## Supplementary Materials

Supporting Table 1: Primers used to generate expression vectors for target protein expression in the apoplast, vacuole and plastids, as well as IgG3 domain exchange variants, starting from two IgG1/3 base constructs (Figure S1).

| Primer ID | Primer name | Sequence | Length [bp] |
|---|---|---|---|
| | Cloning of IgG3 domain exchange variants | | |
| p01 | GA_IgG1_CH2_CH3_frwd | GATACCCCACCGCCGTGTCCAAGATGTCCTGCTCCTGAACTTCTTGGTGGTC | 52 |
| p02 | GA_IgG1_CH2_CH3_rev | TGTAAGTATCTCAGTTCCGAAGGACAAACAG | 31 |
| p03 | GA_IgG1_CH3_frwd | CCTATCGAAAAGACCATCTCTAAGACCAAGGGTCAGCCTAGAGAACCGCAAG | 52 |
| p04 | GA_IgG3_no_CH2_CH3_frwd | CTGTTTGTCCTTCGGAAC | 18 |
| p05 | GA_IgG3_no_CH2_CH3_rev | AGGACATCTTGGACACG | 17 |
| p06 | GA_IgG3_no_CH3_rev | CTTGGTCTTAGAGATGGTCTTTTC | 24 |
| p11 | GA_IgG3_no_Ch3_rev_V2 | CTTGGTCTTAGAGATGGTC | 19 |
| | Cloning of expression vectors targeting mAbs to the vacuole | | |
| p07 | KISIA_IgG1_frwd | TGTCCCCTGGCAAGAAGATTTCTATTGCTTGTTTGTCCTTCGGAACTG | 48 |
| p08 | KISIA_IgG1_rev | AGCAATAGAAATCTTCTTGCCAGGGGACAGAG | 32 |
| p09 | KISIA_IgG3_frwd | TTGAGCCCTGGCAAGAAGATTTCTATTGCTTGTTTGTCCTTCGGAACTG | 49 |
| p10 | KISIA_IgG3_rev | AGCAATAGAAATCTTCTTGCCAGGGCTCAAGC | 32 |

**(Supporting Table 1 continued)**

| Primer ID | Primer name | Sequence | Length [bp] |
|---|---|---|---|
| Cloning of expression vectors targeting mAbs to the plastids | | | |
| p13 | IgG1_3_no_LPH_frwd | ACGTATCCATGGCCCAAGTTCAGCTTCAAGAATC | 34 |
| p14 | IgG1_3_rev | CTATGACTCGAGCTAAGAGCACTCG | 25 |
| p15 | LPH_del_LC_frwd | TCTTACGTGTTGACCCAAGATCC | 23 |
| p16 | LPH_del_LC_rev | TGGCCCAGGGTTGGACTC | 18 |
| Cloning of expression vectors targeting mAbs to the apoplast (deletion of SEKDEL) | | | |
| p17 | KDEL_del_frwd | TGTTTGTCCTTCGGAACTGAG | 21 |
| p18 | KDEL_del_IgG3_rev | CTTGCCAGGGCTCAAGC | 17 |
| p19 | KDEL_del_IgG1_rev | CTTGCCAGGGGACAGAG | 17 |
| Sequencing primers | | | |
| p12 | IgG_sequencing | TTCAAGAACCTCCTGTTCTC | 20 |
| p20 | 35SS_FI | TGACGCACAATCCCACTATC | 20 |
| p21 | 35SS_pA-RI | CCCTTATCTGGGAACTACTC | 20 |

Supporting Table 2: Expression vectors used to assess IgG3 production in *Nicotiana* spp.

| Construct ID | Vector | Antibody scaffold | 5′ UTR | Signal sequences | Target compartment |
|---|---|---|---|---|---|
| 000237 | pTRAc | IgG1 | omega | 2xLPH, SEKDEL | ER |
| 000238 | pTRAc | IgG1 | TL | 2xLPH, SEKDEL | ER |
| 000239 | pTRAc | IgG1 | CHS | 2xLPH, SEKDEL | ER |
| 000240 | pTRAc | IgG3 | omega | 2xLPH, SEKDEL | ER |
| 000241 | pTRAc | IgG3 | TL | 2xLPH, SEKDEL | ER |
| 000242 | pTRAc | IgG3 | CHS | 2xLPH, SEKDEL | ER |
| 000243 | pTRAc | IgG1 | omega | LPH, SEKDEL | ER |
| 000244 | pTRAc | IgG1 | TL | LPH, SEKDEL | ER |
| 000245 | pTRAc | IgG1 | CHS | LPH, SEKDEL | ER |
| 000246 | pTRAc | IgG3 | omega | LPH, SEKDEL | ER |
| 000247 | pTRAc | IgG3 | TL | LPH, SEKDEL | ER |
| 000248 | pTRAc | IgG3 | CHS | LPH, SEKDEL | ER |
| 000249 | pTRAc | IgG1 | omega | LPH, KISIA | Vacuole |
| 000250 | pTRAc | IgG1 | TL | LPH, KISIA | Vacuole |
| 000251 | pTRAc | IgG1 | CHS | LPH, KISIA | Vacuole |
| 000252 | pTRAc | IgG3 | omega | LPH, KISIA | Vacuole |
| 000253 | pTRAc | IgG3 | TL | LPH, KISIA | Vacuole |
| 000254 | pTRAc | IgG3 | CHS | LPH, KISIA | Vacuole |
| 000255 | pTRAc | IgG1 | omega | LPH | Apoplast |
| 000256 | pTRAc | IgG1 | TL | LPH | Apoplast |
| 000257 | pTRAc | IgG1 | CHS | LPH | Apoplast |
| 000258 | pTRAc | IgG3 | omega | LPH | Apoplast |
| 000259 | pTRAc | IgG3 | TL | LPH | Apoplast |
| 000260 | pTRAc | IgG3 | CHS | LPH | Apoplast |
| 000261 | pTRAc | IgG1 | omega | TP | Plastids |
| 000262 | pTRAc | IgG1 | TL | TP | Plastids |

