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Concentration and purification of orotic acid directly from whey with an expanded bed adsorption system

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Abstract

The development of an expanded bed process for the concentration and purification of orotic acid directly from whey is described. Different commercially available adsorbents were tested in series of pilot batch adsorption experiments to determine the most suitable separation system. Best results were achieved using a weak anion-exchange matrix. An elution protocol was established using $MgCl_2$ as eluting agent to recover the adsorbed orotic acid with ~85% yield and 10-fold concentration. Purified orotic acid was precipitated under acid conditions with a yield of 95%.

Keywords: Whey; Expanded bed chromatography; Orotic acid; Vitamins

1. Introduction

Pyrimidine derivatives constitute an important class of compounds with physiological effects. Orotic acid, an uracil derivative known as vitamin B_{13}, is a key intermediate in the biosynthetic pathway of pyrimidines. For instance it is used in multivitamin products and dietary supplements. Biscaro and Bellone [1] first discovered orotic acid in milk. Average amounts were 70–75 mg l^{-1} of orotic acid in cows milk [2].

Milk processing industries in Germany produce ~$10 \cdot 10^9$ kg of whey every year. Valuable ingredients such as lactose, lactalbumin and lactoferrin are separated from whey in special processing steps. A method for the separation of orotic acid has not yet been reported, although its price of €20–40 kg^{-1} seems attractive.

Typically the purification of compounds from particle-containing crude liquids involves different pre-treatment steps such as centrifugation and/or microfiltration prior to chromatography. Compared to traditional processes, direct adsorption from untreated crude liquids offers a significant decrease in time and costs due to reduction in the overall number of purification steps. In this case, expanded bed adsorption (EBA) is the method of choice.

In fluidized bed techniques, the target substance binds to the adsorbent while particles pass through unhindered. EBA combines clearing up, concentration and adsorptive purification in a single step. Various applications using EBA have been published describing, for example, the direct extraction and purification of monoclonal antibodies [5] and recombinant proteins [6], and enzymes from crude yeast extract [3,7] or *Escherichia coli* homogenates [4].

The same principles as in packed bed chromatography were employed for the selection of the most suitable adsorbent for EBA. Ion exchanger or hydrophobic adsorbents can be used for the purification of

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a large number of products. But due to their low specificity these adsorbents are soon saturated with other compounds, whereas immobilized metal ion affinity chromatography (IMAC) shows a very high specificity to the target substance which is able to bind to metal ions resulting in high loading capacities and purification factors.

In this paper an attempt was made to establish a simple, practical and cheap EBA protocol for the concentration and purification of orotic acid directly from whey by comparing different adsorbents and determine the binding and elution characteristics of the target substance. Finally purified orotic acid was precipitated to obtain crystallized product.

2. Experimental

2.1. Materials

Whey used in this work was a gift from Milchwerke Schwaben (Neu-Ulm, Germany). The average amount of orotic acid was 70–75 mg l^{-1}.

The strong anion exchanger Dowex 1-X2 and the weak anion exchanger Dowex MWA-1 were purchased from Dowex (Dow Corning, MI, USA). Fractogel EMD Chelat with the metal chelating ligand imidodiacetic acid attached to it was purchased from VWR International (Darmstadt, Germany). All other chemicals used were obtained from Sigma–Aldrich (Seelze, Germany).

2.2. Analysis of orotic acid and protein quantification

The determination of orotic acid was performed by reversed-phase ion-pair HPLC. The high-performance liquid chromatography system from Beckman Instruments (München, Germany) consisted of a gradient pump, an interface and an UV-variable wavelength detector. Sample injection (Rheodyne valve, 20-μl filling loop) was done by an autosampler from Bio-Rad (München, Germany). Measurements were performed on a NovaPak C_{18} column (150×3.9 mm, 4 μm) from Waters (Eschborn, Germany) at an eluent flow rate of 1.0 ml min^{-1} and UV detection at a fixed wavelength of 280 nm. The mobile phase consisted of 10 m*M* KH_2PO_4, 5 m*M* tetrabutylammonium hydrogensulfate (TBAHS) pH 4.0, containing 1% (v/v) methanol.

