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# (Immuno)affinity chromatography: a versatile tool for fast and selective purification, concentration, isolation and analysis

M.M. Rhemrev-Boom [a,*], M. Yates [b], M. Rudolph [b], M. Raedts [c]

[a] *ResQ lab BV, Kolderveense Bovenboer 14, 7948 LV Nijeveen, The Netherlands*
[b] *ICN Biomedicals GmbH, Thüringer Strasse 15, 37269 Eschwege, Germany*
[c] *Sepragen, Ralreiger 33, 7827 VC Emmen, The Netherlands*

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**Abstract**

Today, thanks to the availability of tailor made biomolecules with the desired biological functions, separations based upon (immuno)affinity techniques are more and more common in a large field of applications. By using the high selectivity of biomolecules (antibodies, receptors, specific proteins), this technique offers the possibility of isolating compounds from complex samples with a selectivity which cannot be achieved by other chromatographic methods. In order to succeed, however, the solid phase support for the immobilisation of the ligand of interest plays a prominent role. For this reason, numerous supports have already been introduced while research on new materials with additional advantages is continued. Here, a new solid phase support will be discussed for (immuno)affinity applications. This material demonstrates low non-specific adsorption and high ligand accessibility, which enables an enhanced selectivity and capacity. Because the material is available in large quantities and exhibits superb mechanical and physical stability, selective isolations have been performed on analytical as well as preparative scale. To demonstrate the potential of this new support, several applications will be presented. Based upon immunoaffinity, two applications for the determination of oestradiol in serum respectively vitamin B12 in fermentation broth will be presented. Regarding affinity chromatography, an enzyme reactor in which the enzyme glucose oxidase is immobilised on the new material, is made for the detection of glucose by Flow Injection Analysis and electrochemical detection. Next, to isolate, identify and test components on their xeno-oestrogenic activity, an affinity column is produced in which human oestrogen receptor is covalently coupled. Several components are screened on their biological activity and the results obtained will be presented here. 

*Keywords:* Affinity support; Immunoaffinity support; Oestradiol; Vitamin B12; Human estrogen receptor

## 1. Introduction

Undoubtedly, (immuno)affinity chromatography is one of the most powerful techniques to

* Corresponding author. Fax: +31-522-492619.
*E-mail address:* m.mrhemrevboom@freeler.nl (M.M. Rhemrev-Boom).