**(Supporting Table 2 continued)**

| Construct ID | Vector | Antibody scaffold | 5′ UTR | Signal sequences | Target compartment |
|---|---|---|---|---|---|
| 000263 | pTRAc | IgG1 | CHS | TP | Plastids |
| 000264 | pTRAc | IgG3 | omega | TP | Plastids |
| 000265 | pTRAc | IgG3 | TL | TP | Plastids |
| 000266 | pTRAc | IgG3 | CHS | TP | Plastids |
| 000267 | pTRAc | IgG3+IgG1CH3 | omega | 2xLPH, SEKDEL | ER |
| 000268 | pTRAc | IgG3+IgG1CH3 | TL | 2xLPH, SEKDEL | ER |
| 000269 | pTRAc | IgG3+IgG1CH3 | CHS | 2xLPH, SEKDEL | ER |
| 000270 | pTRAc | IgG3+IgG1CH2-3 | omega | 2xLPH, SEKDEL | ER |
| 000271 | pTRAc | IgG3+IgG1CH2-3 | TL | 2xLPH, SEKDEL | ER |
| 000272 | pTRAc | IgG3+IgG1CH2-3 | CHS | 2xLPH, SEKDEL | ER |
| 000273 | pTRAc | IgG3+IgG1CH3 | omega | LPH, SEKDEL | ER |
| 000274 | pTRAc | IgG3+IgG1CH3 | TL | LPH, SEKDEL | ER |
| 000275 | pTRAc | IgG3+IgG1CH3 | CHS | LPH, SEKDEL | ER |
| 000276 | pTRAc | IgG3+IgG1CH2-3 | omega | LPH, SEKDEL | ER |
| 000277 | pTRAc | IgG3+IgG1CH2-3 | TL | LPH, SEKDEL | ER |
| 000278 | pTRAc | IgG3+IgG1CH2-3 | CHS | LPH, SEKDEL | ER |
| 000279 | pTRAc | IgG3+IgG1CH3 | omega | LPH, KISIA | Vacuole |
| 000280 | pTRAc | IgG3+IgG1CH3 | TL | LPH, KISIA | Vacuole |
| 000281 | pTRAc | IgG3+IgG1CH3 | CHS | LPH, KISIA | Vacuole |
| 000282 | pTRAc | IgG3+IgG1CH2-3 | omega | LPH, KISIA | Vacuole |
| 000283 | pTRAc | IgG3+IgG1CH2-3 | TL | LPH, KISIA | Vacuole |
| 000284 | pTRAc | IgG3+IgG1CH2-3 | CHS | LPH, KISIA | Vacuole |
| 000285 | pTRAc | IgG3+IgG1CH3 | omega | LPH | Apoplast |
| 000286 | pTRAc | IgG3+IgG1CH3 | TL | LPH | Apoplast |
| 000287 | pTRAc | IgG3+IgG1CH3 | CHS | LPH | Apoplast |
| 000288 | pTRAc | IgG3+IgG1CH2-3 | omega | LPH | Apoplast |

**(Supporting Table 2 continued)**

| Construct ID | Vector | Antibody scaffold | 5′ UTR | Signal sequences | Target compartment |
|---|---|---|---|---|---|
| 000289 | pTRAc | IgG3+IgG1CH2-3 | TL | LPH | Apoplast |
| 000290 | pTRAc | IgG3+IgG1CH2-3 | CHS | LPH | Apoplast |
| 000291 | pTRAc | IgG3+IgG1CH3 | omega | TP | Plastids |
| 000292 | pTRAc | IgG3+IgG1CH3 | TL | TP | Plastids |
| 000293 | pTRAc | IgG3+IgG1CH3 | CHS | TP | Plastids |
| 000294 | pTRAc | IgG3+IgG1CH2-3 | omega | TP | Plastids |
| 000295 | pTRAc | IgG3+IgG1CH2-3 | TL | TP | Plastids |
| 000296 | pTRAc | IgG3+IgG1CH2-3 | CHS | TP | Plastids |

CHS = *Petroselinum hortense* chalcone synthase gene 5′ UTR, ER = endoplasmic reticulum, IgG1 = immunoglobulin G subclass 1, IgG3 = immunoglobulin G subclass 3, LPH = leader peptide of the antibody mAb24 heavy chain, omega = omega prime sequence from tobacco mosaic virus, SEKDEL = signal for target protein retention in the ER, TL = tobacco etch virus leader sequence, TP = transit peptide from ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit of *Solanum tuberosum*.

