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Understanding the protein extraction potential of tomato leaves (*Solanum lycopersicum*) through mass balance modelling

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ABSTRACT

Tomato leaves, the main by-product from tomato production, are a potential protein source. In this study, proteins were extracted from tomato leaves collected from different positions of the plant (top, middle and bottom) using three different purification methods: acid precipitation, ethanol precipitation and dialysis. The protein extraction process was evaluated in terms of protein yield and purity. In addition, mass balances were established to estimate the potential additional protein recovery as well as to identify the limiting factors of the extraction. Significant protein loss (0.62 g/g total proteins) occurred during the initial processing steps, which was mainly attributed to the water absorbed by the fibrous pulp and the presence of large quantities of insoluble proteins. Part of the loss may be recovered from the pulp and thus considered attainable. However, most of these proteins were inevitably lost, due to their insolubility. In general, low protein yields were found for all leaves when aiming for a protein extract with high purity, with some variations in different leaves. Top (young) leaves resulted in the highest protein yield, suggesting the presence of more intact proteins. Acid precipitation resulted in the highest protein purity, suggesting a high selectivity for proteins.

1. Introduction

There is a growing need for diversified protein sources to support the protein transition (Boland et al., 2013). Green leaves, including those from agricultural by-products like tomato leaves, have potential due to their high protein content and wide availability. In the past decade, global tomato production has exceeded 160 million tons annually (FAOSTAT, 2023), making tomato one of the most-produced vegetables in the world. However, the fruits only represent up to about 60% of the plant mass (Taylor & Fraser, 2011). The remaining plant material, primarily being leaves and stems, are typically considered as waste. These materials are mostly discarded (Fernández-Gómez, Díaz-Raviña, Romero, & Nogales, 2013), or in some cases composted. Up to 28% of the dry matter in tomato leaves is proteins (Abo Bakr, Mohamed, & Moustafa, 1982; Yu, Kleuter, Taghian Dinani, Trindade, & van der Goot, 2022), making tomato leaves a potential protein source for the food and feed industry.

Most of the proteins (about 80%) in leaves are located in chloroplasts (Fiorentini & Galoppini, 1983). About half of these proteins are soluble, with RuBisCo (ribulose bisphosphate carboxylase/oxygenase) being the major soluble protein (Ellis, 1979; Fiorentini & Galoppini, 1983). The rest of the chloroplast proteins are mostly insoluble proteins located at the thylakoid membrane. Leaf proteins carry important functions for the plant. In case of tomato plants, proteins in young leaves are primarily responsible for photosynthesis to support the growth of leaves and the plant. When leaves age, (part of) the photosynthetic proteins are degraded into peptides and free amino acids, which are then transported back to other organs such as fruits and young vegetative tissues as nutritional support (leaf senescence) (Havé, Marmagne, Chardon, & Masclaux-Daubresse, 2017). This change in protein composition can be reflected in leaves collected from different positions of the tomato plant (i.e., top, middle and bottom) (Yu et al., 2022). As a consequence, the extraction of proteins can be influenced.

Making foods requires ingredients that have reasonable concentrations. High concentration is typically achieved through extractive purification. When taking the example of soy proteins, one of the most

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well-known plant proteins (Arora et al., 2023), two ways to purify proteins from soy meal are generally used on industrial scale: ethanol washing and acid precipitation (Alibhai, Mondor, Moresoli, Ippersiel, & Lamarche, 2006; Deak & Johnson, 2007). During ethanol washing, the protein solubility is lowered, keeping the proteins together with insoluble components, while soluble components are washed away. This process often results in a moderately concentrated protein fraction. During acid precipitation, proteins are first solubilized in an alkaline solution and separated from insoluble components. The solution is then acidified to insolubilize proteins, hence separating proteins from other soluble components, generating a relatively pure protein fraction. Often, these (insolubilized) proteins are neutralized again to recover protein solubility. The current extraction techniques for leaf proteins are mainly adapted from those fractionation processes applied for storage proteins from seeds or legumes, like soybean (Tamayo Tenorio, Kyriakopoulou, Suarez-Garcia, van den Berg, & van der Goot, 2018). Hence, leaf protein extraction usually starts with a cell disruption step in which liquid from the leaf cells are separated from insoluble fibers (Edwards et al., 1975; Hadidi, Ibarz, Conde, & Pagan, 2019; Hojilla-Evangelista, Selling, Hatfield, & Digman, 2017; Martin, Castellani, de Jong, Bovetto, & Schmitt, 2019). This results in a green juice containing soluble and dispersed proteins (e.g., thylakoid membrane proteins) and other components. The next step is to separate the thylakoid membrane proteins from the soluble proteins. Thylakoid membrane proteins, due to their associations with chlorophyll, lipid and some anti-nutritional factors, have intensive green color and a grassy taste (Fiorentini & Galoppini, 1983; Santamaría-Fernández & Lübeck, 2020). This hinders their application in foods. Common methods to enhance the separation of thylakoid membrane proteins from soluble proteins are mild heating (Martin, Nieuwland, & De Jong, 2014) or high speed centrifugation (Tanambell, Møller, Corredig, & Dalsgaard, 2022). As next step, ethanol or a series of pH adjustments have been used to purify leaf proteins (Akyüz & Ersus, 2021; Ayim, Ma, Alenyorege, Ali, & Donkor, 2018; Hadidi et al., 2019; Hojilla-Evangelista et al., 2017; Yu et al., 2022). In addition, filtration (Ducrocq et al., 2022; Martin et al., 2019) and chromatography (Martin et al., 2014) have also been applied for this.

So far, many researchers proposed ways to assess the protein extraction. Tamayo Tenorio et al. (2018) introduced the use of a yield-purity diagram, in which the protein purity was plotted against the total protein yield. As indicated by Tamayo Tenorio et al. (2018), for leafy biomasses, only low protein yields (0.1 g/g total proteins) were achieved when aiming at high purity (60 to 80%). This indicates a low extraction efficiency, especially when compared to the yields (0.5 to 0.6 g/g total proteins) of other protein-rich crops, such as soy, pea and other legumes at similar purity. Although the yield-purity diagram is a handy tool to compare extraction efficiency between different crops, it does not identify the limiting factors during the extraction process.

