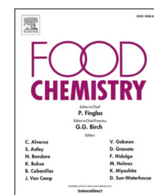
Food Chemistry 406 (2023) 135072

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



The role of plant age and leaf position on protein extraction and phenolic compounds removal from tomato (*Solanum lycopersicum*) leaves using food-grade solvents

Yafei Yu[a], Marietheres Kleuter[b], Somayeh Taghian Dinani[a], Luisa M. Trindade[b], Atze Jan van der Goot[a,*]

[a] *Laboratory of Food Process Engineering, Wageningen University, PO Box 17, 6700 AA Wageningen, the Netherlands*
[b] *Laboratory of Plant Breeding, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, the Netherlands*

ARTICLE INFO

Keywords:
Agricultural side stream
Tomato leaves
Leaf proteins
Plant age
Leaf position
Protein extraction

ABSTRACT

The large availability and considerable amount of proteins (approx. 30 % on dry matter) make tomato leaves attractive as a potential new protein source. In this study, the feasibility of extracting proteins and removing phenolic compounds from tomato leaves using food-grade solvents as function of plant age and leaf position was investigated. Water and 50–50 % ethanol–water were used. We found that most proteins (>70 mg/g leaf protein) remained in the pellet after extraction. The protein purity of the dry matter present in the supernatant did not exceed the original leaf protein content. Additionally, leaf position had stronger effect than plant age on the leaf protein content and extraction yield. Ethanol-water was more efficient in removing phenolic compounds than water. The most phenolic compounds was removed from the top leaves. For future processing, the diversity of leaves has to be considered when striving for full utilization of tomato plants (fruits and leaves).

1. Introduction

Tomatoes are largely produced and consumed around the world. In 2020, 18 million tons of tomatoes were produced worldwide (FAOSTAT, 2020). Around 40 % of tomato plants is considered as waste and discarded during the production and processing of tomatoes (Taylor & Fraser, 2011). Particularly, tomatoes grown in the greenhouses generate approximately 49 tons of by-products per hectare greenhouse per year, which is almost twice the amount (28.5 t ha^{-1} $year^{-1}$) when compared with the by-product production of other plants in the greenhouses (Fernández-Gómez, Díaz-Raviña, Romero, & Nogales, 2013). The leafy by-products contain considerable amount of proteins. About 400 to 600 kg per hectare of proteins is present in the leafy by-products, which is fairly comparable to the protein production of soy (450–600 kg/ha) and cereals (approx. 570 kg/ha) (van Krimpen, Bikker, Van Der Meer, van der Peet-Schwering, & Vereijken, 2013). Tomato leaves are also rich sources of photochemical, phenolic compounds and flavonoids (Arab et al., 2019). Green leaves have been extensively exploited for its potential to be used in foods (Akyüz & Ersus, 2021; Coldebella et al., 2013; Martin et al., 2014, 2019; Tamayo Tenorio, Schreuders, Zisopoulos, Boom, & van der Goot, 2017; Vergara-Barberán, Lerma-García, Herrero-Martínez, & Simó-Alfonso, 2015), however no commercial processes nor products are available at the moment. Tamayo Tenorio, Kyriakopoulou, Suarez-Garcia, van den Berg, and van der Goot (2018) pointed out the difficulty to extract high purity functional proteins with high yield from green leaves, hence the yield-purity dilemma. This dilemma explains why no products from green leaves have been commercialized yet. However, the vast amount of leaves merits investigation to find new processing routes.

The extraction procedures of proteins from green leaves often consist of the following steps: tissue disruption, protein solubilization, protein purification and protein concentration (Tamayo Tenorio et al., 2018). Protein solubilization is considered the most crucial and limiting step. Alkaline extraction is often explored for this purpose. Such step is based on the standard procedure for industrial production of soy and other plant protein isolates. However, alkaline extraction involves high usage of chemicals and requires removal of chemicals or reaction products (often salts) before application in foods. Furthermore, harsh conditions

* Corresponding author at: PO Box 17, 6700 AA Wageningen, the Netherland.
E-mail addresses: yafei.yu-woudstra@wur.nl (Y. Yu), marietheres.kleuter@wur.nl (M. Kleuter), somayeh.taghiandinani@wur.nl (S. Taghian Dinani), luisa.trindade@wur.nl (L.M. Trindade), atzejan.vandergoot@wur.nl (A.J. van der Goot).

https://doi.org/10.1016/j.foodchem.2022.135072
Received 21 June 2022; Received in revised form 8 November 2022; Accepted 23 November 2022
Available online 28 November 2022
0308-8146/© 2022 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

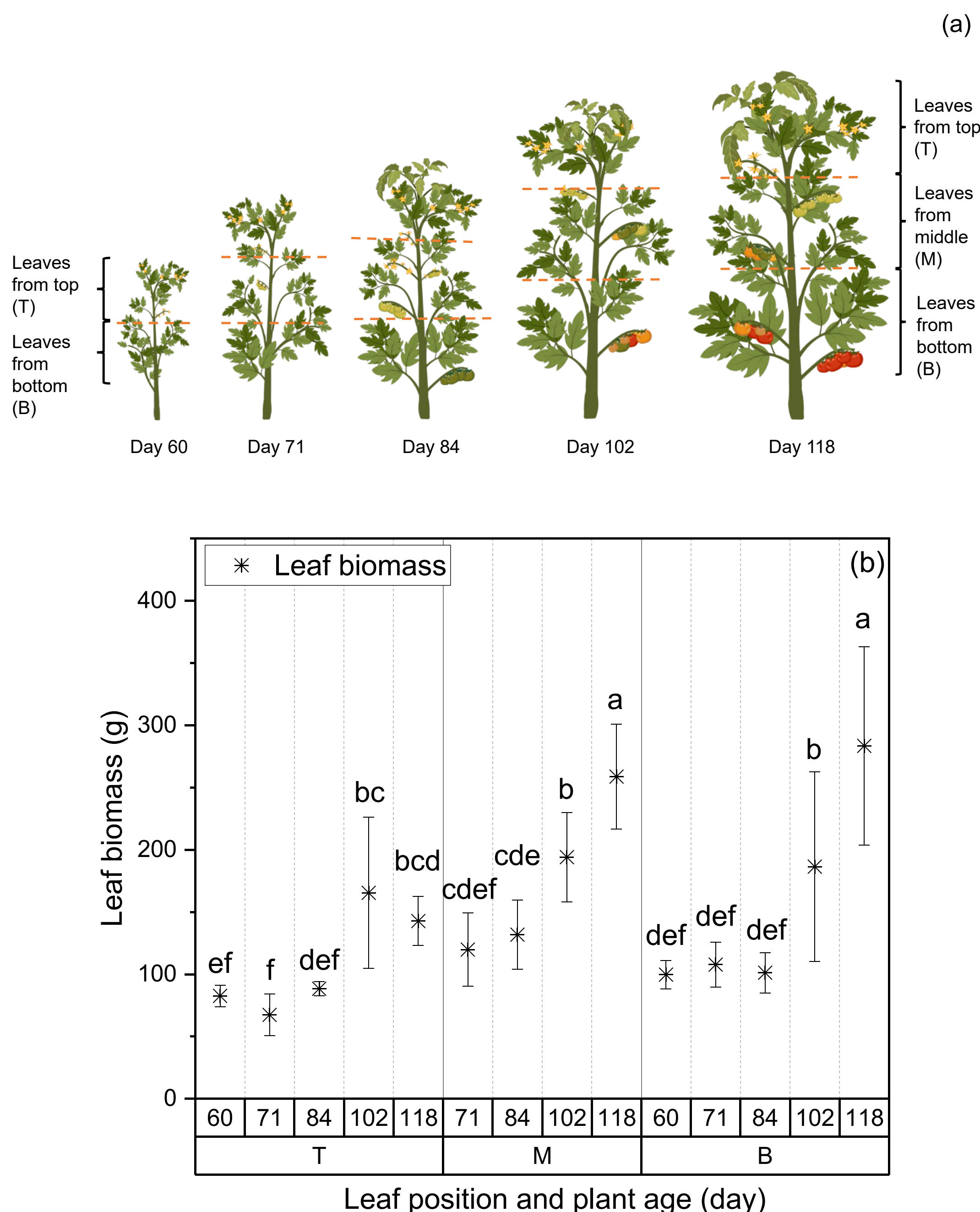


Fig. 1. Schematic demonstration of tomato leaves collection, the tomato plant images were created with BioRender.com (a); leaf biomass at different plant ages and leaf positions (b). In this study, the plant age was defined with the days after sowing of tomato seeds; while the leaf position was defined with the positions where leaves were collected from one plant at one plant age. T, M and B stand for leaves collected from the top, middle and bottom positions of the plants, respectively. Different small upper letters indicate significant difference at $P \leq 0.05$. Data points can be found in Supplementary material Table S.6. *Print in color.*

