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FROM BITTER TO BETTER: OPTIMIZING POLYPHENOL EXTRACTION FROM YELLOW PEA (*PISUM SATIVUM* L.) USING NATURAL DEEP EUTECTIC SOLVENTS (NADES) FOR IMPROVED PLANT PROTEIN PRODUCTS

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SUPPLEMENTARY MATERIAL

22 pages, 8 figures, 9 tables

INTRODUCTION AND OUTLINE

The present document includes descriptions of additional experiments related to the main work. Firstly, additional plots of the rate constants and distribution coefficients for the first- and second-order modeling of the particle size- and temperature-dependent extraction experiments described in the main manuscript are provided, accompanied by the results of the Bayesian information criterion (BIC) test for this data. This is followed by additional information relevant to the color analysis of extracts obtained throughout the work, with solvent and material (dis)coloration as a rudimentary indicator of the extraction process. This kinetic study is complemented with a further visualization of the extraction process with Safranin-stained solvents, showing how different solvents penetrate pea tissue at different rates. Finally, the Supplementary Material concludes with the methodology and results of an experiment that sought to determine the extraction kinetics of polyphenols and proteins to a commercial pea protein concentrate (PPC). With this experiment, the kinetic modeling approach employed in the primary work could not be applied as extraction rates were too fast to be captured experimentally. However, due to the insights gained on working with this material and the commercial relevance of PPC as a co-product of pea starch refining, the data is included within this document.

KINETIC MODELING RATE CONSTANTS AND DSTRIBUTION COEFFICIENTS

The dataset for the plotted rate constants (k_a) and maximum concentrations ($K_D \cdot c_s$) for the various extraction experiments of total phenolic compounds (TPC) (quercetin equivalents, QE) and proteins (bovine serum albumin equivalents, BSAE) from yellow peas, outlined in the main manuscript, is completed below in **Figures S1 to S5**. Rate constants and maximum concentrations for the first- and second order models used to describe experimental outcomes follow the same trends. Generally, this data reveals that increasing water content and temperature and decreasing particle size leads to quicker TPC and protein extraction. Water content for efficient separation of polyphenols and proteins is especially relevant to larger particle sizes; smaller particle sizes show high-rate constants and high extraction yields for TPC and proteins regardless of water content. Temperature's effect on extraction efficiency does not strictly follow Arrhenius trends, where higher temperature would always lead to quicker extraction rates, due to the competing effects of protein aggregation or starch gelatinization at higher temperatures.

Figure S1.

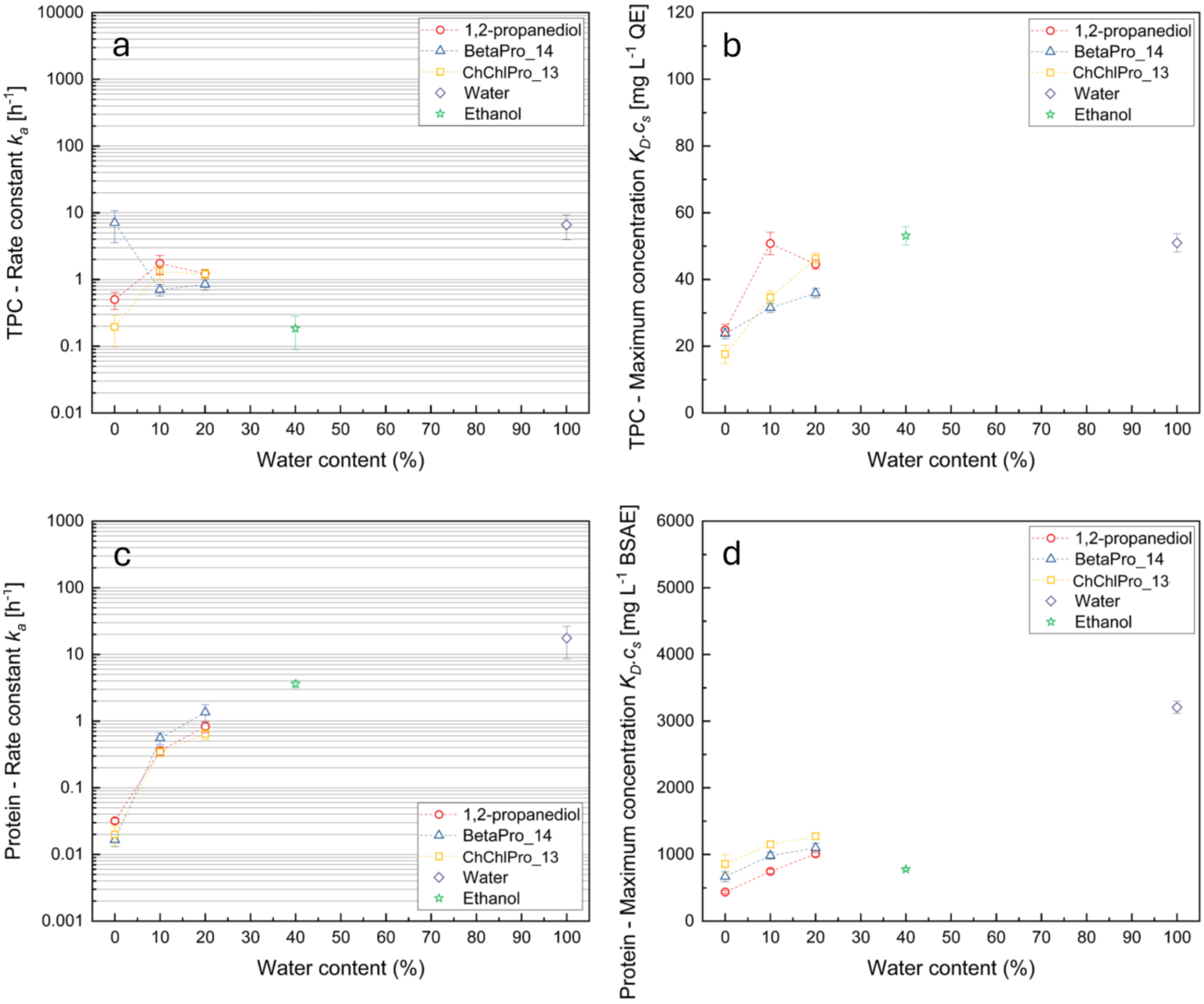

Figure S1. Rate constants k_a and maximum concentration $K_D{\cdot}c_s$ from the first-order model for the different solvent dilutions used for the extraction of TPC and protein from peas: (a) TPC, rate constant k_a (b) TPC, maximum concentration $K_D{\cdot}c_s$, (c) protein, rate constant k_a, and (d) protein, maximum concentration $K_D{\cdot}c_s$. Error bars represent the standard error of the parameter estimates and dashed lines are used to guide the eye.