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selectively isolate and/or concentrate minor components of interest from a complex mixture [1]. Its selectivity is derived from the use of an immobilised specific biomolecule, the affinity ligand, on a suitable solid phase support. Ideally, a sample passed through an (immuno)affinity column, separates into two bands. The first band elutes with a capacity ratio $k' = 0$ and contains all the compounds which do not bind to the affinity ligand. The second band, containing only the analyt, should be strongly adsorbed to the ligand and should not elute. Increasing the mobile phase strength by a change of pH or other parameter causes the analyt peak to elute [2–4]. If used in combination with other modes of chromatography, for instance high performance liquid chromatography (HPLC), the immunoaffinity column can serve as selective on-line pre-cleanup step for the isolation of a group of compounds which are captured by one or more immobilised antibodies. After elution of the captured analytes, quantitative analysis can be performed by HPLC. Via this way the advantages of both techniques, high selectivity as well as high precision, are combined [5]. Today, a variety of biomolecules with the desired (biological) characteristics can be produced even at large scale. As a result, techniques involving this type of separations become more and more accessible in a large field of applications. As a result, there is an ongoing need for solid phase supports, which meet the specific criteria set for these different types of applications. In general, these solid phase supports should be chemically and physically stable, possess good mechanical strength to allow high flow rates and should have low non-specific adsorption and/or interaction [6]. Polymer flexibility and immobilisation of ligands of interest should be such that high ligand accessibility is achieved and the properties of the ligand are not destroyed [7]. For applications on analytical scale, parameters such as particle size and pore-size distribution have to be taken into account. Additionally, low cost, high capacity, maximum throughput and their ability to withstand regenerations and cleaning procedures are important parameters to consider for applications on preparative scale. During the past a wide variety of materials, including organic and inorganic polymers, have been used for the design of these solid phase supports. In general, a solid phase support includes two aspects: the base support and the stationary phase that is chemically or physically immobilised onto the core and carries the necessary functions. The base support plays a dominant role in the mechanical, chemical and thermal stability of packing materials. One of the first materials introduced were natural polysaccharides, such as agarose, cellulose and crosslinked dextran [8]. These materials are stable over a wide pH-range (pH 3–13) and possess a high content of hydroxyl groups available for activation and derivatisation. This hydrophilic surface generally does not interact with proteins and shows low non-specific adsorption. A major drawback is their poor mechanical strength related to their swelling ability. Other organic polymers are based on synthetic polymers, such as polyacrylamide, polyacrylate and polyvinyl polymer [9]. Although more resistant to pressure than polysaccharides, in comparison to inorganic materials they exhibit a lower pressure tolerance. In addition, some of these materials shows swelling differences in the presence of organic solvents, a broader pore-size distribution, decreased efficiency and non-specific interaction due to the hydrophobic character of these materials. The inorganic polymer silica is, undoubtedly, the most widely used chromatographic material. Silica is very stable under pressure and can easily be derivatized to introduce functional ligands [10]. Unfortunately, it is unstable at mild alkaline pH values and dissolves drastically above pH 8. In addition, non-specific interactions occur above pH 4 between deprotonated silanol groups and the basic parts of for instance biomolecules. However, to minimise this non-selective adsorption and to introduce functional groups, a variety of protocols have beendeveloped throughout the years to modify the surface of these supports. Surface modification can be performed either by chemical modification or physical adsorption of polymers. Most chemical reactions on silica or on other metal oxides involve the use of organosilanes that react with hydroxyl groups present on the surface [11,12]. Although numerous attempts have been reported to improve hydrolytic stabil-

ity, ionic interactions of biomolecules with residual silanols are still a matter of concern. A more simple way to effectively shield the surface is by physical adsorption of polymers [13]. After crosslinking of the polymer chain, leakage of polymer can be minimised whereas the stability of the coating is increased [14]. To circumvent, however, non-selective adsorption, it has to be emphasised that only hydrophilic polymers are to be considered. Polymers, which are by means suitable for this purpose, are water-soluble polymers such as (poly)ethyleneimine [15] and modified dextran or agarose [16]. So far, these polymers have been used mainly for the synthesis of a variety of ion exchange media. Only limited work has been reported on the application of these materials for (immuno)affinity purposes [17]. But, in order to be able to covalently bind a ligand of interest, further activation of the surface will be necessary. For the immobilisation of proteins, receptors and enzymes, good coupling procedures have been described by the use of aldehyde activated supports [18]. Coupling can be performed under physiological conditions by the formation of a stable amide-bond resulting in a high capacity and stable affinity support. For the coupling of antibodies, in general, site-directed immobilisation is preferred [19]. Only via this way the antibody can be oriented such that its bivalent binding potential for antigen can be realised fully. Hydrazide activated supports permit the coupling of aldehyde- or ketone-containing ligands through the formation of stable hydrazone linkages. By mildly oxidising the sugar residues from the heavy chains in the $CH_2$ domain of the antibody, formyl groups will be generated, which can be used for site-directed immobilisation of the antibody. Generally, this method results in the coupling of intact antibody molecules and usually gives the highest yield of antigen binding site activity [20]. Besides these activated sites, in order to immobilise these 'bulky' ligands, spacer arms between the core and the activated site are necessary to provide a greater steric accommodation and high ligand accessibility [21,22]. Inevitably, the use of hydrophilic spacer arms is preferred in order to prevent non-selective adsorption.