Supporting Table 3: Model factors with a significant influence on IgG accumulation and integrity during transient expression in BY-2 PCPs as identified by restricted maximum likelihood analysis.

| | **IgG accumulation** | | | **IgG integrity** | |
|---|---|---|---|---|---|
| **Source** | **F-value** | **p-value** | **Source** | **F-value** | **p-value** |
| Whole plot | 0.31 | 0.6759 | Whole plot | 1.21 | 0.2733 |
| A (PCP incubation time) | 0.31 | 0.6759 | A (PCP incubation time) | 1.21 | 0.2733 |
| Subplot | 61.48 | <0.0001 | Subplot | 27.48 | <0.0001 |
| B (BY-2 cultivation time) | 181.16 | <0.0001 | B (BY-2 cultivation time) | 12.19 | 0.0006 |
| C (compartment) | 164.45 | <0.0001 | C (compartment) | 126.00 | <0.0001 |
| D (IgG scaffold) | 96.44 | <0.0001 | D (IgG scaffold) | 21.96 | <0.0001 |
| AB | 7.67 | 0.0062 | AC | 3.36 | 0.0111 |
| AC | 11.37 | <0.0001 | CD | 4.76 | <0.0001 |
| AD | 11.15 | <0.0001 | | | |
| BC | 18.68 | <0.0001 | | | |
| CD | 17.06 | <0.0001 | | | |
| $B^2$ | 36.94 | <0.0001 | | | |
| Coefficients of determination | $R^2$ | 0.9233 | Coefficients of determination | $R^2$ | 0.7765 |
| | Adjusted $R^2$ | 0.9079 | | Adjusted $R^2$ | 0.7444 |
| | Predicted $R^2$ | N.A. | | Predicted $R^2$ | N.A. |

Factors with a non-significant influence on the responses ($p > 0.05$) were removed from the models unless required to maintain the model hierarchy. A predicted $R^2$ is not available for split-plot designs because this metric cannot be calculated. With a difference < 0.2, the values of $R^2$ and adjusted $R^2$ were in reasonable agreement.

Supporting Table 4: Model factors with a significant influence on IgG purity and recovery after protein G chromatography as identified by analysis of variance (ANOVA).

| | **IgG3 purity** | | | **IgG3 recovery** | |
|---|---|---|---|---|---|
| **Source** | **F-value** | **p-value** | **Source** | **F-value** | **p-value** |
| Model | 17.93 | <0.0001 | Model | 7.62 | 0.0005 |
| A (wash buffer pH) | 0.40 | 0.5365 | B (wash buffer conductivity) | 2.66 | 0.1165 |
| B (wash buffer conductivity) | 0.18 | 0.6785 | C (elution buffer pH) | 4.93 | 0.0366 |
| C (elution buffer pH) | 3.29 | 0.0848 | D (elution buffer conductivity) | 13.69 | 0.0012 |
| D (elution buffer conductivity) | 66.77 | <0.0001 | BD | 5.43 | 0.0289 |
| AD | 9.06 | 0.0069 | | | |
| BD | 17.61 | 0.0004 | | | |
| $D^2$ | 17.60 | 0.0004 | | | |
| Coefficients of determination | $R^2$ | 0.8625 | Coefficients of determination | $R^2$ | 0.5699 |
| | Adjusted $R^2$ | 0.8144 | | Adjusted $R^2$ | 0.4951 |
| | Predicted $R^2$ | 0.6313 | | Predicted $R^2$ | 0.2813 |

Factors with a non-significant influence on the responses ($p > 0.05$) were removed from the models unless required to maintain the model hierarchy. With a difference < 0.2, the values of $R^2$, the adjusted $R^2$ and the predicted $R^2$ were in reasonable agreement in the model describing IgG3 purity. The adjusted and predicted $R^2$ in the model describing IgG3 recovery differed by > 0.2, indicating potential issues with the model or data. We hence confirmed the model predictions with additional verification runs (Figure S5).