Figure S2.

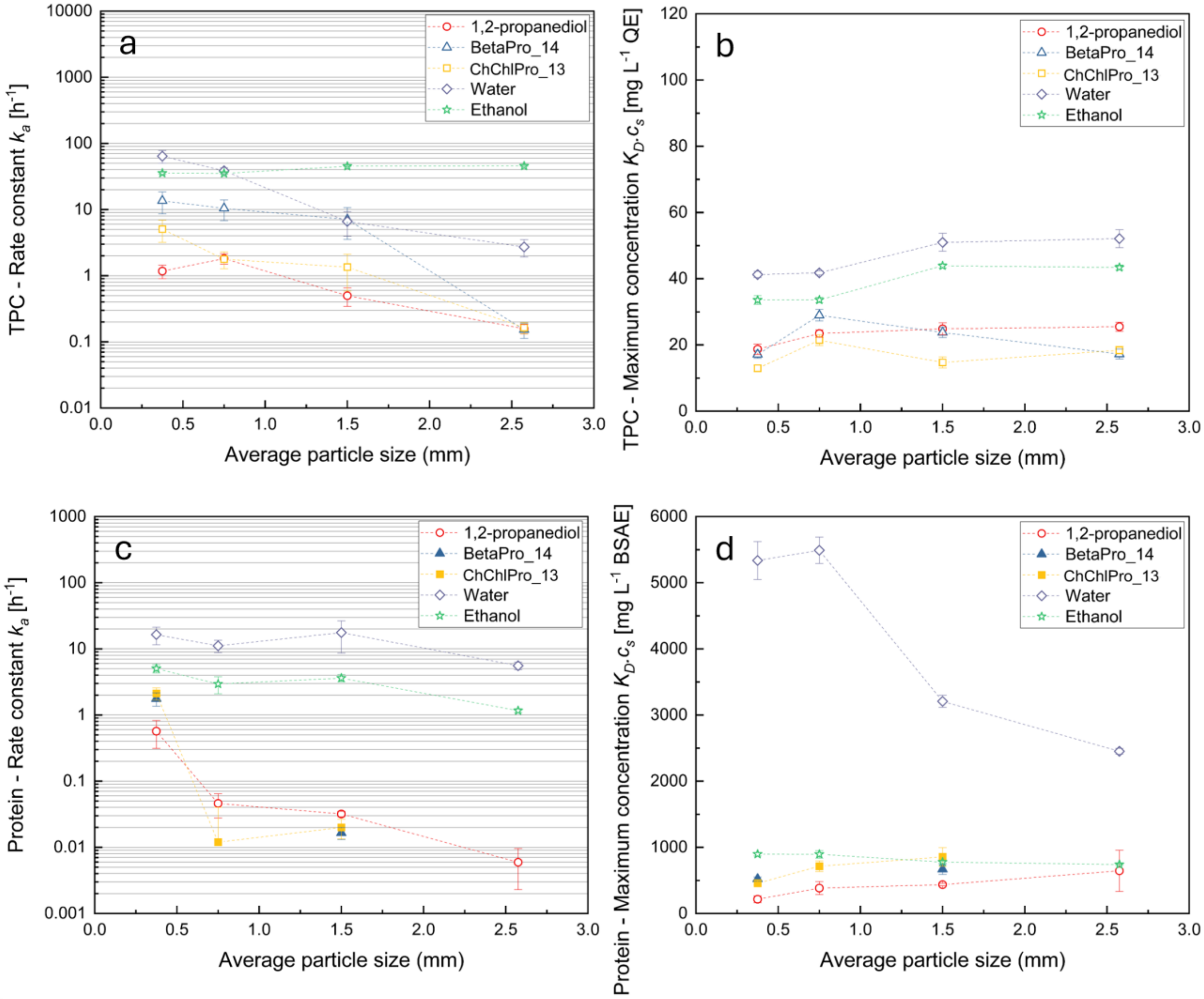

Figure S2. Rate constants k_a and maximum concentration $K_D \cdot c_s$ from the first-order model for the different particle size fractions used for the extraction of TPC and protein from peas: (a) TPC, rate constant k_a (b) TPC, maximum concentration $K_D \cdot c_s$, (c) protein, rate constant k_a, and (d) protein, maximum concentration $K_D \cdot c_s$. Error bars represent the standard error of the parameter estimates, dashed lines are used to guide the eye, and solid symbols indicate incomplete data sets due to poor model fit.

Figure S3.

