0}$ was only used as a reference for the fitted value.

2.5. General procedure of octylphenol desorption from fibres and total extraction from aqueous samples

After performing the experiments described in Sections 2.2 and 2.4, the octylphenol in a fibre was desorbed by inserting the whole fibre exposed-side down in a 250-μL glass insert. The insert was placed on top of a loose rubber septum on the bottom of a 1.8-mL glass vial, to ensure airtight closure. Twenty microliters of ethylacetate was added to the fibre. The vials were then subjected to an ultrasound bath for 15 min in a Transsonic T 460 (Elma Hans Schmidbauer, Singen, Germany). This procedure was found to extract close to 100% of the octylphenol, as a second extraction step delivered less than the detection limit (<0.1% of the amount extracted in the first step).

Aqueous octylphenol samples, taken during the experiments, were subjected to total extraction with ethylacetate in a 1:1 volume ratio by vigorously shaking the mixture for 10 min at 900 rpm on an IKA®-Schüttler MTS 4 (Janke & Kunkel, Staufen, Germany). This procedure was also found to extract close to 100% of the octylphenol, as a second extraction step delivered less than the detection limit (i.e. <1% of the amount extracted in the first step). Generally, 20 μL of the ethylacetate fraction was then transferred to a 250-μL glass insert in a 1.8-mL glass vial with a septum on the bottom. However, for samples with expected octylphenol concentrations below the detection limit, 1.4 mL of the ethylacetate fraction was evaporated under nitrogen flow. The octylphenol was then redissolved in 40 μL of ethylacetate (concentration factor of 35), of which 20 μL was then sampled for analysis. This procedure gave a recovery of 104%.

2.6. GC–MS analytical procedure

Derivatisation and analysis of the octylphenol samples was based on the method of Mol et al. [13]. To all 20-μL extracts, 25 μL was added of a 4:1 (v/v) mixture of MTBSTFA and a solution of 2.2×10^{-6} M PCB #138 and 4.9×10^{-6} M TCP in ethylacetate. The sample was heated at 75 °C for 3 h to maximise derivatisation. Identification and quantification were carried out on a Varian (Walnut Creek, CA, USA) GC–MS consisting of a 3400 CX gas chromatograph, a 8200 CX autosampler and a Saturn 2000 iontrap detector. Injections (on-column; 1 uL) were made on a 1078-type temperature programmable injector equipped with an on-column liner. The temperature during injection was programmed as follows: initial temperature 55 °C, initial hold 0.1 min, program rate 300 °C/min, final temperature 325 °C, final hold 15 min. Separation was performed with a 30 m

capillary column (J&W, Folsom, CA, USA; type CB5-ms, ID 0.25 mm, df 0.25 μm) in combination with a 2-m retention gap (J&W, Folsom, CA, USA; uncoated deactivated fused silica, ID 0.53 mm). The temperature of the column was programmed as follows: initial temperature 70 °C, initial hold 2 min, program rate 10 °C/min, final temperature 280 °C, final hold 5 min. Ion-trap conditions were as follows: transferline temperature 275 °C, manifold temperature 45 °C, trap temperature 250 °C. Measuring mode was the SIS (selected ion storage) range mode, with a scan time of 0.6 s. Data handling was performed with Saturn GC/MS workstation software, version 5.52.

The detection limit was $\sim 5 \times 10^{-8}$ M (determined as 5 times the signal-to-noise ratio). The reproducibility (standard deviation divided by the mean) was determined from 10 injections of the lowest standard (near the detection limit) and found to be 7.5%. At higher concentrations the reproducibility was better. Peak areas of octylphenol were divided by peak areas of PCB #138 to correct for volume-variations. Obtained ratios were calibrated with ratios from octylphenol standards prepared directly in ethylacetate.