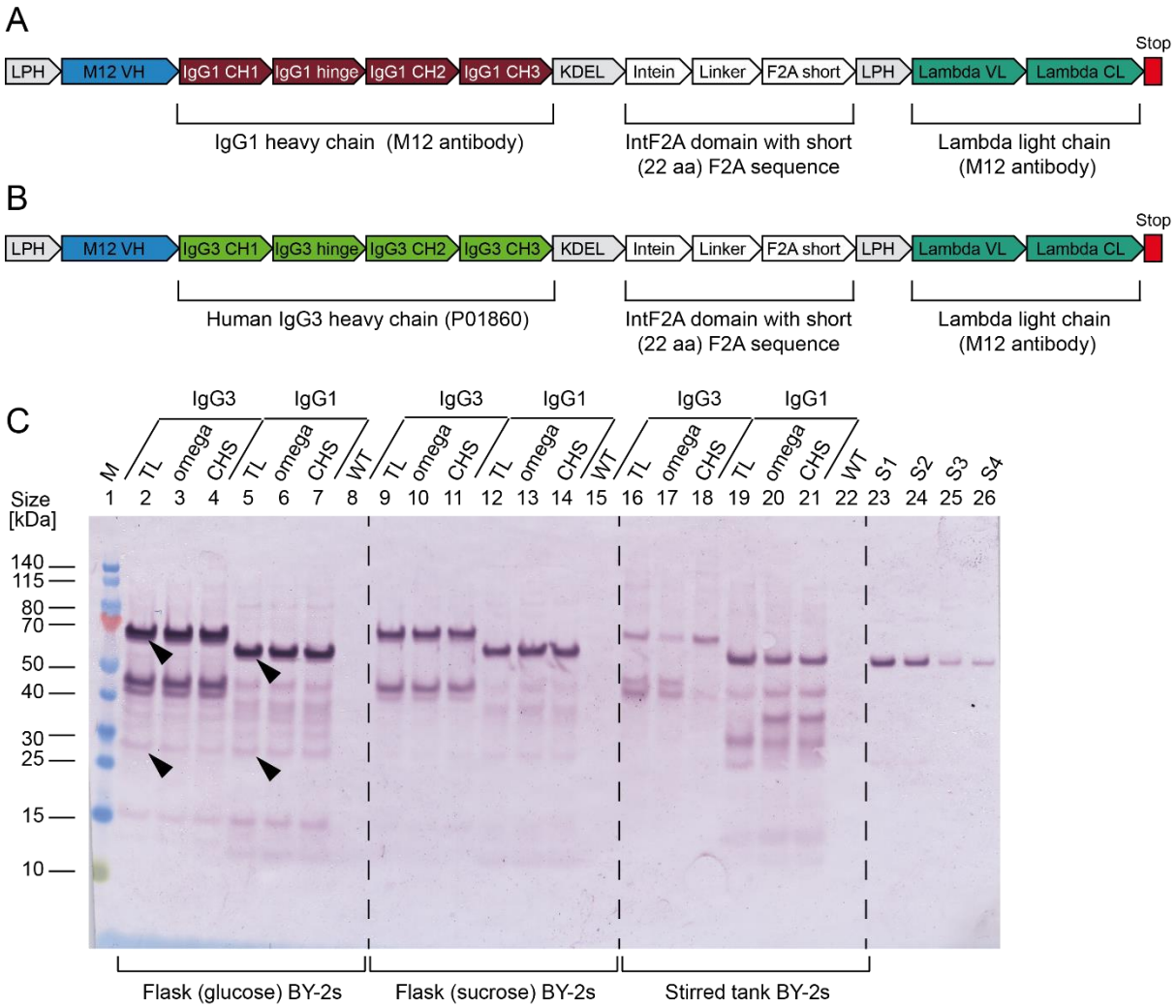



Figure S1: Design of base constructs for IgG1 and IgG3 retained in the ER and assessment of construct functionality by transient expression in BY-2 PCPs. A, B. IgG1 (A) and IgG3 (B) expression cassettes featuring a self-cleaving linker composed of an intein and an F2A peptide [58] facilitating single-promoter transcription and stoichiometric translation of the mAb heavy and light chains. Deviating from the literature, a short F2A peptide (22 amino acids) was used. The CDRs directed against *S. aureus* alpha toxin [60] were introduced into the variable domains of the M12 antibody [33]. C. Investigation of IgG1 and IgG3 integrity by western blotting using an AP-labeled polyclonal goat anti-human IgG. BY-2 cells were cultivated for 168 h and PCPs were incubated for 72 h. Black block arrows indicate the expected molecular mass of the heavy and light chains. CHS – *Petroselinum hortense* chalcone synthase gene 5′ UTR, omega – omega prime sequence from tobacco mosaic virus,

TL – tobacco etch virus leader sequence, WT – wild-type PCP extract, S1 to S4 – IgG1 standard (2G12, Fraunhofer IME) at concentrations of 14.0, 7.0, 5.0 and 2.5 mg $L^{-1}$. CH – constant heavy chain antibody domain, CL – constant light chain antibody domain, ER – endoplasmic reticulum, LPH – N-terminal leader peptide of the antibody mAb24 heavy chain, PCP – plant cell pack, VH – variable heavy chain antibody domain, VL – variable light chain antibody domain.

