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# Production and characterization of a genetically engineered anti-caffeine camelid antibody and its use in immunoaffinity chromatography☆

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ABSTRACT

This work demonstrates the feasibility of using a camelid single domain antibody for immunoaffinity chromatographic separation of small molecules. An anti-caffeine VHH antibody was produced by grafting the complementarity determining sequences of a previously generated antibody onto an anti-RNase A antibody scaffold, followed by expression in *E. coli*. Analysis of the binding properties of the antibody by ELISA and fluorescence-based thermal shift assays showed that it recognizes not only caffeine, but also theophylline, theobromine, and paraxanthine, albeit with lower affinity. Further investigation of the effect of environmental conditions, i.e., temperature, pH, and ionic strength, on the antibody using these methods provided useful information about potential elution conditions to be used in chromatographic applications. Immobilization of the VHH onto a high flow-through synthetic support material resulted in a stationary phase capable of separating caffeine and its metabolites.



## 1. Introduction

Antibodies are the most widely used affinity reagents in biotechnology, biomedicine, and related areas. This is based on the fact that these proteins may be raised against virtually any molecule of interest and that they can bind to such targets with unsurpassed specificity and high affinity. Applications range from diagnostics, e.g., immunoassays, arrays, and sensors, to therapeutics, e.g., biodrugs and cancer-specific drug delivery agents [1–6]. As early as five decades ago, solid-phase immobilized antibodies were first utilized for the adsorption and separation, respectively, of protein antigens [7–10]. Although, especially in the early days of immunoaffinity chromatography, polyclonal antibodies have successfully been used for the separation and purification of both high- and low-molecular weight targets, monoclonal antibodies quickly became the receptor molecules of choice after their introduction by Köhler and Milstein in 1975 [11]. Not only are hybridoma cell lines potentially "immortal," but also produced antibodies are homogenous with regard to their binding and structural properties. Despite their usefulness [12–14], antibody-based stationary phases suffer from a number of shortcomings common to any protein-based affinity system. The susceptibility of these biomolecules to microbial degradation and denaturation caused by environmental parameters such as temperature and pH typically results in rather limited column lifetimes and reusability. As a consequence of the sheer size of a typical immunoglobulin (the most widely used are immunoglobulins G, IgG, with a MW of about 150 kDa), and the fact that it only possesses two binding sites near its N-termini, immunoaffinity stationary phases, furthermore, have a relatively low capacity.

Different approaches can be taken to increase the number of active immobilized binding sites that are available for interaction with analytes dissolved in the mobile phase. Several studies have demonstrated that higher binding capacities can be achieved if antibodies are linked to solid supports in a way that exposes their binding sites to the mobile phase [15–18].

Increased amounts of available binding sites per gram of support material should also be attained if the size of the immobilized receptor is reduced. IgGs are comprised of four polypeptide chains, namely two identical pairs of "heavy" and "light" chains, which are associated through noncovalent interactions as well as disulfide bonds [19]. The two binding sites are formed from six complementarity determining regions (CDRs) in the variable domains, which are located towards the N-termini of the protein chains. Millions of unique amino acid sequences in the variable domains allow for a vast array of possible binding site specificities. The constant

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domains on the other hand, are sequentially much more conserved and serve as structural scaffolding for the antibody. In addition, it is via the constant domains that antibodies may interact with other components of the immune system, e.g., the complement system. Since the antigen-binding properties of immunoglobulins, thus, reside in a relatively small portion of the overall large biomolecule, removal of "unnecessary" domains (i.e., the constant domains) should allow immobilization of antigen-binding sites at a higher density and, in turn, result in increased column capacities. Antigen-binding fragments (Fab), which comprise the $V_H$, $C_{H1}$, $V_L$ and $C_L$ domains and have a molecular weight of about 50 kDa, can be produced from immunoglobulins by proteolytic digestion [20–22]. However, yields are generally rather low making this strategy impractical for routine applications. Although Fab and other fragments such as single chain antibody fragments (scFv) may also be produced by molecular biological techniques [23,24] expression of functional fragments is often challenging, especially since Fab tend to form inclusion bodies in *E. coli*. Nevertheless, several studies have reported the successful use of antibody fragments in chromatographic applications [25–28].

In 1993, Hamers-Casterman et al. discovered a class of antibodies in the sera of camels and llamas that differ significantly from the aforementioned structural characteristics of other immunoglobulins [29]. These camelid antibodies are comprised of only two heavy chains, and their two antigen-binding domains (VHH), which are formed by three CDRs each, do not possess or require light chains for their interaction with antigens. While the complete antibody has a molecular weight of about 100 kDa, a VHH is significantly smaller with a MW of about 15 kDa. Nevertheless, it has been found that camelid antibodies are capable of forming highly specific and strong interactions with their binding partners, which may be biological macromolecules or small molecules [30,31].

Camelid antibodies possess several potential advantages over IgG antibodies with respect to their application in biotechnology and biomedicine; they are much smaller in size, have a relatively high stability and solubility, are relatively easy to express by bacterial expression, and can be readily produced by combinatorial methods such as phage display [32]. VHH domains have recently been shown, in a preclinical mouse model, to be more potent therapeutics for the treatment of collagen-induced rheumatoid arthritis than conventional antibody-based clinical therapies [33]. They have, furthermore been utilized for the isolation of serum albumin and IgG from human blood [34]. However, the utility of camelid VHH antibodies for the isolation of small molecules by chromatographic techniques has not been previously demonstrated.

Here, we report the molecular biological production of an anti-caffeine VHH antibody, its characterization by enzyme-linked immunosorbent assays (ELISAs) and fluorescence-based thermal stability tests, as well as its application for immunoaffinity chromatographic separation and detection of caffeine (MW 194.2 Da) and its metabolites.

The antibody used in this study was created by grafting the CDRs of an anti-caffeine VHH antibody recently generated by Ladenson et al. [35] onto a previously produced anti-RNase A antibody [36,37]. Ladenson et al. reported that their antibody was suitable for ELISA determination of caffeine concentrations in beverages and exhibited increased thermal stability compared to commercially available mouse monoclonal antibodies [35]. The development of techniques suitable for detecting and isolating caffeine and caffeine-metabolites is of great interest as these methylxanthines are contained in many foods and beverages. Caffeine is well known to be a powerful stimulant of the nervous system and to have performance-enhancing effects [38–40]. In humans, caffeine is metabolized by cytochrome P450 enzymes to paraxanthine (80%), theobromine (12%), and theophylline (7%) [38,41].