3. Results and discussion

3.1. Effect of BSA on absorption profile of octylphenol

The depletion of octylphenol in the solution by the PDMS fibre was found to be 3.1%. This is below the limit of 5% depletion at which we consider the depletion to become significant. Therefore, the found depletion was acceptable. The three absorption profiles of octylphenol, measured in the presence of different concentrations of BSA, are depicted in Fig. 1. The heights of the curves in Fig. 1A or the values for $k_1 ff_L$ in Table 1 should not be compared, as these are dependent on the total concentration and the free fraction of octylphenol. The free fractions were unknown and the total concentration of octylphenol was not the same for all the curves in order to enable detection in all three cases.

The lower pH of the control profile (5.9) is not expected to have had an influence on the ionisation of octylphenol, as the pK_a of octylphenol is expected to be >10.3, based on the pK_a values of phenol and *p*-methylphenol [15]. Thus, the difference in pH is not expected to have had an effect on the amounts extracted.

What can be compared is the time to equilibrium or the values for k_2. These show that a low BSA concentration might have a slight effect on the uptake rate, but a high BSA concentration, in combination with a high level of octylphenol, has an enormous effect on the equilibration time. This is in contrast to our earlier finding that the uptake of estradiol ($\log K_{ow} = 4.2$) into a polyacrylate fibre is not affected by the presence of protein [14]. This difference can be explained by the higher hydrophobicity of octylphenol (calculated $\log K_{ow} = 5.42$, by KOWWIN of the US EPA) and the higher diffusion coefficient in PDMS than

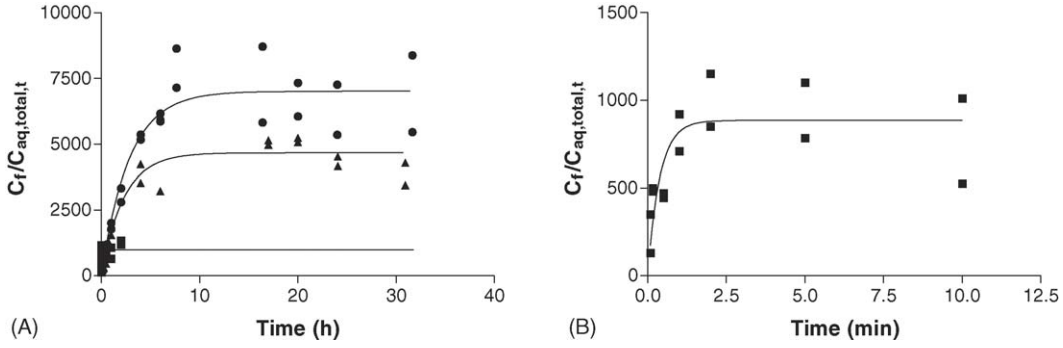

Fig. 1. (A) Absorption profiles of octylphenol in 7-μm PDMS fibres, in presence of no (●), 1×10^{-8} M (▲) or 1×10^{-4} M (■) BSA. (B) An enlargement of the 1×10^{-4} M BSA-curve. $C_{aq,total,t}$ is the total aqueous concentration measured after absorption time *t*.

Table 1
Kinetic constants of the absorption profiles of octylphenol into 7-μm PDMS in presence of various concentrations of BSA

	BSA concentrations		
	0 M	1×10^{-8} M	1×10^{-4} M
$ff_L k_1$ (min^{-1})[a]	41.1	33.9	2.06×10^3
Standard error $ff_L k_1$ (min^{-1})	6.8	5.7	0.65×10^3
95% confidence limits $ff_L k_1$	26.7–55.4	22.1–45.8	0.70×10^3–3.43×10^3
k^2 (min^{-1})[a]	0.0059	0.0073	2.19
Standard error k^2 (min^{-1})	0.0011	0.0013	0.74
95% confidence limits k^2	0.0035–0.0082	0.0045–0.0101	0.64–3.7
n	20	24	20
r^2	0.842	0.920	0.575

[a] ff_L = free fraction of ligand, k_1 = uptake rate constant, k_2 = release rate constant.

in polyacrylate [16], making the transport in the aqueous diffusion layer the rate-limiting step in the uptake of octylphenol in PDMS. Thus, a diffusion layer effect is possible (as explained in the introduction and in Heringa et al. [9]). The effect is so much larger at the high concentration of BSA and octylphenol, because then a large buffer of bound octylphenol is available in the aqueous diffusion layer for desorption at depletion.