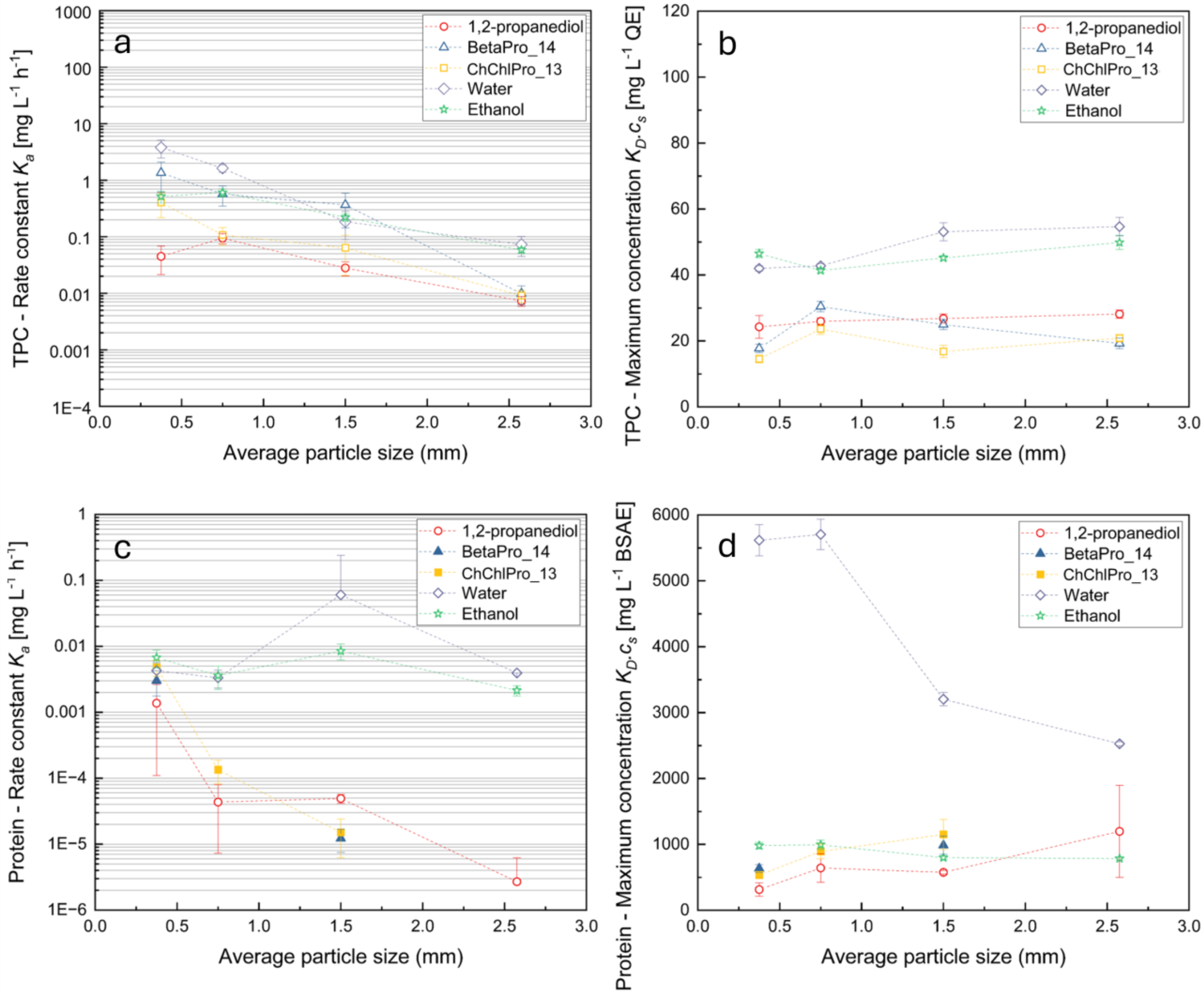

Figure S3. Rate constants K_a and maximum concentration $K_D \cdot c_s$ from the second-order model for the different particle size fractions used for the extraction of TPC and protein from peas: (a) TPC, rate constant K_a (b) TPC, maximum concentration $K_D \cdot c_s$, (c) protein, rate constant K_a, and (d) protein, maximum concentration $K_D \cdot c_s$. Error bars represent the standard error of the parameter estimates, dashed lines are used to guide the eye, and solid symbols indicate incomplete data sets due to poor model fit.

Figure S4.