might denature proteins and reduce protein solubility (Peng, Kersten, Kyriakopoulou, & van der Goot, 2020). The latter is not desired since the aim of most protein extraction procedure is to generate soluble protein fractions, considering that protein solubility is considered as an important property to allow successful application in foods. Recently, a water-only process was proposed by Möller, Li, van der Goot, and van der Padt (2022) to fractionate functional ingredients amongst others from yellow pea, considering the use of other solvents or chemicals hinders or even prevents the application of extracted components in foods due to safety, cost or sustainability reasons.

The presence of phenolic compounds in leaves leads to additional challenges in protein extraction from leaves. Phenolic compounds can interact with proteins, especially at high pH (Keppler, Schwarz, & van der Goot, 2020). The protein-phenol interactions can lead to brown color formation (Narváez-Cuenca, Vincken, & Gruppen, 2013), changes in protein structure (Jia, Gao, Hao, & Tang, 2017), reduced protein solubility (Prigent, Voragen, Visser, van Koningsveld, & Gruppen, 2007) and eventually compromised protein yield and quality. Washing with a mixture of ethanol and water is often suggested as food-grade solvent to remove phenolic compounds (Chemat, Vian, & Cravotto, 2012). Pre-treatment by using 50 % ethanol reduced 59 % browning during protein extraction from tea leaves (Zhang, van Krimpen, Sanders, & Bruins, 2016). In sunflower meal, aqueous ethanol washing under optimal conditions can lead to maximal removal of phenolic compounds, while

retaining of proteins (Jia et al., 2021).

In greenhouses, tomato plants usually grow until 3.5 to 4 m on a vertical wire. Since tomato plants generally grow vertically upwards, the leaves on the top are younger and they receive more sunlight. The leaves on the middle and bottom are older and they receive less sunlight. During the growth of tomato plants, new leaves that grow from the side branches are usually removed together with old bottom leaves (pruning) to enhance fruit yield as well as to reduce plant diseases (Heuvelink, Bakker, Elings, Kaarsemaker, & Marcelis, 2005). The pruning is carried out throughout the whole growth period, leaves are therefore collected from different positions and from plants at different developmental stages (different plant age). The quantity of removed leaves during pruning is however minor compared to the vast amount once tomatoes are harvested from the plants. After tomato harvest, leaves from every position on the plants (at one plant age) become available for future processing. Previous researches showed that plant age influences the protein content and total phenolic compounds in some plants. For instance, the protein of white and red clovers decreased significantly with plant age (Sincik, Bilgili, Uzun, & Acikgoz, 2007). For rapeseed leaves, the protein content reached maximum at full flowering stage (Sincik et al., 2007) and followed by a decrease of 31 % as the plants further aged (Yu, Bals, Grimi, & Vorobiev, 2015). Similarly, the total phenolic compounds in rapeseed leaves (Yu et al., 2015) and sugarcane stems (Qudsieh, Yusof, Osman, & Abdul Rahman, 2002) decreased significantly towards the plant maturity. In case of tomato leaves, it is not clear to the authors how the plant age and leaf position affect the protein content and the subsequential extractions of proteins and phenolic compounds. The age of plants and the position of leaves become relevant when developing processes that aim for an efficient use of total biomass (fruits and leaves) from tomato plants.

The aim of this paper is therefore to investigate the feasibility of extracting proteins and removing phenolic-compounds with food-grade solvents from tomato leaves as a function of plant age and leaf position. With this study, we hope to gain more insights on utilizing tomato leaves as potential protein source for food applications.

2. Material and methods

2.1. Plant materials and chemicals

Tomato plants (*Solanum lycopersicum*, genotype: Moneymaker) were grown in April to August 2021 in the greenhouse (Wageningen University, the Netherlands). The tomato seeds were sowed on 30th April into soil (Lensli, Bleiswijk, Netherlands). No additional fertilizers were used. The seedlings were watered regularly to allow germination and initial growth. After 10 days growing on soil, plants are transferred to Grodan rockwool blocks (ROCKWOOL B.V., Roermond, Netherlands). Each rockwool block only contained one plant (Supplementary Material, Figure S.2). The plants are then placed on a big table with sufficient space in between for maximum light exposure (Supplementary Material, Figure S.2). Leaves do not touch each other. Additionally, the table was flooded once per day with water containing nutrients. Artificial light was applied for 2 h per day to ensure a photoperiod of approx. 16 h per day. In the beginning of June, the plants were transferred into slabs, which contained more rockwool. On the slabs, each plant was hooked to a wire to grow vertically upwards. The distance between the plant stems was averagely 25 cm. Water and nutrient supply were given directly to the plants by the black small hoses connected to the slabs (Supplementary Material, Figure S.3). Water and fertilization supply were given 14 times per day. Artificial light was applied for 3 h per day. On slabs, the length of the plants increased and the size of individual leaf increased, which resulted in uneven light exposure on different positions of the plants. It is generally true that leaves on the top half of the plants received more light than the ones on the bottom half. In addition, on day 60 and 71, the size of leaves on top half of the plants increased over time, resulting in growing canopy size. Hence the leaves on the bottom half of the plants received uneven light since they grew underneath the canopy. From day 84 onwards, the leaves on the top half of the plants reached maximum size and therefore the size of their canopy remained constant. This resulted in even light exposure to the leaves on the bottom half of the plants. From April onwards, side shoots were pruned. Once the plants reached 17 to 18 leaves, old leaves on the bottom were also pruned. In average, one plant generated 3 new leaves per week, resulting in the removal of 3 old leaves from the bottom. Detailed summary of temperature and humidity in the greenhouse, irrigation and the use of nutrients and fertilizers can be found in Supplementary Material, Tables S.1–S.3.

In this study, the plant age was defined with the days after sowing of tomato seeds; while the leaf position was defined with the positions where leaves were collected from one plant at one plant age. Leaves were collected on day 60, 71, 84, 102 and 118 after sowing. The plant ages included in this study represented different developmental stages of the tomato plants. At each plant age, new developments on the plants were observed as follows: day 60 (flowering), day 71 (small green fruits), day 84 (green fruits), day 102 (fruits turning red) and day 118 (red fruits). These different developmental stages were schematically visualized in Fig. 1a. To guarantee the collection of enough material for analysis and to ensure the inclusion of physiological difference in leaves, leaves were divided based on their positions on the plants. This method was adapted from Qudsieh et al. (2002). On day 60, plants were equally divided into two parts (top and bottom) based on the total length of the plant. On day 71, 84, 102 and 118, plants were equally divided into three parts (top, middle and bottom) based on the total length of the plants. Leaves from each part were collected separately (Fig. 1a). Rachises, petioles and petiolules were removed from the leaves (Supplementary Material, Figure S.1). The mass of leaves was recorded. At each plant age, leaves were collected from 3 individual tomato plants. Thus, 15 plants were used in this study. Fresh leaves contained 11.04 ± 1.13 % of dry matter. All leaves were stored at −20 °C within approximately 2 h after harvest. The frozen leaves were then freeze dried with Epsilon 2-10D LSCplus (Martin Christ, Germany) to a final dry matter of 95.6 ± 2.20 %. The freeze-dried leaves were grinded by using a lab-scale mortar and pestle. The freeze-dried and ground leaves were stored at −20 °C for maximal 2 month until further analysis.

