Phosphate-Free Buffer Systems A New Frontier for Apatite Chromatography

Pete Gagnon, Validated Biosystems

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Hydroxyapatite and phosphate

For more than a half century, elution of hydroxyapatite has been believed to require phosphate.

The few exceptions include small alkaline proteins, such as lysozyme and ribonuclease, that can be eluted with salts such as sodium chloride.

M. Gorbunoff, 1984, Anal. Biochem., 136 425-432; T. Kawasaki, 1991, J. Cromatogr., 544 147-184

Hydroxyapatite and phosphate

Some studies have exploited chlorides or acetates in combination with sodium or potassium phosphate to achieve unique selectivities.

Chlorides and acetates selectively weaken ionic interactions (phosphoryl cation exchange and calcium anion exchange) but leave calcium metal affinity relatively unaffected.

Phosphate has been relied upon to suspend the calcium affinity component so that elution can occur.

Hydroxyapatite and phosphate

Recent studies have exploited nonionic organic polymers in combination with phosphates and other salts to achieve unique selectivities.

Polyethylene glycol (PEG) selectively enhances retention in proportion to protein size.

But these applications too have ultimately relied on phosphate for elution.

Hydroxyapatite and sulfate

The Gorbunoff publications that have guided the evolution of hydroxyapatite for the last twenty five years evaluated the effects of many different salts, but did not include sulfates.

Why? Gorbunoff observed: "…SO_3H do not form complexes with calcium…" This suggests that sulfates should be unable to elute calcium affinity interactions.

M. Gorbunoff, 1984, Anal. Biochem., 136 425-432; M. Gorbunoff, 1984, Anal. Biochem., 136 433-439; M. Gorbunoff and S. Timasheff, 1984, Anal. Biochem,. 136 440-445

Hydroxyapatite and sulfate

Later authors in prominent purification texts also discouraged the use of sulfate, stating that: "The presence of … $(NH_4)_2SO_4$ seems not to affect the elution [of hydroxyapatite]."

A major review of HA in the Journal of Chromatography, describing the effects of multiple salts on more than 20 different solutes, did not include sulfates.

E. Karlsson, L. Ryden, J. Brewer, 1989, *in* Protein Purification: Principles, High Resolution Methods, and Applications, J-C. Jansson and L. Ryden, Eds., VCH Publishers, pp. 138-9
T. Kawasaki, 1991, J. Chromatogr., 544 147-184

Potential limitations of sulfate

- *Sulfate salts are well known for their ability to precipitate proteins.*
- *This creates a risk of precipitation for proteins that may elute at high sulfate concentrations.*

- *Preliminary studies indicate that sulfate lacks the HA-stabilizing properties of phosphate.*
- *This means that phosphate or calcium needs to be included in the buffers and samples to maintain column stability.*

Hydroxyapatite and sulfate

"Always listen to experts. They'll tell you what can't be done and why… Then do it."

–Lazarus Long (Robert A. Heinlein)

"The pessimist sees the glass as half empty. The optimist drinks it anyway."

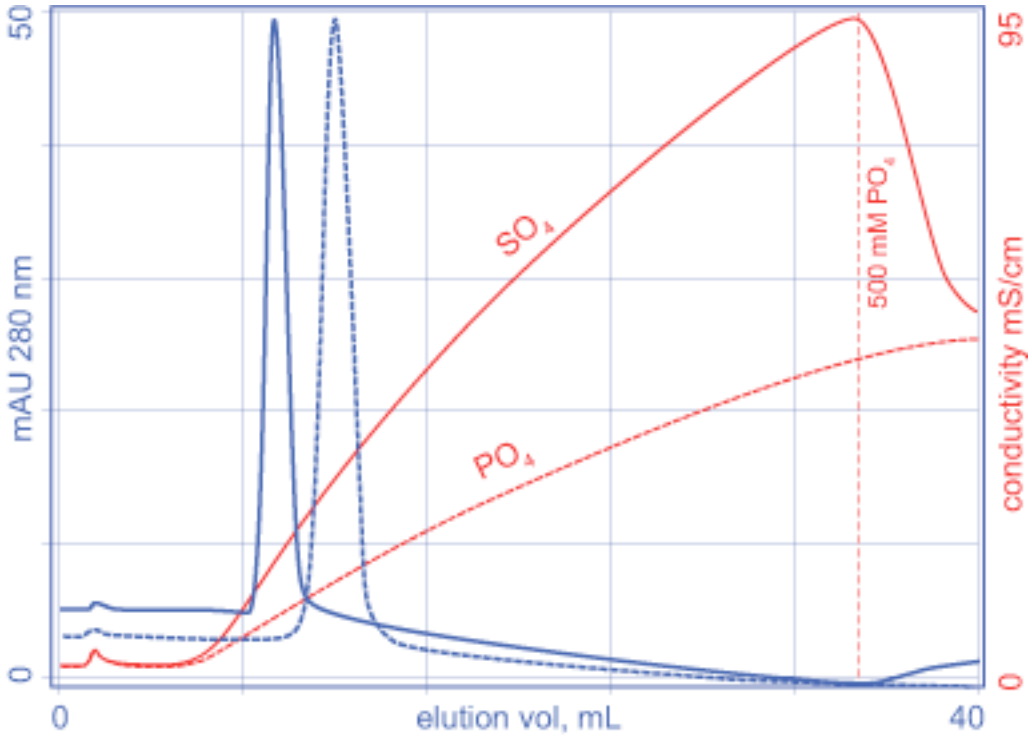
–Bullwinkle J. Moose (Jay Ward)

"Jump in! The water is fine."

–Carcharodon carcharias

Phosphate versus sulfate

Lysozyme



CHT™ Type I, 40 µm

MediaScout™ 5 x 100 (2 mL)

2 mL/min, 600 cm/hr

A: 25 mM $NaPO_4$, pH 7

B1: 500 mM $NaPO_4$, pH 7

B2: A + 1 M Na_2SO_4, pH 7

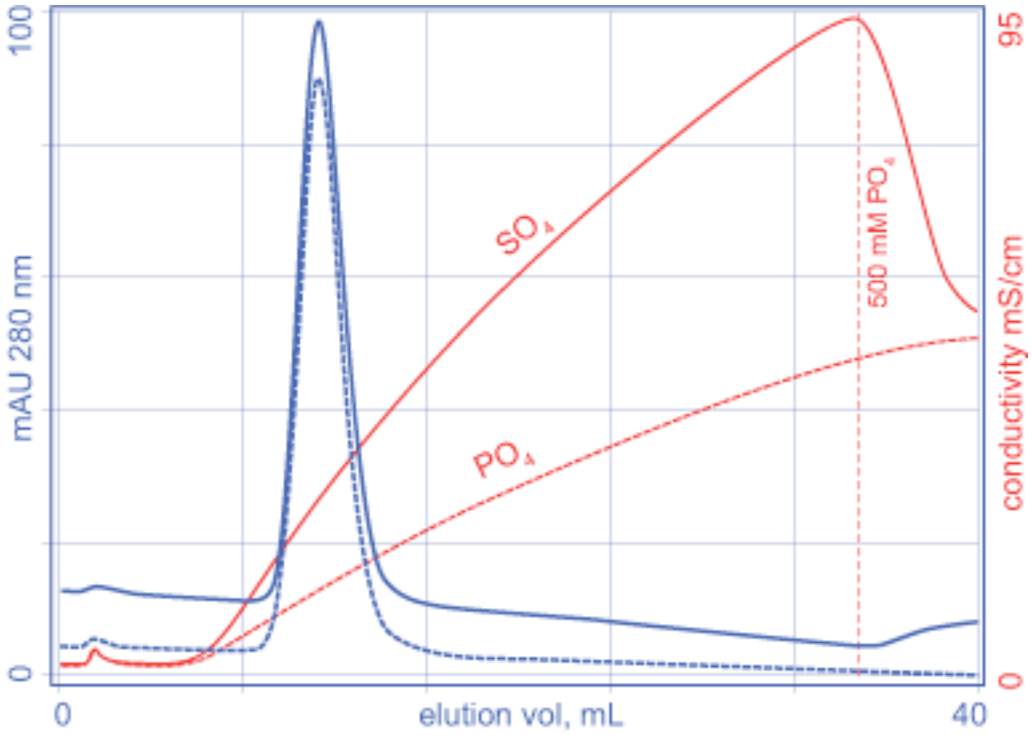
Elute: 15 CVLG A > B

Clean: 500 mM $NaPO_4$, pH 7

Lysozyme binds HA dominantly through phosphoryl cation exchange. Elution occurs at about 100 mM phosphate, or 160 mM sulfate. Elution at higher conductivity in sulfate suggests the influence of an additional binding mechanism, which is assumed to be calcium affinity, and which explains the results as reflecting lower calcium affinity of sulfate.