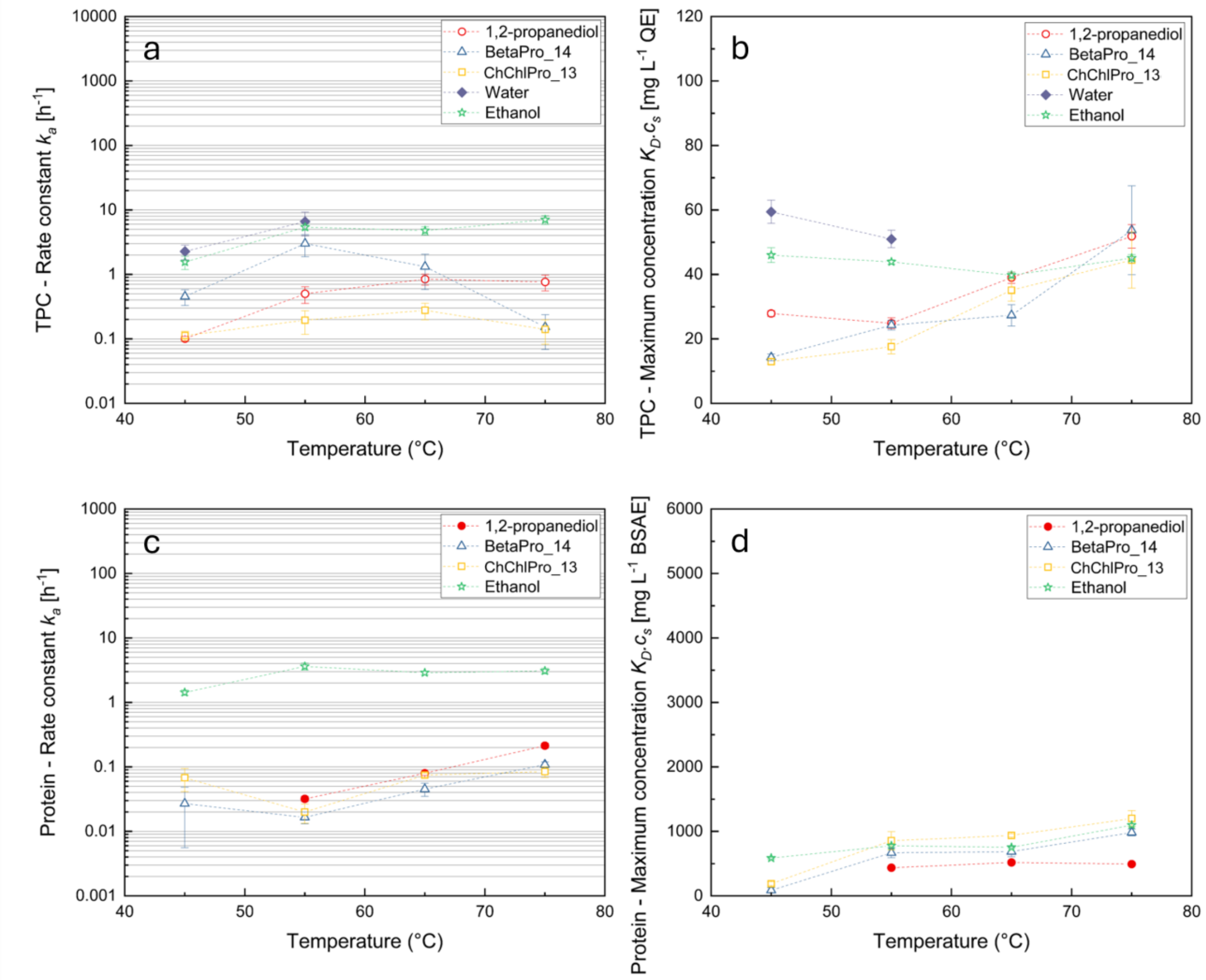

Figure S4. Rate constants k_a and maximum concentration $K_D{\cdot}c_s$ from the first-order model for the different temperatures used for the extraction of TPC and protein from peas: (a) TPC, rate constant k_a (b) TPC, maximum concentration $K_D{\cdot}c_s$, (c) protein, rate constant k_a, and (d) protein, maximum concentration $K_D{\cdot}c_s$. Error bars represent the standard error of the parameter estimates, dashed lines are used to guide the eye, and solid symbols indicate incomplete data sets due to poor model fit.

Figure S5.

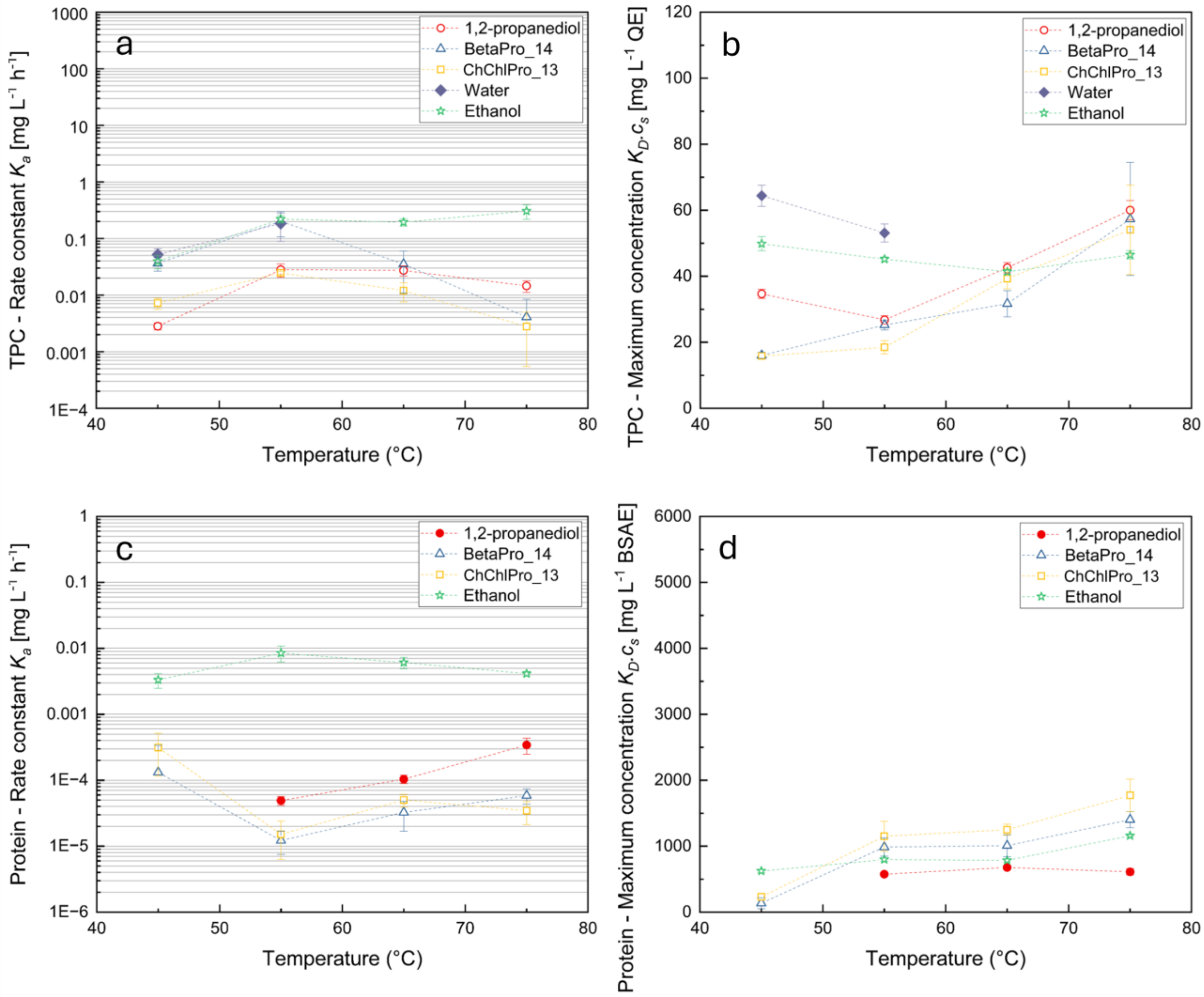

Figure S5. Rate constants K_a and maximum concentration $K_D \cdot c_s$ from the second-order model for the different temperatures used for the extraction of TPC and protein from peas: (a) TPC, rate constant K_a (b) TPC, maximum concentration $K_D \cdot c_s$, (c) protein, rate constant K_a, and (d) protein, maximum concentration $K_D \cdot c_s$. Error bars represent the standard error of the parameter estimates, dashed lines are used to guide the eye, and solid symbols indicate incomplete data sets due to poor model fit.

BAYESIAN INFORMATION CRITERION TEST

The Bayesian information criterion (BIC) test reveals a preference for the second-order model to best describe the experimental data. When the BIC difference is greater than 10 the model is decisively correct; this occurs rarely with the present data, indicating a general slight preference for the second-order model. **Tables S1 to S4** detail the results of the BIC comparison of the first- and second-order models for TPC and protein extraction from peas according to particle size and temperature.