L-aspartic acid and 2-mercaptoethanol were purchased from Sigma-Aldrich (St Louis, MO, USA). Absolute ethanol and acetic acid were purchased from Merck KGaA (Darmstadt, Germany). Folin-Ciocalteu reagent was purchased from MP Biomedicals (Illkirch, France). Sodium carbonate anhydrous was purchased from VWR Chemicals (Darmstadt, Germany). Sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1 % SDS, 26.3 % w/v glycerol, 0.01 % bromophenol blue), running buffer, Precision Plus Protein Dual Color Standards and Bio-safe Coomassie Staining buffer for SDS-PAGE were purchased from Bio-Rad Laboratories (Hercules, USA). Ultrapure water (MilliQ water) was purified by using a Milli-Q IQ 7000 Ultrapure Lab Water System (Merck KGaA, Darmstadt, Germany).

2.2. Protein extraction and phenolic compounds removal

The method was adapted from Kiskini, Vissers, Vincken, Gruppen, and Wierenga (2016). MilliQ water and 50–50 % ethanol–water (0.5 % v/v acetic acid) were used. Acetic acid was added according to the method for minimizing protein loss during extraction. The 50–50 % ethanol–water (0.5 % v/v acetic acid) was referred as ethanol–water in this study. Freeze-dried and ground leaves (0.1 g) were mixed with MilliQ water or ethanol–water at a ratio of 1:10 g/mL. The mixture was incubated in a thermomixer (700 rpm, 4 °C, 30 min) in the dark and centrifuged at 8,000 g for 5 min. The supernatant was carefully separated from the pellet by pipetting. Afterwards, 1 mL of fresh solvent was added to the pellet and the same incubation procedure was followed. This step was repeated 4 times. The supernatant from every step was combined and filtered (0.45 μm cellulose filter) to yield a final

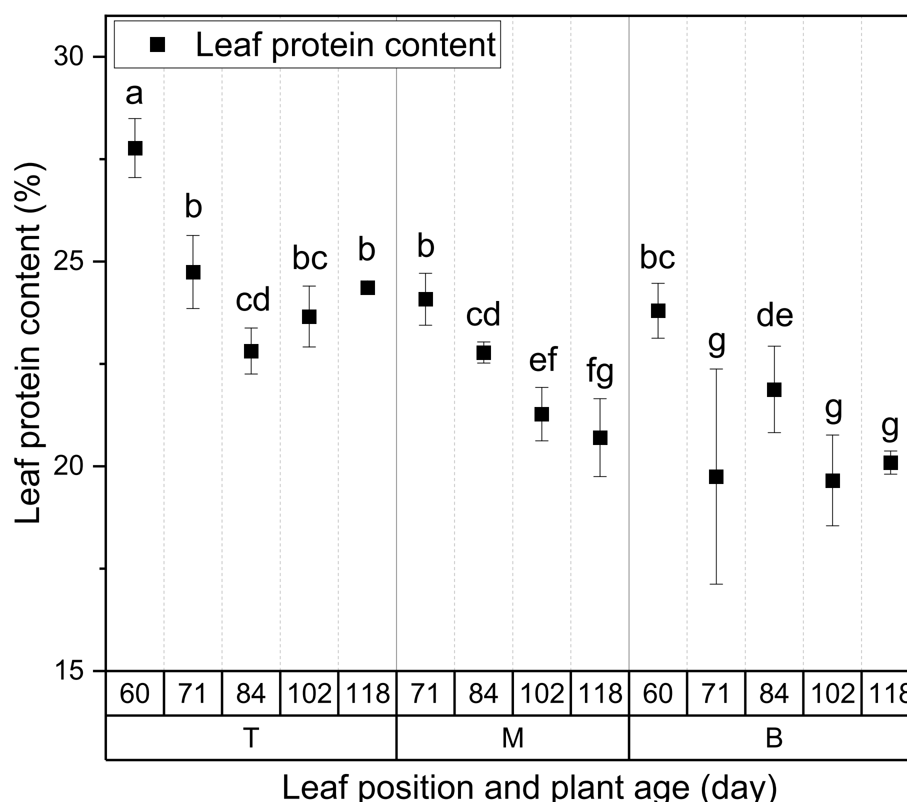


Fig. 2. Protein content of leaves with different plant ages and leaf positions. T, M and B stand for leaves collected from the top, middle and bottom positions of the plants, respectively. Different small upper letters indicate significant difference at $P \leq 0.05$. Data points can be found in Supplementary material, Table S.6.

supernatant. The final supernatant was stored in a dark fridge (4 °C) overnight due to its sensitivity to light and temperature, and the phenolic compounds content was measured within one day after extraction. The final pellet was dried overnight in a heating evaporator (Reacti-Therm III #TS-18823, Thermo Scientific, US) at 25 °C with N_2 gas to ensure the evaporation of solvent. The dried pellets were stored at −20 °C until further analysis. The final supernatant and final pellet were further referred to as supernatant and pellet in this study.

Preliminary experiments were carried out to screen the type of solvents for maximal removal of phenolic compounds. Leaf samples from day 60 were used. The same procedure as abovementioned was followed but with pure ethanol, 50–50 % methanol–water and pure methanol (all with 0.5 % acetic acid). Pure methanol removed the same amount of phenolic compounds as the ethanol–water (Supplementary Material, Figure S.4). However, high concentration of methanol resulted in a darker color (Supplementary Material, Figure S.4) and possibly more saponin contaminants (Vissers et al., 2017). The ethanol–water was therefore chosen in this study.

2.3. Phenolic compounds content determination

The total phenolic compounds content in the supernatants was determined based on the Folin-Ciocalteau method (Slinkard & Singleton, 1977). The calibration curve was made with gallic acid solutions with concentrations of 0 to 1 mg/mL in MiliQ water or ethanol–water. Supernatants after extraction (100 μL) were added to 7.9 mL MilliQ water and mixed thoroughly with a vortex. Then, 500 μL of Folin-Ciocalteu reagent and 1.5 mL of 20 % (w/v) sodium carbonate were added to the mixture and mixed thoroughly. The new mixture was incubated in a water bath at 40℃ for 30 min. The absorbance of different mixtures was measured by using a UV/Vis spectrophotometer (DR 600, HACH, the Netherlands) at 750 nm wavelength. Each measurement was repeated twice. The total phenolic compounds content was expressed as mg/g dried leaves. All phenolic compounds determinations were carried out within 1 day after extraction.

2.4. Dry matter content determination

The dry matter content of ground freeze-dried leaves was determined by leaving them in a hot air oven at 105 °C overnight. Due to the limited sample size, the dry matter content of the dried pellets obtained after evaporation was not measured. Thus, we assumed 100 % dry matter content for all dried pellets. For the supernatants, 1 mL of each sample was taken and dried overnight in a heating evaporator at 25 °C with N_2 gas till constant weight. The dry matter content of supernatants was determined by the weight loss during evaporation.

2.5. Protein content determination

The total nitrogen content of ground freeze-dried leaves and dried pellets were determined by using the Dumas nitrogen combustion method with a rapid N exceed® analyzer (Elementar, Langenselbold, Germany). Around 50 to 100 mg of sample was weighed in a tin foil sheet and closed tightly without headspace, the sample was then combusted at 900 °C in the presence of oxygen. L-aspartic acid was used as the standard. Due to the presence of non-protein nitrogen, a conversion factor of 4.4 was used to estimate the protein content for both freeze-dried leaves and pellets (Kiskini et al., 2016; Milton & Dintzis, 1981). The protein content of each leaf sample was measured twice. The protein content of the supernatants was derived using mass balance:

$$m_{pt,sup} = m_{pt,leaf} - m_{pt,pellet} \tag{1}$$

Where $m_{pt,leaf}$, $m_{pt,sup}$ and $m_{pt,pellet}$ are the mass of total proteins in dried tomato leaves, supernatant and pellet on dry basis, respectively.