## 2. Materials and methods

### 2.1. Chemicals and reagents

*N*,*N*′-Disuccinimidyl carbonate (DSC), *N*,*N*′-dicyclohexylcarbodiimide (DCC), and dimethylaminopyridine (DMAP) were from Nova Biochem (La Jolla, CA). Tris(hydroxymethyl)aminomethane (Tris), bovine serum albumin (BSA), gelatin, caffeine, theobromine, paraxanthine, and theophylline were all purchased from Sigma (St. Louis, MO). *N*-Hydroxysuccinimide (NHS) and 1,2-phenylenediamine (OPD) were from Aldrich (Milwaukee, WI), and theophylline-7-acetic acid from Fluka (Allentown, PA). The streptavidin-horseradish peroxidase (HRP) conjugate and Precise Precast Protein Gels 4–20% (15-wells) were obtained from Pierce (Rockford, IL). Biotin sulfo-NHS was graciously provided by Ed Fujimoto from Campbell Science (Rockford, IL). LB Agar, Miller (molecular genetics powder) and LB Broth, Miller (molecular genetics granular) were purchased from Fisher Bioreagents (Fair Lawn, NJ). Broad Range Protein Molecular Markers were from Promega (Madison, WI). POROS-OH was purchased from PerSeptive Biosystems (Cambridge, MA). Imidazole was from ACROS/Fisher (Fair Lawn, NJ). Oligonucleotides were purchased from Sigma-Genosys (St. Louis, MO), and SYPRO Orange dye (5000×) was from Invitrogen (Carlsbad, CA). The pET-21a expression vector was purchased from Novagen (Gibbstown, NJ). Wizard SV Gel and PCR Clean-up System and Wizard Plus SV Minipreps kits were obtained from Promega (Madison, WI). Calf intestinal alkaline phosphatase (CIAP) was purchased from New England Biolabs (Ipswich, MA). XL1-Blue supercompetent cells were from Stratagene (La Jolla, CA). HPLC grade methanol was purchased from Fluka (Allentown, PA); other organic solvents were also HPLC grade and obtained from Sigma (St. Louis, MO). Inorganic salts were from ACROS/Fisher (Fair Lawn, NJ); all other chemicals were from Sigma (St. Louis, MO). Water was purified using a MilliQ water purification system (Millipore, Bedford, MA). Phosphate buffered saline (PBS) was prepared according to Ref. [42] and adjusted to pH 7.4 with 0.1N HCl.

### 2.2. Production of the camelid anti-caffeine VHH antibody

#### 2.2.1. Design and assembly of the antibody expression construct

Starting with a pHFT-VHHR expression vector containing a gene encoding an anti-RNase A VHH antibody (cAb-RN05) [36,37], which was kindly provided by S. Koide (University of Chicago), the VHH gene segment was isolated through double digestion with the restriction enzymes NdeI and XhoI. This VHH gene segment included an N-terminal $(His)_6$-Flag-Tev purification tag (Fig. 1). Similarly, the expression vector pET-21a(+) was digested with NdeI and XhoI. Each sample was gel-purified using a Wizard SV Gel and PCR Clean-up System. Next, the digested vector was dephosphorylated using CIAP. The cAb-RN05 VHH gene was then ligated into the dephosphorylated vector and the newly acquired plasmid, p21-RN05, was transformed into XL1-Blue and selected on LB-Amp agar plates. Several colonies were used to start overnight cultures (LB, 100 μg/ml ampicillin) followed by plasmid isolation. DNA sequence analysis of the plasmid was performed at the CRC-DNA Sequencing Facility at the University of Chicago.

The new p21-RN05 construct served as the framework to build the anti-caffeine VHH antibody. Kunkel mutagenesis [43] was performed to substitute the anti-caffeine CDR regions into the p21-RN05 framework. Briefly, p21-RN05 was introduced into CJ236 cells to generate uracil containing parent DNA which was followed by infection with M13KO7 helper phage to generate single strand-dUTP parent DNA. Three Kunkel primers (Table 1) were then used to introduce the new CDR sequences. Parent DNA was removed by transformation of the Kunkel reaction into $Ung^+$ XL1-Blue *E.*

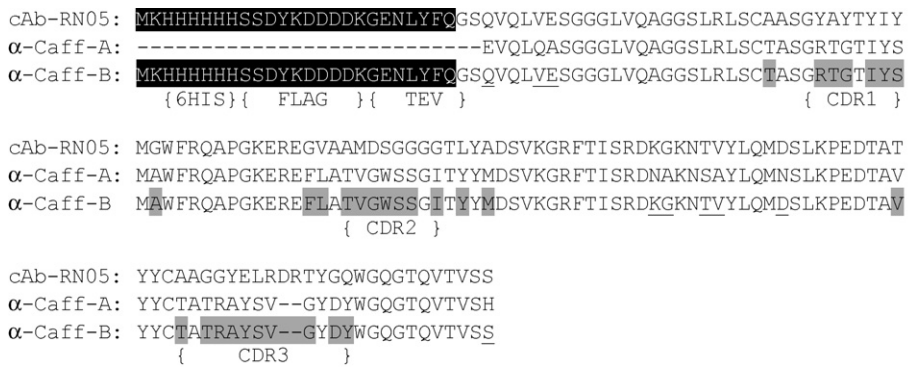


**Fig. 1.** Sequence alignment of the anti-RNase A VHH (cAB-RN05), the anti-caffeine VHH (α-Caff-A) produced by Ladenson et al. [35], and the anti-caffeine VHH produced here (α-Caff-B). Amino acid positions that were changed to produce α-Caff-B are highlighted in grey, while sequence differences in non-CDR regions are underlined. Dashes indicate gaps in the amino acid sequence. The N-terminal $(His)_6$-Flag-Tev tag was used for protein purification. The tobacco etch virus (TEV) sequence allowed removal of the sequence indicated by inverted text colors.

*coli*. DNA sequencing was performed to ensure correct sequences throughout the VHH gene.

### 2.2.2. Expression and purification

The pET-21A vector containing the anti-caffeine $(His)_6$-Flag-Tev-VHH sequence was transformed into BL21(DE3) *E. coli* and selected on LB-Amp (100 μg/ml) plates. An initial 5 ml overnight culture grown at 37 °C was used to start a 50 ml subculture, followed by a final inoculation of a 1 l culture. Antibody expression was induced with 1 mM β-D-thiogalactoside at mid-log phase ($OD_{600} \sim 0.5$–$0.8$). The culture was grown overnight at 20 °C with shaking (235 rpm). The cells were harvested by centrifugation (17,700 × *g* for 15 min) and pellets were stored in a −20 °C freezer overnight. The cell pellet was resuspended in 10 mM Tris and sonicated for two 1 min on/1 min off cycles with a Model 60 Sonic Dismembrator (Fisher Scientific, Fair Lawn, NJ) at an output power of 21 W. The lysed cells were centrifuged (22,700 × *g* for 15 min) and the soluble fraction saved. The soluble fraction was first purified on a 5 ml Histrap HP column (GE Healthcare, Piscataway, NJ) using a 70 ml linear gradient between running buffer (20 mM imidazole, 50 mM disodium hydrogen phosphate/HCl, 500 mM NaCl, pH 8.0) and elution buffer (500 mM imidazole, 50 mM disodium hydrogen phosphate/HCl, 500 mM NaCl, pH 8.0). The protein eluted as a lone peak, which was subsequently pooled and run on a HiLoad 26/60 Superdex-75 size exclusion column (GE Healthcare, Piscataway, NJ) using 10 mM Tris/HCl, 300 mM NaCl, pH 8.0. The anti-caffeine antibody was then pooled and supplemented with EDTA (final concentration 100 mM) in preparation for the tobacco etch virus (TEV) protease reaction which was used to remove the $(His)_6$-Flag-Tev purification tag (Fig. 1). Tev protease is a highly specific protease, which recognizes the sequence Glu-Asn-Leu-Tyr-Phe-Gln-Gly and leaves behind a single N-terminal Gly on VHH. One mg of $His_6$-TEV protease was added to 50 mg of antibody and the reaction was incubated at 4 °C for 6 h with stirring. TEV cleavage was at least 90% efficient as judged by size exclusion chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis. Anti-caffeine VHH antibody (without the tag) was isolated by passing the TEV reaction over the HisTrap column and collecting the flow through.