Table S1. Comparison of the first- and second-order models for TPC extraction as a function of particle size using the Bayesian Information Criterion (BIC)

Solvent	Particle Size	Model BIC		
		First Order	Second Order	Difference
1,2-propanediol	Extra-large	18.2	11.8 [a]	6.2
	Large	26.9	18.8 [a]	8.1
	Medium	33.5	24.6 [a]	8.9
	Small	12.6 [a]	14.0	1.4
BetaPro_14	Extra-large	21.2	17.2 [a]	4.0
	Large	33.7	30.7 [a]	3.0
	Medium	56.2	49.2 [a]	7.0
	Small	22.4	22.1 [a]	0.3
ChChlPro_13	Extra-large	16.8	10.4 [a]	6.3
	Large	32.4	29.3 [a]	3.1
	Medium	43.8	37.1 [a]	6.7
	Small	17.2	13.5 [a]	3.7
Water	Extra-large	42.9	41.0 [a]	1.9
	Large	44.9	42.1 [a]	2.8
	Medium	28.8	26.8 [a]	2.1
	Small	20.1	16.3 [a]	3.8
Ethanol	Extra-large	16.8	12.0 [a]	4.8
	Large	16.6	13.5 [a]	3.1
	Medium	34.7	28.0 [a]	6.7
	Small	25.2	19.1 [a]	6.0

[a] A lower BIC value indicates the model that is more likely to be correct.

Table S2. Comparison of the first- and second-order models for protein extraction as a function of particle size using the Bayesian Information Criterion (BIC)

Solvent	Particle Size	Model BIC		
		First Order	Second Order	Difference
1,2-propanediol	Extra-large	57.2 [a]	57.4	0.2
	Large	56.3	51.1 [a]	5.1
	Medium	90.8 [a]	91.0	0.2
	Small	58.1	58.0 [a]	0.1
BetaPro_14	Extra-large	29.1	29.1	0.0
	Large	66.8	65.6 [a]	1.2
	Medium	38.3 [a]	38.4	0.1
	Small	73.6	73.1 [a]	0.5
ChChlPro_13	Extra-large	48.4	48.4	0.0
	Large	32.4	29.3 [a]	3.1
	Medium	127.1	123.5 [a]	3.6
	Small	69.9	64.8 [a]	5.0
Water	Extra-large	100.7	88.7 [a]	12.1 [b]
	Large	115.8 [a]	116.2	0.4
	Medium	198.3 [a]	198.9	0.6
	Small	122.0	115.6	6.4
Ethanol	Extra-large	68.2 [a]	79.1	10.9 [b]
	Large	77.8 [a]	85.6	7.8
	Medium	155.9	152.9 [a]	3.0
	Small	76.5 [a]	82.2	5.7

[a] A lower BIC value indicates the model that is more likely to be correct.

[b] An absolute BIC difference greater than 10 indicates the model that is decisively correct

Table S3. Comparison of the first- and second-order models for TPC extraction as a function of temperature using the Bayesian Information Criterion (BIC)

Solvent	Temperature	Model BIC		
	°C	First Order	Second Order	Difference
1,2-propanediol	45	3.8	0.7[a]	3.1
	55	16.4	14.2[a]	2.2
	65	26.5	20.3[a]	6.2
	75	28.7	19.9[a]	8.8
BetaPro_14	45	13.9	6.4[a]	7.5
	55	31.9	28.6[a]	3.3
	65	39.9	36.9[a]	3.0
	75	36.4	35.4[a]	1.0
ChChlPro_13	45	2.3	-3.4[a]	5.7
	55	29.9	26.7[a]	3.2
	65	31.6	27.7[a]	4.0
	75	33.0	32.2[a]	0.9
Water	45	29.6	24.2[a]	5.4
	55	44.9	42.1[a]	2.8
	65	45.1[a]	45.3	0.2
	75	34.0	34.0	0.0
Ethanol	45	33.8	28.2[a]	5.7
	55	16.6	13.5[a]	3.1
	65	19.4	11.9[a]	7.5
	75	18.4[a]	18.8	0.4

[a] A lower BIC value indicates the model that is more likely to be correct.

Table S4. Comparison of the first- and second-order models for protein extraction as a function of temperature using the Bayesian Information Criterion (BIC)

Solvent	Temperature	Model BIC		
	°C	First Order	Second Order	Difference
1,2-propanediol	45	62.0	48.8 [a]	13.2 [b]
	55	56.3	51.1 [a]	5.1
	65	51.8	50.9 [a]	1.0
	75	50.1 [a]	56.2	6.1
BetaPro_14	45	41.2	4.2	0.0
	55	66.8	65.6 [a]	1.2
	65	68.2 [a]	68.5	0.3
	75	57.1 [a]	59.4	2.3
ChChlPro_13	45	55.4	53.8 [a]	1.6
	55	78.3	77.2 [a]	1.1
	65	54.7 [a]	61.9	7.2
	75	59.0	58.7 [a]	0.3
Ethanol	45	63.8 [a]	72.8	9.1
	55	77.8 [a]	85.6	7.8
	65	60.3 [a]	71.4	11.1 [b]
	75	64.4	56.1 [a]	8.3

[a] A lower BIC value indicates the model that is more likely to be correct.

[b] An absolute BIC difference greater than 10 indicates the model that is decisively correct

COLOR ANALYSIS OF LIQUID EXTRACTS

The pea extracts' color development over a period of 5 h is shown in Tables S1 to S5. The measurements were performed with a calibrated colorimeter and the color difference ΔE^*_{ab} was calculated with respect to the time at which the extraction started (0 h). Samples were placed in a clear glass Petri dish at a thickness of approximately 1 cm on a pure white background. Combinations of solvents [water, 60% ethanol, betaine and 1,2-propanediol (1:4), choline chloride and 1,2-propanediol (1:3), and 1,2-propanediol] and group pea particle size fractions [small ($0.25 \leq x < 0.5$ mm), medium ($0.5 \leq x < 1$ mm), large ($1 \leq x < 2$ mm), extra-large ($2 \leq x < 3.15$ mm)] were used for the color analysis.