The protein yield of supernatant and pellet were calculated as follows:

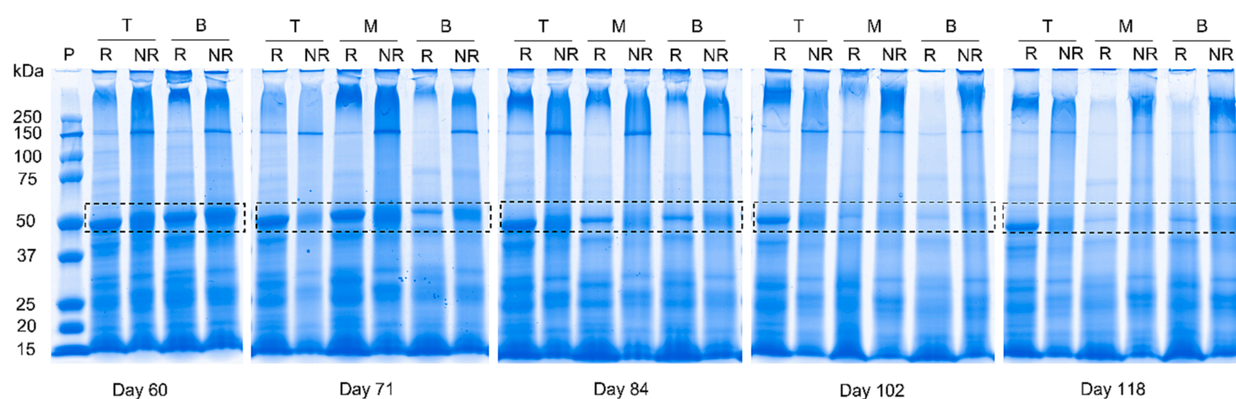


Fig. 3. SDS-PAGE analysis of leaves with different plant ages and leaf positions under reducing (R) and non-reducing (NR) conditions. P stands for pre-stained molecular marker. T, M and B stand for leaves collected from the top, middle and bottom positions of the plants, respectively. The dashed line areas indicate the large subunits of RuBisCo. *Print in color.*

$$Protein\ yield\ (mg/g\ leaf\ protein) = m_{pt,sup}/m_{pt,leaf}\ or\ m_{pt,pellet}/m_{pt,leaf} \quad (2)$$

The protein content of leaves and of the supernatant and pellet (further referred to as purity) were calculated as follows:

$$Protein\ content\ (\%) = \mathrm{m}_{pt,leaf}/\mathrm{m}_{DM,leaf}*100\% \quad (3)$$

$$Protein\ purity\ (\%) = \mathrm{m}_{pt,sup}/\mathrm{m}_{DM,sup}*100\%\ or\ \mathrm{m}_{pt,pellet}/\mathrm{m}_{DM,pellet}*100\% \quad (4)$$

Where $m_{DM,\mathrm{leaf}}$, $m_{DM,\mathrm{sup}}$ and $m_{DM,\mathrm{pellet}}$ represent the mass of dry matter in dried tomato leaves, supernatant and pellet, respectively. As indicated by the equations, the protein content of leaves can be named as protein purity of leaves. However protein content is common term used for raw material, while protein purity is a term used for certain component in an ingredient or a fraction. We therefore to use protein content to describe leaves and protein purity to describe the supernatants.

2.6. Protein composition determination

The protein composition determination of leaves was carried out by using SDS polyacrylamide gel electrophoresis (SDS-PAGE) on a Bio-Rad Mini-Protein cell (Bio-Rad Laboratories, Hercules, USA) under reducing and non-reducing conditions. The method was adapted from Tenorio, Gieteling, De Jong, Boom and Van Der Goot (2016). For reduced gels, sample buffer was mixed with 2-mercaptoethanol at ratio of 19: 1 (v/v). For non-reduced gels, sample buffer was directly used. For both reduced and non-reduced gels, freeze-dried leaves were dissolved in a designated sample buffer. The mixture was then diluted two times with MilliQ water to make a final protein concentration in dispersion of 5 mg/mL. The new mixture was heated at 95 °C for 10 min in a heating block and then centrifuged at 10,000 g for 5 min. After that, 15 μL of the supernatants and 10 μL of the Precision Plus Protein Dual Color Standards were loaded on a 10 % Mini-PROTEAN® TGX™ Precast gel (Bio-Rad Laboratories, Hercules, USA) in different lanes. The electrophoresis was carried out at 200 V for approximately 1 h. The gel was washed three times with MilliQ water and stained with the Bio-safe Coomassie Stain. Excess stain was removed with MilliQ water. The GS-900 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, USA) was used for gel imagining.

2.7. Statistical analysis

The statistical analysis in this study was carried out by using IBM SPSS statistics, version 25.0 (IBM, Armonk, US). Significant differences were analyzed with a univariate general linear model with the Duncan test. Differences were considered significant when $P \leq 0.05$ and were shown as the small upper letters in the figures. In other words, if two data points carry the same letter, there is no significant statistical difference between these two points. On the contrary, if two data points carry a different letter, there is significant statistical difference between these two points.

3. Results and discussion

3.1. Leaf protein content

The effect of plant age and leaf position on the protein content of tomato leaves is summarized in Fig. 2. In this study, the plant age was defined with the days after sowing of tomato seeds; while the leaf position was defined with the positions where leaves were collected from one plant at one plant age. It was observed that the overall level of the protein content in the leaves decreased with plant age (Fig. 2). The highest protein content (27.8 ± 0.72 %) was found in leaves on the top of the plants on day 60. The protein content of the top leaves decreased to 24.7 ± 0.89 % on day 71 and remained roughly constant when the plant age further increased. A similar trend was observed for the protein content of leaves from the bottom. The protein content of the middle leaves however continuously decreased from 24.1 ± 0.63 % to 20.7 ± 0.95 % from day 71 to day 118.

The leaf position had a stronger effect on the protein content than the plant age (Fig. 2). Especially the protein content in leaves collected from higher positions was higher than the protein content in leaves from lower positions. For instance, the top leaves on day 60 contained 4 % more proteins than that of bottom leaves at the same plant age. This trend was observed at every plant age.

The decrease of leaf protein content with plant age was also reported for other leafy materials, such as white and red clovers and rapeseed leaves (Sincik et al., 2007; Yu et al., 2015). One of the possible explanations for such age related change of protein content in leaves is protein senesce. This is a process where protein degradation happens in aging and inefficient photosynthetic organs (leaves). The degradation products including peptides and free amino acids are then transferred to support organs such as fruits with additional nutrients, leaving leaves with less total proteins (Havé, Marmagne, Chardon, & Masclaux-Daubresse, 2017). This study revealed that such changes in total protein content of tomato leaves is visible in terms of both leaf position and plant age (Fig. 2). As mentioned in section 2.1, at day 60, the tomato plants were divided into two parts (top and bottom) based on the total length of the plants. On day 71, 84, 102 and 118, the plants were divided into three parts (top, middle and bottom) based on the total length of the plants. The leaves were then harvested from each part. Since the plants continued to grow taller over time, the mass of leaves from each position continuously increased (Fig. 1b), which reduces the effect of leaf position on the protein content of each part. All of these observations make it crucial to consider plant age and leaf position when collecting leaves. It