Prior to injection 500 μl of the sample were deproteinated by adding 100 μl 12% (w/v) trichloric acid (TCA) [2].

Protein determination was carried out using the Roti-Quant® Coomassie reagent from Roth (Karlsruhe, Germany) according to the standard protocol with bovine serum albumin as standard substance.

2.3. Batch adsorption method

Both ion exchangers were prepared by washing with four bed volumes (BVs) of distilled water, three BVs of 1 *M* HCl and once again with four BVs of distilled water.

Fractogel EMD Chelat was first washed with 4 BVs distilled water, then loaded using 250 m*M* $MgCl_2$. To remove weakly bound metal ions, the adsorbent was washed with 100 m*M* sodium acetate, 0.5 *M* NaCl pH 4.0 and finally with 4 BVs of distilled water [4].

For the determination of the binding capacity for orotic acid, different amounts of prepared adsorbent (0–700 mg) were incubated in 2 ml whey at room temperature. After 30 min the concentration of orotic acid in the supernatant was measured.

To investigate the appropriate desorption conditions for the ion exchanger, loaded adsorbent was first washed with distilled water and afterwards incubated for 15 min in different concentrated solutions of HCl (0.01–2.5 *M*) and/or $MgCl_2$ (0.01–2.5 *M*). The wash and elution volumes were collected and investigated for orotic acid concentration.

2.4. Expanded bed adsorption method

Expanded bed adsorption was carried out in a Pharmacia Biotech (Uppsala, Sweden) XK26 column (40×2.6 cm) using Dowex MWA-1 as adsorbent (75 g, ~80 ml). The column was connected to a peristaltic pump from Brown Biotech International (Melsungen, Germany) and to a FRAC-100 fraction collector from Pharmacia Biotech.

The settled bed height of the adsorbent was 15 cm. The adsorbent was first washed with 10 BVs of 2 *M* HCl and afterwards with the same volume of distilled water using a flow velocity of 10.7 cm min^{-1}.

For sample application different temperatures and flow velocities were tested. Prior to feed application the expanded bed was allowed to stabilize for at least 30 min.

Weakly retained material and particles had been washed out with distilled water (8 BVs) at the same flow rate used for feed application. The loaded adsorbent was then allowed to settle down and the column adaptor was lowered to the surface of the sedimented bed. Flow was reversed and captured substances were eluted at a flow rate of 5 ml min^{-1} using a step gradient starting with 0.1 *M* $MgCl_2$. After an elution volume of 100 ml the concentration of $MgCl_2$ in the buffer was increased to 1.5 *M*. The pH of the elution buffer was set to pH 1. Applying these elution conditions the adsorbent was directly regenerated.

During sample application, wash and elution step fractions were collected and analysed for total protein and orotic acid content.

2.5. Precipitation of orotic acid

Purified orotic acid was precipitated by adding 2.5 *M* HCl to the sample up to 10% (v/v) at 4 °C. After centrifugation the precipitant was dried at 50 °C overnight.

For quantification a small amount of pellet was resolved in 1 *M* NaOH and the concentration of orotic acid was measured by HPLC as described above.

3. Results and discussion

3.1. Batch adsorption experiments

To optimize binding, wash and desorption conditions several batch adsorption experiments with different amounts of adsorbents were performed. It was observed that ~90% of orotic acid was adsorbed using 300 mg of ion exchanger per milliliter whey. The disadvantage in using a strong ion exchanger is the increasing co-adsorption (3-fold) of other UV-active substances along with orotic acid. In comparison, using the same amount of Fractogel EMD Chelat only ~20% of orotic acid was adsorbed (Table 1). Therefore, further experiments were performed using the weak ion exchanger.

Weakly bound substances or particles were removed by washing the adsorbent with water without any loss of bound orotic acid. It was shown that ~65% of bound orotic acid was released in one elution step by increasing the ion strength from 0.03 to 7.5 mol l^{-1}. By repeating this step twice ~96% of the adsorbed target substance was desorbed; by decreasing the pH, 43% of adsorbed orotic acid could be desorbed.

On the basis of these data expanded bed experiments were carried out, using the weak ion exchanger Dowex MWA-1 as stationary phase.