The colorimeter output data in terms of color difference, ΔE^*_{ab}. In the $L^*a^*b^*$ color space, the color difference ΔE^*_{ab} indicates the degree of color difference (but not the direction of change), where , L^* represents lightness from white ($L^* = 100$) to black ($L^* = 0$), while a^* and b^* stand for chromaticity coordinates with different hues: $+a^*$ represents the red direction, $-a^*$ the green, $+b^*$ the yellow, and $-b^*$ the blue (**Equation S1**):

$$\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2(\Delta a^*)^2(\Delta b^*)^2} \qquad \text{(S1)}$$

Then, color difference is then converted to RGB values using MATLAB's open-source code "rgb2lab" (The MathWorks, Inc., Natick, Massachusetts, USA). Both the digital representations of the RGB color of the solvent and the color difference values (ΔE^*_{ab}) for the solid-liquid batch extraction experiments with the different size fractions of ground pea particles and solvents are detailed in **Tables S5 to S9** below.

Table S5. Solvent color difference (ΔE^*_{ab}) for solid-liquid batch extraction with ground pea particles and water as solvent.

	Small		Medium		Large		Extra large	
Sampling time (h)	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)
0	⬜		⬜		⬜		⬜	
1	🟫	12.16	🟫	9.40	🟫	15.26	🟫	11.75
3	🟫	13.03	🟫	13.54	🟫	14.68	🟫	14.49
5	🟫	12.21	🟫	10.19	🟨	5.83	🟫	15.31

Table S6. Solvent color difference (ΔE^*_{ab}) for solid-liquid batch extraction with ground pea particles and 60% ethanol as solvent.

Sampling time (h)	Small		Medium		Large		Extra large	
	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)
0	⬜		⬜		⬜		⬜	
1	🟨	5.67	🟨	2.31	🟨	2.84	🟨	5.00
3	🟨	5.29	🟨	4.47	🟨	5.15	🟨	4.48
5	🟨	5.39	🟨	5.96	🟫	10.99	🟨	4.61

Table S7. Solvent color difference (ΔE^*_{ab}) for solid-liquid batch extraction with ground pea particles and BetaPro_14 as solvent.

Sampling time (h)	Small		Medium		Large		Extra large	
	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)
0	⬜		⬜		⬜		⬜	
1	🟨	11.84	⬜	4.28	⬜	4.56	⬜	2.72
3	🟨	11.21	⬜	6.33	⬜	5.25	⬜	2.64
5	🟨	11.59	⬜	6.76	⬜	5.78	⬜	3.30

Table S8. Solvent color difference (ΔE^*_{ab}) for solid-liquid batch extraction with ground pea particles and ChChlPro_13 as solvent.

Sampling time (h)	Small		Medium		Large		Extra large	
	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)
0	⬜		⬜		⬜		⬜	
1	🟨	9.96	⬜	1.24	⬜	1.32	⬜	0.32
3	🟨	8.52	⬜	3.51	⬜	0.93	⬜	0.91
5	🟨	8.87	⬜	4.24	⬜	0.32	⬜	1.19

Table S9. Solvent color difference (ΔE^*_{ab}) for solid-liquid batch extraction with ground pea particles and 1,2-propanediol as solvent.

Sampling time (h)	Small		Medium		Large		Extra large	
	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)	Color	ΔE^*_{ab} (%)
0	■		■		■		■	
1	■	7.61	■	1.63	■	1.43	■	1.15
3	■	6.87	■	-0.32	■	-0.21	■	1.03
5	■	7.31	■	-0.56	■	-0.59	■	0.77

The results of the color analysis of spent solvents used in this work provides a visual element to support quantitative measurements mase in the establishment of the kinetic models. Smaller particles sizes facilitate faster and more exhaustive solute extraction, whereas larger particle sizes are associated with a less efficient, slower process. Moreover, NADES and 1,2-propanediol are generally slower to extract colored solutes than water or 60% ethanol, especially with larger particle size fractions. Importantly, color analysis is a suitable visual indicator of extraction yield but cannot capture the nuanced nature of the extraction of many different polyphenolic compounds and proteins, some which lead to solvent discoloration, and some that do not. For example, this may explain why water samples exhibit a greater ΔE^*_{ab} than all other samples, as they simply tend more towards a brownish discoloration as opposed to a green-yellow discoloration. Water is rather more effective for protein extraction than polyphenol extraction, as demonstrated by the quantitative data presented in the main manuscript. Moreover, enzymatic reactions that may lead to solvent discoloration occur more readily in water than in 1,2-propanediol or NADES, and this may also contribute to greater ΔE^*_{ab} values [Sajib *et al.*, 2020].

VISUALIZATION OF SOLVENT PENETRATION WITH STAINING

Solvent migration into whole peas was examined by soaking the materials in the different solvents [1:10 solid-to-liquid ratio (*w/*w)] colored with 0.05% (*v/v*) Safranin stain (Euromex Microscopen B.V., Duiven, Netherlands). The following stained solvents were analyzed: water, 60% ethanol, betaine and 1,2-propanediol (1:4), choline chloride and ,2-propanediol (1:3), and 1,2-propanediol. For the sake of improved solvent penetration into the solid matrix, the two NADES and 1,2-propanediol solvents were diluted with 20% water, as water addition helps to hydrate the matrix and facilitate diffusion of the molecules and dye. This corresponds with the higher extraction rates observed with hydrated NADES in the batch extraction experiments described in the main manuscript. The experiment was carried out for 1 h and 1.75 h periods. After this, the peas were removed from the solvent and embedded in Stearin wax (Glorex AG, Füllinsdorf, Switzerland). The samples were hardened in the fridge before being cut with a microtome revealing cross-sections of the peas. Stained pea cross-sections were photographed under a Leica S6 D Greenough stereo-microscope equipped with a MC170 HD 5 MP camera (Leica Microsystems, Wetzlar, Germany).

Solvent migration into whole peas was examined by soaking the materials in the different solvents colored with Safranin stain. The general differential equation to model extraction kinetics described in the main manuscript is the combination of all rate constants that can explain the experimental data. In this sense, it is expected that staining the solvents used in this work with a highly-soluble dye, then immersing peas in this solution, adds a visual element to the first step of the mass transfer process: solvent penetration into the solids [Wang *et al.,* 2023].

