



Biorefining green triticale grass as a flavonoid-rich source of protein for sustainable food systems

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ABSTRACT

Green biomass serves as an eco-friendly, plant-derived substitute for conventional protein sources. Leaf protein concentrate (LPC) not only acts as a viable alternative to animal-derived proteins but also contains essential vitamins and bioactive compounds providing nutraceutical advantages. The extraction technique plays a critical role in maximizing LPC yield. In this study, green juice derived from the wet pressing of green triticale biomass was divided into two aliquots, each subjected to distinct processing techniques for LPC isolation. One portion underwent direct thermal coagulation via microwave irradiation, followed by vacuum filtration, yielding green LPC (MW-GLPC) and its brown juice (GJ-BJ). The other was first centrifuged to remove large photosynthetic complexes, producing yellow juice that was subsequently thermally coagulated and vacuum filtered to obtain yellow LPC (YLPC) and its brown juice (YJ-BJ).

The crude protein content in the MW-GLPC fraction (38.44 g 100 g⁻¹ DW) was higher than the raw green juice (16.38 g 100 g⁻¹ DW). YLPC fraction, obtained by incorporating a centrifugation step into the process, resulted in a significantly increase in crude protein (67.22 g 100 g⁻¹ DW). For fractions of brown juice (BJ), the crude protein content differed depending on the processing technique, with GJ-BJ exhibiting 0.73 g 100 g⁻¹ FW and YJ-BJ displaying 1.06 g 100 g⁻¹ FW. Size exclusion chromatography (SEC) indicated that BJ primarily contained oligopeptides ranging from 200 to 3000 Da.

Phytochemical assessments demonstrated that YLPC exhibits the highest concentration of some beneficial bioactive compounds, such as luteolin (27.2 µg g⁻¹), and isovitexin (111.6 µg g⁻¹). These findings are consistent with results obtained from the *Drosophila melanogaster* model under high-sugar conditions designed to simulate high-sugar-induced stress. Flies supplemented with a concentration of 20% YLPC demonstrated a 10.52% increase in viability relative to the control group, thereby indicating the beneficial potential of YLPC in high-sugar containing environments.

1. Introduction

Ensuring a stable and predictable supply of protein for the food and feed industries is one of the most important challenges facing agriculture today and likely tomorrow. The dependence on traditional animal-based

protein sources, such as meat, dairy, and fish, has some environmental and economic implications (Agudelo Higueta et al., 2023). Animal agriculture is resource-intensive, contributing significantly to deforestation, greenhouse gas emissions, and biodiversity loss, while also consuming extensive amounts of water, land, and energy. Additionally,

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the rising costs of animal feed and the unpredictability of market conditions further complicate the sustainability of these protein sources (FAO, 2013).

Taking these considerations into account, it is essential to seek alternative protein sources that offer health, environmental, and economic benefits. Seeds and protein-rich crops (such as legumes and cereals) represent a viable, plant-based solution to this challenge. Seeds contribute over half of the global consumption of dietary protein and energy, highlighting their significant economic, cultural, and nutritional value. They can contain up to 41.3 % protein by dry weight of different seed species (Elamine et al, 2022).

Legumes serve as rich, sustainable protein sources. They play a pivotal role in enhancing soil fertility through biological nitrogen fixation, thereby diminishing reliance on synthetic fertilizers. Similarly, cereals provide an accessible and cost-efficient source of plant-based protein (Romanyà & Casals, 2020).

However, legumes and cereals do not only contain protein in their seeds and fruits. Their green biomass also contains significant amounts of protein. Green biomass refers to the nutrient-rich green parts of plants, such as leaves and grass, which are primarily used as animal feed for their protein content. As a renewable and abundant resource, it requires fewer natural inputs compared to animal farming, resulting in a lower carbon footprint (Poore & Nemecek, 2018). The use of green biomass also mitigates the environmental impact of protein production by reducing greenhouse gas emissions and water usage (Schader et al., 2015).