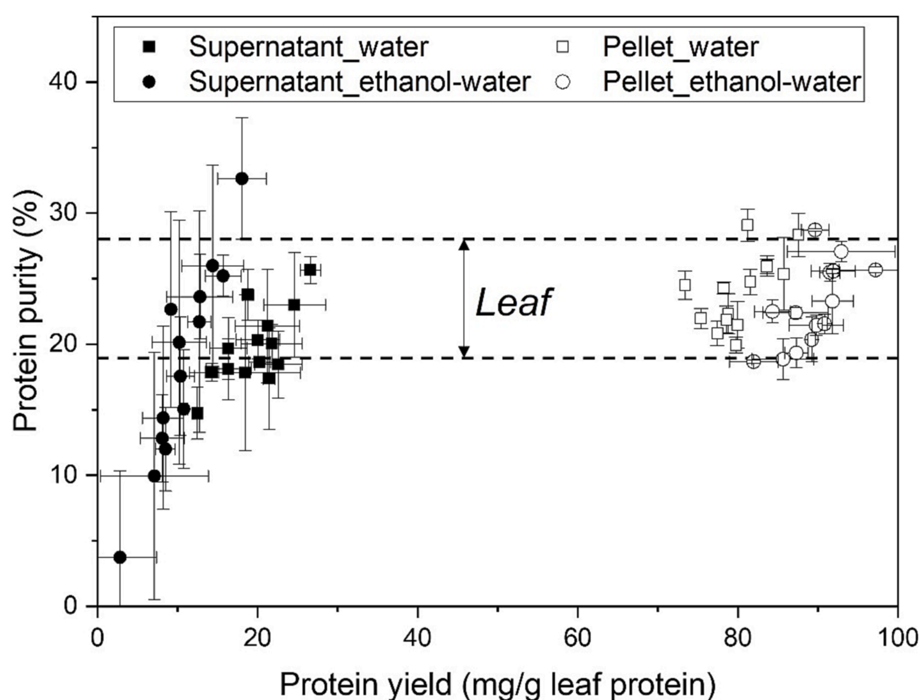


Fig. 4. Protein yield-purity diagram after extraction with water or ethanol–water from leaves with different plant ages and leaf positions. The dashed line area represents the range of leaf protein content. Data points can be found in Supplementary material Table S.4.

would be interesting to investigate in details how the protein senesce affect the protein content and the sequential protein extraction from tomato leaves. More precise collection of leaves should be therefore carried out.

3.2. Leaf protein composition

The SDS-PAGE profile for reduced and non-reduced gels of all leaf materials were similar (Fig. 3). The most distinctive band for both gel conditions was around 50 kDa, which corresponds to the large subunit of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) from tomatoes (UniProtKB - P27065 (RBL_SOLLC), 2006). The large subunit of RuBisCO with similar molecular weight (50 – 55 kDa) has been reported for sugar beet (Kiskini et al., 2016), alfalfa (Tanambell, Møller, Corredig, & Dalsgaard, 2022) and red clover (Amer, Juul, Møller, Møller, & Dalsgaard, 2021). Compared to the reduced gels, the band at 50 kDa was slightly more smeared and the area above 250 kDa was more intensive for the non-reduced gels. The latter phenomenon could be accumulation of big molecular weight molecules that could not be separated on the gel due to the absence of 2-mercaptoethanol under the non-reducing condition. Such accumulation was also reported by Tanambell et al. (2022). Bands in the range of 25 to 37 kDa were observed for both reduced and non-reduced gels. Those bands were believed to be associated with thylakoid membranes (Kiskini et al., 2016). Bands at around 150 and 75 kDa were also visible. It is not clear to the authors which types of proteins those bands referred to. It is generally accepted that RuBisCo and thylakoid membrane proteins represents most of the proteins (approx. 80 % of total proteins) present in the chloroplasts in green leaves (Fiorentini & Galoppini, 1983). The presence of those proteins in tomato leaves was confirmed by this study.

When focused on the reduced gels, only slight differences were observed in terms of plant age and leaf position (Fig. 3). The large subunit of RuBisCO from bottom leaves on day 71 was less intensive compared to the middle and the top leaves at the same plant age. Similar results were observed for the middle and bottom leaves on day 84, 102 and 118 in comparison to the top leaves at each plant age. The reduced band intensity could be contributed by reduced amount of RuBisCO in leaf materials due to protein senesce or difference of protein solubility in the sample buffer.

3.3. Leaf protein extraction

The efficiency and selectivity of water and ethanol–water for protein extraction from tomato leaves were analyzed by using the protein yield-purity diagram (Fig. 4). The protein yield reflects the capability of the solvent to separate proteins from the plant matrix, while the protein purity reflects the selectivity of the solvent to separate proteins from other soluble components.

Fig. 4 reveals that most of the protein remained in the pellets after extraction. In the case of water extraction, about 12 to 27 mg/g leaf protein were extracted in the supernatants from leaves with various ages and collected positions. This means that at least 73 mg/g leaf protein remained in the pellets. The low protein yield indicated that water was inefficient of extracting all proteins from tomato leaves during extraction. This could be caused by interactions between proteins and other components in the leaves or during extraction (Tamayo Tenorio et al., 2016), which limits the protein solubility. Additionally, when water was mixed with tomato leaves, the pH of the mixture became 5.33 ± 0.05 due to the acidic nature of the leaves, regardless of the age. The isoelectric points (pIs) of RuBisCO were reported to be in the range of 4.4 to 4.7 (Bahr, Bourque, & Smith, 1977). Rubisco can take up to 50 % of total proteins in C3 plants like tomatoes (Hilditch, Jones, Balding, Smith, & Rogers, 1991). It is possible that the low pH led to a lower protein solubility and thus lower extraction yield.

At least 82 mg/g leaf protein remained in the pellet after ethanol–-water extraction (Fig. 4). This was expected because proteins generally become more insoluble in the presence of ethanol due to the lower polarity of ethanol in comparison to water. In addition to the acidic nature of the tomato leaves, the presence of acetic acid also contributed to the lower protein solubility as the pH of the supernatants was 4.23 ± 0.09 when ethanol–water was used. The high amount of remaining proteins in the pellets indicated that neither water nor ethanol–water could efficiently extract the proteins from the plant matrix.