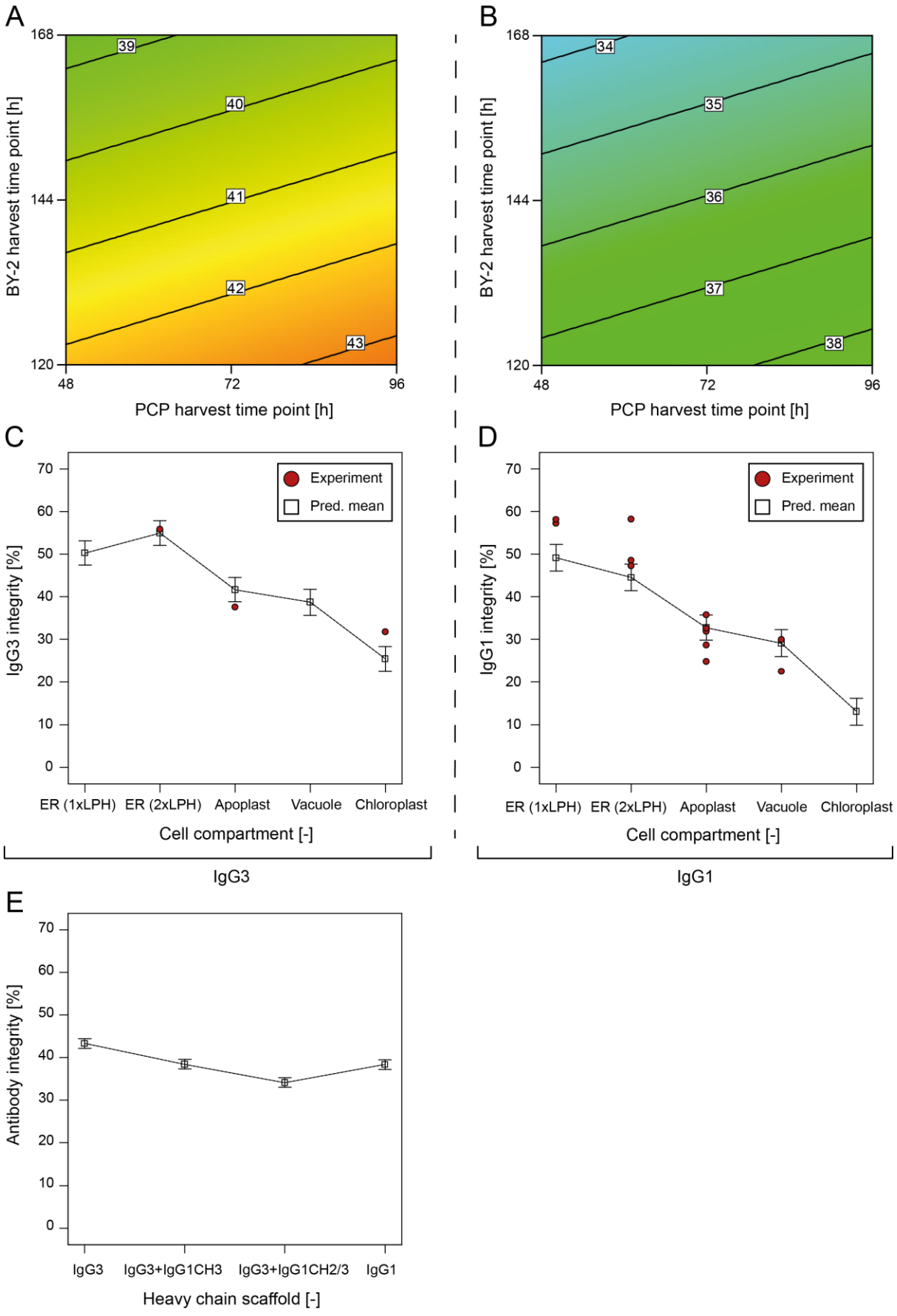
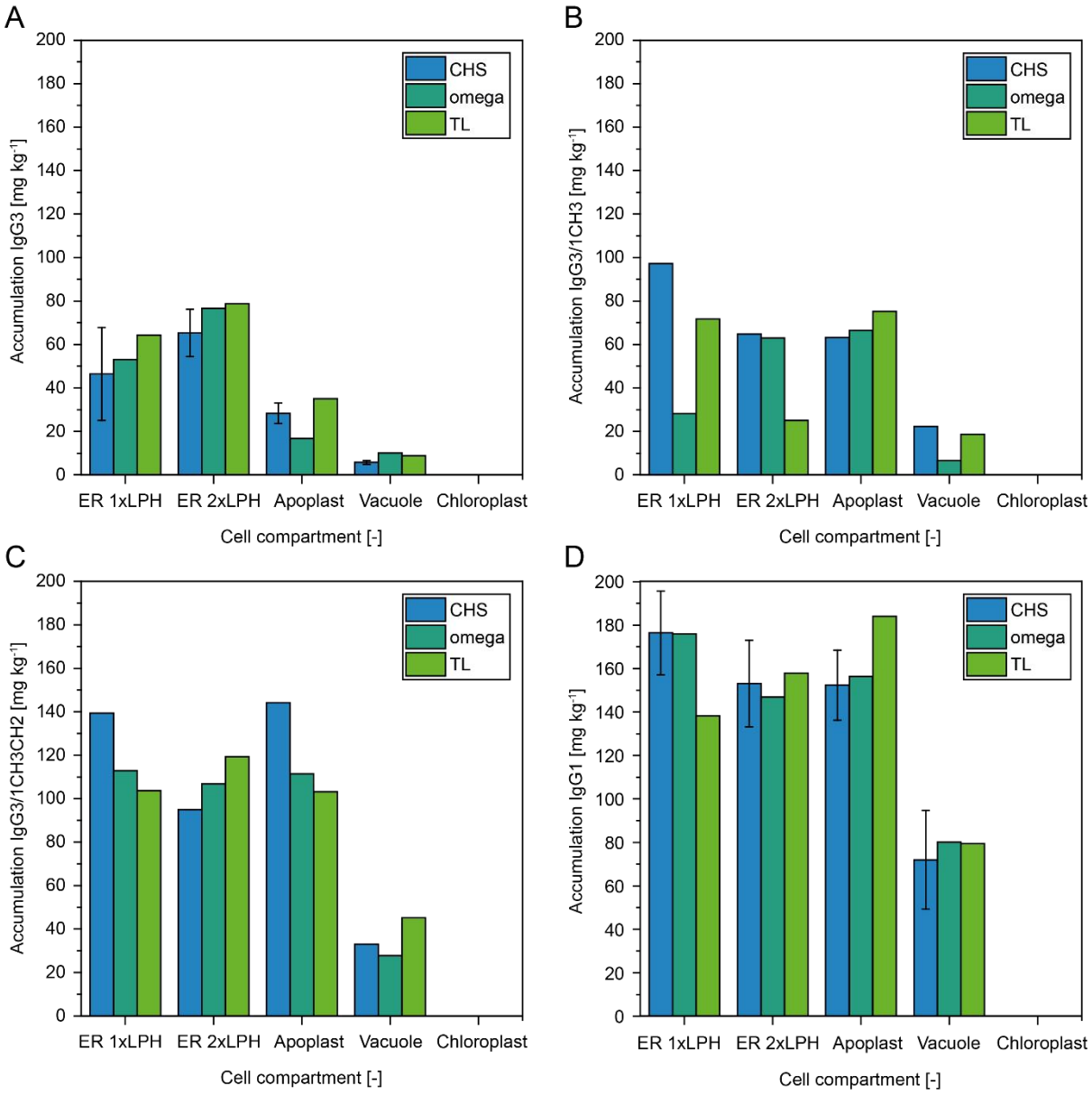