Phosphate versus sulfate

Monoclonal IgG



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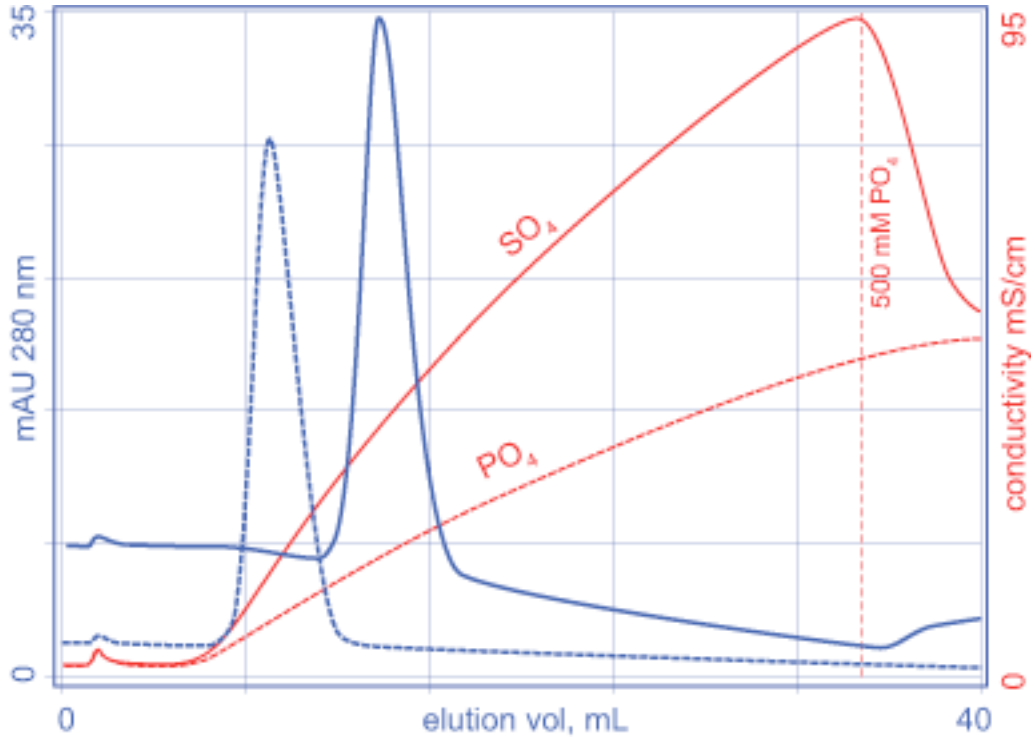
Elute: 15 CVLG A > B

Clean: 500 mM $NaPO_4$, pH 7

The eluting sulfate concentration for this antibody is roughly twice its elution concentration in phosphate. Since the higher conductivity must reduce the contribution of ionic binding, the implication is that later elution reflects sulfate's lower calcium affinity.

Phosphate versus sulfate

BSA



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B2: A + 1 M Na_2SO_4, pH 7

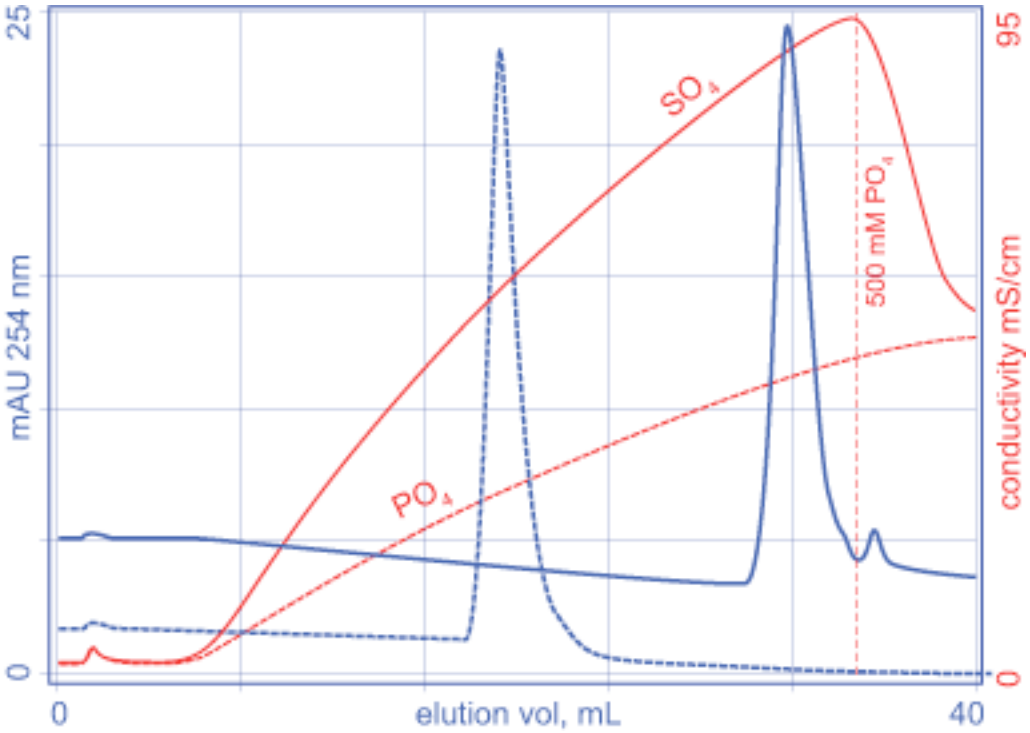
Elute: 15 CVLG A > B

Clean: 500 mM $NaPO_4$, pH 7

Albumin binds dominantly by calcium affinity with additional energy from anion exchange. Given that higher conductivity should weaken anion exchange binding, the relatively high elution concentration in sulfate again suggests that sulfate has weaker calcium affinity.

Phosphate versus sulfate

DNA



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B1: 500 mM $NaPO_4$, pH 7

B2: A + 1 M Na_2SO_4, pH 7

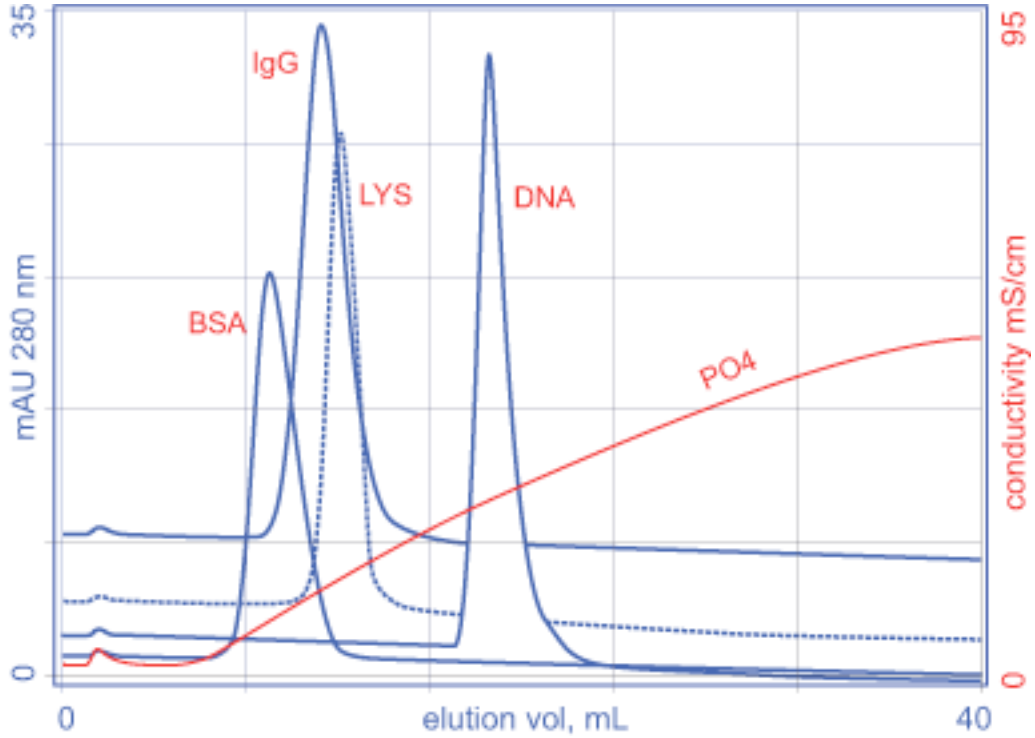
Elute: 15 CVLG A > B

Clean: 500 mM $NaPO_4$, pH 7

DNA binds dominantly through calcium metal affinity. While cation exchange repulsion and anion exchange attraction may affect binding characteristics in low molarity phosphate, the high elution conductivity in sulfate should largely eliminate both.