The aim of this study is therefore to establish a method to identify the limiting factors of leaf protein extraction. Proteins were extracted from fresh tomato leaves collected from different positions (top, middle and bottom) of the plant using three different purification methods (acid precipitation, ethanol precipitation and dialysis). Mass balances of these processes were established with the aim to estimate the location of losses and identify potential ways to enhance the recovery.

2. Material and methods

2.1. Plant material and chemicals

Tomato plants (*Solanum lycopersicum*, cultivar: Moneymaker) were grown from June to October 2022 in the greenhouse (Wageningen University, the Netherlands). Similar growing conditions were applied as described by Yu et al. (2023, 2022). Detailed information regarding temperature and humidity in the greenhouse, the use of nutrition, pesticides and insecticides during the plant growth can be found in Supplementary material Fig. S1, Table S1 and S2, respectively. Leaves were harvested on 20th of October in 2022 (114 day after seed sowing). The tomato plants were around 3 m tall and contained both green and mature fruits. Each plant contained 15 to 21 leaves. Based on the total number of leaves, the plants were divided equally into three parts, representing the top, middle and bottom position of the plant (Qudsieh, Yusof, Osman, & Abdul Rahman, 2002; Yu et al., 2022). Leaves from each position were collected separately. Rachises, petioles and petiolules were removed from the leaves (Altartouri, Abu Maizer, Idais, Tamimi, & Arafeh, 2015), after which the rest of the leaves were manually mixed and used as study material. In total, leaves from 30 plants were harvested and mixed. The total weight of leaves from different positions were 1899 g (top), 4073 g (middle) and 5044 g (bottom), respectively. The harvest was carried out in one day and the harvested leaves were stored at 4 °C in dark conditions for 16 h before protein extraction.

Ethanol (96%) was purchased from VWR Chemicals (Paris, France). Hydrochloric acid was purchased from Actu-All Chemicals BV (Hoogeveen, the Netherlands). Sodium hydroxide was purchased from Merck KGaA (Darmstadt, Germany). D-Methionine (>98%, HPLC grade) and cellulose were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Milli-Q water was purified by Milli-Q IQ 7000 118 Ultrapure Lab Water System (Merck KGaA, Darmstadt, Germany).

2.2. Protein extraction

Protein extraction was carried out in three steps: juicing, centrifugation and protein purification. All experiments were performed three times. The protein extraction process is schematically presented in Fig. 1.

Step 1 Juicing

Leaves from the top (T), middle (M) and bottom (B) positions of the plants were juiced using a twin-screw Angel Juicer II 7500 (Angel Juicers, Queensland, Australia). From each position, a dark green juice (GJ) and (fibrous) pulp were generated and separately collected and weighed. The pulp was frozen (−20 °C) and freeze dried (Epsilon 2-10D LSCplus, Martin Christ, Osterode, Germany).

Step 2 Centrifugation

The GJ was centrifuged (15,000 *g*, 4 °C, 30 min) and the supernatant was separated from pellet 1 (P1) by pouring carefully. The supernatants

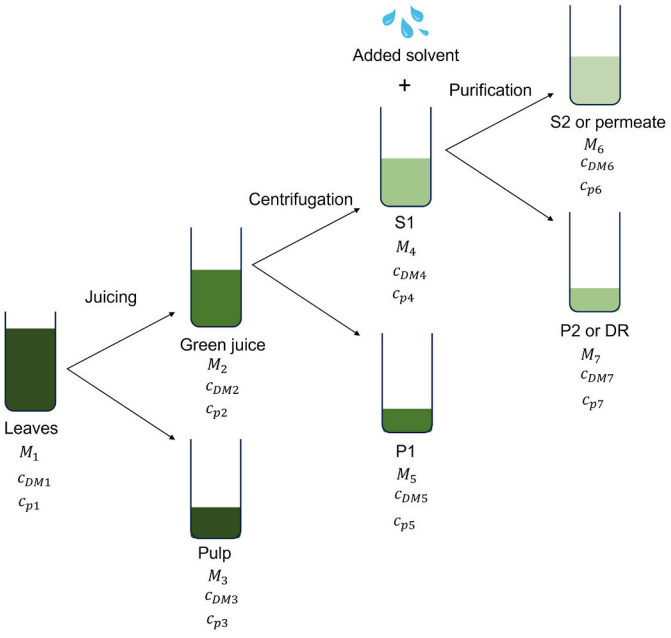

Fig. 1. Schematic demonstration of protein extraction process and measured values for each fraction obtained during the processing.

generated in this step were further referred to as supernatant 1 (S1) in this study. The S1 and P1 were separately collected and weighed. The P1 was frozen (−20 °C) and freeze dried.

Step 3 Purification

Based on the total weight of S1 from each leaf material, S1 was equally divided into three aliquots. The proteins in each aliquot were purified by acid precipitation, ethanol precipitation or dialysis.

In case of the acid precipitation, the pH of the first S1 aliquot was adjusted to 3.5 with 5 M hydrochloric acid. This pH was selected as the precipitation pH due to the net zero zeta potential value of the proteins at this pH (Zetasizer Ultra, Malvern Panalytical Ltd., Malvern, UK) (Supplementary material, Fig. S2). The volume of the hydrochloric acid was measured by noting the total pipetting volume. The mixture was then allowed to precipitate at 4 °C in dark conditions for 13 h, and subsequently centrifuged (15,000 *g*, 20 min, 4 °C). Supernatant 2 was separated from pellet 2 by careful pouring. Both fractions were separately collected and weighed. Subsequently, pellet 2 was re-dispersed in 100 g MilliQ water, and the pH of the dispersion was adjusted to 7 with 6 M sodium hydroxide. The volume of the sodium hydroxide was measured by noting the total pipetting volume. Finally, supernatants 2 and dispersions were frozen at −20 °C and freeze dried. The freeze dried supernatant 2 and dispersions are further referred to as acid precipitation supernatant 2 (AS2) and pellet 2 (AP).