Key crops for green biorefining, include both perennial and annual legumes and grasses. Such as triticale, which is a hybrid cereal. It is obtained from the crossing of wheat and rye, recognized primarily as a fodder grain. Triticale crops are sown in autumn and they are able to be harvested in the early spring, effectively avoiding the critical dry season. This timing minimizes competition with other strategic crops and provides green forage following the extended winter months. (Domokos-Szabolcsy et al., 2023). Notably, about 57–74% of the total crude protein of triticale green biomass could be extracted by using mechanical pressing (Jørgensen et al., 2020).

In agricultural practice, green grasses are primarily used in ensiled

form for animal feed. However, in the context of an increasingly resource-constrained world, green grasses represents a forward-thinking solution of sustainable protein source for humans as well (Pap et al., 2024).

While producing leaf protein concentrate from green biomass, in general, the first step is the mechanical liquid/solid separation resulting liquid soluble protein enriched juice and solid pulp rich in fiber and insoluble protein. The goal of secondary processing is then to prepare value-added products from the green juice, using various methods such as thermal coagulation and centrifugation (Gaffey et al., 2023). Through advanced extraction methods, leaf protein concentrate (LPC) is produced by green biomass, which not only serves as a substitute for animal-derived proteins but also offers a rich quantity of vitamins and phytochemicals with significant nutritional and medicinal benefits. The cultivation of green biomass for LPC production promotes sustainable agricultural practices, reducing the environmental impact associated with conventional livestock farming. Thus, green biomass and its derivatives stand as vital components in the future of nutrition and sustainable food systems. Even though current European Union novel food regulations limit the commercial use of it, proteins derived from green biomass show strong potential as future food and feed content, driving growing interest in exploring their applications (Pap et al., 2024). It can be further processed to obtain white-yellow protein, a purified fraction with reduced chlorophyll and anti-nutritional compounds, enhancing its digestibility, amino acid bioavailability, and suitability for human consumption in functional food applications (Domokos-Szabolcsy et al., 2023).

The challenge to be addressed is to test the applicability of these high-protein and phytonutrient containing green biomass to the human diet. These tests can be conducted on model organisms such as *Drosophila melanogaster* (fruit fly) to test the safety and toxicological properties of product-candidates before they are offered to humans. *Drosophila* is a widely preferred model organism for such toxicity and safety tests due to its rapid reproductive cycle and well-understood genetic structure. The genetic, physiological and biochemical effects of substances in a food can be examined via *Drosophila* tests (Günes, 2015). In the present study, we hypothesized that fractionated triticale