The protein purity of the supernatants and the pellets was compared

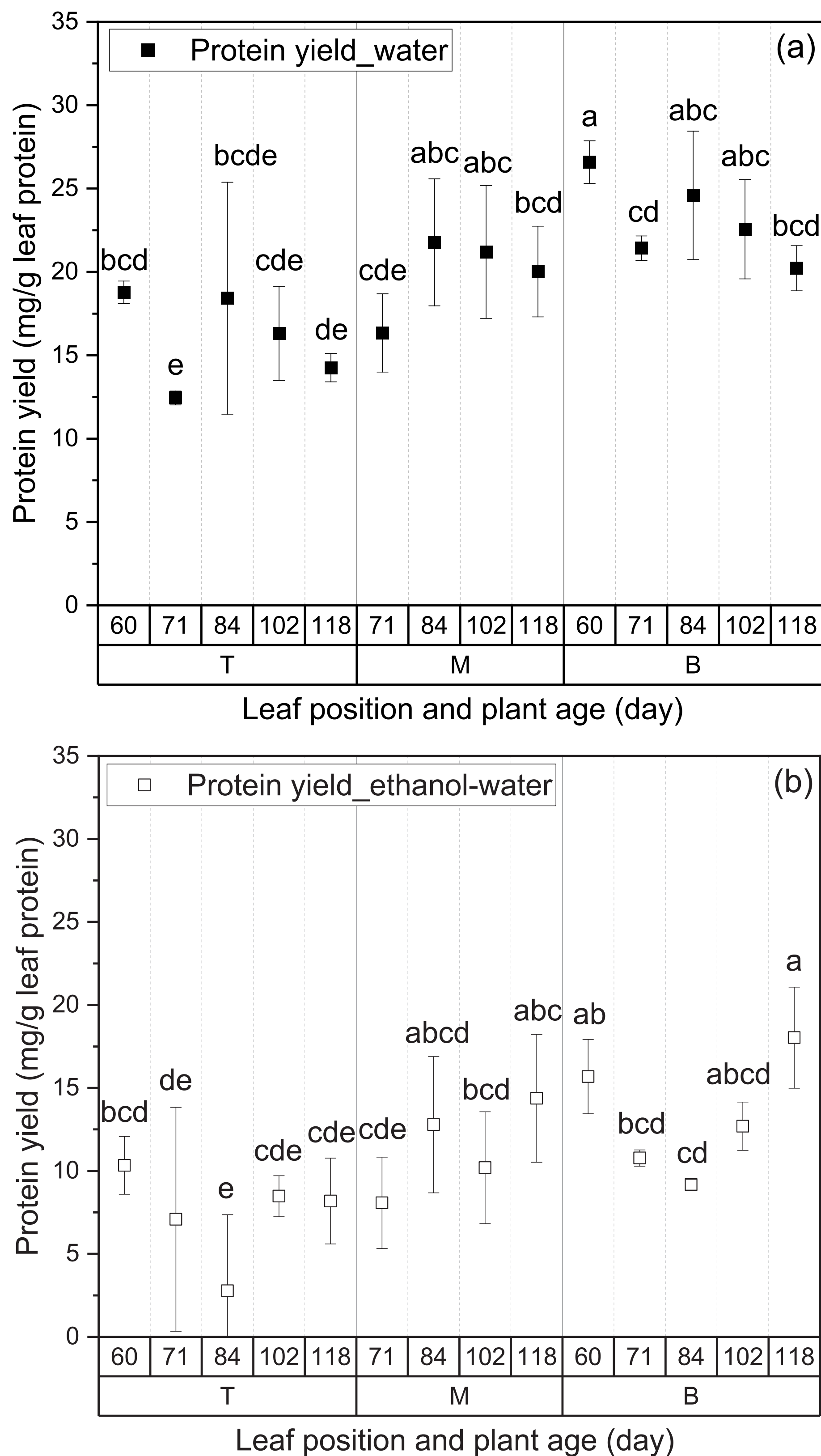


Fig. 5. Protein yield of supernatants after extraction with water (a) or ethanol–water (b) from leaves with different plant ages and leaf positions. T, M and B stand for leaves collected from the top, middle and bottom positions of the plants, respectively. Different small upper letters indicate significant difference at $P \leq 0.05$. Data points can be found in Supplementary material Table S.4.

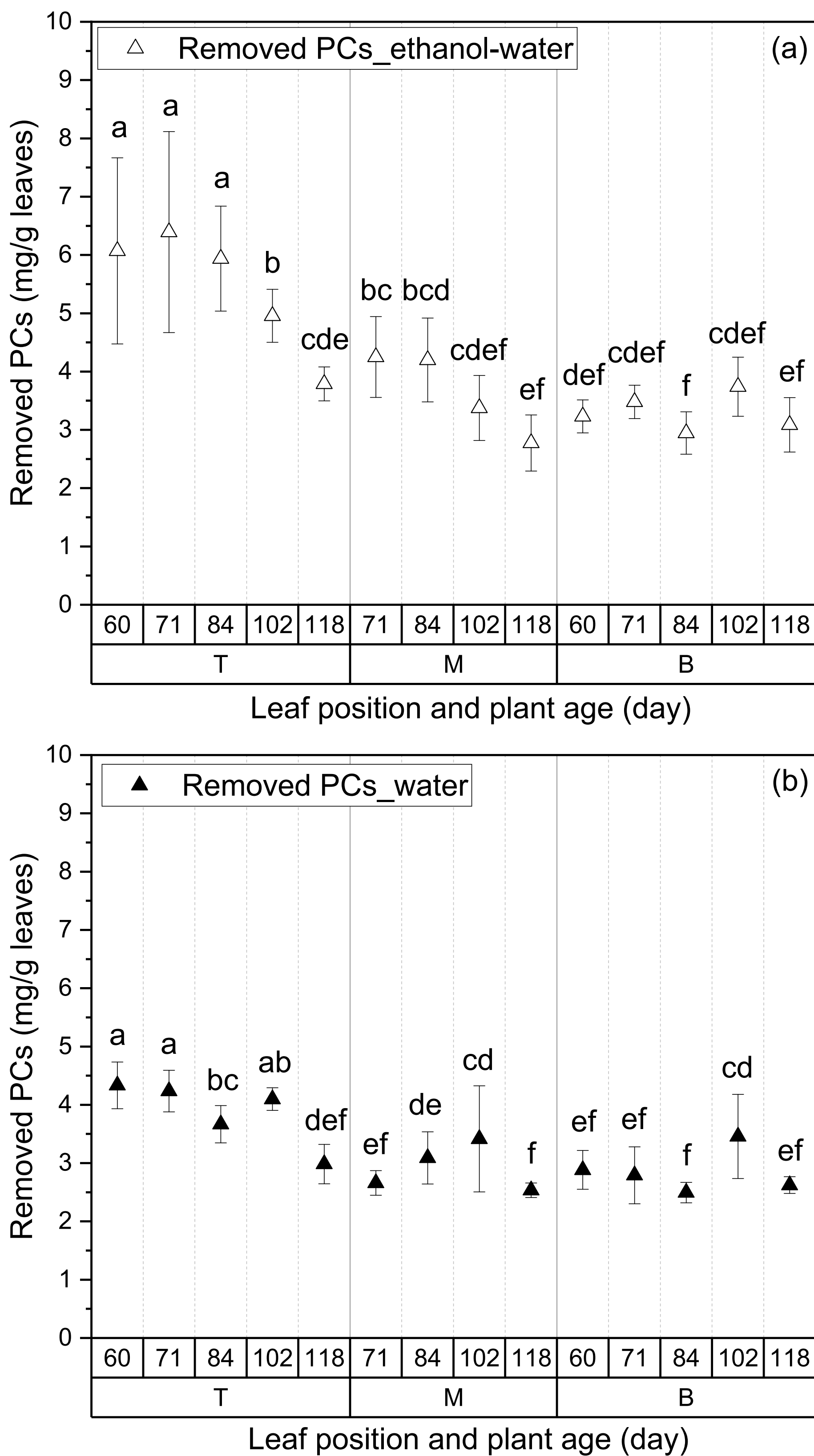


Fig. 6. The amount of removed phenolic compounds in supernatants after extraction with ethanol–water (a) or water (b) from leaves with different plant ages and leaf positions. PCs stands for phenolic compounds; T, M and B stand for leaves collected from the top, middle and bottom positions of the plants, respectively. Different small upper letters indicate significant difference at $P \leq 0.05$. Data points can be found in Supplementary Material Table S.5.

with the protein content of the leaves (Fig. 4). As indicated in section 2.5, although the protein content of leaves shared the same equation as the protein purity of leaves. Protein content is more commonly used for raw material, while protein purity is more commonly used for target component in an ingredient or a fraction. When water was used as extraction solvent, the protein purity of both supernatants and pellets was similar to the protein content in the leaves. This similarity suggested co-extraction of other soluble components such as water-soluble carbohydrates and phenolic compounds, resulting in no further concentration of proteins. When ethanol–water was used, the protein purity of the supernatants was somewhat lower than the protein content of leaves, indicating some selectivity of ethanol–water to other soluble components rather than to proteins. The protein purity of pellets after ethanol–water extraction was similar to the protein content of leaves.

The protein yield and purity of the supernatants (soluble fractions) in this study were to be found in the same range as protein fractions from multiple leafy sources (Tamayo Tenorio et al., 2018). This reflects the general dilemma of protein extraction from green leaves. As discussed by Tamayo Tenorio et al. (2018), this dilemma indicates that current protein extraction techniques are not optimized for leafy material yet. However, the abundant availability of leaves as side stream makes better insights in protein extraction and application relevant to probe its potential as novel protein source.

The protein yield was influenced by the leaf position (Fig. 5a and Fig. 5b, respectively). For example, Fig. 5a shows that when water was used for extraction, 19 mg/g leaf protein was extracted from the top leaves on day 60. Significantly more proteins (27 mg/g leaf protein) was extracted from bottom leaves at the same plant age. The same effect was observed for leaves on day 71. Although the difference of protein yield between leaf position for leaves at 84, 102 and 118 day was not significant, the protein yield followed an increasing trend as the leaf position changed downwards. When ethanol–water was used for extraction (Fig. 5b), significantly more proteins were extracted from the middle and bottom leaves than from top leaves on days 84, 102 and 118; while the difference of protein yield between leaf position on day 60 and 71 was not significant. We hypothesized that the effect of leaf position on the protein yield of supernatants might be linked to protein senesce. As a result, leaves on the lower positions of the plants probably contained more peptides and free amino acids and less intact proteins than leaves from the higher positions of the plants. Thus, the higher yields in the supernatants can be expected to be the consequences of higher solubilities of peptides and free amino acids in both water and ethanol–water.