In case of the ethanol precipitation, ethanol (96%) was mixed with the second S1 aliquot to a final concentration of 25% ethanol (*v*/v). This concentration was selected since it resulted in the highest protein purity, amongst all tested concentrations (85%, 70%, 50% and 25% (v/v)), during the preliminary experiments (Supplementary material, Fig. S3). The volume of the added 96% ethanol was measured by noting the total pipetting volume. The mixture was allowed to precipitate at 4 °C in dark conditions for 13 h, and subsequently centrifuged (15,000 *g*, 20 min, 4 °C). Supernatant 2 was separated from pellet 2 by pouring carefully, after which both fractions were separately collected and weighed. Then, the pellet 2 was directly frozen at −20 °C. Ethanol was first removed from supernatant 2 with a Rotary Evaporator RC900 (40 °C and 17 kPa, KNF, Freiburg im Breisgau, Germany) before freezing. The frozen supernatant 2 and pellet 2 were subsequently freeze dried and are further referred to as ethanol precipitation supernatant 2 (ES2) and pellet 2 (EP).

In case of the dialysis, the third S1 aliquot was poured into 35 mm diameter SnakeSkin™ dialysis tubes with a 3.5 kD molecular weight cut-off (Thermos Scientific, Rockford, USA). The S1 samples were dialyzed against demineralized water at 4 °C in dark conditions for 68 h, with the demineralized water being refreshed every 17 h. After dialysis, retentate was collected, frozen at −20 °C and freeze dried. The freeze dried dialysis retentate is referred to as DR.

2.3. Compositional analysis

The dry matter of all leaves and fractions generated during protein extraction (GJ, pulp, S1, P1, AS2, AP, ES2, EP and DR) was defined as the weight after freeze drying. Together with the wet mass, the dry matter content was used to create mass flows of extraction processes, using the e!Sankey 5 program (iPoint-systems gmbh, Hamburg, Germany). The results are presented in Fig. 3.

The total nitrogen content of all leaves and fractions was measured with the Dumas nitrogen combustion method using a FlashSmart™ Elemental Analyzer (ThermoFisher Scientific, MA, USA). Around 10 mg of sample was weighed in a tin foil cup and closed tightly without headspace. All samples were combusted at 950 °C in the presence of oxygen. A calibration curve was made with *D*-methylthionine in the range of 1 to 15 mg, with cellulose was used as blank. Every sample was measured three times. Although a lower nitrogen-to-protein conversion factor (e.g., 4.4) could be more accurate for leaves and leaf products (Kiskini, Vissers, Vincken, Gruppen, & Wierenga, 2016; Milton & Dintzis, 1981), 6.25 was used in this study to allow easy comparison with previous studies (Tamayo Tenorio et al., 2018). The protein content of top, middle and bottom leaves were 27.8 ± 1%, 27.0 ± 1% and 23.9 ± 1.1%, respectively.

2.4. Statistical analysis

Statistical analyses were carried out by using IBM SPSS statistics, version 28.0.0.0 (IBM, Armonk, US). Significant differences were analyzed with one-way ANOVA using a multivariate general linear model and with the Duncan test. Differences were considered significant when $P \leq 0.05$ and were shown as the small upper letters.

3. Theory and calculation

3.1. Yield-purity calculation and mass balance establishment

The protein extraction was first evaluated using protein yield and purity, which were calculated based on measured data. Fractions obtained at every processing step were given a number, hence 1, 2, 3, 4, 5, 6 and 7 represent leaves, green juice, pulp, S1, P1, supernatant or permeate and pellet or DR after protein purification, respectively. As shown in Fig. 1, the measured data for each fraction included the total weight, dry weight and protein purity:

$$\text{Total weight (g)} = M \tag{1}$$

$$\text{Dry weight (g)} = Mx_{DM} \tag{2}$$

Where M is the mass (g) of a fraction, x is the mass fraction or concentration (g/g) of a component in a fraction, DM is the total dry matter (sum of all components) in a fraction.

The protein purity (of e.g., S1) can be expressed as:

$$\text{Protein purity (\%)} = \frac{x_{p4}}{x_{DM4}} * 100 \tag{3}$$

The protein yield can be expressed in two ways. The first way is to compare the proteins in a obtained fraction with the total proteins in leaves (i.e., total protein yield). The second way is to compare with the proteins in the starting material of each processing step (i.e., step-wise protein yield). An example of calculating both protein yields of S1 is given:

$$\textit{Total protein yield (g/g)} = \frac{M_4 x_{p4}}{M_1 x_{p1}} \tag{4}$$

$$\textit{Step} - \textit{wise protein yield (g/g)} = \frac{M_4 x_{p4}}{M_2 x_{p2}} \tag{5}$$

Where x_p represents the mass fraction (g/g) of proteins in a fraction.

The protein yield(s) and purity of every fraction obtained during the protein extraction process were calculated. The results are presented in Fig. 4 (with protein purity as a function of total protein yield). The step-wise protein yield was used to estimate the attainable yield, of which the procedure is described below:

The objective of the extraction process is obtaining the proteins in liquid or dispersed phases, which are present in the green juice, S1 and P2 or DR. Hence, pulp, P1 and S2 or permeate are considered to be side-streams. For the calculation in this section, the focus was on fractions that contain solid particles and insoluble components (e.g., pulp, P1 and P2). According to Peters, Vergeldt, Boom, and van der Goot (2017), solid particles and insoluble components may absorb water upon hydration. Therefore, it is likely that part of the soluble components can dissolve in this absorbed water. This part is considered to be an attainable loss. It is important to note that the liquid phase in the pulp (and the green juice) also contains dispersed (undissolved and insoluble) proteins, because juicing just separates the large pulp fraction from the juice. In the second and third processing steps, high speed centrifugation separated the soluble proteins from the insoluble proteins. Therefore, for instance, S1

is considered to contain soluble proteins and the insoluble proteins are assumed to all ended up in P1. In other words, the pellets obtained during protein extraction contain an insoluble phase with only insoluble components (B), as well as a liquid phase containing water and soluble or dispersed components (A) (Fig. 2).