The fact that the fibres were exposed in a non-agitated system has probably also played a significant role in the size of the diffusion layer effect, because in such an exposure system the diffusion layer is thicker than in a system where the solution is stirred. However, as we wanted this method to be applicable in cell culture plates for in vitro estrogenicity assays, where stirring cannot be performed, we had to study this still-standing situation.

An effect like this has been reported by others before [12,17,18] and the solution to this problem is to perform all analyses at extraction equilibrium. In that case, there is no difference anymore in the fibre concentration in samples with and samples without binding matrix. In the case of octylphenol, this means leaving the fibre in the sample for absorption for at least 16 h (Fig. 1).

3.2. Competitive ELISA

The ELISA showed that 68 ± 8 ng of HSA was present on each of the exposed fibres. With a molecular weight of human albumin of 65 kDa [19], this corresponds to 1.0 ± 0.1 pmol of HSA per fibre. Thus, fouling of the fibre by protein adsorption does take place, as reported by Poon et al. [20,21] and suggested by Escher et al. [22]. According to Heringa et al. [9], fouling could have two types of effects on the uptake of compounds into the fibre coating: either the fouling blocks uptake or the adsorbed protein adds to the measured amount of compound by its bound ligands.

If the fouling were to block uptake, we would have observed an increased time to equilibrium in absorption profiles of the previous experiment. As the presence of protein showed a decrease of the equilibration time, we doubt that of this type of effect by the fouling occurred. To verify if fouling could increase the measured amount of octylphenol in the fibre, we compared the amount that could theoretically be present on the adsorbed protein with the amount of octylphenol absorbed in the fibre. Assuming there is one binding site for octylphenol per albumin molecule, there could maximally be 1.0 pmol octylphenol bound to the protein on the fibre. This is insignificant when the fibre contains $\geq$100 pmol or when $C_f \geq 7 \times 10^{-3}$ M for this particular fibre. This is the case at free aqueous octylphenol concentrations $\geq 1 \times 10^{-6}$ M (the fibre–water partition coefficient (K_{spme}) is 6.9×10^3) for this particular fibre. Therefore, this effect will not have been of influence on the results of the previous experiment. However, even at lower free aqueous concentrations, the adsorbed amount may be insignificant, depending on the occupancy of the adsorbed protein and whether the adsorbed amount of protein depends on its aqueous concentration.

For better consistency and more direct validity of the fouling results for SPME use in in vitro cultures (which usually contain BSA and not HSA in the culture medium), it would have been preferable to perform the ELISA with BSA. However, no competitive-ELISA kit for BSA could be found. As it is expected that the fouling is caused by non-specific binding and that the non-specific binding properties will not differ significantly between BSA and HSA, extrapolation of the fouling results with HSA to BSA should be possible.

3.3. Determination of K_a of octylphenol for BSA

The analysis of the water samples gave an average total concentration of 6.7×10^{-7} M octylphenol and this value was used for L_{total} in the model. The results of the experiment and the manual fit to the experimental data are shown in Fig. 2. The model visually fits the data well, although no goodness of fit-value was obtained, as Berkeley Madonna does not provide this. The K_a was determined to be 1×10^7 M^{-1}. As the number of significant digits shows, the precision was not high, as this value had to be determined from a manual fit.

A manual determination of the goodness of fit (gof) by calculation of the root-mean-square of the residuals (Eq. (5)) results in a value of 3.5×10^{-4} M. In this equation, n is the number of residuals summed. Similarly, the standard deviation (s) of the data points from the model (Eq. (6)) was calculated to be 3.6×10^{-4} M.

$$\text{gof} = \sqrt{\frac{\sum (C_f\,(\text{data}) - C_f\,(\text{model}))^2}{n}} \quad (5)$$

$$s = \sqrt{\frac{\sum (C_f\,(\text{data}) - C_f\,(\text{model}))^2}{n-1}} \quad (6)$$