Finally, the antibody was again purified on a Superdex-75 size exclusion column. Protein concentrations and expression yields were determined via absorbance measurement at 280 nm using extinction coefficients of 33,015 and 30,035 $M^{-1}\,cm^{-1}$ for the antibody with and without tag, respectively. Both extinction coefficients were determined following the method of Pace et al. [44].

## 2.3. Antibody characterization

### 2.3.1. Enzyme-linked immunosorbent assays

*2.3.1.1. Synthesis of a BSA-caffeine conjugate.* To enable characterization of the binding properties of the camelid VHH antibody using ELISA, a BSA-caffeine conjugate was produced for use as microtiter plate coating antigen. This was accomplished following a two step procedure. First, an NHS ester of theophylline-7-acetic acid was synthesized that was subsequently conjugated to BSA via accessible amines on the protein surface. To a cooled solution of 2 g theophylline-7-acetic acid (8.4 mmol) in 70 ml of dry dimethylformamide, 1.9 g DCC (10 mmol) and 1.15 g of NHS (10 mmol) were added slowly. After 5 min, the reaction turned turbid and was removed from the ice bath. The reaction was checked after 4 h by thin layer chromatography using chloroform:methanol (1:3) as solvent to verify formation of the product. The precipitate was filtered and the remaining solution lyophilized. Twenty-eight milligrams of BSA were dissolved in 4 ml of PBS. Ten milligrams of the theophylline-7-acetic acid NHS ester were dissolved in 0.5 ml dimethylsulfoxide and added to the protein solution. The mixture was stirred overnight at 4 °C and then dialyzed against PBS. The protein–caffeine conjugate was characterized spectroscopically [45] and found to contain between 6 and 8 caffeine residues per molecule of BSA.

*2.3.1.2. Antibody biotinylation.* A portion of the camelid anti-caffeine VHH antibody was biotinylated to enable detection of its binding to antigen-coated microtiter plates in ELISAs using streptavidin-HRP. The biotinylation procedure was as follows: to a 250 μl fraction of antibody in PBS (1 mg/ml) 1 mg of sulfo-NHS biotin was added; the mixture was stirred for 4 h at 4 °C. The solution was then dialyzed against three changes of PBS. Following dialysis, the antibody concentration was determined by spectrophotometric analysis to be 0.96 mg/ml.

*2.3.1.3. ELISA protocols.* Ninety-six-well MaxiSorp polystyrene microtiter plates (Nunc; Rochester, NY) were coated with solutions of the BSA–caffeine conjugate (2 μg/ml; 100 μl/well) in 50 mM carbonate buffer, pH 9.6. The conjugated protein was allowed to adsorb overnight at 4 °C. After each step of the protocol, plates were washed

**Table 1**
Kunkel primers used for introducing the anti-caffeine VHH antibody CDRs.

| | |
|---|---|
| CDR1 | 5′-TGGTGCTTGACGGAACCAAGCCATGGAGTAGATGGTACCGGTACGACCGGAAGCGGTGCAGCTCAGACGCAG-3′ |
| CDR2 | 5′-CGACCTTTTACGCTGTCCATGTAGTAGGTGATACCGGAGGACCAACCAACGGTAGCCAGGAATTCACGTTCTTTACC-3′ |
| CDR3 | 5′-GGGTGCCTTGACCCCAGTAGTCGTAACCAACGGAGTAAGCACGGGTAGCGGTGCAGTAGTAAACTGCGGTGTCTTCTGG-3′ |

three times with PBS/0.05% Tween 20. Unoccupied sites on the plastic surface were blocked with a 1% gelatin solution in PBS/0.05% Tween 20 (250 μl/well) by incubation at 37 °C for 2 h. Serial dilutions of the biotinylated antibody stock solution were prepared in PBS for use in a noncompetitive ELISA to obtain a binding curve. One hundred microliter samples of each dilution were applied per well and incubated for 2 h at room temperature. After washing, HRP-labeled streptavidin (1 mg/ml) was applied at a 1/10,000 effective dilution in PBS. One hundred microliters per well were applied and the plate was incubated for 15 min at room temperature. The substrate solution was prepared by adding 8 mg of OPD and 13 μl of 30% hydrogen peroxide to 20 ml of citrate/phosphate buffer. One hundred microliters of the OPD solution was added per well; HRP converts OPD to 2,2-diamino-azo-benzene, a colored product. The reaction was stopped by acidification with 1N sulfuric acid (100 μl/well). Absorbances were determined at 492 nm in a FLUOstar 403 Galaxy plate reader from BMG Labtechnologies (Offenburg, Germany).

For competitive ELISAs, the plates were coated and blocked as described above for noncompetitive ELISAs. Serial dilutions of competitor in PBS were applied in triplicate onto the plate (50 μl/well), followed by addition of antibody at a constant concentration (1/1500 effective dilution of stock) in PBS (50 μl/well). The HRP-streptavidin incubation and color formation steps were carried out as described for noncompetitive ELISAs.

*2.3.1.4. Evaluation of potential elution solvents.* The efficacy of potential elution solvents to be used in chromatographic applications was evaluated using noncompetitive ELISAs. The following solvents were investigated: 4 M NaCl, 10 mM ammonium hydroxide, 6 M guanidine/HCl, 10% dioxane, 100 mM citric acid, deionized water, 10% ethanol, 1 M acetic acid, 10% 1-propanol, 100 mM triethyl amine (TEA), and 10 mM phosphoric acid. After binding of the antibody, the microtiter plate was incubated with the elution solvents for 15 min at room temperature. Antibody which remained bound after this step was detected with HRP-labeled streptavidin. PBS was used as a reference buffer.

This study was extended to further investigate the effect of pH on antibody binding. For that, the aforementioned experiment was performed using 10 mM phosphate buffers in the pH range of 2.5–7.5 as potential elution solvent.

It should be noted that under the experimental conditions used here none of the potential elution solvents removes the coating or blocking reagents from the ELISA plate.