Phosphate versus sulfate

Composite view, phosphate



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2 mL/min, 600 cm/hr

A: 25 mM $NaPO_4$ pH 7

B: 500 mM $NaPO_4$ pH 7

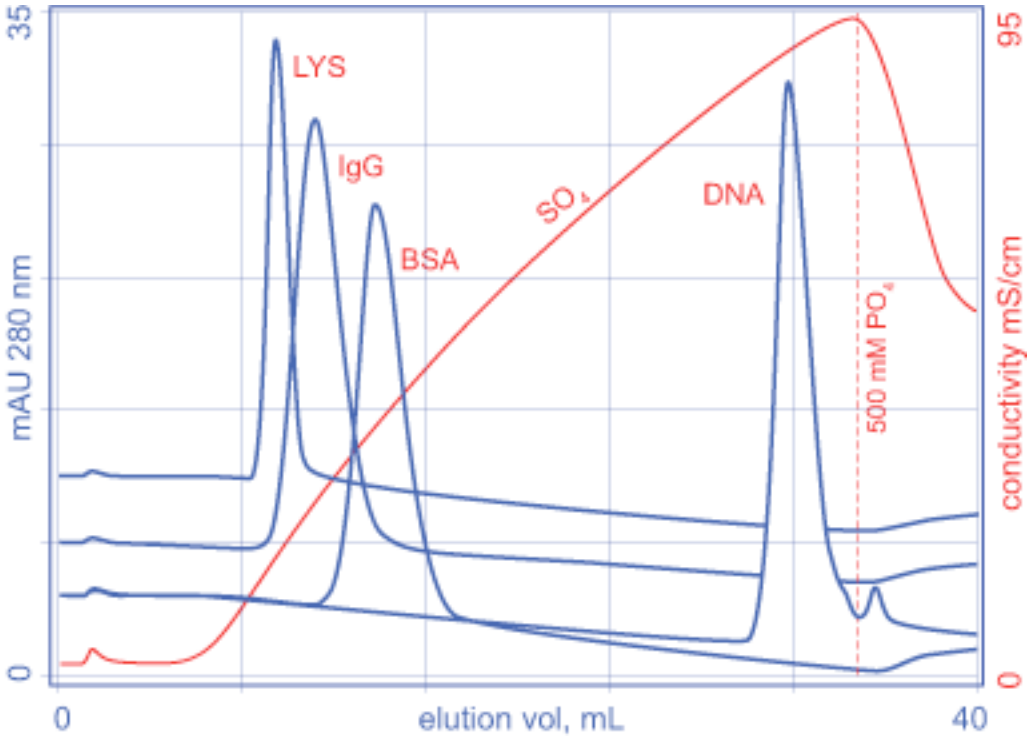
Elute: 15 CVLG A > B

Clean: 500 mM $NaPO_4$ pH 7

Most proteins elute at less than150 mM phosphate. Resolution can be improved with shallow gradients but only up to a point, and bear the disadvantage of broader more dilute peaks. DNA separation from proteins is better than anion exchange but not extraordinary.

Phosphate versus sulfate

Composite view, sulfate



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2 mL/min, 600 cm/hr

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B: A + 1 M Na_2SO_4, pH 7

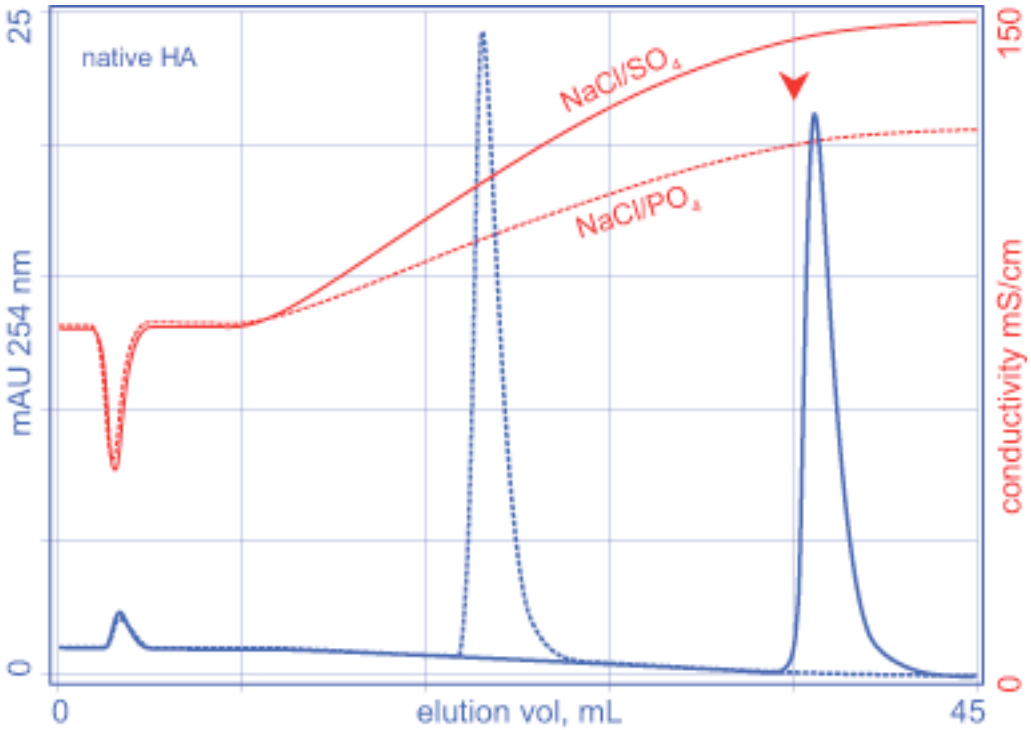
Elute: 15 CVLG A > B

Clean: 500 mM $NaPO_4$, pH 7

Selectivity is strongly altered and separation is improved in the sulfate gradient. Note the transposition in binding order, with greater enhancement in proportion to the contribution of calcium affinity. DNA separation is extraordinary.

Phosphate versus sulfate

DNA, SO_4 v PO_4 in the presence of 1 M NaCl



CHT Type I, 40 µm

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2 mL/min, 600 cm/hr

A: 10 mM $NaPO_4$,
1 M NaCl pH 7

B1: A + 500 mM $NaPO_4$ pH 7

B2: A + 1 M Na_2SO_4 pH 7

Elute: 15 CVLG A > B

Arrow indicates programmed end of gradient

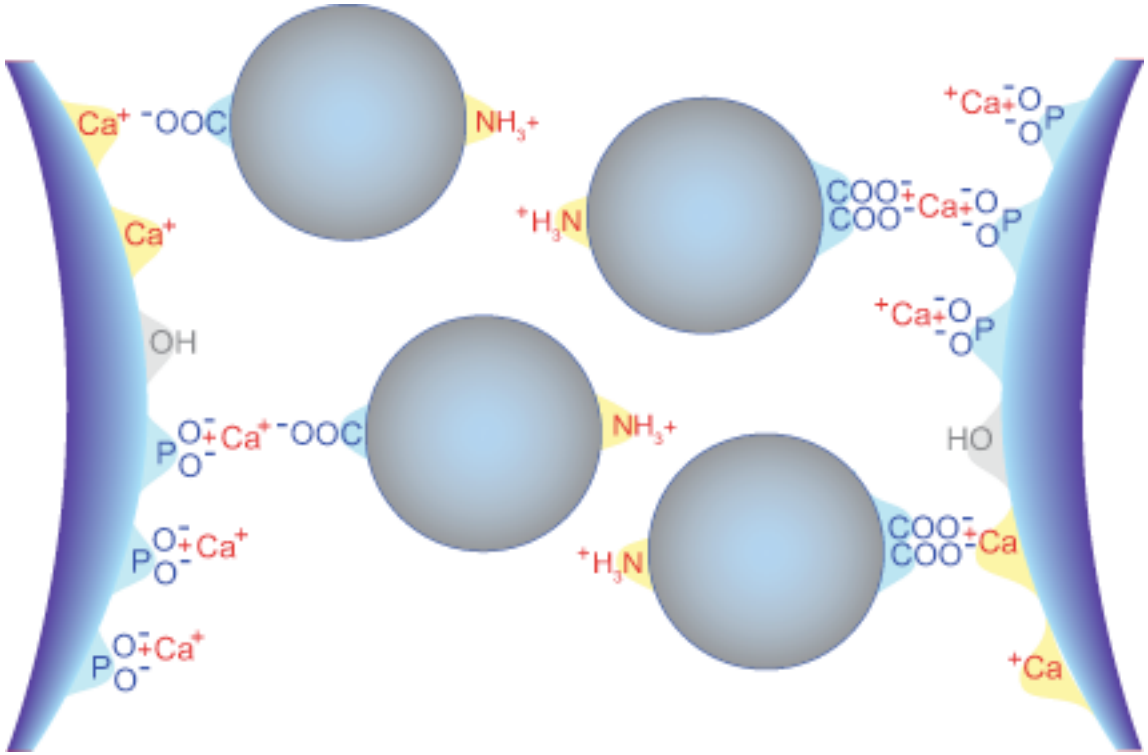
Running gradients in 1.0 M sodium chloride is intended to eliminate most ionic interactions, which should leave calcium affinity as the sole retention mechanism. This allows direct comparison of relative calcium affinities for phosphate and sulfate.