3.4. Phenolic compounds removal

Fig. 6a and b present the amount of removed phenolic compounds in the supernatants after extraction by water and ethanol–water from leaves with different plant ages and leaf positions. Compared to water, ethanol–water removed much more phenolic compounds from all leaf samples, regardless of the plant age and leaf position (Fig. 6a and b). This finding is aligned with previous studies where most polyphenols (78 g/kg) were extracted using 50 % ethanol–water from green tea residues (Zhang et al., 2016), Feijoa fruit waste (Sun-Waterhouse, Wang, Waterhouse, & Wadhwa, 2013) and mate tea (Turkmen, Sari, & Velioglu, 2006) with the least ethanol addition. Further increase of ethanol concentration to 60 % or 80 % led to similar extraction of phenolic compounds but increased co-extraction of other soluble components and more pigments (Zhang et al., 2016). In addition, Jia et al. (2021) reported that the first extraction step removed most of the phenolic compounds from sunflower kernels, while the effect of additional steps was limited, especially after 3 steps. Since a five-step extraction was applied in this study, we therefore assumed that most of the phenolic compounds were extracted after such a procedure. The extracted phenolic compounds can be used as functional ingredients for food applications (Sun-Waterhouse et al., 2013).

The extraction of total phenolic compounds was influenced by plant age (Fig. 6a). For instance, 6.07 ± 1.60 mg/g leaf phenolic compounds were extracted with ethanol–water from top leaves on day 60, while slightly more phenolic compounds were extracted from top leaves on day 71 (Fig. 6a). The least amount of phenolic compounds was extracted from top leaves from the oldest plant (118 day). The extraction of phenolic compounds was even more influenced by leaf position (Fig. 6a). In comparison to the top leaves, significantly less phenolic compounds were extracted from the bottom leaves on day 60. The effect of plant age and leaf position on the amount of extracted phenolic compounds was also observed when water was used, although at a lower overall level (Fig. 6b). Such effect on total phenolic compounds was also reported for other plant sources. The total phenolic compounds in rapeseed leaves reached maximum (2.06 g/100 g dry matter) after a rapid plant growth, and followed by a decrease to 0.54 g/100 g towards the end of the plant maturity (Yu et al., 2015). The top part of sugarcane stem contained significantly higher content of tannin than the middle and bottom parts. The total tannin of these three parts decreased significantly as the plants further matured (Qudsieh et al., 2002).

4. Conclusions

The large availability and considerable amount of proteins (approx. 30 % on dry matter) make leaves from tomato plants a very interesting source of plant proteins. This study revealed that water and ethanol–-water had limited efficiency and selectivity for protein extraction from tomato leaves. As a result, the extraction yield was low and the protein was not concentrated during extraction as compared to the leaf material. The ethanol–water was however efficient for phenolic compounds removal. In addition, this study quantified the diversity of tomato leaves as sources of proteins and phenolic compounds. It is thus important for future processing to realize that the leaves from tomato plants are not a homogenous mass. Consequentially, protein content and protein extraction yield vary with age of the plants and positions of the leaves. The highest extraction yield was found when leaves from bottom of the plants were used, while leaves from top contained more proteins. In the meanwhile, the most phenolic compounds was extracted from the top leaves. We therefore concluded that it might be interesting to consider tomato leaves as sources of both proteins and phenolic compounds. The diversity of leaves has to be considered when developing processes that aim for an efficient use of total biomass (hence fruits and leaves) produced by tomato plants.

CRediT authorship contribution statement

Yafei Yu: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft. **Marietheres Kleuter:** Resources, Writing – review & editing. **Somayeh Taghian Dinani:** Conceptualization, Supervision, Writing – review & editing. **Luisa M. Trindade:** Supervision, Writing – review & editing. **Atze Jan van der Goot:** Conceptualization, Supervision, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Wouter de Groot and Jos Sewalt for their help in the lab.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.135072.

References

Akyüz, A., & Ersus, S. (2021). Optimization of enzyme assisted extraction of protein from the sugar beet (Beta vulgaris L.) leaves for alternative plant protein concentrate production. *Food Chemistry, 335*(July 2020), Article 127673. https://doi.org/10.1016/j.foodchem.2020.127673

Amer, B., Juul, L., Møller, A. H., Møller, H. S., & Dalsgaard, T. K. (2021). Improved solubility of proteins from white and red clover – inhibition of redox enzymes. *International Journal of Food Science and Technology, 56*(1), 302–311. https://doi.org/10.1111/ijfs.14632

Arab, M., Bahramian, B., Schindeler, A., Valtchev, P., Dehghani, F., & McConchie, R. (2019). Extraction of phytochemicals from tomato leaf waste using subcritical carbon dioxide. *Innovative Food Science and Emerging Technologies, 57*(July), Article 102204. https://doi.org/10.1016/j.ifset.2019.102204

Bahr, J. T., Bourque, D. P., & Smith, H. J. (1977). Solubility properties of fraction I proteins of maize, cotton, spinach, and tobacco. *Journal of Agricultural and Food Chemistry, 25*(4), 783–789. https://doi.org/10.1021/jf60212a027

Chemat, F., Vian, M. A., & Cravotto, G. (2012). Green extraction of natural products: Concept and principles. *International Journal of Molecular Sciences, 13*(7), 8615. https://doi.org/10.3390/IJMS13078615

Coldebella, P. F., Gomes, S. D., Evarini, J. A., Cereda, M. P., Coelho, S. R. M., & Coldebella, A. (2013). Evaluation of protein extraction methods to obtain protein concentrate from cassava leaf. *Engenharia Agricola, 33*(6), 1223–1233. https://doi.org/10.1590/S0100-69162013000600015

FAOSTAT. (2020). https://www.fao.org/faostat/en/#data/QCL.

Fernández-Gómez, M. J., Díaz-Raviña, M., Romero, E., & Nogales, R. (2013). Recycling of environmentally problematic plant wastes generated from greenhouse tomato crops through vermicomposting. *International Journal of Environmental Science and Technology, 10*(4), 697–708. https://doi.org/10.1007/s13762-013-0239-7

Fiorentini, R., & Galoppini, C. (1983). The proteins from leaves. *Qualitas Plantarum Plant Foods for Human Nutrition, 32*(3–4), 335–350. https://doi.org/10.1007/BF01091193

Havé, M., Marmagne, A., Chardon, F., & Masclaux-Daubresse, C. (2017). Nitrogen remobilization during leaf senescence: Lessons from Arabidopsis to crops. *Journal of Experimental Botany, 68*(10), 2513–2529. https://doi.org/10.1093/jxb/erw365

Heuvelink, E., Bakker, M. J., Elings, A., Kaarsemaker, R., & Marcelis, L. F. M. (2005). Effect of leaf area on tomato yield. *Acta Horticulturae, 691*, 43–50. https://doi.org/10.17660/ACTAHORTIC.2005.691.2.

Hilditch, C. M., Jones, P. B., Balding, P., Smith, A. J., & Rogers, L. J. (1991). Rubilose bisphosphate carboxylases from macroalgae: Proteolysis during extraction and properties of th enzyme from Porphyra umbilicalis. *Phytochemistry, 30*(3), 745–750. https://doi.org/10.1016/0031-9422(91)85245-U.