### 2.3.2. Thermal stability screens

Fluorescence-based thermal melts were performed using a Bio-Rad IQ5 real-time PCR instrument (Bio-Rad Laboratories, Hercules, CA). All thermal melts were run in 96-well thin-wall PCR plates with a total solution volume of 25 μl. Unless otherwise stated, samples were prepared in 50 mM phosphate buffer containing 150 mM NaCl (pH 7), antibody at a concentration of 4 μM, and 4× SYPRO Orange dye (1/1250 dilution of dye stock). Thermal shift assays were performed by adding ligand (caffeine, theophylline, theobromine, or paraxanthine) to a single well at a final concentration of 500 μM. To assess antibody stability at different pH values, samples were prepared in the following buffers: 50 mM glycine/HCl, pH 1–3, 50 mM sodium acetate/HCl, pH 4–6, 50 mM sodium phosphate, pH 7–8, and 50 mM glycine/NaOH, pH 9–12. Experiments were also performed using a range of NaCl concentrations (0, 150, 500, and 1000 mM). Thermal melts were performed by raising the temperature from 25 to 85 °C at 0.5 °C intervals every 30 s. Wavelengths of 490 and 575 nm were used as excitation and emission wavelengths, respectively. Protein melts were run in triplicate (except for pH/salt screens). The midpoint of the unfolding transition ($T_m$) was estimated by determining the maximum of the first derivative. Standard deviations of the determined $T_m$ were typically less then 1 °C with an average of 0.5 °C.

## 2.4. Chromatography

### 2.4.1. Stationary phase and column preparation

Two grams of POROS-OH (20 μm particles) were reacted with 350 mg DSC and 287 mg DMAP in 17.5 ml dry acetone for 1.5 h at 4 °C. The activated support material contained 5.1 μmol active sites/g of support material as determined spectrophotometrically following the procedure by Wilchek and Miron [46]. A fraction was reacted overnight with 5 mg of the camelid anti-caffeine VHH antibody in PBS at 4 °C under salting-out conditions, i.e., in the presence of 0.125 M sodium sulfate. Remaining active groups on the support were quenched for 1 h by treatment with 0.1 M Tris-buffer, pH 7.4. After extensive washing with PBS, the amount of immobilized VHH antibody was determined spectrophotometrically as the difference of the absorbance (at 280 nm) of the antibody solution before and after the immobilization step, and was found to be 17 mg/g of support material.

A stainless steel column (3.2 mm × 50 mm) was slurry packed with the VHH antibody-derivatized support in PBS at 160 bar using an Alltech Slurry Packer Model 1666 (Alltech, Deerfield, IL). No leakage of antibody was detected during column equilibration. The column was stored under azide-containing PBS at 4 °C only when not used for an extended period of time.

### 2.4.2. HPLC conditions

The chromatographic system used in this study comprised a Hitachi L-7100 pump with a degasser, an L-7400 UV-detector equipped with an analytical flow cell, and a D-7000 interface with System Manager V 4.0 software. Injections were performed using a Rheodyne 7725i injection valve (Hitachi, Naperville, IL) equipped with a 20 μl loop.

Unless stated otherwise, all chromatographic separations were performed at room temperature under isocratic conditions using PBS, pH 7.4, as mobile phase. Flow rates used in this study ranged from 0.5 to 4 ml/min. All analytes were detected utilizing their UV-absorbance at 272 nm. The elution order was determined by injection of the individual analyte as well as by spiking. The void volume for the determination of chromatographic data was measured using buffer [47]. For the short retention times obtained in this study (especially for paraxanthine and theobromine), errors in the determination of the void volume affect the calculation of chromatographic parameters. Slight variations may be explained by this fact. To ensure reproducibility of results, all measurements were carried out at least in triplicate. Standard deviation of chromatographic parameters was typically less than 5%.

The effect of the flow rate on antibody-based chromatographic retention was investigated using theobromine and paraxanthine as model analytes and flow rates between 0.5 and 4 ml/min. The temperature dependence of the chromatographic retention of paraxanthine was studied between 5 and 35 °C at 2 ml/min. The antibody column and buffer reservoir were immersed in a water bath (Haake, Berlin, Germany), and the temperature was increased in increments of 5 °C. The antibody column was equilibrated for at least 30 min at a flow rate of 2 ml/min before the corresponding series of injections were performed at the respective temperature. To study the influence of the ionic strength on the chromatographic retention of paraxanthine, the molarity of NaCl in a 10 mM phosphate buffer was changed by adding varying amounts of the salt to a solution containing 10 mM $K_2HPO_4$ and $KH_2PO_4$, which was adjusted to pH 7.4. The concentration of NaCl ranged from 0 to 250 mM. The antibody column was equilibrated for at least 30 min at a flow rate of 2 ml/min before the corresponding series of injections were performed.

The separation of a mixture of caffeine, theophylline, and paraxanthine was accomplished at a flow rate of 2 ml/min using a pulse gradient. Paraxanthine and theophylline were separated using 100% PBS, pH 7.4, as mobile phase from $t = 0$ min to $t = 9$ min. The mobile phase was switched to 100% 10 mM phosphate buffer, pH 2.5, from $t = 9.1$ min to $t = 12$ min in order to elute caffeine. The mobile phase changed back to 100% PBS, pH 7.4, from $t = 12.1$ min to the end of the run. Blank injections of PBS were employed to subtract baseline absorbance changes caused by solvent mixing.

## 3. Results and discussion

### 3.1. Generation, expression, and purification of the camelid anti-caffeine VHH antibody

The anti-caffeine antibody used in this study was created utilizing solely molecular biological techniques by replacing the CDRs of a previously produced anti-RNase A antibody (cAb-RN05) [36,37] with the CDR sequences of a camelid anti-caffeine antibody that had been generated recently by Ladenson et al. [35]. Fig. 1 shows a sequence alignment of the VHH antibodies. While the three CDRs of the anti-RNase A antibody were completely replaced with those of the anti-caffeine antibody, nine non-CDR residues remained unchanged after CDR "grafting." Based on existing structural data [37], these non-substituted residues are located in framework regions which are not in close proximity to the binding interface. It was therefore assumed that these residues would not play a significant role in caffeine binding. It is noteworthy that the results of this study later confirmed the validity of this assumption as the engineered antibody possessed binding activity.

Following insertion of the antibody-encoding sequence into an appropriate vector, the anti-caffeine VHH antibody was expressed at high levels in BL21 (DE3) *E. coli* cells. Interestingly, the antibody was expressed in soluble form, which is notable, as many VHH antibodies form insoluble inclusion bodies; refolding procedures, which are typically required to obtain functional antibody, thus, were unnecessary. Purification of the anti-caffeine antibody was facilitated by the presence of an N-terminal $(His)_6$-Flag-Tev tag, which enabled an initial purification on a nickel column. Further purification was achieved by size exclusion chromatography on a Superdex-75 column, from which the protein eluted as a single peak. An advantage of having the N-terminal TEV cleavage site is that, upon enzymatic removal of the His tag, the final product possesses only an N-terminal glycine remnant of the original tag preceding the VHH gene. Based on SDS–PAGE analysis (Fig. 2), the obtained antibody was highly pure and had an estimated molecular weight of about 13 kDa, which is in good agreement with the calculated value of 13.1 kDa. Following these procedures, a total of about 50–60 mg of highly pure, soluble anti-caffeine VHH antibody could be produced per liter of culture.

### 3.2. Antibody characterization

#### 3.2.1. Determination of antibody specificity by ELISA

A typical direct immunoassay setup is comprised of a surface-immobilized antigen, a primary antibody that can bind to it, and a secondary, labeled antibody that specifically binds to the primary receptor. For detection, characteristic properties of the label, e.g., radioactivity or fluorescence, are utilized. In an ELISA, the label is an enzyme such as HRP that can convert a substrate to a colored product, whose absorbance at a certain wavelength is detected.