Within these assumptions, mass balances were established for the extraction process, with the aim to identify limiting factors in each processing step. Then, the (maximum) attainable yield can be calculated by quantifying the mass of soluble or dispersed proteins in the liquid phase of the pellet. The mass of soluble and dispersed proteins in the pellets depends on two factors: the quantity of insoluble components and the swelling capacity of those insoluble components.

To establish the mass balances, several additional assumptions were made:

1) The dry matter in leaves was simplified to proteins and carbohydrates, as these are the two dominant components in leaves (Tamayo Tenorio et al., 2018). The protein content on dry basis was defined to be 28%, 27% and 24% for top, middle and bottom leaves, respectively (mean values from experiments, Section 2.3). The carbohydrate content, representing all non-protein components, was therefore calculated to be 72%, 73% and 76% in corresponding leaves.
2) No loss of mass occurred during the processing.
3) The mass fraction of the soluble or dispersed components in the supernatant is the same as that in the liquid phase in the pellet.

An example of calculating the lost dispersed proteins in the first processing step (i.e., juicing) is given in the Supplementary material Eq. S1 to S10. When assuming that the lost dispersed proteins in phase A is recoverable, the attainable protein yield (in first processing step) can be written as:

$$\text{Attainable yield } (g/g) = \frac{M_3 x_{p2}(x_{DM3} - x_{DM2})}{M_1 x_{p1}(1 - x_{DM2})}\zeta \quad (6)$$

Where ζ is the swelling capacity (g/g) of insoluble components in phase B.

The rest of (insoluble) proteins in phase B is not attainable, which was referred to as the inevitable loss. When there are no insoluble components ($x_{DM,3} = x_{DM,2}$) or the insoluble components do not absorb water ($\zeta = 0$), the inevitable loss (in first processing step) equates to the step-wise protein yield (Eq. 5) of pulp:

$$\text{Attainable yield} + \text{Inevitable loss} = \text{Step} - \text{wise protein yield} \quad (7)$$

To summarize, in the first two processing steps, it was assumed that the soluble or dispersed proteins in liquid phase A in the pellet can be extracted with additional processing (detailed discussion in Section 4.3), while the insoluble proteins in phase B are inevitable loss. In the last processing step, it was assumed that the proteins in supernatants or permeate are inevitable loss, since they do not precipitate or that they pass through the dialysis membrane. The results of attainable yield and inevitable loss of each step are presented in Fig. 6. When considering the attainable yield of each step, a theoretical protein yield-purity diagram can be obtained, which is presented in Fig. 7.

4. Results and discussion

4.1. Mass flows during protein extraction

Tomato leaves have a high moisture content (88%) (Fig. 3), which is a common feature for leafy biomasses (Abdollahi et al., 2019; Tamayo Tenorio et al., 2018). The first processing step in extraction is juicing, which led to a division of about 6 to 4 of dry matter from tomato leaves to GJ and pulp. GJ contained liquid with dispersed particles, including cell wall debris and insoluble thylakoid membranes. This division is due to the fact that twin screw press only roughly separates large solids from the liquid, and does not prevent small particles from entering the juice. The pulp mainly contained fibers from the cell wall, but retained quite some juice since fibers and other insoluble components have the capacity to hold water (Peters et al., 2017). As next step, high speed centrifugation led to a similar division of about 6 to 4 of dry matter from GJ to S1 and P1. Here, high speed centrifugation was used to effectively separate the insoluble components from the liquid, resulting in S1, which contained just water and soluble components. P1 mainly

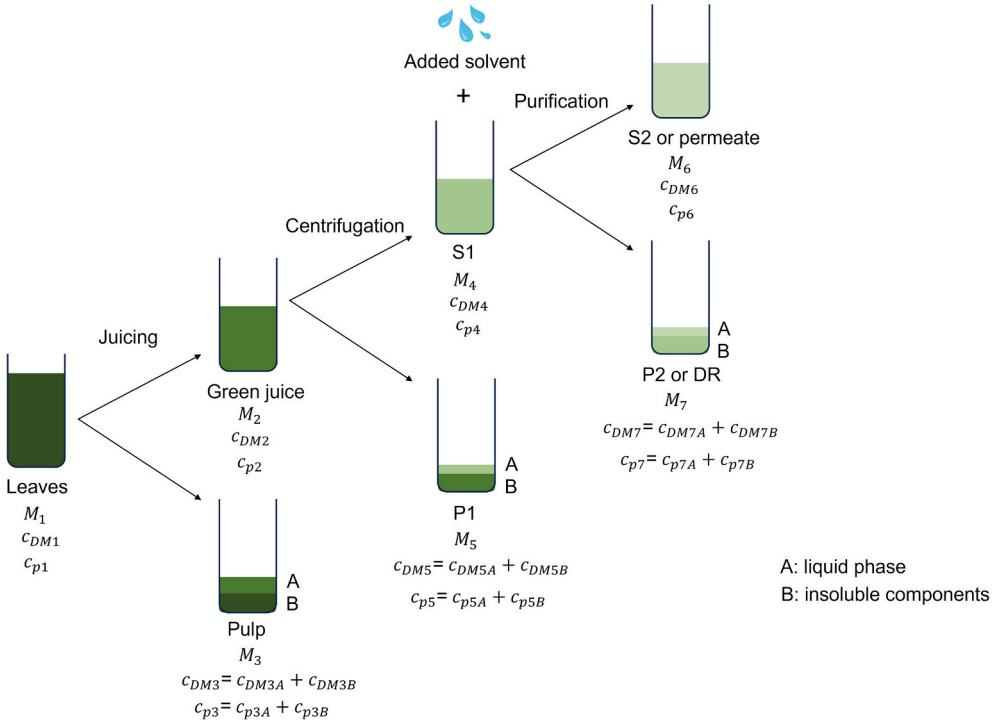

Fig. 2. Schematic demonstration of fractions obtained during protein extraction and their theoretical composition.