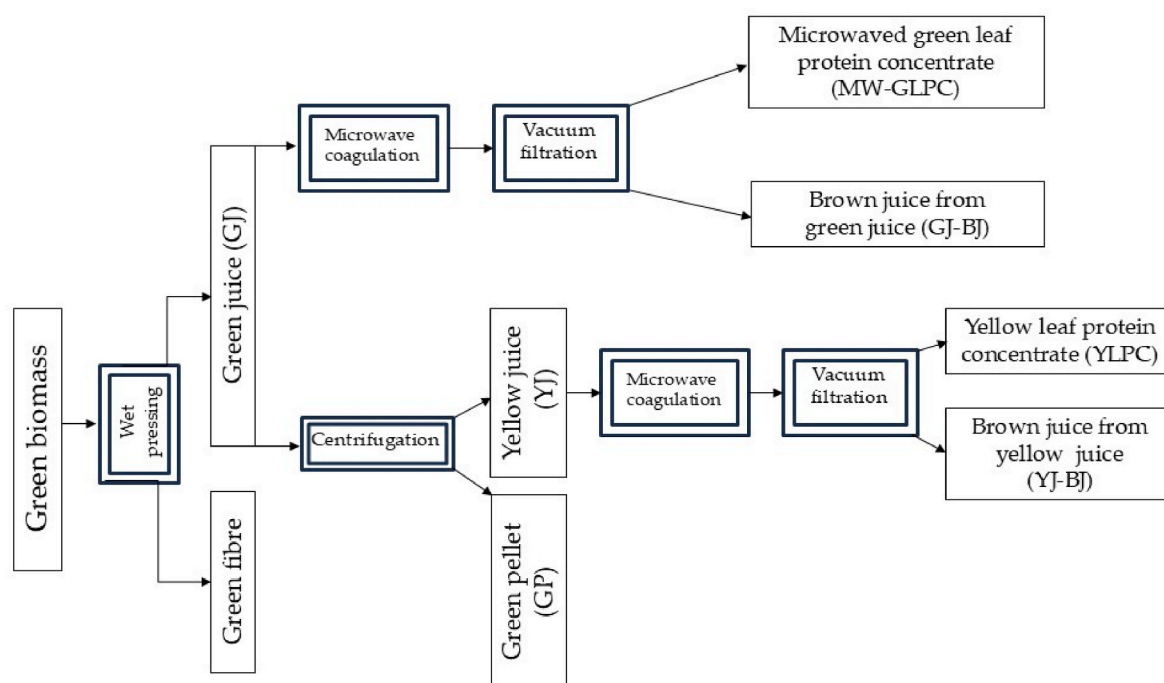


Fig. 1. Schematic flow chart of applied processes to obtain microwaved green leaf protein concentrate (MW-GLPC); brown juice from green juice (GJ-BJ); yellow leaf protein concentrate (YLPC); brown juice from yellow juice (YJ-BJ); green pellet (GP).

LPC would enhance *Drosophila* survival under stress.

In this study, the potential effects of different fractions derived from triticale green leaves on the survival rate and viability of fruit flies were investigated. These findings may contribute to development of affordable and sustainable plant-based protein sources for use in the human diet.

2. Materials and methods

2.1. Experimental design of growth conditions

The winter triticale (\times *Triticosecale* Wittm. var. 'Dimenzio') was grown in the demonstration garden of the University of Debrecen, Debrecen (47° 32' 0" N, 21° 38' 0" E) from autumn 2022 through spring 2023. The triticale seeds were sown using a 4:1 ratio, following standard green forage cultivation practices. The field trial followed a Randomized Complete Block Design (RCBD) with three replications. Each plot covered an area of 2.0 \times 2.5 m², with a 0.5 m row spacing between plots. Sowing occurred in October 2022, and the fresh biomass was collected in May 2023, at the stage when the triticale's vegetative shoots were fully developed but before flowering. The crop was grown under rainfed conditions, with additional nutrients provided during the growth period.

2.2. Processing of fresh green biomass

Triticale's fresh green biomass was harvested early in the morning, during cooler temperatures, to reduce degradation of leaf proteins by proteases. The harvested biomass was immediately transported to the laboratory in an icebox. There, 1 kg of the fresh material was mechanically pressed using a twin-screw juicer (Angel Juicer 5500, Angel Ltd, South Korea) to obtain a protein-rich green juice (GJ). This process also produced a fiber-rich pressed cake as a by-product. The entire procedure was carried out in triplicate. The green juice was then analyzed for its pH (Mettler Toledo S20 Seven Easy pH meter, Switzerland), Brix value (RBR32-ATC manual refractometer, Polling, Germany), and electrical conductivity (EC) (Thermo Scientific Orion 209A + portable conductivity meter, Germany).

2.3. Isolation of soluble proteins in green juice

The thermal coagulation of proteins in green juice was performed in a pulsed, rotating tray microwave device (Samsung, China). The green juice was poured into wide-mouthed beakers and placed in the microwave oven. Based on the literature and our previous experience, 80 + 2 °C is required for the coagulation of cytoplasmic and chloroplast

Table 1

Elution profile of amino acid separation: Solvent A: 100 % AccQ-tag Ultra eluent A; Solvent B: 10 % AccQ-tag Ultra eluent B in LC-MS grade water; Solvent C: LC-MS grade water; Solvent D: 100 % AccQ-tag Ultra eluent B.