This number of 3.6×10^{-4} M does not say very much by itself, as it is dependent on the height of the values of C_f. Relat-

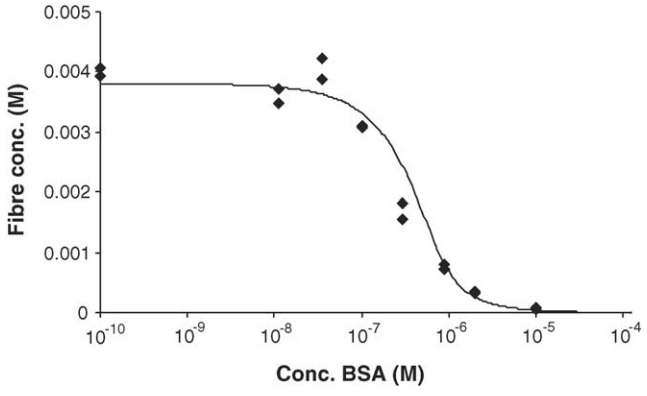

Fig. 2. Fibre concentrations of octylphenol against BSA concentration to determine the affinity constant. Dots represent measured data, of which the first pair are measured in absence of BSA, but as it is impossible to take the log of zero, the BSA concentration was artificially set at 1×10^{-10} M. The line represents the manually fitted model, with $L_{total} = 6.7 \times 10^{-7}$ M, $C_{f,0} = 0.0038$, and $K_a = 1 \times 10^7$ M^{-1}.

ing the standard deviation to the measured C_f values, we find that it is 9–540% of the measured C_f values. Obviously, the use of absolute residuals does not give a representative idea of the goodness of fit. Instead, some form of weighting should be used, but manual calculations of this form are beyond our league. Therefore, we are not able to provide a representative goodness of fit value. The determined value of the association constant should thus only be used as an indication of the albumin binding affinity of octylphenol.

Although the highest BSA concentration tested was 50-fold lower than the in vivo serum concentration of 5×10^{-4} M [23], the obtained K_a value is representative for the affinity in vivo, as the data almost completely cover the binding curve (see Fig. 2). Higher BSA concentrations were expected to create detection problems with octylphenol and would not have changed the outcome of the determination.

Teeguarden and Barton [24] calculated that the association constant of octylphenol for human albumin should be $2 \times 10^6\,M^{-1}$ in their computational model in order to obtain the 0.3% free octylphenol in human male serum reported by others. This deducted value is reasonably comparable to our experimental value, considering the difference in method of determination, the assumptions that were made for their determinations and the difference in species (human versus bovine) of the albumin.

The affinity of octylphenol for BSA is much larger than the reported affinities of other xeno-estrogenic compounds for albumin: $8.9 \times 10^3\,M^{-1}$ for *p*,*p*′-DDT and HSA [25] and $2.5 \times 10^5\,M^{-1}$ for diethylstilbestrol (DES) and BSA [26], for example. Additionally, an affinity in the order of 10^7 seems quite high for such an unspecific protein as BSA. Perhaps the assumption of one binding site per BSA molecule was not correct, although steroid hormones appear to have only one binding site on albumin [27]. As octylphenol has enough similarity to estradiol to be able to bind to the estrogen receptor, we have assumed octylphenol would have estradiol-like binding properties for albumin, too. Therefore, we made the assumption of one binding site. However, the structure of octylphenol, with the octyl-chain on one end and a hydrophilic hydroxide group on the other end, may also be sufficiently similar to a fatty acid to allow octylphenol to bind to the multiple fatty acid binding sites on albumin. Long-chain fatty acids, like other target compounds of albumin such as bilirubin and hematin, have albumin binding affinities in the order of 10^7. This is another indication that octylphenol might act as a fatty acid in the binding to albumin.

These speculations need to be verified in, for example, crystallographic studies, before strong conclusions can be drawn on the binding affinity of octylphenol for BSA. What is clear, however, is that a very large proportion of octylphenol will be bound to albumin in serum-containing culture medium or in blood.