A
168
144
120
BY-2 harvest time point [h]
39
40
41
42
43
48
72
96
PCP harvest time point [h]
B
168
144
120
BY-2 harvest time point [h]
34
35
36
37
38
48
72
96
PCP harvest time point [h]
C
70
60
50
40
30
20
10
0
IgG3 integrity [%]
Experiment
Pred. mean
ER (1xLPH)
ER (2xLPH)
Apoplast
Vacuole
Chloroplast
Cell compartment [-]
IgG3
D
70
60
50
40
30
20
10
0
IgG1 integrity [%]
Experiment
Pred. mean
ER (1xLPH)
ER (2xLPH)
Apoplast
Vacuole
Chloroplast
Cell compartment [-]
IgG1
E
70
60
50
40
30
20
10
0
Antibody integrity [%]
IgG3
IgG3+IgG1CH3
IgG3+IgG1CH2/3
IgG1
Heavy chain scaffold [-]

Figure S2: Predictive model for compartment-specific IgG1 and IgG3 antibody integrity. A-D. IgG3 integrity dependent on plant cell (pack) harvest times averaged over all compartments (A) and per compartment at 168 h BY-2 and 96 h PCP harvest (C). IgG1 integrity dependent on plant cell (pack) harvest times (B) and compartment at 168 h BY-2 and 96 h PCP harvest (D). E. Effect of heavy chain scaffold (Figure 1B) on mAb integrity. Numbers inside the plot in panels (A) and (B) represent mAb iso-integrity lines. The mAb integrity was assessed by densitometric analysis of silver-stained LDS gels using AIDA software. Error bars in C–E represent least significant difference (LSD) bars around the model predicted means (dots). CH – constant heavy chain antibody domain, LPH – N-terminal leader peptide of the antibody mAb24 heavy chain, PCP – plant cell pack.


A
B
C
D
CHS
omega
TL
Accumulation IgG3 [mg kg-1]
Accumulation IgG3/1CH3 [mg kg-1]
Accumulation IgG3/1CH3CH2 [mg kg-1]
Accumulation IgG1 [mg kg-1]
ER 1xLPH
ER 2xLPH
Apoplast
Vacuole
Chloroplast
Cell compartment [-]