Time (min)	Flow Rate (mL.min ⁻¹)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Solvent D (%)
0.00	0.65	10.00	0.00	90.00	0.00
0.29	0.65	9.90	0.00	90.10	0.00
3.50	0.65	9.90	0.00	90.10	0.00
4.60	0.65	9.90	25.00	65.10	0.00
5.49	0.65	9.00	80.00	11.00	0.00
7.10	0.65	8.00	25.00	57.90	9.10
7.30	0.65	8.00	15.60	57.90	18.50
7.50	0.65	8.00	12.00	57.90	22.10
8.20	0.65	7.80	0.00	77.20	15.00
8.30	0.65	4.00	0.00	36.30	59.70
8.55	0.65	4.00	0.00	36.30	59.70
8.60	0.65	4.00	65.00	26.00	5.00
9.20	0.65	4.00	60.00	36.00	0.00
9.70	0.65	10.00	0.00	90.00	0.00
10.90	0.65	10.00	0.00	90.00	0.00

proteins from GJ. Reaching 80 + 2 °C took an average of 9.5-10 min, calculated for a volume of ~770 mL GJ. During this time, we stopped the microwave device 3-4 times and stirred the GJ to ensure homogeneous heat distribution, and checked the temperature rise with a digital thermometer (TFA, Germany). In addition, protein coagulation can be monitored with high accuracy visually. We stopped the process when it reached 80 + 2 °C. The green curd-like fraction containing coagulated protein aggregates was easily filtered through a 35 μ m sieve using vacuum filtration (Fári & Domokos-Szabolcsy, 2018), yielding concentrated green leaf protein (MW-GLPC) and brown juice obtained from green juice (GJ-BJ) (Fig. 1). These experimental conditions were selected in accordance with the established methodologies reported in the previous study of Domokos-Szabolcsy et al. (2022). The pH, Brix, and electrical conductivity (EC) of the GJ-BJ were measured, and the fresh weight of the MW-GLPC was recorded. To determine the dry matter content, the MW-GLPC was freeze-dried (Alpha 1-4 LSC basic freeze dryer, Martin Christ Ltd., Germany), and its dry weight was measured. The freeze-dried samples were then crumbled into a fine powder, sealed in sterile bags, and stored at -20 °C for a maximum of 4 months prior to further analysis (Domokos-Szabolcsy et al., 2022).

The green juice was further processed to obtain a more suitable protein concentrate for human consumption, which was a purified fraction with reduced chlorophyll and anti-nutritional compounds, enhancing its digestibility, amino acid bioavailability, and had a white-yellow color. Firstly the green juice was centrifuged (KR25i floor centrifuge, Jouan SA, France) in 5000 rpm (4330 \times g, at the bottom of the tube) at 4 °C for 20 min to isolate white-yellow colored protein from other biomass residues, resulting a liquid fraction called yellow juice (YJ) and a solid fraction called green pellet (GP). Subsequently, the YJ was precipitated at 80 + 2 °C using a microwave coagulation technique as described above. Then it was filtered through a 35 μ m mesh sieve using vacuum filtration, yielding to yellow leaf protein concentrate (YLPC) and brown juice from yellow juice (YJ-BJ). The YLPC and green pellet were freeze dried and grounded. The fresh weight and the dry weight of the YLPC of the LPC was determined to calculate the dry matter content. They were stored at -20 °C as placed in sterile bags for a maximum of 4 months prior to future applications (Domokos-Szabolcsy et al., 2022).

2.4. Determination of crude protein

The Dumas method was applied to measure the nitrogen, protein, and carbon contents of freeze-dried GJ and LPCs and the Elementar Vario Max Cube Analyzer was used (Elementar Analysensysteme GmbH, Germany). From the lyophilized fractions/product candidates (including GJ, GP, MW-LPC, and YLPC) 250 mg DW while from the liquid GJ-BJ and Y-BJ samples 250 mg FW were placed in a combustion tube., These samples were burned in an oxygen-rich environment, the nitrogen was broke down into nitrogen gas (N₂) and carbon into carbon dioxide gas (CO₂). Nitrogen oxides were transformed into nitrogen gas (N₂) in the reduction tube. At the same time, carbon dioxide was trapped in columns for future measurement. The instrument recorded the nitrogen produced, and after heating the CO₂ columns, it assessed the carbon amount. In the end, the nitrogen level was used to find the protein content by multiplying it by 6.25 to determine the total crude protein (Domokos-Szabolcsy et al., 2023).