Since small molecules typically do not adsorb to standard microtiter plate surfaces, a protein conjugate of a caffeine analog was synthesized. For that, the acetic acid derivative of theophylline, theophylline-7-acetic acid, was activated by conversion of the acid into an NHS ester (Fig. 3). Reaction with BSA produced amide bonds between the small molecule and amino groups on the surface of the protein. Spectrophotometric analysis of the product showed that approximately 6–8 residues of small molecule were bound per protein molecule. This conjugate could be used for coating microtiter plates via simple adsorption during overnight incubation in a basic buffer (pH 9.6).

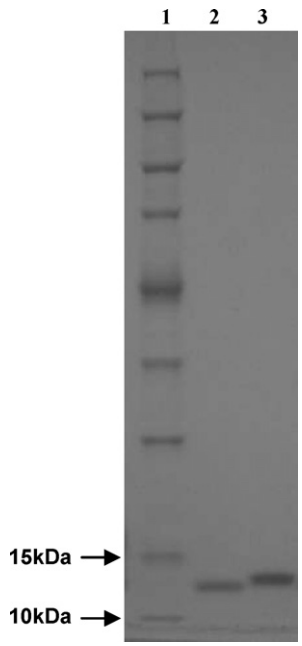


**Fig. 2.** SDS–PAGE gel showing purified anti-caffeine VHH antibody. Lane 1, protein ladder; lane 2, purified anti-caffeine VHH; lane 3, purified anti-RNase A VHH used as control.

Although enzyme-labeled anti-camelid antibodies are commercially available, a different approach was chosen to enable detection. The anti-caffeine VHH antibody was biotinylated using sulfo-NHS biotin, and the biotin-derivatized antibody was detected utilizing streptavidin-conjugated HRP. An advantage of this approach is that complex-formation between biotin and streptavidin occurs rather rapidly, so that the incubation time for the corresponding step in an ELISA can be reduced to ca. 15 min, as compared to 2 h that are typically required when secondary antibodies are used.

In order to test the utility of this assay setup, serial dilutions of the biotinylated camelid anti-caffeine VHH antibody were applied to a microtiter plate that had been coated with the BSA-conjugate. Fig. 4 shows that a classical binding curve was obtained with the BSA-conjugate of theophylline-7-acetic acid, but that the antibody did not bind to the control, i.e., underivatized BSA. This unambiguously proved that the antibody specifically bound to the immobilized small molecule and that the obtained result was not caused by non-specific interactions between the applied antibody and the microtiter plate surface.

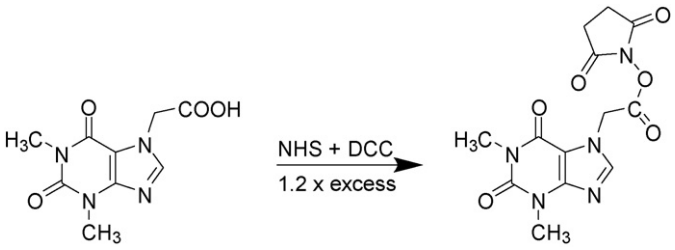


**Fig. 3.** Synthesis of theophylline-7-acetic acid NHS ester.

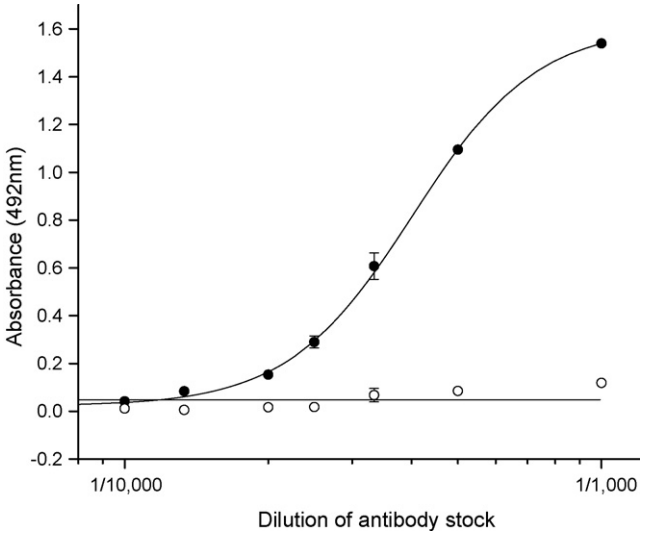


**Fig. 4.** Noncompetitive ELISA results obtained with biotinylated camelid anti-caffeine VHH antibody using a BSA-caffeine conjugate (●) or BSA (○) as solid phase coatings. Data points represent means of triplicate determinations. Missing error bars are obscured by the symbols.

Competitive ELISAs were then performed in order to assess relative affinities of the antibody for caffeine and its metabolites. As seen in Fig. 5, characteristic inhibition curves were obtained with all compounds tested. Caffeine showed the strongest interaction with the antibody as indicated by its relatively low $IC_{50}$ value of 1.2 μM. The $IC_{50}$ value represents the concentration of competitor causing 50% inhibition of antibody binding to a solid-phase immobilized antigen and is a measure of the relative affinity between antibody and competitor. Theophylline was bound more weakly by the antibody ($IC_{50}$ = 29 μM); it should be noted that the observed difference in affinity compared to caffeine is based solely on the loss of one methyl group at N-7 of the purine structure (Fig. 6). The results obtained with paraxanthine and theobromine, furthermore, showed that a loss of the other two methyl groups of caffeine has an even more dramatic effect on antibody affinity, as the $IC_{50}$ values of paraxanthine and theobromine were determined to be 340 and 400 μM, respectively.

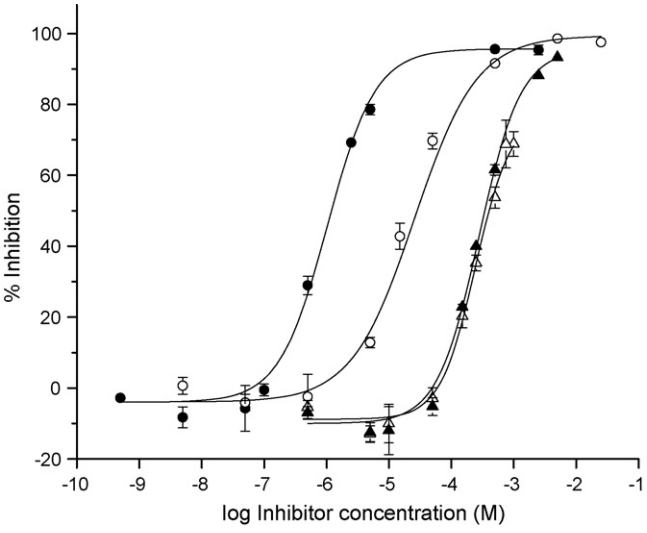


**Fig. 5.** Competitive ELISA results obtained with camelid anti-caffeine VHH antibody at a fixed concentration and varying concentrations of caffeine (●), theophylline (○), paraxanthine (▲), and theobromine (△), respectively, used as competitors. Error bars indicate standard deviations of triplicate determinations. Missing error bars are obscured by symbols.