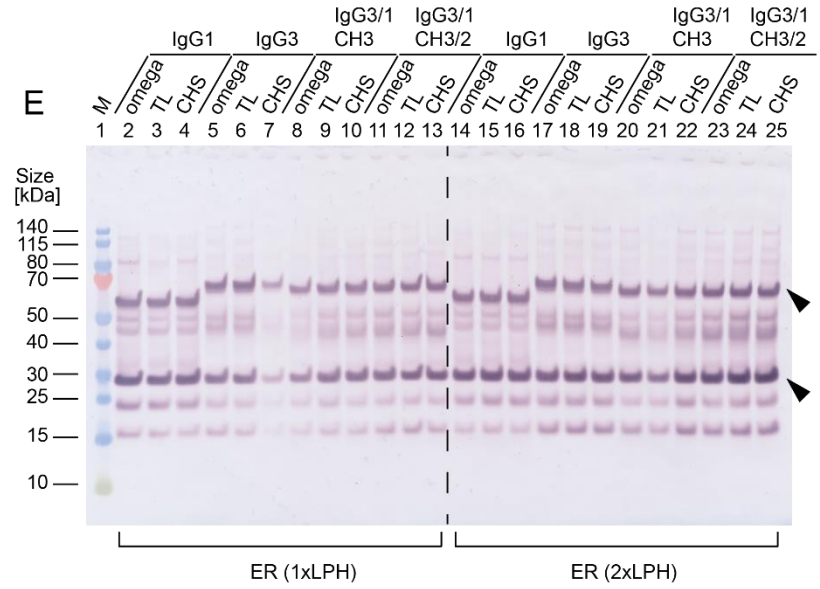

E
IgG1
IgG3
IgG3/1 CH3
IgG3/1 CH3/2
M
omega
TL
CHS
Size [kDa]
140
115
80
70
50
40
30
25
15
10
ER (1xLPH)
ER (2xLPH)

Figure S3: Accumulation and integrity of IgG1 and IgG3 variants in BY-2 PCPs.
A-D. Accumulation of IgG3 (A), IgG3 variant with IgG1 CH3 domain (B), IgG3 variant featuring IgG1 CH2 and CH3 domains (C) and IgG1 (D) dependent on the subcellular compartment and 5′ UTR. Even though silver-stained LDS gels indicated the presence of ~10% intact mAbs when targeting the chloroplasts (Figure S2), these mAbs did not bind to a protein G-coated SPR sensor, indicating that chloroplast-derived mAbs were not functional. Error bars indicate the standard deviation (n = 3 PCPs). BY-2 cells were cultivated for 168 h and PCPs were incubated for 96 h. E. Integrity of ER-targeted IgG1 control and IgG3 variants detected with AP-labeled polyclonal goat anti-human IgG and goat anti-human Ig λ antibodies on a western blot. Black block arrows indicate the expected molecular mass of heavy and light chains. CHS – *Petroselinum hortense* chalcone synthase gene 5′ UTR, LPH – leader peptide of the antibody mAb24 heavy chain, omega – omega prime sequence from tobacco mosaic virus, TL – tobacco etch virus leader sequence. CH – constant heavy chain antibody domain, PCP – plant cell pack.