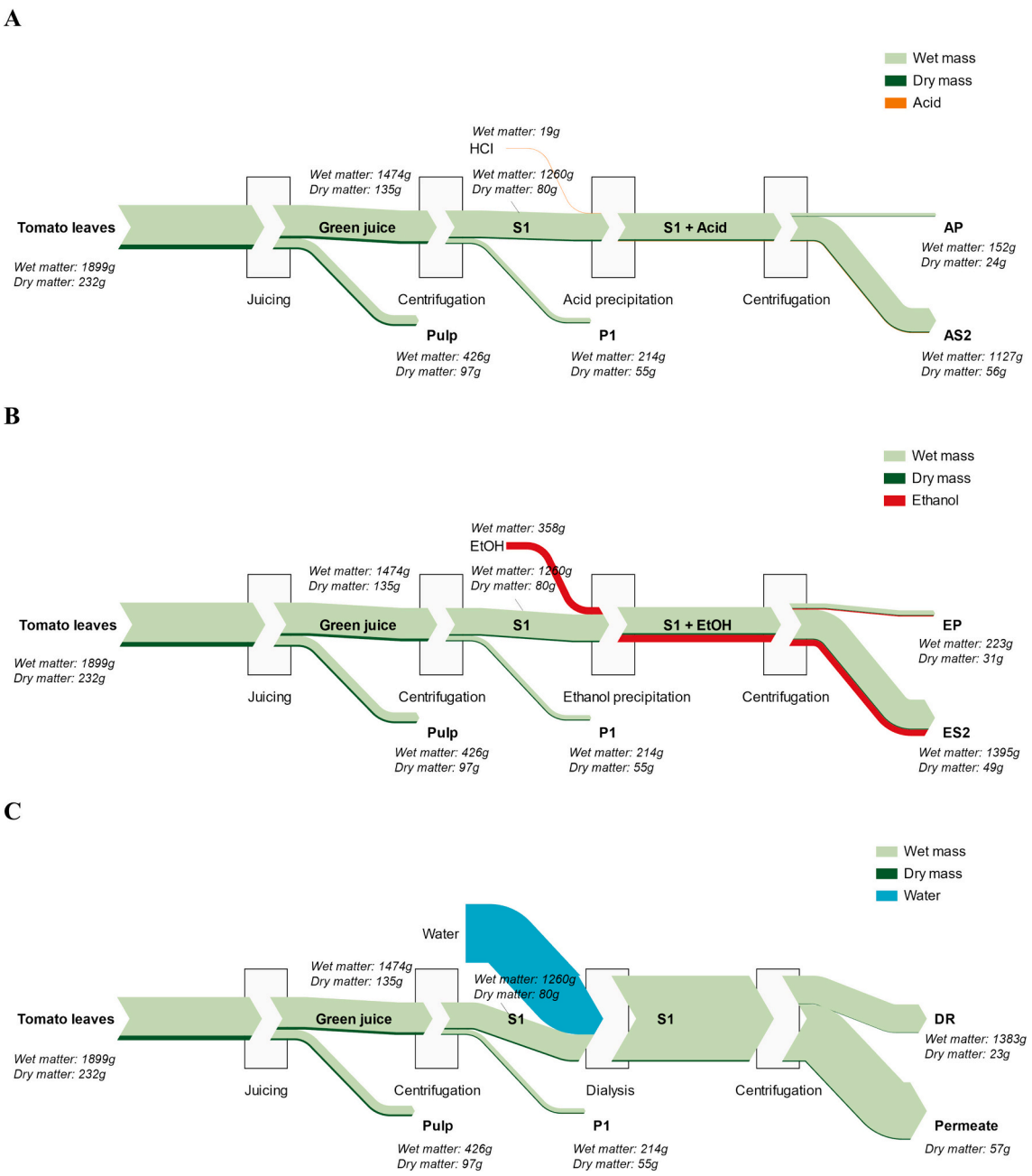

Fig. 3. Wet and dry mass flows of protein extraction with acid precipitation (A), ethanol precipitation (B) and dialysis (C) as purification methods. Data derived from top leaves are presented as example. Only measured data are indicated on the graph. For dialysis, the thickness of the net wet matter flow of the added water and permeate were estimated based on the measured dry matter in DR and permeate.

contained insoluble components from the thylakoid membranes. However, similar as in the pulp, it is suspected that some soluble components are also present in P1, due to water absorption by the insoluble components. In this study, three additional purification steps were assessed, namely acid precipitation, ethanol precipitation and dialysis. As can be seen in Fig. 3, the final purification step resulted in further division of the dry matter over the various output streams, thereby lowering the overall yield to about 0.1 g/g of total dry matter from tomato leaves in the final products (AP, EP or DR respectively).

4.2. Protein yield-purity diagram

The protein yield and purity of fractions obtained during protein extraction are presented in Fig. 4. When starting from leaves, it becomes clear that the first two processing steps (juicing and centrifugation) resulted in a mostly horizontal shift of the points in the diagram, suggesting significant reduction in protein yield with a minimal impact on protein purity. To be more specific, the juicing step resulted in a large division of total leaf proteins (1 g/g) into GJ (on average 0.73 g/g) and fibrous pulp (on average 0.27 g/g). In the meanwhile, a slight protein enrichment was observed as the protein purity of GJ (on average 32.8%) was somewhat higher than the original protein content of leaves (on average 26.2%). As the next step, the centrifugation of GJ resulted in a further division of the proteins into S1 and P1 (on average 0.38 and 0.35 g/g), with average purities of 27.9 and 41.2%, respectively. The fact that the protein purity of S1 is lower than that of the GJ suggests that part of the proteins present in the GJ were not fully soluble, but present as dispersed particles, while a larger fraction of the non-protein