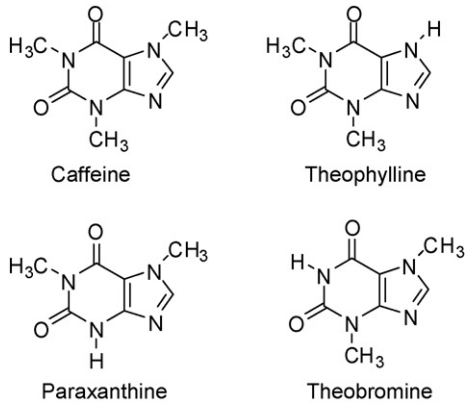


**Fig. 6.** Chemical structures of caffeine and its metabolites.

### 3.2.2. Evaluation of potential elution solvents by ELISA

The efficacy of various solvents that are traditionally used for elution in classical affinity chromatography on the binding properties of the camelid anti-caffeine VHH antibody were investigated using noncompetitive ELISAs as described in the materials and methods section. The percentage of antibody remaining on the plate after incubation with each solvent at room temperature for 15 min is presented in Fig. 7. Among the most effective elution solvents were 6 M guanidine HCl, 1 M acetic acid, 100 mM citric acid, and 10 mM phosphoric acid. As guanidine HCl is chaotropic and has a strong denaturing effect on proteins, it was not considered as an elution solvent. Interestingly, all other effective solvents were acids. It was also found that neither organic solvents (at a concentration of 10%) nor basic conditions resulted in any significant loss of antibody binding; also high or low ionic strength conditions did not cause any loss in binding. It was therefore decided to further investigate the effect of acidic pH values on antibody binding. For that, the test was repeated using 10 mM phosphate buffer in the pH range between 2.5 and 7.5 as elution solvents. As seen in Fig. 8, solutions with a pH value lower than 4 significantly reduced antibody binding.

### 3.2.3. Fluorescence-based thermal stability experiments

One of the greatest disadvantages of protein-based affinity chromatography columns is their susceptibility to denaturation caused by unfavorable environmental conditions. In order to assess, e.g.,

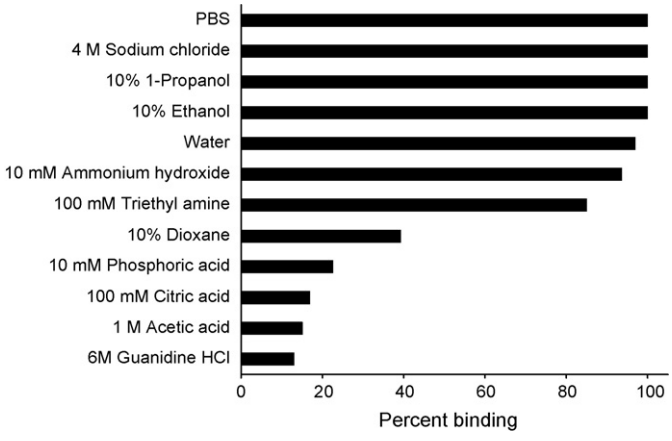


**Fig. 7.** Efficacy of potential elution solvents as determined by noncompetitive ELISA. After binding of the primary antibody, the microtiter plate was incubated with elution solvents for 15 min at room temperature. Antibody which remained bound to the surface-immobilized antigen was detected with HRP-labeled streptavidin. PBS was used as a reference buffer.

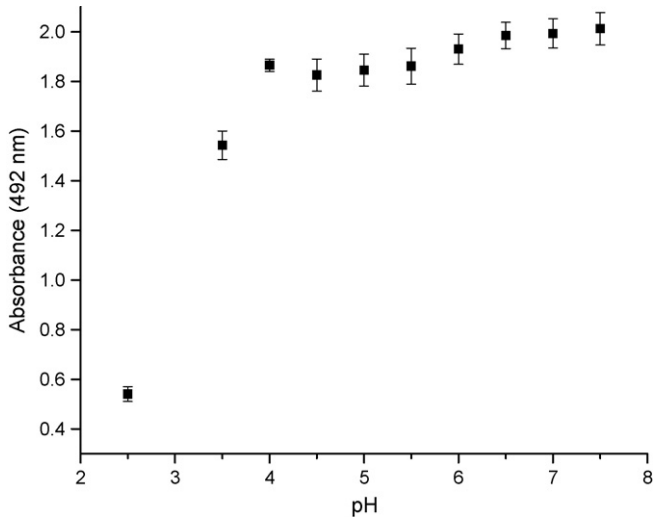


**Fig. 8.** Effect of the pH of potential elution solvents as determined by noncompetitive ELISA. After binding of the primary antibody, the microtiter plate was incubated with the elution solvents for 15 min at room temperature. Antibody which remained bound to the surface-immobilized antigen was detected with HRP-labeled streptavidin.

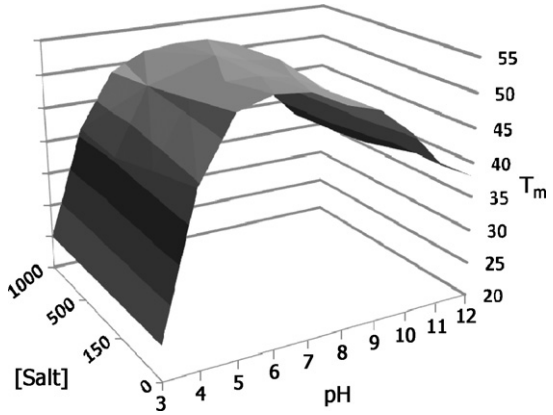


**Fig. 10.** Stability surface (as measured by $T_m$ values) for the anti-caffeine antibody as a function of pH and salt.

the utility of potential elution conditions it is, therefore, of great relevance to investigate the effect of parameters such as temperature, pH, and ionic strength on protein stability.

High-throughput fluorescence-based thermal stability experiments have emerged as a useful tool to gauge protein stability in applications including drug [48] and protein stabilization agent screening [49,50]. Furthermore, they can be used to measure or rank relative affinities of ligands for a particular protein without requiring extensive assay development [51]. These studies take advantage of the spectral properties of environmentally sensitive fluorescent dyes (e.g., Sypro Orange, ANS, Ref. [52]) that serve as indicators of protein unfolding. If a protein unfolds under certain experimental conditions, hydrophobic amino acid residues previously buried in the interior of the protein become surface-exposed and accessible to both solvent and dye, which results in an observable change in fluorescence.

Here, fluorescence-based thermal melts were used to investigate the temperature and pH stability of the anti-caffeine VHH antibody and to obtain further information on its binding specificity. Fig. 9 shows plots of the raw data for thermal unfolding of the anti-caffeine VHH antibody alone and with the four different ligands caffeine, theophylline, theobromine, and paraxanthine. The apparent mid-point of the unfolding transition (i.e., the $T_m$) for the unliganded antibody was 56 °C. Although this value is rather low in comparison to previously reported $T_m$ values for single domain VHH antibodies, which range between 60 and 75 °C [53], the antibody's thermostability is comparable to that of typical monoclonal antibodies. The results shown in Fig. 9, furthermore, demonstrate that ligand binding stabilizes the native structure of the antibody and leads to elevated $T_m$ values. Based on the thermal shift assays, which were performed in the presence of a constant 500 μM ligand concentration, the antibody exhibits the highest affinity for caffeine, followed by theophylline, paraxanthine, and theobromine. A requirement for this correlation to be true is that the binding enthalpies must be similar [54]; this, however, is likely a safe assumption considering the structural similarity between the ligands. It should be noted, furthermore, that the observed affinity ranking is consistent with the ELISA results.