In this article a new solid phase support will be presented for the purpose of immunoaffinity and affinity separations. The material is based upon alumina and undoubtedly possesses of a high mechanical and physical stability, and compared to silica, a significantly higher chemical stability. Although for high resolution separations in HPLC, silica is superior above alumina, however, for the purpose of (immuno)affinity, these properties are not essential. Additionally, its low price and the fact that it is produced in large quantity enables its use for large-scale separations. The stationary phase consists of a crosslinked water-soluble polymer, to which a 13-atom hydrophilic spacer and the desired activation chemistry (aldehyde versus hydrazine) are coupled. Via this way a material is obtained which demonstrates a good mechanical, physical and chemical stability, low non-selective interaction and a guaranteed high ligand accessibility. To our knowledge no other work has been reported on the use of alumina for the purpose of immunoaffinity and affinity separations. The potential of this new material is demonstrated here by presenting a variety of applications based upon immunoaffinity or affinity chromatography.

## 2. Experiments

### 2.1. Apparatus

In order to test the different (immuno)affinity columns, a model 110B liquid chromatography equipped with a model 163 variable UV–VIS detector (Beckman) or a model DECADE electrochemical detector (Antec Leyden, Leiden, The Netherlands) was used. Injection was carried out by means of a model 210A Sample Injection Valve (50 ul loopvolume) (Beckman, Leiden, The Netherlands). The peaks were monitored by means of a model Chrom-Jet integrator (Interscience, Breda, The Netherlands). The (immuno)-affinity column was coupled on- and off-line with the analytical system by means of a Rheodyne two-position six-way valve (Bester, Amsterdam, The Netherlands). For the separation of the eluted peaks, an analytical column, Inertsil,

150 × 4.6 mm, 5 μm (Chrompack, Vlissingen, The Netherlands), was used.

### 2.2. Materials

Sodium-meta-periodate, sodiumcyanoborohydride, disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium chloride were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Hydrochloric acid, potassium thiocyanate, water (HPLC-grade) and methanol (HPLC-grade) were obtained from Boom (Meppel, The Netherlands). These chemicals were used for coupling of the ligand of interest respectively buffer solutions during the coupling and elution in a chromatographic assay.

For the determination of oestradiol in serum by means of immunoaffinity chromatography, β-oestradiol was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), whereas anti-oestradiol was purchased from Nuclilab (Ede, The Netherlands).

For the vitamin B12 assay by means of immunoaffinity chromatography, cyanocobalamin (vitamin B12) as well as anti-cyanocobalamin was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). For the determination of glucose by means of Flow Injection Analysis (FIA) in combination with electrochemical detection, glucose was purchased from Boom (Meppel, The Netherlands), whereas the enzyme glucose oxidase was obtained from Boehringer Mannheim (Almere, The Netherlands). For the environmental screening on xeno-oestrogenic activity, β-oestradiol was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), whereas Human Oestrogen Receptor (hER) was obtained from 10 P's (Breda, The Netherlands). The compounds butylbenzylphthalate, bis(2-ethylhexyl)phthalate and 3-OH-bis-(ethylhexyl)-phthalate were a gift from R.I.V.M. (Bilthoven, The Netherlands) and were tested for their xeno-oestrogenic activity.

Coupling of the ligand of interest was performed in bulk on RiFlex-ALD (62–200 μm) for affinity respectively RiFlex-HY (62–200 μm) for immunoaffinity chromatography. Both materials were produced in house by ResQ lab (Nijeveen, The Netherlands). The (immuno)-affinity media obtained was packed in a 50 × 4.6 mm empty PEEK columns at a flow rate of 10 ml/min with water as eluens. Suspensions were made in water, whereas packaging was performed according to the procedures described for packing of media with the Self Packing Device (Boehringer Mannheim, Almere, The Netherlands).

## 3. Procedures

### 3.1. Coupling of anti-oestradiol

Anti-oestradiol (200 μg) was dissolved in 500 μl 0.1 M phosphate buffer pH 7.0. Towards this solution 10 mg of sodium periodate was added and dissolved by gently mixing. The reaction was allowed to stand for 30 min at 4°C in the dark. The solution was desalted by ultrafiltration at 5000 g for 30 min at 4°C with a Centrisart C30 filter (Sartorius, Nieuwegein, The Netherlands). The supernatant obtained was dissolved in 1 ml of 0.1 M phosphate buffer pH 7,0 and was added to 1.4 g of RiFlex HY activated alumina. The media was washed prior to coupling with 0.1 M phosphate buffer pH 7,0. The reaction was allowed to complete by stirring overnight at room temperature. After coupling, the media was washed with 0.1 M phosphate buffer pH 7.0.