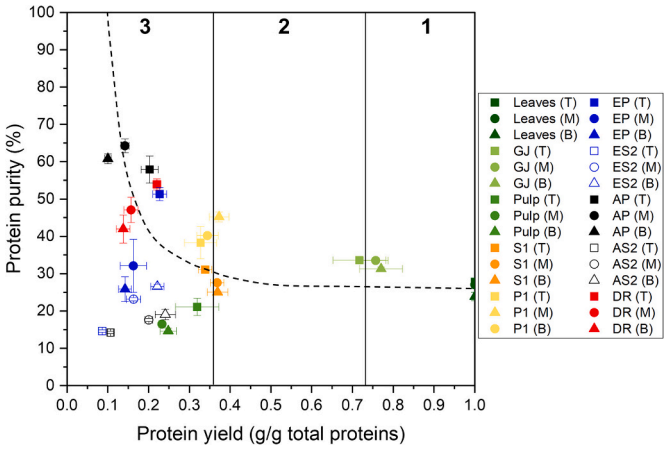

Fig. 4. Total protein yield and purity of all obtained fractions during protein extraction. The number 1, 2 and 3 represent the three processing steps (i.e., juicing, centrifugation and purification, respectively). T, M, and B stand for leaves collected from the top, middle, and bottom positions of the plant. The black dotted line indicates the direction of shift for all data points from step 1 to step 3.

components (i.e., carbohydrates) was soluble and thus ended up in S1. Since the subsequent protein purification aimed to purify proteins in dispersed or liquid phases, it becomes clear that only 0.38 g/g total proteins in tomato leaves were available for the further purification. In other words, the other 0.62 g/g total proteins were lost already from the initial two processing steps (Fig. 4). When comparing the data to the yield-purity diagram of leafy biomasses proposed by Tamayo Tenorio et al. (2018), a similar behavior was observed. Moreover, the fact that the significant loss of proteins occurred during the initial processing makes it inevitable that the final extraction yield is low. Fig. 4 also shows that the processing itself plays a more important role in determining the direction of shift in the yield-purity diagram than the differences in leaves. It explains why leaves from different positions did not lead to variations in the initial processing (Fig. 4).

As a consequence of the significant protein loss from the first two steps, the last processing step (protein purification) resulted in a vertical shift of points in Fig. 4, rather than horizontal. Thus this step mostly influenced the protein purity (from around 14% to 64%), with only a small effect on the yield (from around 0.1 to 0.2 g/g). When zooming into this step, it was then observed that the protein yield was mainly influenced by the leaf position (Fig. 5a). For instance, the protein yield was highest for the top leaves (on average 0.2 g/g) and lower for middle and bottom leaves (on average 0.15 and 0.13 g/g, respectively), independent of the purification method. It is hypothesized that this decrease in protein yield was due to the protein degradation during leaf senescence (Havé et al., 2017). The degradation products, with smaller molecular weights, readily pass through the dialysis membrane, resulting in a decreased protein yield. Besides, they do not precipitate in ethanol or at low pH. In previous work, similar effects were observed on protein yield of different tomato leaves (Kleuter et al., 2024; Yu et al., 2022). The leaf position also influenced the protein purity, although this effect was smaller than that of the purification method (Fig. 5b). When purified by ethanol precipitation and dialysis, proteins from the top leaves had higher protein purity as compared to those from middle and bottom leaves. On an overall level, dialysis resulted in higher protein purity than ethanol precipitation, indicating low selectivity of ethanol to soluble proteins in leaves. The low selectivity was likely due to the co-precipitation of other soluble components, such as oligosaccharides (Ku, Jansen, Oles, Lazar, & Rader, 2003). Acid precipitation resulted in the highest protein purity, independent of the leaf position (57.9, 64.3 and 60.8% for top, middle and bottom leaves). Thus acid precipitation was the most selective method to purify soluble proteins, probably due

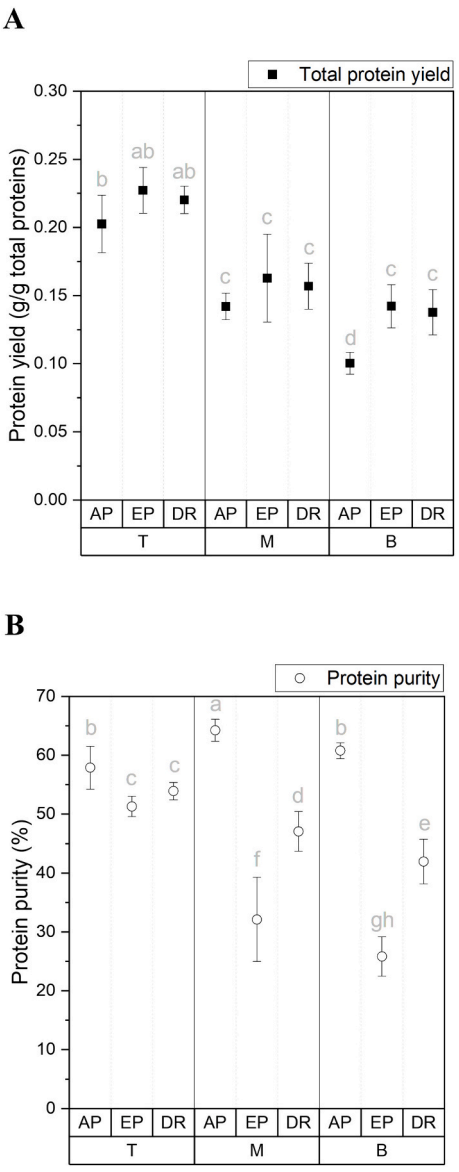

Fig. 5. Total protein yield (A) and purity (B) of AP, EP and DR. T, M, and B stand for leaves collected from the top, middle, and bottom positions of the plant.

to the fact that pH 3.5 was close to the isoelectric point of these proteins (Supplementary material Fig. S2).