To further investigate the antibody's tolerance to extremes of pH and ionic strength, additional stability screens were performed over a wide range of pH (1–12) and salt concentrations (0–1000 mM). Fig. 10 presents a surface plot of determined antibody $T_m$ values. Perhaps not surprising, the maximum $T_m$ was found to be between pH 6 and 9, which is around physiological pH. Most noticeable is

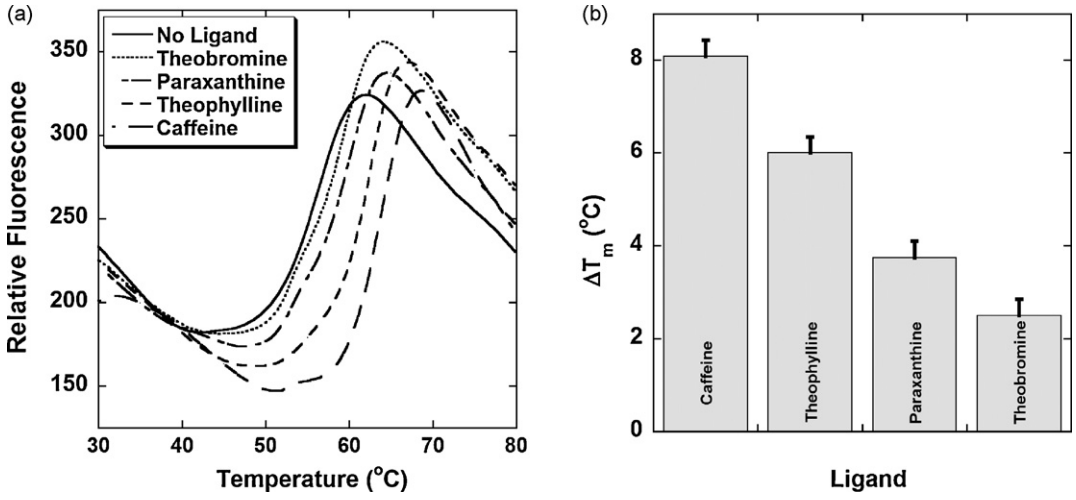


**Fig. 9.** Fluorescence-based thermal melt assays performed with anti-caffeine VHH antibody. (a) Thermal melt raw data displaying the fluorescence signal as a function of temperature. Assays were performed with unliganded antibody or antibody in the presence of ligand (at 500 μM) as indicated. (b) Temperature shift values ($\Delta T_m$) for the four methylxanthines.

the considerable drop in $T_m$ as the pH decreases below pH 4. In fact, the unfolding transitions were not fully captured below pH 3 and are therefore not included in the plot (i.e., $T_m$ values were below 25 °C). At elevated pH (pH ≥ 10) there is also a drop in $T_m$, although not to the same extent as observed under acidic conditions. Salt concentration (NaCl concentrations up to 1 M) did not appear to appreciably change the $T_m$ at any pH value. All these results are in good agreement with the findings of the ELISA experiments.

### 3.3. Chromatographic studies

The application of single domain VHH antibodies for chromatographic separation of low-molecular weight molecules is attractive for several reasons. Their limited size results in increased column capacities and they can be readily expressed in high yields in *E. coli*. In addition, it has been shown that they possess higher thermodynamic stability compared to conventional antibody fragments [53]. The VHH domain employed here, furthermore, was solely produced by molecular biological techniques. Thus, no proteolytic digestion, which is typically applied to obtain antibody fragments from whole immunoglobulins, had to be performed.

Five milligrams of camelid anti-caffeine VHH antibody were immobilized onto DSC-activated POROS and slurry packed into a stainless steel column (3.2 mm × 50 mm). The coupling chemistry applied here can be performed under mild buffer conditions and leads to the formation of a stable, uncharged carbamate bond between stationary phase and protein. Yields are typically high and leakage of antibody is negligible. In this study, more than 99% of the total amount of antibody invested was linked to the support as was determined spectrophotometrically by measuring the difference of the absorbance (at 280 nm) of the antibody solution before and after the immobilization step (data not shown).

In order to separate caffeine and its metabolites using HPLC, differences in the antibody's affinity toward each individual analyte had to be considered. While lower affinity compounds require conditions favoring association with the antibody to achieve retention on the column, higher affinity compounds may call for different conditions that reduce their interaction with the immobilized antibody and enable elution. It has previously been found that flow rate is one of the chromatographic parameters that can be modulated to adjust elution conditions to the strength of interaction between immobilized antibodies and analytes [55–57]. To investigate the effect of flow rate, retention of the model analyte theobromine was examined between 0.5 and 4 ml/min using PBS as mobile phase. The POROS support material utilized here is particularly valuable for such studies as it allows use of a wide range of flow rates without causing a significant increase in backpressure [58,59]. While higher flow rates led to an improvement in peak shape (not shown), changes in the flow rate had virtually no effect on the retention factor $k$ obtained with theobromine ($k$ = 3.23). It should be noted that theobromine has the lowest affinity toward the antibody of all compounds investigated in this study. Using anti-amino acid antibodies and amino acid analytes, it had been demonstrated previously that an effect of the flow rate on analyte retention is dependent on the affinity between the immobilized antibody and its binding partner [57]. A comparison of the retention factors obtained with paraxanthine, which is bound somewhat more strongly by the antibody, seems to support this observation as the retention factor for this compound decreased from $k$ = 4.87 at 0.5 ml/min to $k$ = 4.49 at 4 ml/min. The difference in retention between theobromine and paraxanthine, though, was not sufficient to allow separation of these compounds, which coeluted as one peak.

The effect of temperature on the interaction between paraxanthine and the immunoaffinity stationary phase was evaluated at temperatures between 5 and 35 °C. This variable showed the greatest effect on analyte retention, with lower temperatures favoring the interaction, thus leading to increased retention factors. The Van't Hoff plot of ln $k$ as a function of $1/T$ has a positive slope (Fig. 11), indicating that the interaction between the VHH antibody and paraxanthine is enthalpically favored. However, a regression line of all points deviates from linearity ($y = 4.63x - 14.63$; $R^2 = 0.9759$). The apparent deviation from linearity, in particular the downward curvature, is likely indicative of the presence of a significant negative change in heat capacity ($\Delta C_p$) upon binding of paraxanthine to the antibody [60]; a large and negative $\Delta C_p$ may suggest a considerable change in surface area or additional linked equilibria [61].