3.2. Expanded bed experiments

Frontal analysis was employed to determine the volume of untreated whey that can be applied per volume of adsorbent before significant breakthrough of orotic acid occurs. Adsorption in an expanded bed is a process which is controlled by the residence time of the target molecule in the column. Hence, two different flow velocities were compared (Fig. 1).

At a flow velocity of 10.7 cm min^{-1}, the breakthrough of total protein had already occurred when ~250 ml of whey was applied to the column. After the application of 1800 ml whey, ~70% of the initial orotic acid concentration could be detected at the column outflow. Under these conditions the dynamic capacity of Dowex MWA-1 for orotic acid at 5%

Table 1
Results of the batch adsorption experiments

Adsorbent	Amount of adsorbent (mg ml^{-1} whey)	Adsorbed orotic acid (%)	Adsorbed impurities (%)
Dowex 1-X2	300	90	75
Dowex MWA-1	300	90	25
Fractogel EMD Chelat	300	20	NE[a]

[a] NE=not estimated.

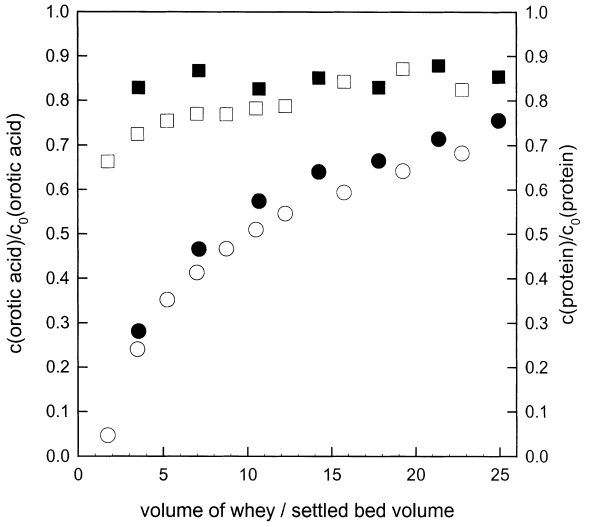

Fig. 1. Comparison of breakthrough profiles of orotic acid in expanded bed adsorption onto Dowex MWA-1 using two different flow velocities at room temperature (column size 40×2.6 cm, settled bed height 15 cm of adsorbent material). Orotic acid (circle) and total protein (square) in the effluent; closed symbols refer to a flow velocity of 10.7 cm min^{-1}, open symbols refer to a flow velocity of 5.3 cm min^{-1}.

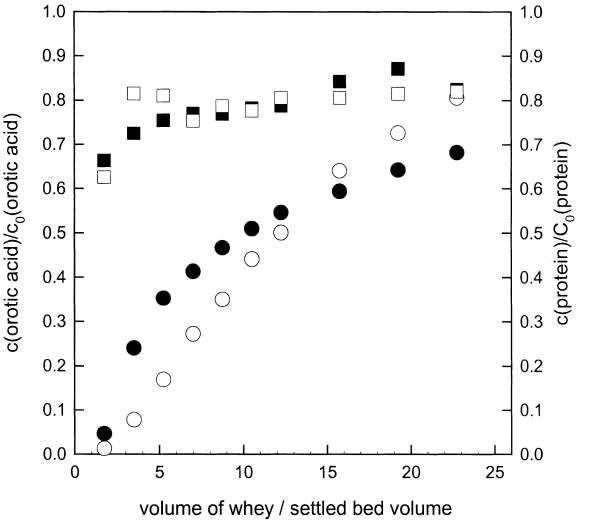

Fig. 2. Breakthrough curves for orotic acid and total protein at room temperature and 40 °C at a flow velocity of 5.3 cm min^{-1} (column size 40×2.6 cm, settled bed height 15 cm of adsorbent material). Orotic acid (circle) and total protein (square) in the effluent; closed symbols refer to room temperature, open symbols refer to a temperature of 40 °C.

breakthrough was calculated to be 0.15 mg ml^{-1} adsorbent. At 70% breakthrough the dynamic binding capacity was 1.7 mg ml^{-1}. By reducing the flow velocity to 5.3 cm min^{-1}, the breakthrough of total protein occurred after 400–450-ml feed application. However, no significant change in the dynamic binding capacity for orotic acid could be achieved. A further increase in residence time by decreasing the flow velocity may have a negative effect on bed stability, as a result of decreased expansion. The low binding capacities for orotic acid may be due to the saturation of binding sites with other small charged molecules in whey.