Phosphate versus sulfate

- *Later elution of all tested solutes indicates that sulfate has weaker calcium affinity than phosphate.*
- *DNA elution at 1.0 M sulfate versus 200 mM phosphate (in the presence of 1.0 M NaCl) suggests that the differential in calcium affinity is about 5x.*
- *Later elution corresponds with higher conductivity, which means that the relative contribution of ion exchange is diminished for all solutes.*
- *As illustrated in the composite profiles, these differences create a unique window of selectivity.*

Ca-hydroxyapatite

Calcium converts primary phosphates into secondary calcium groups. This abolishes phosphoryl cation exchange but increases the number of sites available for retention by calcium affinity and anion exchange.



Ca-hydroxyapatite

*Ca-Ha has been neglected as a practical tool, probably because phosphate cannot be used to elute it.**

Phosphate has such a high affinity for calcium that even low concentrations, such as 10 mM, remove secondary calcium, restoring HA to its native form.

Adding soluble calcium to phosphate buffers to force the equilibrium is impractical because it forms precipitates that clog the column.

**1.0 M $CaCl_2$ has also been shown incapable of eluting most solutes (Gorbunoff).*

Ca-hydroxyapatite

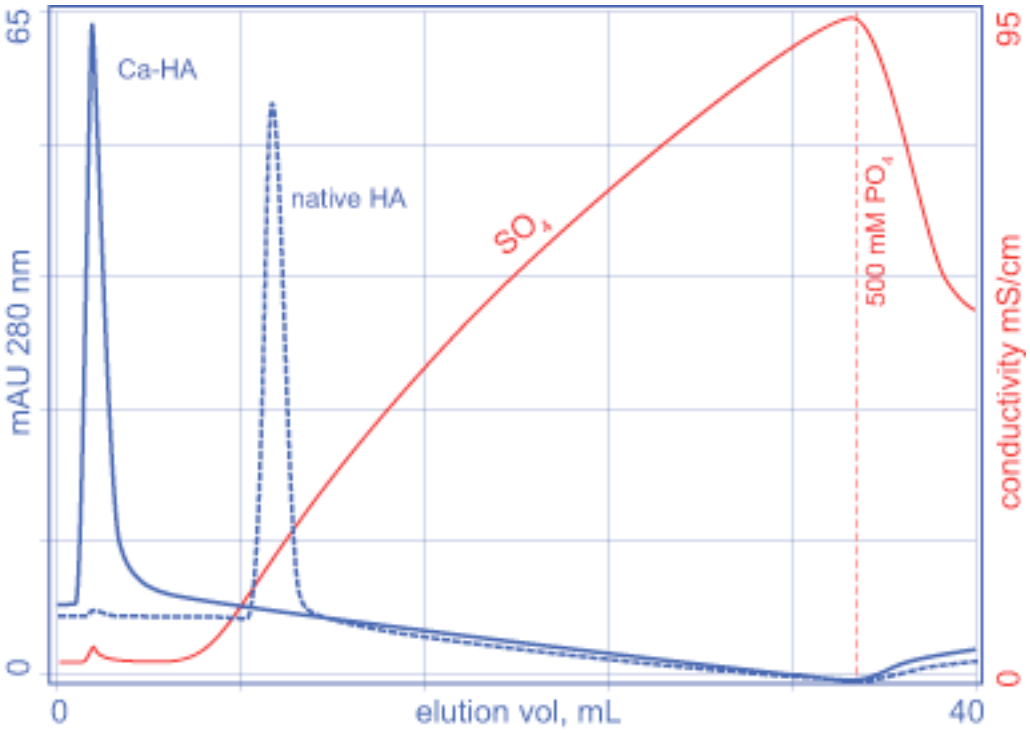
The lower calcium affinity of sulfate however, permits operation of HA in Ca-HA mode.

Sulfate ions are too weak to strip secondary calcium from HA-phosphoryl residues – at least at low sulfate concentrations.

Sulfates do not form precipitates with low concentrations of calcium. This permits the continuous presence of soluble calcium to push the equilibrium towards the Ca-HA form during elution.

Native versus Ca-hydroxyapatite

Lysozyme



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2 mL/min, 600 cm/hr

A1: 25 mM $NaPO_4$, pH 7

B1: A + 1 M Na_2SO_4, pH 7

A2: 50 mM Hepes, 3 mM $CaCl_2$, pH 7

B2: A + 1 M Na_2SO_4, pH 7

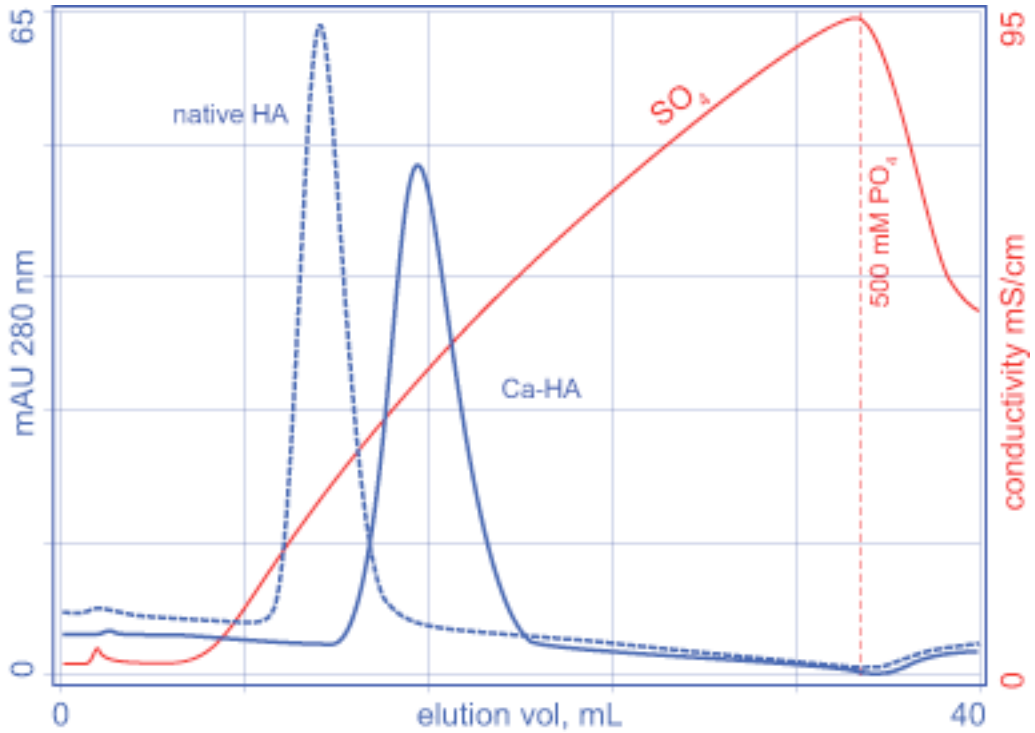
Elute: 15 CVLG A > B

Clean: 500 mM $NaPO_4$, pH 7

The absence of phosphoryl cation exchange groups precludes lysozyme binding to Ca-HA. Tailing of the flow-through peak is consistent with weak calcium affinity, also implied by the comparative elution profiles from native HA in sulfate and phosphate gradients.