### 3.2. Coupling of anti-vitamin B12

Anti-vitamin B12 (150 μg) was dissolved in 500 μl 0.1 M phosphate buffer pH 7.0. Towards this solution 10 mg of sodium periodate was added and dissolved by gently mixing. The reaction was allowed to stand for 30 min at 4°C in the dark. The solution was desalted by ultrafiltration at 5000 g for 30 min at 4°C with a Centrisart C30 filter (Sartorius, Nieuwegein, The Netherlands). The supernatant obtained was dissolved in 1 ml of 0.1 M phosphate buffer pH 7.0 and was added to 1.4 g of RiFlex HY activated alumina. The media was washed prior to coupling with 0.1 M phosphate buffer pH 7.0. The reaction was allowed to complete by stirring overnight at room temperature. After coupling, the media was washed with 0.1 M phosphate buffer pH 7.0.

### 3.3. Coupling of the enzyme glucose oxidase

Glucose oxidase (1 mg) was dissolved in 1 ml of 0.1 M phosphate buffer pH 7.0. Towards this solution 1.4 g of RiFlex-ALD was added and 10 mg of sodium cyanoborohydride. The media was washed prior to coupling with 0.1 M phosphate buffer pH 7.0. The reaction was allowed to complete by stirring overnight at room temperature. After coupling, the media was finally washed with 0.1 M phosphate buffer pH 7.0.

### 3.4. Coupling of hER receptor

Human Oestrogen Receptor (hER) (50 μg) was dissolved in 1 ml of 0.1 M phosphate buffer pH 7.0. Towards this solution 1.4 g of RiFlex-ALD was added and 10 mg of sodium cyanoborohydride. The media was washed prior to coupling with 0.1 M phosphate buffer pH 7.0. The reaction was allowed to complete by stirring overnight at room temperature. After coupling, the media was washed with 0.1 M phosphate buffer pH 7.0.

## 4. Results and discussion

The most conventional way to coat metal oxides is by using the organosilane chemistry. Probably due to the instability of the Si–O–Al bond, only limited work has been published on the preparation of alumina-based phases. Experiments with γ-glycidoxypropyl trimethoxy-silane as coating reagent conducted in our laboratory demonstrated, as expected, the non-selective adsorption of apolar compounds such as testosterone and oestradiol. These results indicated that the alumina surface was not adequately shielded. Better results were obtained when the surface of the alumina particles was coated through physical adsorption of a water-soluble polymer. To circumvent leaching out of adsorbed polymer, the polymer was crosslinked afterwards. The selectivity of the support thus obtained was investigated by conducting similar studies with oestradiol, testosterone and cyanocobalamin. In this case no non-selective adsorption was observed for the compounds tested, and demonstrated thus the complete coating of the surface of the alumina particles. Possibly due to the limited accessibility of 'bulky' molecules, only low capacity affinity columns were obtained with these supports. For this reason, a hydrophilic 13-atom spacer was covalently coupled to the crosslinked polymer, which demonstrated an improvement of the ligand accessibility and enabled the introduction of the activation chemistry necessary for the covalent coupling of biomolecules under physiological conditions. For the coupling of ligands of interest, two different activated media were developed. By covalent coupling of adipic dihydrazide to the spacer atom, a hydrazine activated support (RiFlex-HY) support was thus obtained, which enables the site-directed immobilisation of antibodies. By covalent coupling of glycidol to the spacer atom followed by mild oxidation with sodium metaperiodate, an aldehyde activated support (RiFlex-ALD) was obtained, which can be used for the immobilisation of proteins, enzymes, receptors, etc.