To improve the maximum uptake rate of orotic acid the temperature was increased from room temperature to 40 °C while the flow velocity kept constant at 5.3 cm min^{-1}. Fig. 2 shows the breakthrough profile at higher temperature compared to the curve at room temperature.

Increased temperature improved the binding kinetics for orotic acid. The dynamic binding capacity at 5% breakthrough could be enhanced to 0.3 mg ml^{-1} while the breakthrough curve for total protein was nearly the same.

Washing and elution steps were also performed at 40 °C. The loosely bound material was removed from the bed by washing with 8 sedimented bed volumes (SBVs) of water at the same flow velocity as used during feed application. No orotic acid was found in the effluent during the washing step.

As retention in ion-exchange chromatography is based on charge–charge interactions, elution takes place when the ionic strength of the mobile phase is increased. Salt molecules displace the bound sample molecules back into the mobile phase. The pH of the mobile phase also strongly affects the charge characteristics of sample molecules. Changes in pH can thus be used to weaken or eliminate charge–charge interactions, thereby causing elution. Therefore the pH of the elution buffer was set below the pK_a value of orotic acid (pK_a 2.57) in order to protonate, and thus desorb, orotic acid.

Based on the results of batch adsorption experiments, we used a step-wise elution by increasing the $MgCl_2$ concentration of the elution buffer from 0.1 to 1.5 *M* at a pH of 1. Desorption was performed in packed-bed mode at a flow rate of 5 ml min^{-1}. A typical chromatogram is illustrated in Fig. 3. Most of

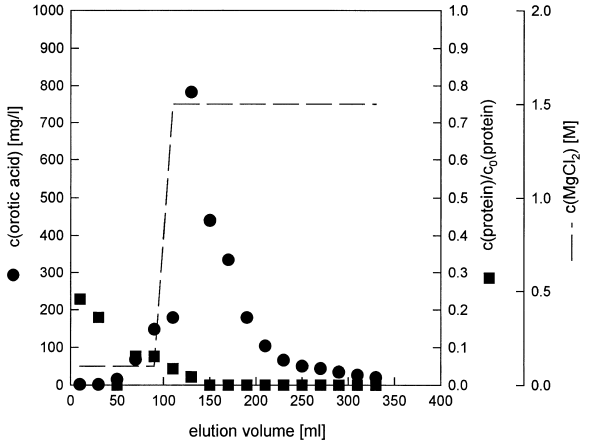

Fig. 3. Step-wise elution of orotic acid from Dowex MWA-1 by increasing the $MgCl_2$ concentration in the elution buffer from 0.1 to 1.5 *M* at a pH of 1. Elution flow rate was 5 ml min^{-1} at 40 °C. Orotic acid concentration in the fraction (●), total protein in the fraction (■).

the bound protein elutes immediately before increasing the ionic strength of the elution buffer. Nevertheless some protein co-elutes with orotic acid (Fig. 3). Recovery of 85% was achieved for bound orotic acid.

Orotic acid is insoluble at pH 0 and 4 °C. Therefore 2.5 *M* HCl was added to the collected and cooled elution fraction to precipitate the target molecule. This gave 95% orotic acid recovery with an absolute amount of orotic acid in the precipitate of 65% (w/w).

4. Conclusions

A protocol for an ion-exchange expanded bed system for the concentration and purification of orotic acid directly from whey was developed. The appropriate conditions for binding, washing and desorption steps were evaluated in batch adsorption experiments using different adsorption materials. Dowex MWA-1, a weak ion exchanger, was successfully employed to isolate orotic acid from whey with a desorption yield of 85%.

Orotic acid could be precipitated by a pH shift to pH 0 at 4 °C achieving a yield of 95%. The complete purification process was realized using inexpensive, commercially available adsorbent materials and equipment. Therefore implementation of this technique in existing whey processing methods could be easily achieved.

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