Figure S6 shows a collection of stereo-microscope images of pea cross-sections following their soaking in Safranin-stained solvents. As is confirmed by the kinetic modeling data, NADES and 1,2-propanediol are visibly more resistant to penetrating the peas than water, despite their 20% water content. For 1,2-propanediol specifically, it is possible to see how only 20% of added water to the solvent already aids in solvent penetration (d and e). With water, it's possible to observe how the process progresses over time, with staining more evident after 1.75 h as opposed to 1 h (a and b). It is equally possible to observe material swelling due to starch hydration, evidenced by larger pea size, softer tissues (the other samples have a glassy appearance), and reduced density (cracks in the material visible in sample b).[Jia *et al.,* 2023] While starch swelling in aqueous solutions may play a role in solvent penetration, these trends may be further attributed to differences in solvent viscosity - a well-known key parameter in the rate and efficiency of solid-liquid extraction processes [Li *et al.,* 2022]. Samples soaked in 60% ethanol were too soft to be effectively separated from the Stearin wax and are consequently not pictured here.

Figure S6.

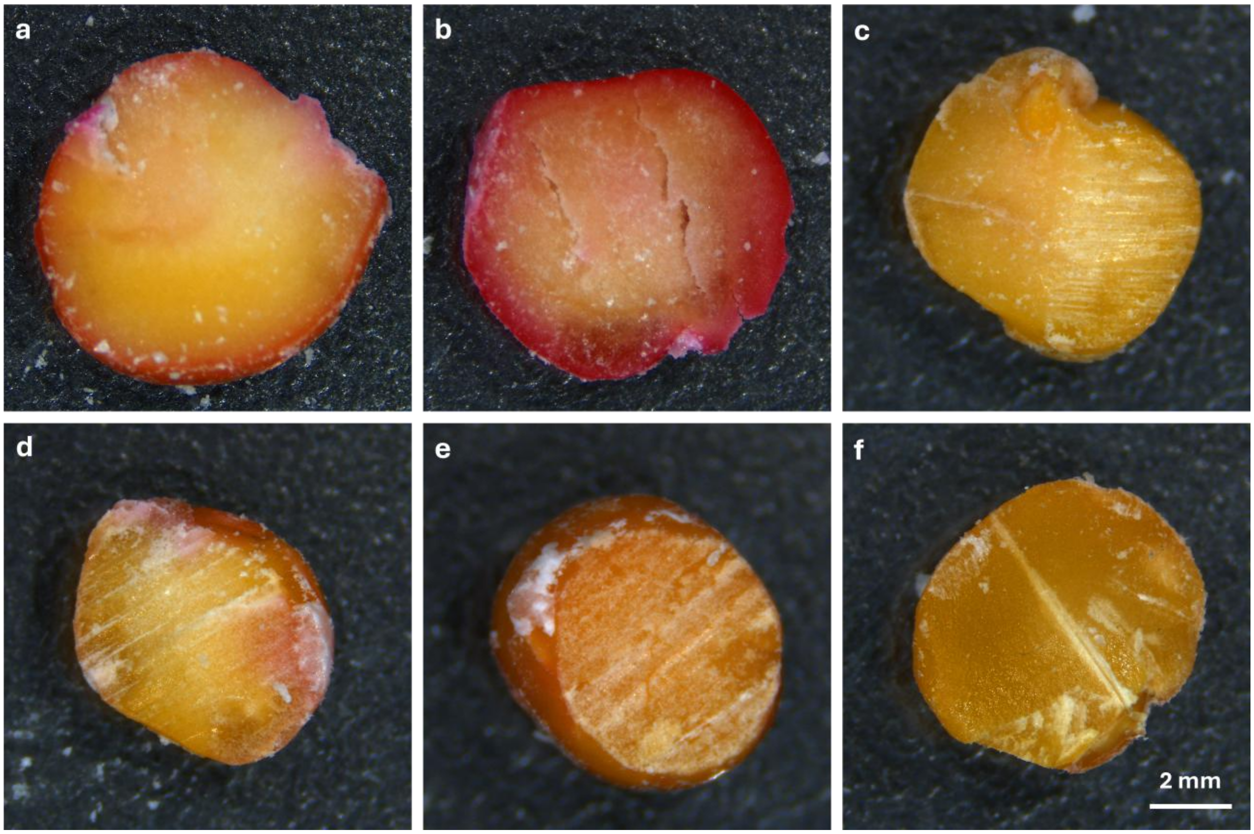

Figure S6. Cross-section stereo-microscope images of yellow peas after soaking in 0.05% (*v/v*) Safranin-solvent solutions as a visualization of solvent penetration: (a) water, 1h, (b) water, 1.75h, (c) choline chloride-1,2-propanediol (1:3) with 20% water, 1h, (d) 1,2-propanediol, 1h, (e) 1,2-propanediol with 20% water, 1h, (f) betaine-1,2-propanediol (1:4) with 20% water, 1h.

REFERENCE EXTRACTION EXPERIMENTS USING COMMERICAL PEA PROTEIN CONCENTRATE

Commercial pea protein concentrate (PPC) from dry fractionation was obtained from Prodapi MV GMbH (Flensberg, Germany). The commercial PPC used for comparison contained 60 g kg^{-1} moisture, 528 g kg^{-1} protein, 55 g kg^{-1} ash, 34 g kg^{-1} fat, and 323 g kg^{-1} other compounds. Compositional analysis of the pea protein concentrate was performed by Amt für Verbraucherschutz (AVS), Steinhausen, Switzerland. Briefly, protein was analysed using the Kjeldahl method, crude fat was extracted with solvent after acid digestion, moisture was determined gravimetrically by heating at 105 °C, and the ash content was determined in a muffle furnace.

For solid-liquid extraction experiments of commercial PPC, this material was used as-is after stirring to ensure homogeneity of the material. Here, the same protocol was used as for the ground pea particles, with minor differences. Notably, small particle size meant that shorter sampling intervals were used (1, 2, 3, 5, 15, 30, 60 minutes). Then, suspended PPC was removed from the solvent by immediately filtering the sample through a 1.0 µm glass fibre syringe filter, effectively stopping the extraction process. Otherwise, the same liquid-to-solid ratio and experimental setup were used, with 1,2-propanediol, betaine and 1,2-propanediol at a 1:4 ratio (BetaPro_14) NADES, choline chloride and 1,2-propanediol at a 1:3 ratio (ChChlPro_13) NADES, 60% ethanol, and water as solvents. No water was added to the 1,2-propanediol or NADES. Temperature was only varied with water as a solvent (35, 45, 55, 65, 75 °C) as it was difficult to press the high-viscosity NADES and 1,2-propanediol through the syringe filters, especially at lower temperatures.