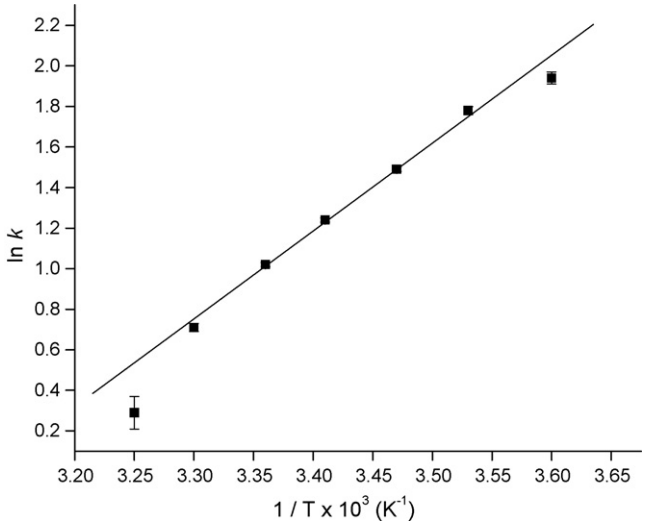


**Fig. 11.** Van't Hoff plot for chromatographic analyses of paraxanthine at 2 ml/min using PBS as mobile phase. Values represent means of triplicate determinations. Missing error bars are obscured by the symbols.

Varying the ionic strength of the mobile phase is known to be a potentially effective tool for modulating the interaction between immunoaffinity stationary phases and analytes. In order to investigate the effect of this chromatographic parameter on the interaction between paraxanthine and the immobilized anti-caffeine VHH antibody, analyses were performed using 10 mM phosphate buffer (pH 7.4) containing varying concentrations of NaCl. As seen in Fig. 12, the retention factor $k$ appears to have a minimum between

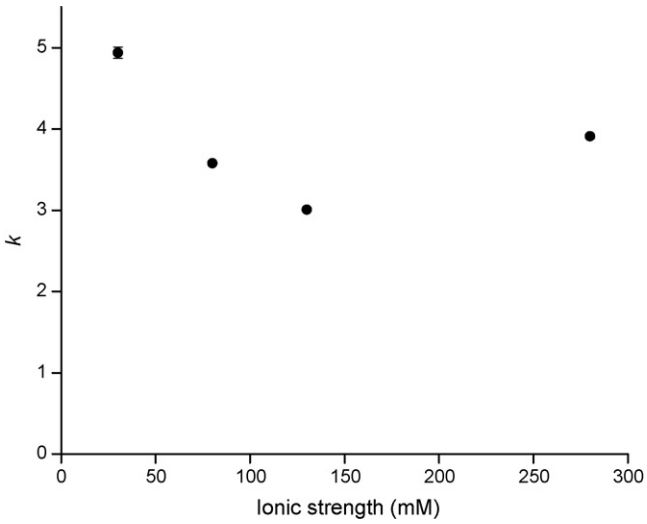


**Fig. 12.** Influence of the ionic strength on the retention factor $k$. Analyses of paraxanthine were performed at 2 ml/min in 10 mM phosphate buffer, pH 7.4, containing varying amounts of NaCl. Values represent means of triplicate determinations. Missing error bars are obscured by the symbols.

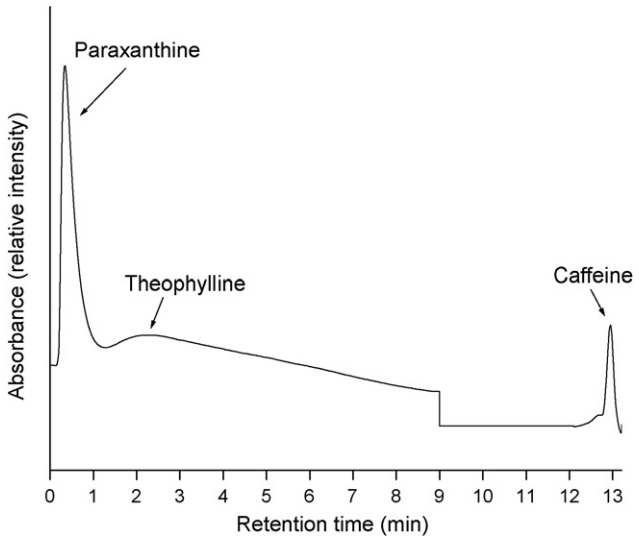


**Fig. 13.** Simultaneous separation and detection of paraxanthine, theophylline, and caffeine. Gradient elution was achieved by applying 100% PBS, pH 7.4, from $t = 0$ min to $t = 9$ min, and 100% 10 mM phosphate buffer, pH 2.5, from $t = 9.1$ min to $t = 12$ min, which was followed by 100% PBS, pH 7.4, from $t = 12.1$ min to the end of the run. Blank injections of PBS were employed to subtract baseline absorbance changes caused by solvent mixing.

100 and 150 mM, which indicates that the effect of ionic strength may be rather complex. While it is well known that electrostatic interactions between analytes and proteins are favored at decreased ionic strength and weakened at high ionic strength, an increase in the salt concentration may also cause dehydration, which, in turn, can increase hydrophobic interactions [62]. Thus, the increase in retention observed at higher ionic strength (i.e., between 250 and 300 mM) seen in Fig. 12 may be attributed to stronger hydrophobic interaction between the antibody binding site and the aromatic analyte.

While the three metabolites of caffeine, i.e., theobromine, paraxanthine, and theophylline (not shown) could all be eluted from the immunoaffinity column under mild isocratic conditions using, e.g., PBS as sole mobile phase, the higher affinity between the immobilized antibody and caffeine required more drastic conditions to achieve elution of this ligand. However, as it was known from both the ELISA and thermal shift assays that the antibody's binding affinity is significantly reduced at pH values below 4, low pH phosphate buffers were tested as elution solvent. It was found that antibody-bound caffeine could successfully be eluted by brief pulses of phosphate buffer of pH 2.5. Fig. 13 shows a separation of paraxanthine, theophylline, and caffeine on the VHH antibody column. While PBS, pH 7.4, was utilized for the separation of paraxanthine and theophylline, 10 mM phosphate buffer, pH 2.5, was applied to elute the more strongly bound caffeine. The gradient only subjected the column to a 3 min pulse of 10 mM phosphate buffer, pH 2.5, which was sufficient to disrupt the interaction between the antibody and this analyte, after which the column was regenerated with PBS. It is noteworthy that the brief pulses of acidic buffer did not significantly reduce binding capacity of the column, which could be reused for at least 25 separation cycles.

## 4. Conclusions

Over the last fifty years, solid-phase immobilized antibodies have become a common and most valuable tool in all areas of life sciences. While the introduction of hybridoma technology resulted in a significant boost of immunoaffinity techniques, more recent advancements in molecular biology have greatly facilitated the production and targeted manipulation of antibody fragments. The availability of high throughput-capable analytical methods, furthermore, aids in the characterization of the biophysical properties of such engineered biomolecules. Due to their unique features, camelid single domain antibodies are particularly attractive receptor molecules for use in affinity techniques. They are relatively small but conformationally stable and can be readily expressed in bacterial cell cultures. This work reports the first use of a camelid VHH antibody for chromatographic separation of small molecules. While here the specific binding properties of an anti-caffeine antibody were utilized for separation of caffeine and related methylxanthines, camelid antibodies with other binding specificities should also be suitable for analogous applications, making this a generally useful approach.

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