An assay, based upon immunoaffinity chromatography, was developed for the determination of oestradiol in serum. By switching the immunoaffinity column on- and off-line with the analytical system via the two-position six-way valve, breakthrough versus capturing of oestradiol by the immunoaffinity column was determined. No breakthrough of oestradiol was found and up to 2 μg of oestradiol was quantitatively captured by the immunoaffinity column. Quantitative elution of the captured oestradiol was possible by means of 80% v/v methanol in water. A typical chromatogram obtained after capturing, elution and analysing of oestradiol by means of immunoaffinity in combination with RP-HPLC is shown in Fig. 1. The precision of the method was determined by analysing a standard solution containing 200 ng/ml of oestradiol. The day-to-day reproducibility, expressed as the relative standard deviation in the peak height after six successive runs with one immunoaffinity column, was found to be 4%. The linearity and limit of detection (LOD) of the assay was found to be 0–2000 ng/ml respectively 25 ng/ml of oestradiol. The sensitivity of the assay could be improved by injecting 1000 μl of sample instead of 50 μl. In

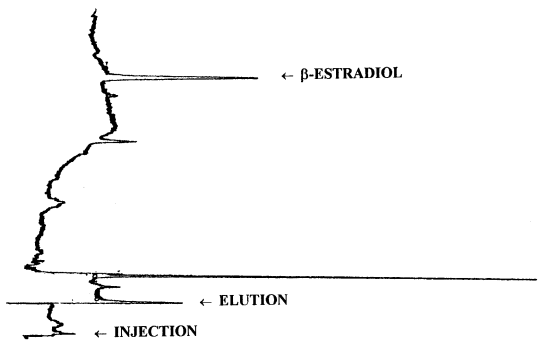


Fig. 1. Analysis of a standard solution of β-estradiol by immunoaffinity chromatography and RP-HPLC. Capturing of antigen: 0.01 M phosphate buffer pH 7.0 (0–5 min); Elution and quantitative analysis of antigen: 80%v/v methanol (5–20 min); injection volume: 1 ml; flow: 1 ml/min; detection: UV–VIS at 230 nm; Retention time of β-estradiol: 15.5 min.

this case a LOD of 2 ng/ml (7.3 nMol/l) of oestradiol could be obtained. The selectivity of the assay was determined by analysing a spiked standard reference serum sample containing a variety of hormones. Although the immobilised antibody did show cross-reactivity (Fig. 2), the content of oestradiol could accurately be determined thanks to the additional separation by means of RP-HPLC. The stability of the immunoaffinity column was tested by analysing weekly both standard solutions as well as samples. During a period of 6 months $\sim 200$ separations have been carried out. During that time the column remained its activity and no significant degradation of the antibody was observed. When not in use, the immunoaffinity column was stored in the refrigerator at 4–8°C.

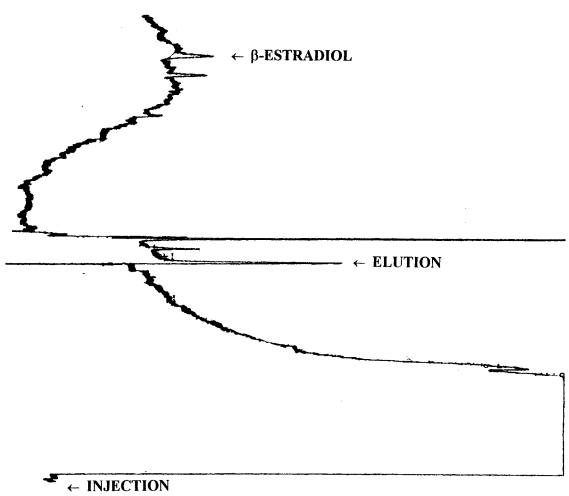


Fig. 2. Analysis of a standard reference serum sample by immunoaffinity chromatography and RP-HPLC (chromatographic conditions: see Fig. 1). The serum sample contains besides β-estradiol, estriol, cortisol, aldosterone, progesterone, testosterone and 17-hydroxyprogesterone.

Because the reference values of oestradiol in serum (0–2 nMol/l) are significantly lower than the sensitivity of the assay presented here, the method will not be suitable as diagnostic tool for the determination of oestradiol in serum. However, this assay typically demonstrates the advantage of combining immunoaffinity with RP-HPLC. A fast and selective cleanup and preconcentration is obtained on-line by means of the immunoaffinity column, whereas the analyt of interest is determined accurately, precise and, thanks to the selective cleanup, within several minutes by means of RP-HPLC.