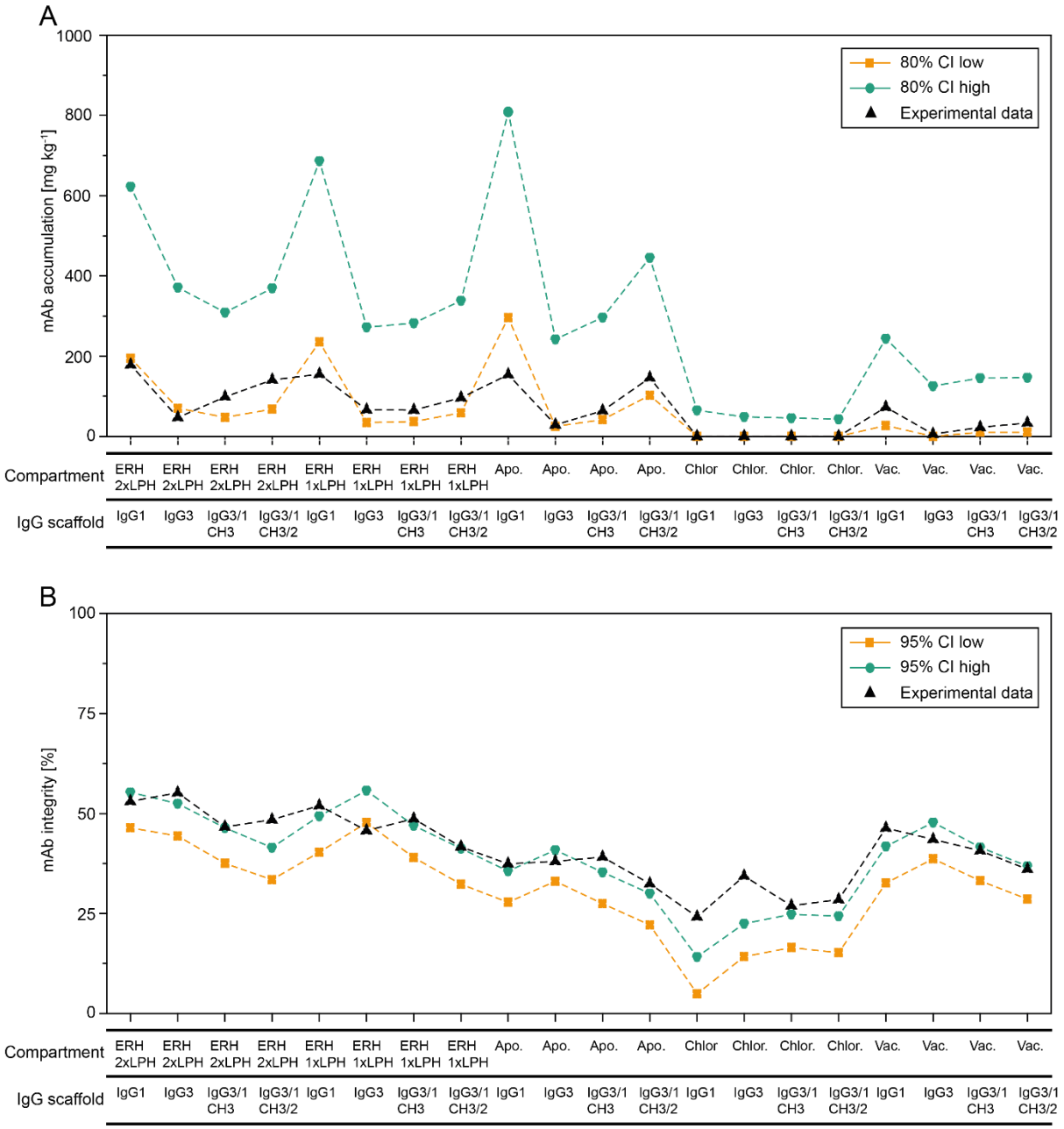



Figure S4: Comparison of model predictions (80% or 95% confidence interval, CI) and verification runs for mAb accumulation and integrity. A. SPR measurement of IgG accumulation dependent on subcellular compartment and IgG scaffold. B. Antibody integrities evaluated by densitometric analysis of silver-stained LDS gels dependent on the subcellular compartment and IgG scaffold. The broad confidence interval for IgG accumulation originated from substantial batch-to-batch variation during expression in PCPs. The data correspond to constructs featuring a CHS 5′ UTR. BY-2 cells were cultivated for

168 h and PCPs were incubated for 96 h. Apo. – apoplast targeting, CH – constant heavy chain antibody domain, ERH – endoplasmic reticulum targeting with hexa-His-tag, LPH – N-terminal leader peptide of the antibody mAb24 heavy chain, PCP – plant cell pack, Vac. – vacuolar targeting.

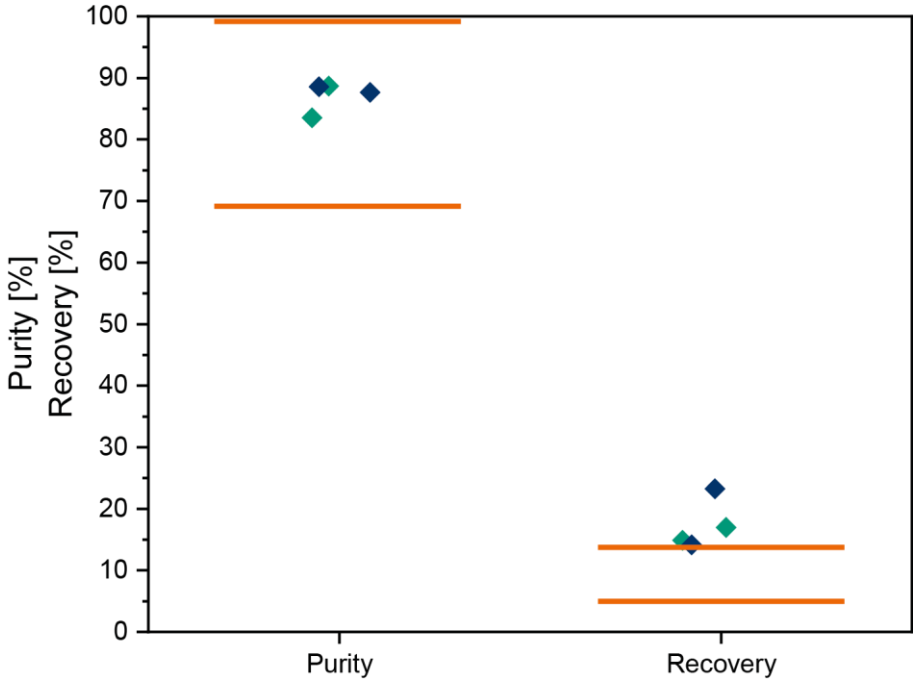



Figure S5: Comparison of model predictions (95% confidence interval, CI, orange lines) and verification runs (green diamonds at 25 mS $cm^{-1}$; blue diamonds at 50 mS $cm^{-1}$) for IgG3 purity and recovery after purification by protein G chromatography. The wash step was performed at pH 8.0 and 5.0 mS $cm^{-1}$ using a contact time of 2 min in all experiments. Elution buffer conductivities up to 25 mS $cm^{-1}$ (always pH 2.0) were used to establish the descriptive models, which cannot be used for predictions at higher conductivities, i.e. at 50 mS $cm^{-1}$ used to test extreme conditions (blue diamonds). Independent of conductivity, model predictions and verification run performance matched well for purity, the model under-predicted the recovery, most likely due to scale-effects