Native versus Ca-hydroxyapatite

Monoclonal IgG



CHT Type I, 40 µm

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2 mL/min, 600 cm/hr

A1: 25 mM $NaPO_4$, pH 7

B1: A + 1 M Na_2SO_4, pH 7

A2: 50 mM Hepes, 3 mM $CaCl_2$, pH 7

B2: A + 1 M Na_2SO_4, pH 7

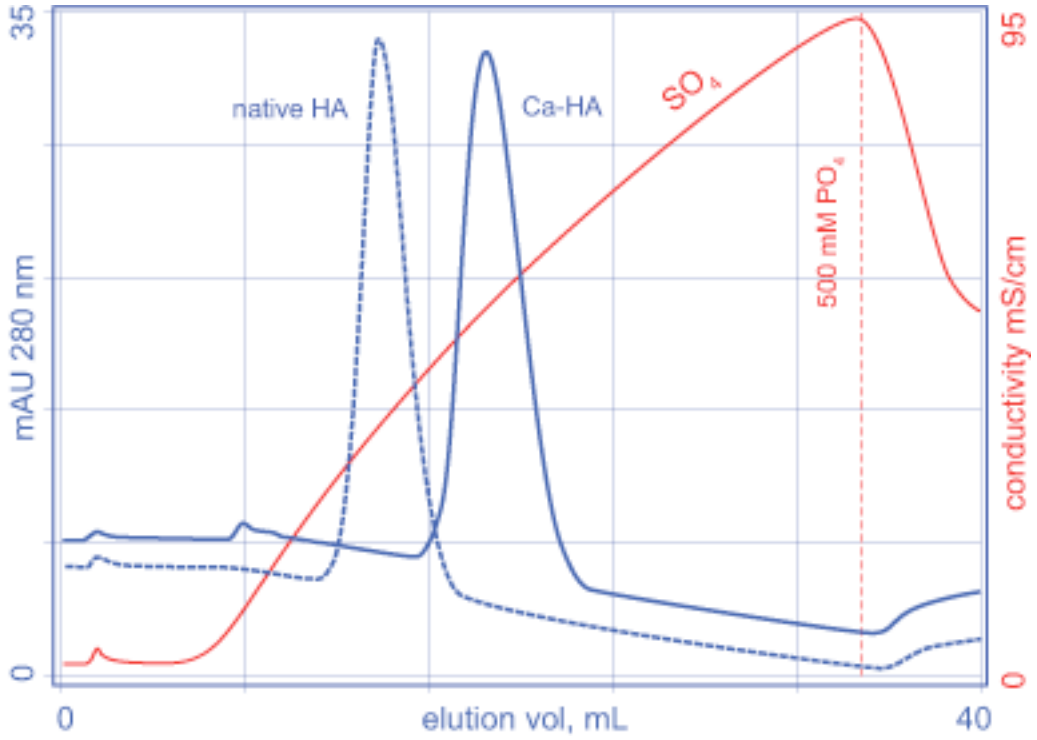
Elute: 15 CVLG A > B

Clean: 500 mM $NaPO_4$, pH 7

Antibody retention is much stronger on Ca-HA despite the loss of phosphoryl cation exchange. Given that 400 mM sulfate should substantially weaken anion exchange binding, increased retention probably reflects an increase in the number of calcium affinity binding sites.

Native versus Ca-hydroxyapatite

BSA



CHT Type I, 40 µm

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2 mL/min, 600 cm/hr

A1: 25 mM $NaPO_4$, pH 7

B1: A + 1 M Na_2SO_4, pH 7

A2: 50 mM Hepes, 3 mM $CaCl_2$, pH 7

B2: A + 1 M Na_2SO_4, pH 7

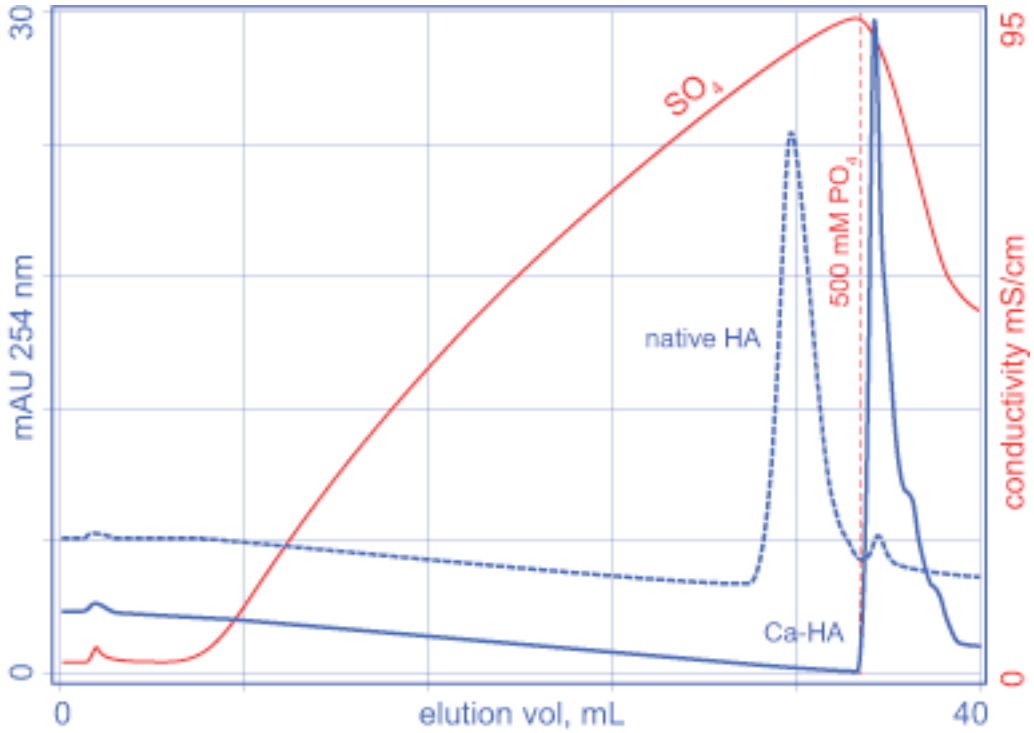
Elute: 15 CVLG A > B

Clean: 500 mM $NaPO_4$, pH 7

Stronger binding of BSA probably reflects enhancement of both calcium anion exchange and affinity, as well as elimination of charge repulsion by native HA phosphate groups.

Native versus Ca-hydroxyapatite

DNA



CHT Type I, 40 µm

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2 mL/min, 600 cm/hr

A1: 25 mM $NaPO_4$, pH 7

B1: A + 1 M Na_2SO_4, pH 7

A2: 50 mM Hepes, 3 mM $CaCl_2$, pH 7

B2: A + 1 M Na_2SO_4, pH 7

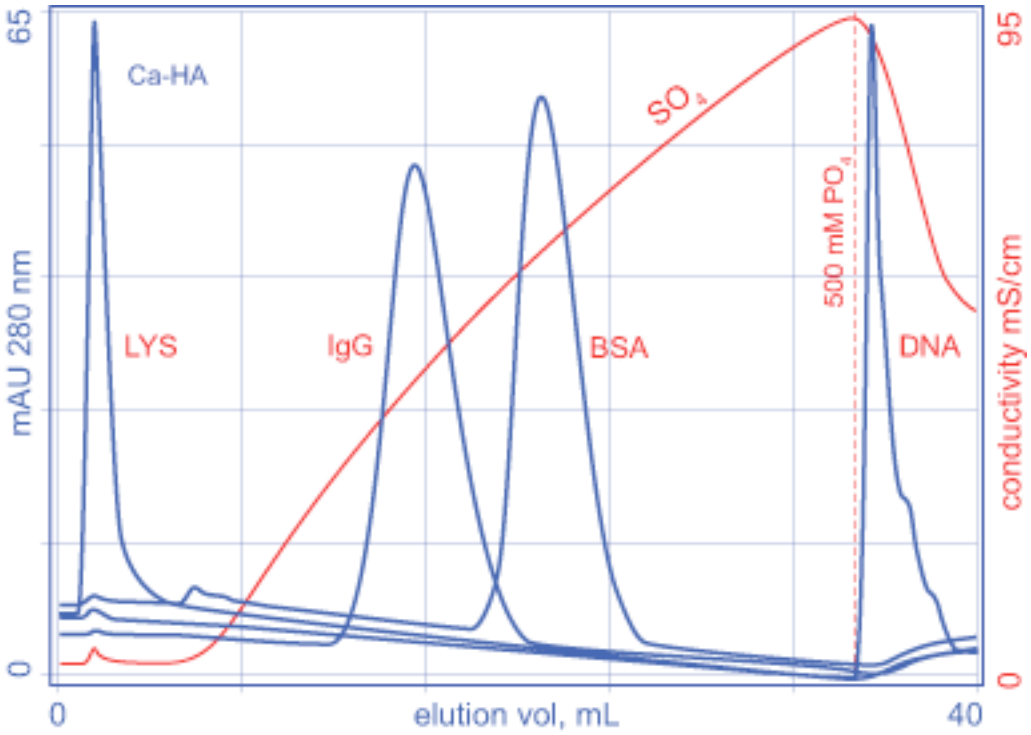
Elute: 15 CVLG A > B

Clean: 500 mM $NaPO_4$, pH 7

DNA fails to elute from Ca-HA in 1.0 M Na_2SO_4. Given the high conductivity, this is assumed to dominantly reflect an increase in the concentration of calcium affinity binding sites. These data also show that calcium remains complexed to HA-phosphate groups in 1.0 M sulfate.