4. Conclusion

Fouling of the fibre occurs, but does not seem to block octylphenol uptake or add sufficient extra analyte to the fibre in the experimental conditions applied here. More study on the influence of analyte and matrix concentration on fouling and the effects of fouling are necessary. In the still-standing, non-agitated setup applied here, there is a large effect of protein presence on the kinetics op octylphenol uptake. This effect can be circumvented by performing analyses at extraction equilibrium, but this finding shows one must stay alert on such effects. Octylphenol has been shown to bind to a large extent to albumin, which stresses the necessity to measure free, available concentrations in toxicological studies with this compound. Such measurements can be performed with the nd-SPME method presented in this paper. This method can be used as a basis for development of free concentration-measurements of other xeno-estrogens.

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We would like to thank Jan van Eijkeren for supervising the mathematics.

Appendix A. Model derivation

Assuming a single binding site per protein, the binding reaction of a ligand (L) to a protein (P) can be formulated as:

$$L_{free} + P_{free} \Leftrightarrow LP \tag{I.1}$$

where L_{free} is the free concentration of ligand, P_{free} is the concentration of unoccupied protein and LP is the concentration of ligand–protein complexes. The Law of Mass Action states that at binding equilibrium, the affinity constant (K_a) can be expressed as in Eq. (I.2) [28,29]:

$$K_a = \frac{LP}{L_{free} P_{free}}. \tag{I.2}$$

The mass balances of a system containing one protein and one ligand are:

$$\begin{aligned} P_{total} &= P_{free} + LP \\ L_{total} &= L_{free} + LP \end{aligned} \tag{I.3}$$

in which P_{total} and L_{total} are the total concentrations of protein and ligand, respectively. If the protein mass balance is used to substitute P_{free} in Eq. (I.2), and the resulting equation is rearranged, Eq. (I.4) can be obtained [28], which corresponds with the Langmuir equation:

$$LP = \frac{K_a L_{free} P_{total}}{1 + K_a L_{free}}. \tag{I.4}$$

This equation can be used to substitute LP in the mass balance equations, to obtain the equations used to determine the affinity constant:

$$\begin{aligned} P_{free} &= P_{total} - LP = P_{total}\left(1 - \frac{K_a L_{free}}{1 + K_a L_{free}}\right), \\ L_{total} &= L_{free} + LP = L_{free}\left(1 + \frac{K_a P_{total}}{1 + K_a L_{free}}\right). \end{aligned} \tag{I.5}$$

Appendix B. Affinity constant determination model script for Berkeley Madonna

Model to determine the affinity constant of a ligand for a protein by measuring free fractions or nd-SPME fibre concentrations in solutions with the same total ligand concentration but different protein concentrations.

```
METHOD RK4

RENAME TIME = log Ptotal                                          ;is log of total concentration of protein
RENAME STARTTIME = log Ptotal0
RENAME STOPTIME = log Ptotalf
RENAME DTMIN = dmin
RENAME DTMAX = dmax
RENAME DTOUT = dout

log Ptotal0 = -10.0
log Ptotalf = 0.0
dmin = 1.e-6
dmax = 1.
dout = 0.01

Ka = 4.3e+6                                                       ;is affinity constant of ligand for protein
Ltotal = 1.0e-6                                                   ;is total ligand concentration
ffL = Lfree/Ltotal                                                ;is free fraction of ligand
ffP = Pfree/Ptotal                                                ;is unoccupied fraction of protein
Cf = ffL*Cf0                                                      ;is fibre concentration at a certain protein concentration
Cf0 = 4.0e-3                                                      ;is fibre concentration at a protein concentration of zero

Ptotal = 10.00**log Ptotal
Pfree = (1.0 - (Ka*Lfree)/(1.0 + Ka*Lfree))*Ptotal                ;is concentration of unoccupied protein

GUESS Lfree = Ltotal/2
ROOTS Lfree = (1.0 + Ka*Ptotal/(1.0 + Ka*Lfree))*Lfree - Ltotal   ;is free concentration of ligand
LIMIT Lfree >= 0.
LIMIT Lfree <= Ltotal
```

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