In conclusion, the results in this study were in-line with previous protein extraction studies on other leafy biomasses (Tamayo Tenorio et al., 2018). A significant loss of proteins was found in the initial processing steps, which consequentially led to a low overall yield, when aiming at higher purity. In addition, small variations were observed in extraction yield and purity caused by differences in leaves, but the overall effect is minor as compared to that of processing methods.

4.3. *Identification of limiting factors during protein extraction*

The attainable yield and inevitable loss were estimated per processing step (Eq. 6 and 7, Section 3.1), with the aim to identify the limiting factor during protein extraction. The results are presented in Fig. 6. In the first two processing steps, not all proteins end up in green juice and S1, resulting in lost proteins in side-streams (pulp and P1). However, these lost proteins not only include insoluble proteins, but also soluble and dispersed proteins, which were suspectedly present in the

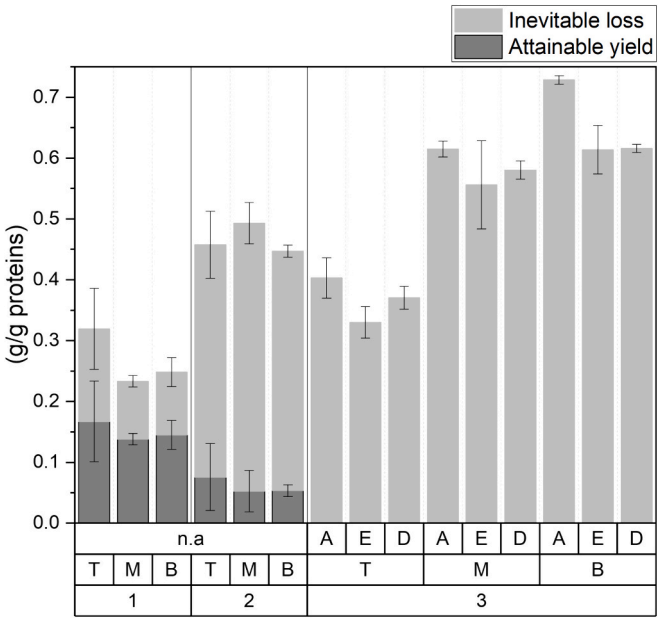

Fig. 6. Attainable yield and inevitable loss in each processing step. 1, 2 and 3 represent the three processing steps (i.e., juicing, centrifugation and purification, respectively) during protein extraction. T, M, and B stand for leaves collected from the top, middle, and bottom positions of the plant. A, E and D stand for acid precipitation, ethanol precipitation and dialysis in the purification step, respectively.

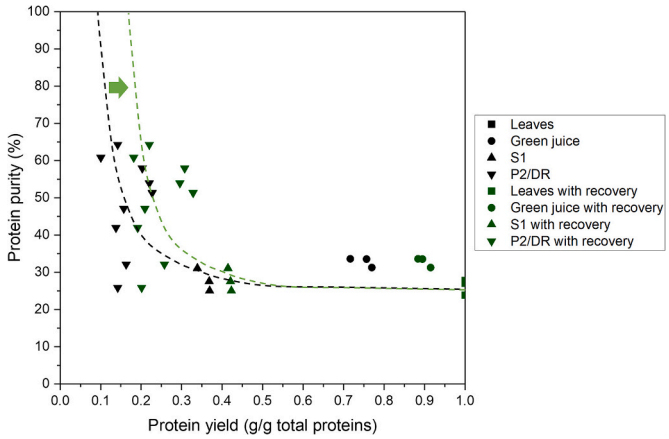

Fig. 7. General properties of protein yield and purity when extracting proteins from tomato leaves. The black dotted line indicates protein extraction without additional attainable yields. The green arrow and green dotted line indicates protein extraction with additional attainable yields. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

liquid phase absorbed by the insoluble components (Fig. 3). These soluble and dispersed proteins in the liquid phase were therefore considered as attainable, leaving only the truly insoluble proteins as inevitable loss. In the last processing step, the targeted proteins end up in the pellet or retentate, by means of precipitation or filtration. Hence, the proteins in the supernatants and permeate were considered as inevitable loss.

In the first processing step, the attainable yield and inevitable loss were on average 0.15 g/g and 0.12 g/g, respectively (Fig. 6). The large proportion of attainable proteins, being dispersed proteins in the liquid phase, is determined by both the net quantity of insoluble components and their swelling capacities. The swelling capacities of the insoluble components in pulp was determined to be 5.3 g water/g fraction (Supplementary material Fig. S4), which is at similar level as what is typically reported for plant material (2 to 4 g water/g fraction) (Peng, Kyriakopoulou, Keppler, Venema, & van der Goot, 2022). To recover these proteins, one possible route is through a secondary pressing. Already 0.13 g/g of the proteins in the pulp can be recovered with one time re-pressing (Knuckles, Bickoff, & Kohler, 1972), and the recovery can be increased to 0.5 g/g when additional water is applied during pressing (Morrison & Pirie, 1961). Here, the fact that additional yield is achieved with additional water during pressing validates the hypothesis that part of the proteins in pulp were soluble or dispersed proteins in the liquid phase. Another route is through multi-step washing, in which the washing liquids used in prior steps can be potentially reused for subsequent washes (Möller, Li, van der Goot, & van der Padt, 2022). An industrial translation of this multi-step washing is a counter-current multi-stage extraction, which is more efficient in energy and solvent usage (Vázquez-León et al., 2019). However, it is important to note that the maximum attainable yield from pulp is only 0.15 g/g. The rest of the 0.12 g/g proteins in pulp were likely bound with cell wall debris (Tamayo Tenorio, Gieteling, De Jong, Boom, & Van Der Goot, 2016), and thus not susceptible to additional washing or re-pressing. In conclusion, in the juicing step, up to 0.15 g/g proteins can be recovered through additional processing and thus considered attainable. The water absorption by the pulp is hence the largest limiting factor in this step, but the overall effect on enhancing the yield is not very large (Fig. 7).