Native versus Ca-hydroxyapatite

Composite view, Ca-HA, SO_4 gradient



CHT Type I, 40 µm

MediaScout 5 x 100 (2 mL)

2 mL/min, 600 cm/hr

A: 50 mM Hepes, 3 mM $CaCl_2$, pH 7

B: A + 1 M Na_2SO_4, pH 7

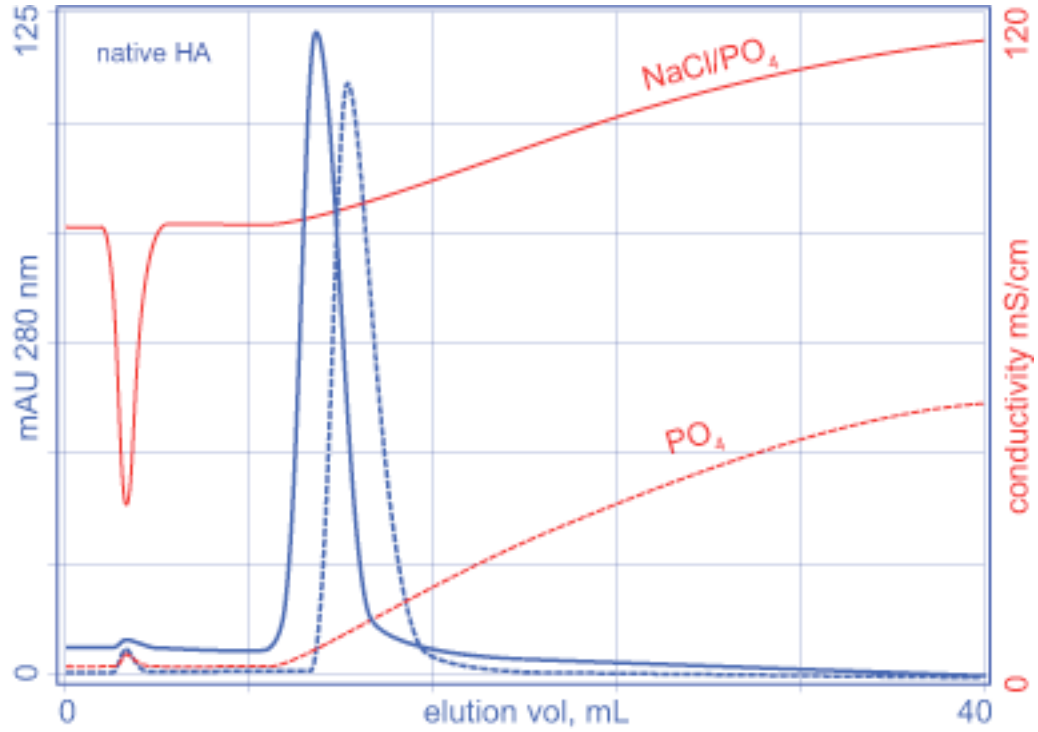
Elute: 15 CVLG A > B

Clean: 500 mM $NaPO_4$, pH 7

This profile demonstrates more effective purification than sulfate gradients on native HA, and vastly superior purification in comparison with phosphate gradients on native HA. This pattern should apply fairly well to most IgG monoclonal antibodies. It also suggests that removal of leached protein A, endotoxin, and enveloped virus should be excellent.

Native versus Ca-hydroxyapatite

The effect of elevated conductivity on IgG retention



CHT Type I, 40 µm

MediaScout 5 x 100 (2 mL)

2 mL/min, 600 cm/hr

A1: 10 mM $NaPO_4$, pH 7

B1: 500 mM $NaPO_4$, pH 7

A2: A1 + 1 M NaCl

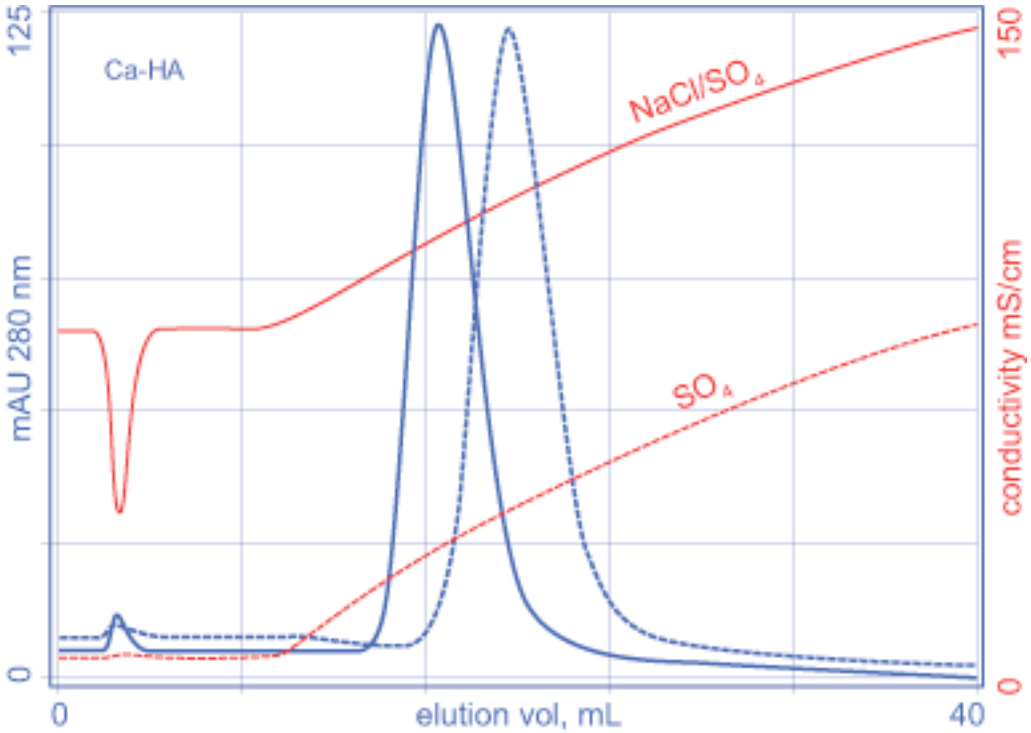
B2: B1 + 1 M NaCl

Elute: 15 CVLG A > B

Most IgG antibodies do not bind in 1.0 M NaCl at 10 mM phosphate. This one is barely retained. Given that calcium affinity is unaffected by NaCl, the reduction is assumed to reflect suspension of phosphoryl cation exchange and calcium anion exchange by the high concentration of NaCl.

Native versus Ca-hydroxyapatite

The effect of elevated conductivity on IgG retention



CHT Type I, 40 µm

MediaScout 5 x 100 (2 mL)

2 mL/min, 600 cm/hr

A1: 50 mM Hepes, 3 mM $CaCl_2$, pH 7

B1: A + 1 M Na_2SO_4, pH 7

A2: A1 + 1 M NaCl

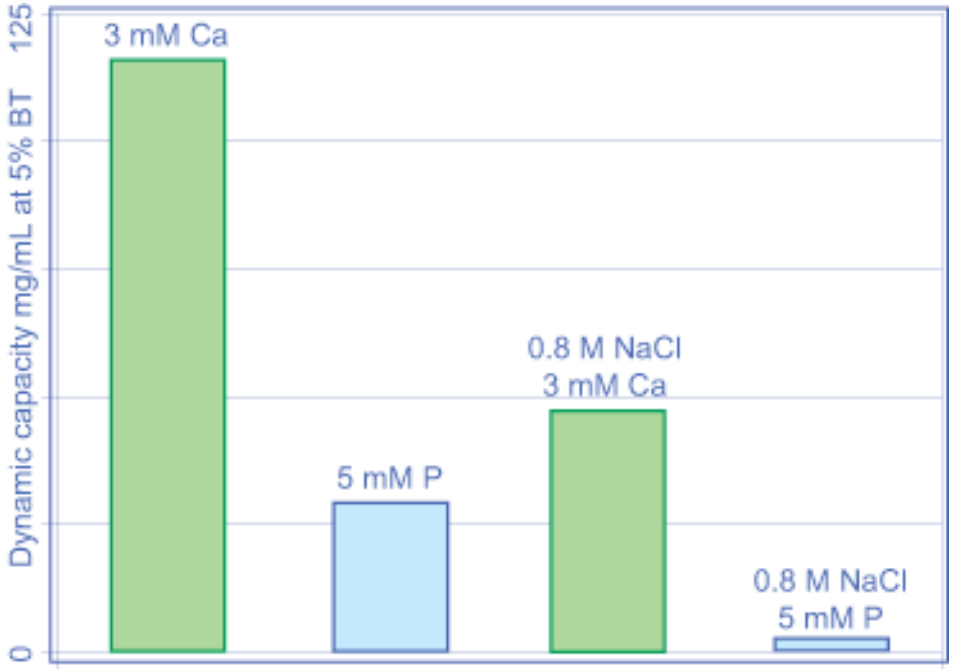
B2: B1 + 1 M NaCl

Elute: 15 CVLG A > B

Antibody retention is reduced on Ca-HA but remains strong. Given that phosphoryl cation exchange is abolished on Ca-HA, weaker retention is assumed to reflect suspension of calcium anion exchange.