A second assay based on immunoaffinity in combination with RP-HPLC was developed for the analysis of vitamin B12 (cyanocobalamin) in fermentation broth. Capturing respectively breakthrough of vitamin B12 was determined in a similar way as described above. The immunoaffinity column quantitatively captured Vitamin B12, whereas quantitative elution could be performed by 30% v/v methanol in water, containing 500 μl of concentrated hydrochloric acid and 150 mM sodium chloride. The precision of the method was determined by analysing a standard solution containing 7.96 μg/ml of vitamin B12. The repeatability, expressed as the relative standard deviation in the peak height after six repetitive injections, was found to be 4%. The linearity of the method was found to be up to 10 mg/l of vitamin B12. Above a concentration of 10 mg/l of vitamin B12 and at an injection volume of 50 μl, breakthrough of vitamin B12 was observed. By analysing fermentation broth samples and comparing the results with those obtained with a validated method, the selectivity of the assay was demonstrated. The deviation between the nominal and calculated results was $< 2\%$, and was therefor found to be well in line with the precision of

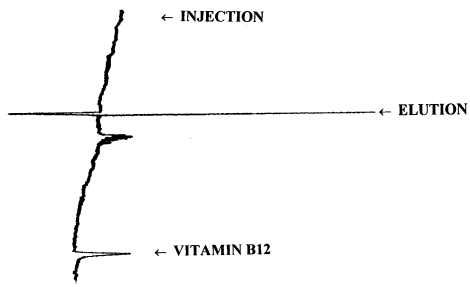


Fig. 3. Analysis of a standard solution of vitamin B12 by immunoaffinity chromatography and RP-HPLC. Capturing of antigen: 0.01 M phosphate buffer pH 7.0 (0–5 min); Elution and quantitative analysis of antigen: 30%v/v methanol and 70%v/v water containing 500 μl concentrated HCL/l + 150 mM NaCl; injection volume: 50 μl; flow: 0.8 ml/min; detection: UV–VIS at 361 nm; Retention time of vitamin B12: 14.4 min.

the method. Based on these results, it was concluded that the method was accurate and selective enough for the determination of vitamin B12 in fermentation broth. An example of a chromatogram of a standard and fermentation broth sample is shown in Fig. 3.

To demonstrate the applicability of the RiFlex-ALD media, several applications have been successively developed, which will be partly demonstrated here. To examine whether or not an enzyme could be coupled without loosing its activity, it was decided to immobilise glucose oxidase in order to determine glucose by means of Flow Injection Analysis (FIA) in combination with electrochemical detection. Especially for process control during biotechnological processes but also for diabetic care, the monitoring of the content of glucose is of importance. The method is based on the fact that the glucose oxidase converts glucose in gluconolactone and $H_2O_2$. The hydrogen peroxide generated in this reaction is then electrochemically determined on a platinum electrode, which is polarised at + 500 mV versus an Ag/AgCl electrode. Samples and standard solutions of glucose were injected every 2 min onto the column and transported at a flow rate of 0.5 ml/min with 0.1 M phosphate buffer pH 7.0 towards the electrochemical detector for detection. The method was found to be linear up to 50 mM of glucose and demonstrated a LOD of 0.2 mM of glucose. The precision of the method, expressed as the relative standard deviation in the peak height after six repetitive injections, was found to be 2%. Although based on these results, the capacity of the 'enzyme reactor' and the performance of the method were demonstrated, the objective of the study was to demonstrate the possibility of coupling biomolecules to the RiFlex-ALD media without loosing their biological activity. For this reason, no additional experiments were conducted to further validate the assay.

Another assay developed with the RiFlex-ALD media was based on the coupling of human Estrogen Receptor (hER). This receptor binds oestradiol and regulates the concentration of oestradiol in the blood of organisms. Oestradiol in turn plays an important role in the reproduction of mammals as well as fishes. Several studies have pointed out the presence of contaminants in the environment, which exhibit affinity with this receptor and affecting in a negative way the reproduction of several species. Due to this phenomenon, the interest in these so-called xeno-oestrogenic compounds increases, however, only a few of tests are available to screen and identify compounds for their oestrogenic activity. By coupling human Estrogen Receptor to a solid phase support, analytes of interest can be selectively isolated from the matrix by means of their biological activity. Compounds with xeno-estrogenic activity will be captured by the hER affinity column, whereas compounds which do not show xeno-estrogenic activity will not be captured by the hER affinity column. Finally, quantitative analysis of the compounds captured can be carried out by means of RP-HPLC.