In the second processing step, the attainable yield and inevitable loss were on average 0.06 g/g and 0.41 g/g, respectively (Fig. 6). The attainable yield was remarkably low, due to the low dry matter in S1 (Fig. 3). Here, it is assumed that the mass fraction of soluble components in S1 equates to that in the liquid phase of P1. The large proportion of water in S1 therefore dictates the most soluble components coming from the green juice, resulting in low quantity of soluble components, including soluble proteins, in P1. As a result, most of the proteins in P1 were insoluble proteins and thus inevitably lost. These insoluble proteins were likely to be the thylakoid membrane proteins. This was supported by their intensive green color and lipid-like appearance (picture not shown), due to their known association with chlorophyll and lipids (Fiorentini & Galoppini, 1983; Tamayo Tenorio et al., 2018). In previous work (Yu et al., 2023), attempts were made to re-solubilize these insoluble proteins by increasing the extraction pH to 10. However, limited effect was observed on the protein yield, while the extracted products exhibited an undesired brown color, which was likely caused by the oxidation of phenolic compounds at this pH (Yu et al., 2023). Additionally, similar attempts were made by Tamayo Tenorio et al. (2016) to re-solubilize the thylakoid membrane proteins from sugar beet leaves through consecutive washing with water and surfactants. The effect of this method was also limited since the total protein yield did not increase after washing. It is suspected that this conclusion also holds for other leafy biomasses. It is important to stress that membrane proteins in leaves are very heterogenous by nature. These proteins are different in size, charge, hydrophobicity, post-translational modification and complexation (Friso et al., 2004; Lin & Guidotti, 2009). Their heterogeneity hence results in poor selectivity when using a single solvent. In addition, their association with lipids, pigments and a broad range of secondary metabolites further complicates the effort to solubilize these membrane proteins with solvents (Carpentier et al., 2005; Rose, Bashir, Giovannoni, Jahn, & Saravanan, 2004). In conclusion, the large amount of insoluble proteins obtained after centrifugation was identified as the major limiting factor in this step, especially given the challenges to change their solubilities.

Finally, in the last processing step, (soluble) proteins from S1 were purified into a protein-rich fraction by either precipitation or dialysis. Therefore, the proteins left in the supernatants or permeate were likely smaller peptides and amino acids that cannot be precipitated or that were filtered out. Here, these proteinaceous components were defined as inevitable loss. As shown in Fig. 6, the amount of this inevitable loss depended on the leaf position. This correlates to the protein yield of AP, EP and DR (Fig. 5), since together with the proteins in AS2, ES2 and

permeate, they account for all proteins in S1. As discussed in Section 4.2, the difference in the amount of inevitable loss in top, middle and bottom leaves is likely attributed to the age-related protein degradation (Havé et al., 2017; Vicente, Morcuende, & Babiano, 2011).

4.4. Reflection on protein extraction from tomato leaves

In this study, a full processing chain from different tomato leaves to dried protein ingredients was analyzed. The results demonstrated that the protein extraction from tomato leaves was coupled with significant protein losses during the initial processing steps, prior to the protein purification. The protein losses were mainly attributed to the water absorption by fibrous pulp and large proportion of insoluble (thylakoid membrane) proteins. It is possible to recover part of these lost proteins by means of multi-step washing (Möller et al., 2022) or a secondary pressing (Knuckles et al., 1972; Morrison & Pirie, 1961). However, the recovery potential is limited as a result of the general properties of protein extraction from tomato leaves (Fig. 7). Part of the leaf proteins were insoluble proteins and so far, no food-grade routes are available to recover these proteins (Tamayo Tenorio et al., 2016; Tamayo Tenorio, Boom, & van der Goot, 2017; Yu et al., 2023). In addition, further purification requires selectivity between proteins and the other components. Here the highest selectivity was achieved with acid precipitation, but this selectivity only holds for part of the proteins.

Thus, on a general level, low extraction yields were obtained when aiming at high purity. In conclusion, in a protein-rich fraction with high purity, only a small fraction of the proteins in tomato leaves will be present. It is suspected that this conclusion is not limited to tomato leaves, since similar observations were made across many types of leafy biomasses when translating reported data to yield and purity (Santamaría-Fernández & Lübeck, 2020; Tamayo Tenorio et al., 2018). The results in this study also demonstrated the limited potential to improve the yields when striving for high purity (Fig. 7). The valorization of tomato leaves would therefore benefit from generation of whole fractions with focus on functionality, instead of isolating small fractions with high purity (Van Der Goot et al., 2016). For example, previous work demonstrated the potential of using tomato leaf juice (S1 in this study) as an enzymatically active fraction for novel applications, which would allow the use of the whole fraction rather than isolating a small fraction of proteins (Yu, Kleuter, America, Trindade, & van der Goot, 2024; Yu, Kleuter, Trindade, & van der Goot, 2024). A similar concept has been proposed for thylakoid membrane fragments (Tenorio, De Jong, Nikiforidis, Boom, & Van Der Goot, 2017) where these fragments were found to stabilize emulsions. This approach utilized the naturally present protein/lipid ratio and their interactions, thus allowing the use of whole thylakoid membrane instead of isolating individual component.

The last point to discuss is the applicability of the approach presented in this study. The estimation of the attainable yield was based on the fact that insoluble components hold water and therefore soluble proteins. This concept is valid for many types of alternative protein sources can thus be used to estimate the potential improvement of their extraction processes. By knowing the potential improvement, one can identify the limiting factors in a separation process.

CRediT authorship contribution statement

Yafei Yu: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marietheres Kleuter:** Writing – review & editing, Resources. **Ranqi Zhang:** Methodology, Investigation, Data curation. **Luisa M. Trindade:** Writing – review & editing, Supervision, Resources. **Remko M. Boom:** Writing – review & editing, Conceptualization. **Atze Jan van der Goot:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2024.103790.

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