Native versus Ca-hydroxyapatite

The effect of elevated conductivity on IgG binding capacity



CHT Type I, 40 µm

MediaScout 5 x 50 (1 mL)

1 mL/min, 300 cm/hr

A: 50 mM MES, pH 6.7

plus solutes as indicated

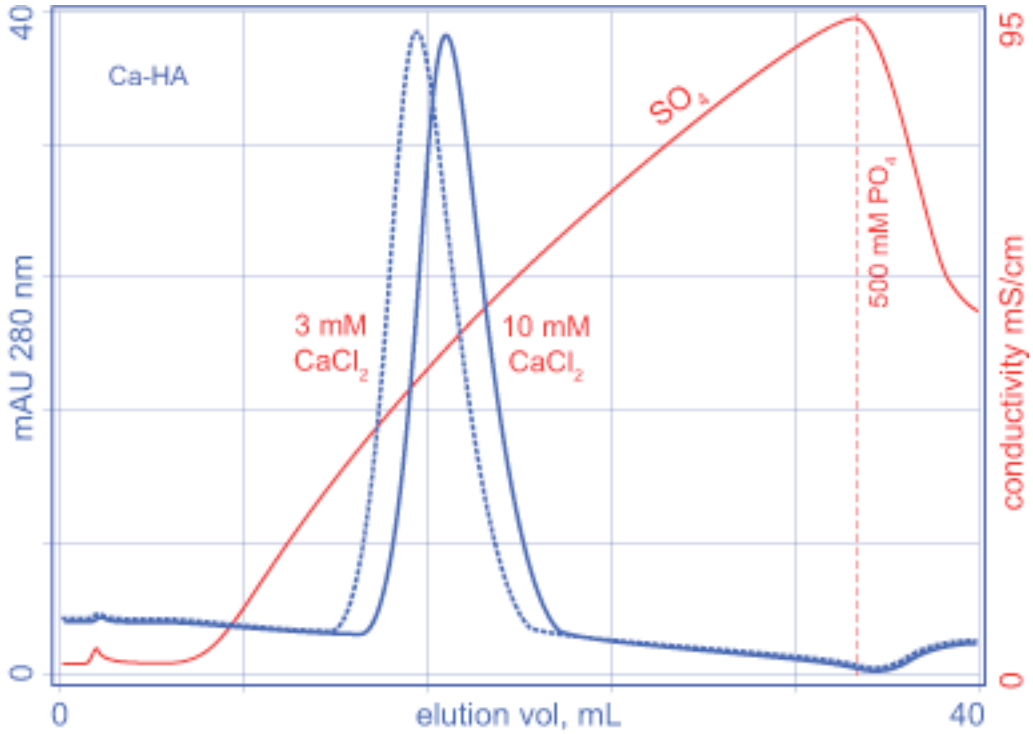
Protein A purified IgG in 0.1 M arginine, 50 mM acetate, 50 mM Tris, pH 6.7

Load by in-line dilution, 1 part sample, 4 parts buffer A

The same trends are revealed in IgG binding capacity. Ca-Ha supports more than 4 times greater capacity than native HA in the absence of NaCl, but also offers nearly twice as much capacity in 0.8 M NaCl as native HA offers in the absence of NaCl.

Stability of Ca-HA

The effect of soluble calcium concentration on IgG elution



CHT Type I, 40 µm

MediaScout 5 x 100 (2 mL)

2 mL/min, 600 cm/hr

A1: 50 mM Hepes, 3 mM $CaCl_2$, pH 7

A2: 50 mM Hepes, 10 mM $CaCl_2$, pH 7

B: A + 1 M Na_2SO_4, pH 7

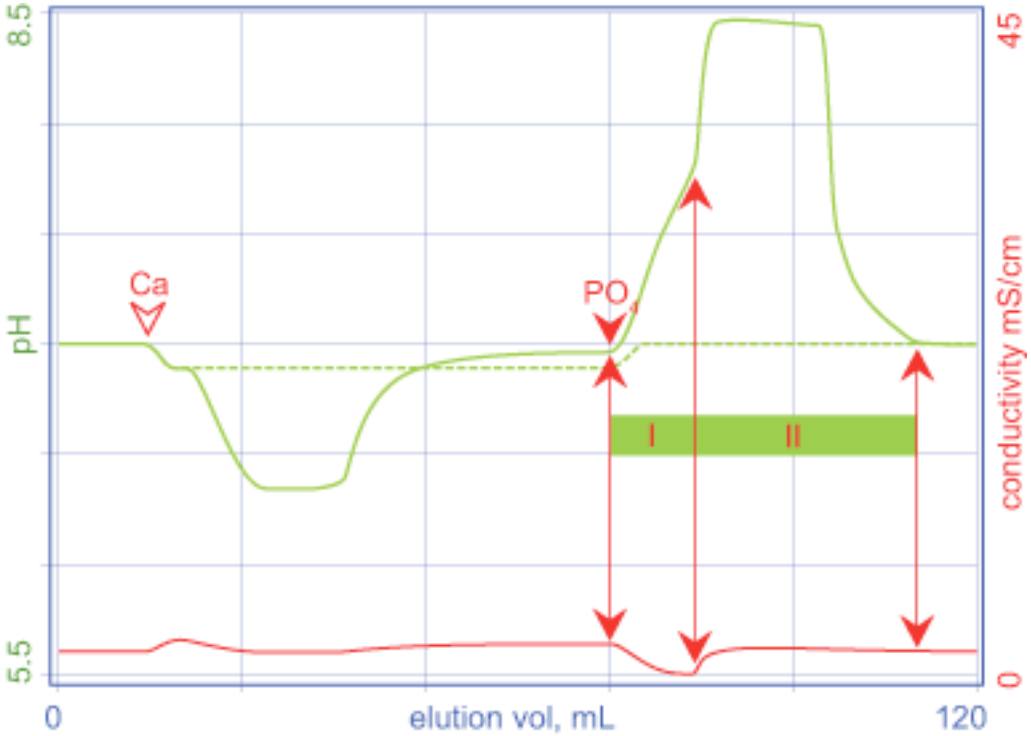
Elute: 15 CVLG A > B

Clean: 500 mM $NaPO_4$, pH 7

Increased retention in the presence of a higher calcium concentration suggests that at least some secondary calcium is removed from Ca-HA by elevated sulfate concentrations. It also suggests that increasing calcium along with sulfate might better stabilize the Ca-HA form.

Stability of Ca-HA in phosphate

Native HA > Ca-HA > native HA



CHT Type I, 40 µm

MediaScout 5 x 100 (2 mL)

5 mL/min, 1500 cm/hr

A: 10 mM $NaPO_4$, pH 7

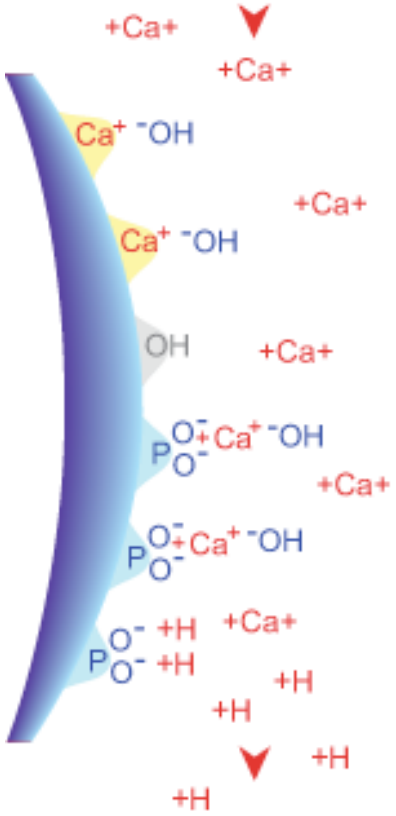
B: 50 mM Hepes, 5 mM $CaCl_2$, pH 7

C: 10 mM $NaPO_4$, pH 7

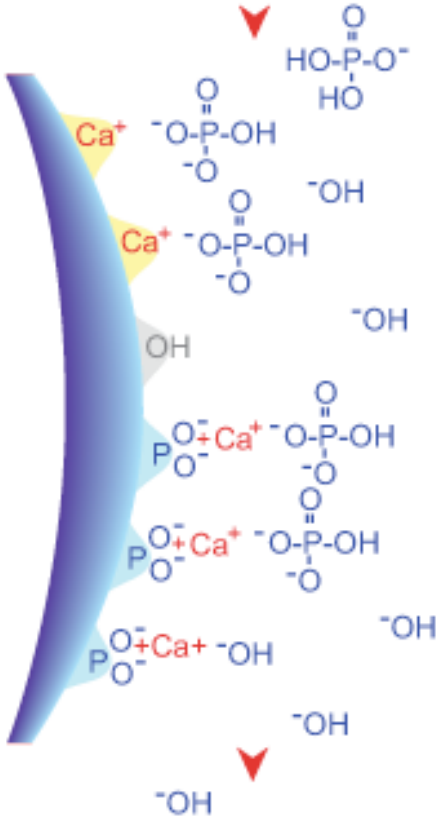
Conversion of native to Ca-HA is accompanied by a pH drop (proportional to calcium concentration). Note the biphasic pH increase with introduction of phosphate, and the loss of conductivity during phase I. The dashed profile indicates pH with the column off-line.