To test the xeno-oestrogenic activity of several compounds, the hER affinity media produced here was previously tested for its activity and capacity. Again, by switching the hER-affinity column on- and off-line by means of the two-position six-way valve with the analytical system, breakthrough versus capturing of oestradiol by the hER-affinity column could be determined. Injection of a standard solution containing 4 mg/l of oestradiol demonstrated no breakthrough of oestradiol, and it was concluded that oestradiol was quantitatively captured by the hER-affinity

column. Quantitative elution of oestradiol from the affinity column was found to be possible with 25%v/v 6 M KSCN/50%v/v water/25%v/v methanol, whereas elution of oestradiol from the analytical column was possible with 65%v/v methanol. An example of a typical chromatogram and of the chromatographic conditions used is given in Fig. 4. To examine whether or not the column could distinguish between compounds with or without xeno-estrogenic activity, several detergents were analysed with the assay described here. It was found that a detergent, butylbenzylphthalate with only slightly xeno-estrogenic activity, was captured for only 6%, whereas bis(2-ethylhexyl)phthalate, a detergent that is known to exhibit xeno-estrogenic activity, was captured quantitatively. Its possible metabolite, 3-OH-bis-(ethylhexyl)-phthalate was captured for only 22%. Although the possibilities of this assay is clearly demonstrated, more chemicals need to be tested on their possible xeno-estrogenic activity and the results should be compared with those obtained with in vivo tests. Although this will be done in the near future, these experiments lie, however, beyond the scope of this investigation.

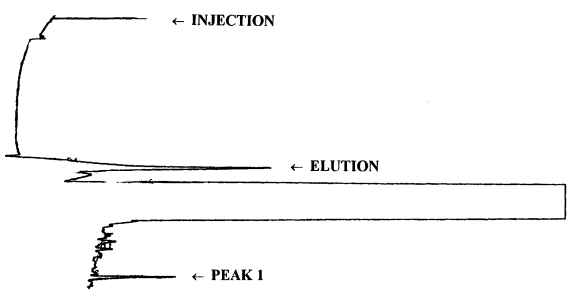


Fig. 4. Analysis of β-oestradiol (peak 1, $t_r = 85.5$ min) by RP-HPLC after being capture and eluted by the hER-affinity column. Capturing: 0.01 M phosphate buffer pH 7.0; Elution: 25%v/v 6 M KSCN, 50%v/v solvent A and 25%v/v methanol; Washing: water; Quantitative analysis: 65%v/v methanol; injection volume: 50 μl; flow: 0.5 ml/min; detection: UV–VIS at 280 nm.

## 5. Conclusions

Coating of metal oxides the conventional way via organosilane chemistry does not lead to the required characteristics; the materials maintain their character and undesirable non-selective adsorption remains. Coating with hydrophilic polymers alters the characteristics of the alumina surface significantly; no non-selective adsorption of nonpolar or polar compounds such as testosterone or oestradiol respectively vitamin B12 was observed. Via this way, it was found to be possible to use alumina as rigid core for the production of immunoaffinity (RiFlex-HY) and affinity (RiFlex-ALD) columns. These media have the advantage of resisting high pressures and organic solvents and can therefore by used in combination with conventional HPLC. For the immobilisation of antibodies, site-directed coupling via a hydrazine-activated media demonstrated good results. For an efficient coupling of the antibody, the use of a spacer was found to be required. Using a hydrophilic 13-atom spacer for this purpose did not lead to non-selective adsorption of apolar or polar compounds like oestradiol or testosterone respectively vitamin B12. It did however improve the ligand accessibility, which resulted in high capacity immunoaffinity media. Coupling of ligands via the amide bond of the aldehyde activated media demonstrated to give high capacity affinity media.

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