This work demonstrated the remarkable influence of particle size and cellular rupture on extraction efficiency. For all solvents, maximum extraction efficiency was reached within the first 5 minutes, often even within the first minute. This was also independent of temperature. For this reason, the first- and second-order models could not be used to describe the experimental data. Instead, **Figures S7 and S8** show the average yield of TPC and proteins across the entire 1h extraction experiment; this value serves as a proxy for the maximum extractable concentrations of analyte ($K_D \cdot c_s$) for different experimental conditions.

This work highlights how with decreasing particle size and an increasing degree of ruptured plant cells, polyphenol extraction becomes a faster process. However, there are still differences between the maximum yields of polyphenols and proteins obtained by different solvents. Water is the most efficient for TPC extraction from PPC, followed by 1,2-propanediol and 60% ethanol (**Figure S7**). Meanwhile, the free proteins of PPC, i.e., not bound within intact cells, as is the case when working with ground pea particles, are much more readily solubilized in water, while overall protein extraction remains low for all other solvents. With water, it's possible to observe how TPC extraction remains steady across a range of temperatures, while protein extraction peaks at 55°C before solubility decreases possibly due to temperature-induced aggregation (**Figure S8**). It's also noteworthy that the high viscosity of the NADES and 1,2-propanediol makes it difficult to recover PPC from these solvents. For these reasons, this work highlighted in the main manuscript focusses on ground pea particles instead.

Figure S7.

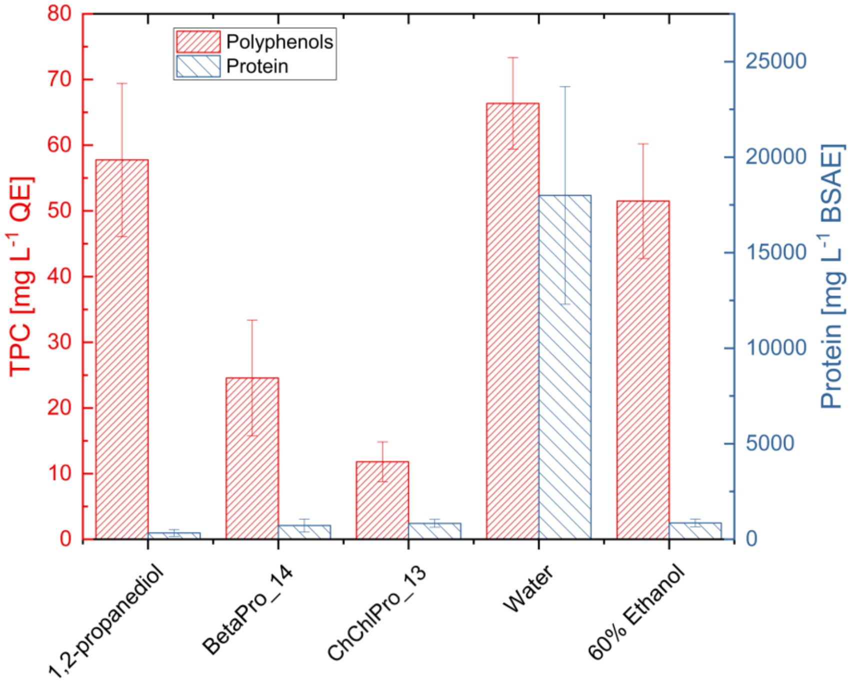

Figure S7. Average extraction of total phenolic compounds (TPC) and protein from commercial pea protein concentrate with 1,2 propanediol, BetaPro_14 and ChChlPro_13 , water, and 60% ethanol over an hour. Plots show mean values and standard deviation (*n* = 3). TPC were calculated as quercetin equivalents (QE) and protein were calculated as bovine serum albumin equivalents (BSAE).

Figure S8.

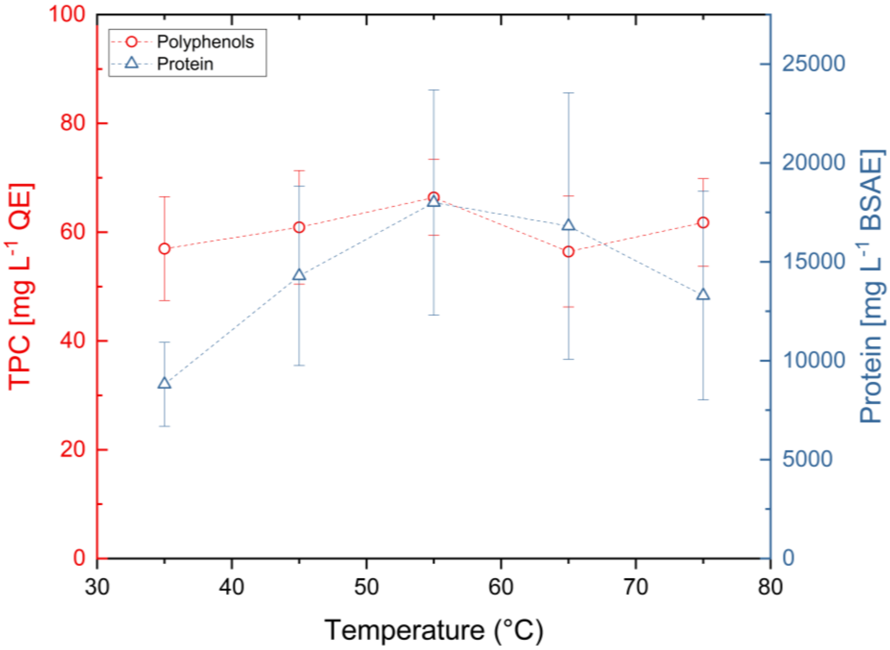

Figure S8. Average extraction of total phenolic compounds (TPC) and protein from commercial pea protein concentrate with water at 35, 45, 55, 65, and 75 °C over an hour. Plots show mean values and standard deviation ($n = 3$). TPC were calculated as quercetin equivalents (QE) and protein were calculated as bovine serum albumin equivalents (BSAE). The dashed line is used to guide the eye.