Jia, J., Gao, X., Hao, M., & Tang, L. (2017). Comparison of binding interaction between β-lactoglobulin and three common polyphenols using multi-spectroscopy and modeling methods. *Food Chemistry, 228*, 143–151. https://doi.org/10.1016/j.foodchem.2017.01.131

Jia, W., Kyriakopoulou, K., Roelofs, B., Ndiaye, M., Vincken, J. P., Keppler, J. K., & van der Goot, A. J. (2021). Removal of phenolic compounds from de-oiled sunflower kernels by aqueous ethanol washing. *Food Chemistry, 362*(October 2020), Article 130204. https://doi.org/10.1016/j.foodchem.2021.130204

Keppler, J. K., Schwarz, K., & van der Goot, A. J. (2020). Covalent modification of food proteins by plant-based ingredients (polyphenols and organosulphur compounds): A commonplace reaction with novel utilization potential. *Trends in Food Science & Technology, 101*(March), 38–49. https://doi.org/10.1016/j.tifs.2020.04.023

Kiskini, A., Vissers, A., Vincken, J. P., Gruppen, H., & Wierenga, P. A. (2016). Effect of Plant Age on the Quantity and Quality of Proteins Extracted from Sugar Beet (Beta vulgaris L.) Leaves. *Journal of Agricultural and Food Chemistry, 64*(44), 8305–8314. https://doi.org/10.1021/acs.jafc.6b03095

Martin, A. H., Castellani, O., de Jong, G. A. H., Bovetto, L., & Schmitt, C. (2019). Comparison of the functional properties of RuBisCO protein isolate extracted from sugar beet leaves with commercial whey protein and soy protein isolates. *Journal of the Science of Food and Agriculture, 99*(4), 1568–1576. https://doi.org/10.1002/jsfa.9335

Martin, A. H., Nieuwland, M., & de Jong, G. A. H. (2014). Characterization of heat-set gels from RuBisCO in comparison to those from other proteins. *Journal of Agricultural and Food Chemistry, 62*(44), 10783–10791. https://doi.org/10.1021/jf502905g

Milton, K., & Dintzis, F. R. (1981). Nitrogen-to-protein conversion factors for tropical plant samples. *Association for Tropical Biology and Conservation, 13*(3), 177–181. https://doi.org/10.2307/2388122

Möller, A. C., Li, J., van der Goot, A. J., & van der Padt, A. (2022). A water-only process to fractionate yellow peas into its constituents. *Innovative Food Science & Emerging Technologies, 75*, Article 102894. https://doi.org/10.1016/J.IFSET.2021.102894

Narváez-Cuenca, C. E., Vincken, J. P., & Gruppen, H. (2013). Quantitative fate of chlorogenic acid during enzymatic browning of potato juice. *Journal of Agricultural and Food Chemistry, 61*(7), 1563–1572. https://doi.org/10.1021/jf305093u

Peng, Y., Kersten, N., Kyriakopoulou, K., & van der Goot, A. J. (2020). Functional properties of mildly fractionated soy protein as influenced by the processing pH. *Journal of Food Engineering, 275*, Article 109875. https://doi.org/10.1016/j.jfoodeng.2019.109875

Prigent, S. V., Voragen, A. G., Visser, A. J., van Koningsveld, G. A., & Gruppen, H. (2007). Covalent interactions between proteins and oxidation products of caffeoylquinic acid (chlorogenic acid). *Journal of the Science of Food and Agriculture, 1243*(July), 1237–1243. https://doi.org/10.1002/jsfa.3011

Qudsieh, H. Y. M., Yusof, S., Osman, A., & Abdul Rahman, R. (2002). Effect of maturity on chlorophyll, tannin, color, and polyphenol oxidase (PPO) activity of sugarcane juice (Saccharum officinarum var. yellow cane). *Journal of Agricultural and Food Chemistry, 50*(6), 1615–1618. https://doi.org/110.1021/jf010959l.

Sincik, M., Bilgili, U., Uzun, A., & Acikgoz, E. (2007). Short communication. Harvest stage effects on forage yield and quality for rape and turnip genotypes. *Spanish Journal of Agricultural Research, 5*(4), 510–516. https://doi.org/10.5424/sjar/2007054-286

Slinkard, K., & Singleton, V. L. (1977). Total phenol analysis: automation and comparison with manual methods. *American Journal of Enology and Viticulture, 28*(1).

Sun-Waterhouse, D., Wang, W., Waterhouse, G. I. N., & Wadhwa, S. S. (2013). Utilisation potential of Feijoa fruit wastes as ingredients for functional foods. *Food and Bioprocess Technology, 6*(12), 3441–3455. https://doi.org/10.1007/s11947-012-0978-3

Tamayo Tenorio, A., Gieteling, J., De Jong, G. A. H., Boom, R. M., & Van Der Goot, A. J. (2016). Recovery of protein from green leaves: Overview of crucial steps for utilisation. *Food Chemistry, 203*, 402–408. https://doi.org/10.1016/j.foodchem.2016.02.092

Tamayo Tenorio, A., Kyriakopoulou, K. E., Suarez-Garcia, E., van den Berg, C., & van der Goot, A. J. (2018). Understanding differences in protein fractionation from conventional crops, and herbaceous and aquatic biomass - Consequences for industrial use. *Trends in Food Science and Technology, 71*(August 2017), 235–245. https://doi.org/10.1016/j.tifs.2017.11.010

Tamayo Tenorio, A., Schreuders, F. K. G., Zisopoulos, F. K., Boom, R. M., & van der Goot, A. J. (2017). Processing concepts for the use of green leaves as raw materials for the food industry. *Journal of Cleaner Production, 164*, 736–748. https://doi.org/10.1016/j.jclepro.2017.06.248

Tanambell, H., Møller, A. H., Corredig, M., & Dalsgaard, T. K. (2022). RuBisCO from alfalfa – native subunits preservation through sodium sulfite addition and reduced solubility after acid precipitation followed by freeze-drying. *Lwt, 154*(September 2021). https://doi.org/10.1016/j.lwt.2021.112682

Taylor, M. A., & Fraser, P. D. (2011). Solanesol: Added value from Solanaceous waste. *Phytochemistry, 72*(11–12), 1323–1327. https://doi.org/10.1016/j.phytochem.2011.03.015

Turkmen, N., Sari, F., & Velioglu, Y. S. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin-Ciocalteu methods. *Food Chemistry, 99*(4), 835–841. https://doi.org/10.1016/J.FOODCHEM.2005.08.034

UniProtKB - P27065 (RBL_SOLLC). (2006). https://www.uniprot.org/uniprot/B6ECN9.

van Krimpen, M. M., Bikker, P., Van Der Meer, I. M., van der Peet-Schwering, C. M. C., & Vereijken, J. M. (2013). Cultivation, processing and nutritional aspects for pigs and poultry of European protein sources as alternatives for imported soybean products. *Wageningen UR Livestock Research, February*, 48.

Vergara-Barberán, M., Lerma-García, M. J., Herrero-Martínez, J. M., & Simó-Alfonso, E. F. (2015). Use of an enzyme-assisted method to improve protein extraction from olive leaves. *Food Chemistry, 169*, 28–33. https://doi.org/10.1016/j.foodchem.2014.07.116

Vissers, A., Kiskini, A., Hilgers, R., Marinea, M., Wierenga, P. A., Gruppen, H., & Vincken, J. P. (2017). Enzymatic browning in sugar beet leaves (Beta vulgaris L.): Influence of Caffeic acid derivatives, oxidative coupling, and coupled oxidation. *Journal of Agricultural and Food Chemistry, 65*(24), 4911–4920. https://doi.org/10.1021/acs.jafc.7b01897

Yu, X., Bals, O., Grimi, N., & Vorobiev, E. (2015). A new way for the oil plant biomass valorization: Polyphenols and proteins extraction from rapeseed stems and leaves assisted by pulsed electric fields. *Industrial Crops and Products, 74*, 309–318. https://doi.org/10.1016/j.indcrop.2015.03.045

Zhang, C., van Krimpen, M. M., Sanders, J. P. M., & Bruins, M. E. (2016). Improving yield and composition of protein concentrates from green tea residue in an agri-food supply chain: Effect of pre-treatment. *Food and Bioproducts Processing, 100*, 92–101. https://doi.org/10.1016/j.fbp.2016.06.001