Stability of Ca-HA in phosphate

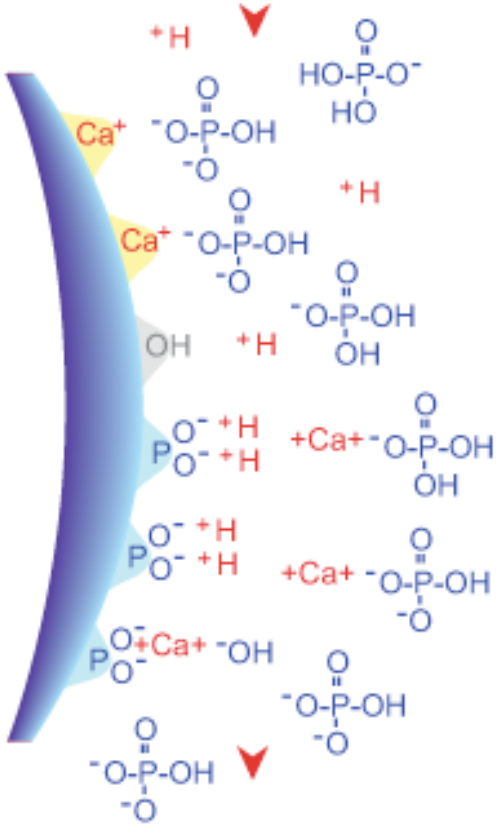
Formation of Ca-HA



Reversion: phase I

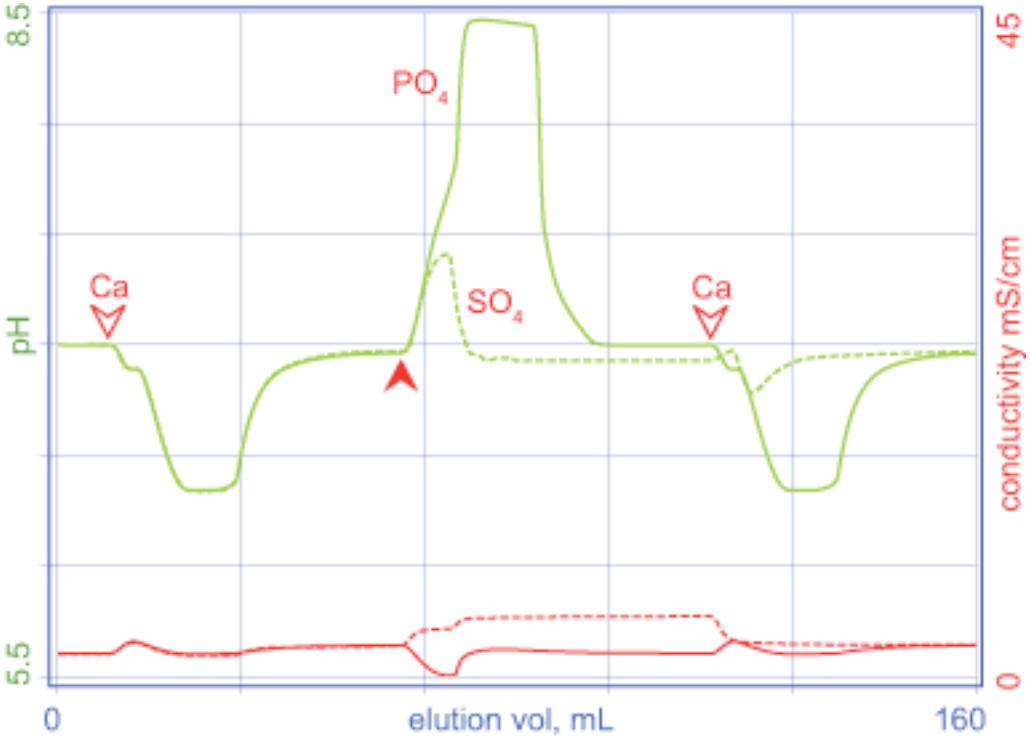


Reversion: phase II



Stability of Ca-HA in sulfate

Comparison of phosphate and sulfate effects on Ca-HA



CHT Type I, 40 µm

MediaScout 5 x 100 (2 mL)

5 mL/min, 1500 cm/hr

A: 10 mM $NaPO_4$, pH 7

B: 50 mM Hepes, 5 mM $CaCl_2$, pH 7

C1: 10 mM $NaPO_4$, pH 7

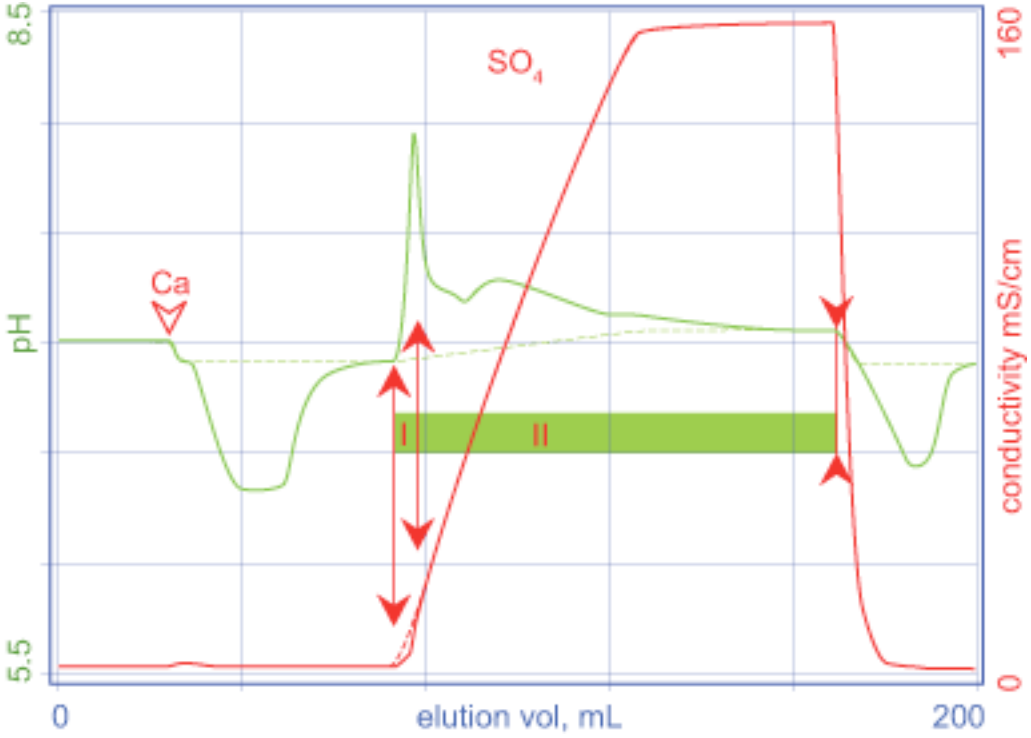
C2: 50 mM Hepes, 5 mM $CaCl_2$, 10 mM ammonium sulfate, pH 7

D: 50 mM Hepes, 5 mM $CaCl_2$, pH 7

Note the relatively small phase I pH excursion with 10 mM sulfate, and the proportionally smaller pH drop upon reintroduction of equilibration buffer. These results are consistent with protein elution behavior indicating that sulfate has relatively low calcium affinity.

Stability of Ca-HA in sulfate

Sulfate effects on Ca-HA



CHT Type I, 40 µm

MediaScout 5 x 100 (2 mL)

5 mL/min, 1500 cm/hr

A: 10 mM $NaPO_4$, pH 7

B: 50 mM Hepes, 5 mM $CaCl_2$, pH 7

C: 50 mM Hepes, 5 mM $CaCl_2$ 1 M ammonium sulfate, pH 7

D: elute 25 CVLG B > C

E: 50 mM Hepes, 5 mM $CaCl_2$, pH 7

The pH profile suggests that substantial secondary calcium is removed during the gradient, but even prolonged washing in 1.0 M sulfate is insufficient to remove it completely. Note in particular that the pH drop associated with restoration of Ca-HA is smaller than the pH drop with initial formation of Ca-HA. Note also the aberration in the conductivity profile at phase I.

Conclusions

Sulfate and calcium create entire new realms of selectivity for hydroxyapatite.

This has practical value in three contexts:

- *They provide new tools for understanding how hydroxyapatite works, in general and for specific solutes.*

- *They provide new tools to optimize existing hydroxyapatite applications.*

- *They enable new hydroxyapatite applications.*

Conclusions

Sulfate gradients on native HA support more effective separations than phosphate gradients.

Sulfate gradients on Ca-HA support even more effective separations than sulfate on native HA.

Depending on your interests, these enhancements can improve purification of proteins, plasmids, or vaccines.

Also expect sulfate gradients to support more effective fractionation of phosphoproteins from unphosphorylated proteins, especially on Ca-HA.

Conclusions

The ability of Ca-HA to support higher binding capacities than native HA – especially at elevated conductivity values – makes HA more attractive as an intermediate or polishing purification tool.

It may allow HA to become a competitive option for IgG capture – without dilution – from CCS, potentially enabling a new generation of non-protein A based purification procedures.

The option of binding in Ca-HA mode then reverting to native HA makes it possible to achieve high binding capacity, then choose the elution selectivity that best serves a particular application.

Acknowledgements

Sincere thanks to Avid BioServices for providing monoclonal antibodies, to Bio-Rad Laboratories for providing ceramic hydroxyapatite, and to ATOLL for packing hydroxyapatite into their MediaScout columns. Special thanks to the Process Applications group at Bio-Rad for preliminary information on the effect of sulfates on stability of hydroxyapatite.

Copies of this presentation can be downloaded at www.validated.com