2.5. Determination of amino acid composition

The amino acid composition was determined using ultrahigh-pressure liquid chromatography (UHPLC) on a Waters Acquity H-Class Plus UPLC system (Waters, Milford, MA, USA). Preparing samples, 20 mg of powdered sample was placed in a 50 mL digestion tube.

Hydrolysis was performed with 6000 mol m⁻³ HCl in a microwave digestion unit (CEM MARS One, Matthews, NC, USA) for 1 h. Subsequently, the pH of the hydrolyzed sample was adjusted using 6000 mol

m^{-3} NaOH, followed by filtration through a 3 kDa PES membrane filter (VWR International, Radnor, PA, USA).

Amino acid separation was accomplished via AccQTag pre-column derivatization chemistry. The hydrolyzed and neutralized samples were then treated with an AccQ-Tag Ultra derivatization reagent kit (Waters, Millford, MA, USA) in accordance with the manufacturer's protocol (Domokos-Szabolcsy et al., 2022).

Derivatized amino acids were resolved on an AccQ-tag Ultra C18 column (1.7 μ m; 2.1 mm \times 100 mm, Waters, Milford, MA, USA) equipped with an Accquity in-line filter (0.2 μ m; 2.1 mm, Waters, Millford, MA, USA). Chromatographic separation was conducted using a gradient elution (11 min, 0.100 mL min^{-1}) with the following eluents: Eluent A (100% AccQ-tag Ultra eluent A), Eluent B (10% AccQ-tag Ultra eluent B in LC-MS grade water), Eluent C (LC-MS grade water), Eluent D (100% AccQ-tag Ultra eluent B). The column temperature was maintained at 54 °C. Data analysis was performed using Waters Empower 3 software. The elution profile for the UPLC separation was demonstrated in Table 1, following the method described by Domokos-Szabolcsy et al. (2022).

2.6. Determination of protein expression pattern by SDS-PAGE

The alterations in protein expression across the fractions were examined using one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE), based on the study of Domokos-Szabolcsy et al. (2024). For preparing the samples, 10 mg of freeze-dried material was combined with 250 μ L of Laemmli 4x buffer (composition per 100 mL: 25 mL of 1M Tris-HCl, 8 g sodium dodecyl sulfate (SDS), 40 mL glycerol, 0.3 g Dithiothreitol (DTT), and bromophenol blue). The mixtures were agitated by vortexing and heated at 95 °C for 5 min. Subsequently, they underwent centrifugation for 15 min at 13,000 rpm (16060 \times g, at the bottom of the tube) and 4 °C. The resulting supernatant was collected for upcoming analysis.

The SDS-PAGE was conducted using a discontinuous polyacrylamide gel in a vertical electrophoresis setup. The gel was assembled in a Mini-Protean Tetra Cell system (Bio-Rad Inc., Hercules, MI, USA), with a 12.5% resolving gel and a 9% stacking gel. Following electrophoresis, the gels were treated with Coomassie G250 staining solution. Finally, protein bands were visualized and assessed using the BioRad ChemiDoc MP Imaging System.

2.7. Determination of quantitative phytochemical analysis

From the lyophilized sample, 250 mg was extracted with a methanol-water solution (70:30). The mixture was shaken and then subjected to an ultrasonic bath at 21 °C for 2 h in the dark. Afterward, it was centrifuged for 5 min at 6000 rpm (2800 \times g, at the bottom of the tube) and 4 °C. The supernatant was filtered through a 0.22 μ m PTFE filter to prepare the sample for analysis.

Targeted phytochemical quantification followed the methodology outlined by Domokos-Szabolcsy et al. (2023), employing a Dionex UltiMate 3000 UHPLC system coupled with UHPLC-HR-ESI-Orbitrap/MS (Thermo Fisher, Waltham, MA, USA). Compound identification was achieved by cross-referencing molecular weights, retention times, and fragmentation profiles via Thermo Trace Finder 2.1 software. Quantitation was performed using an externally derived calibration curve. The standard compounds used for quantification included: Astragalín (analytical standard), Isovitexin (analytical standard), Riboflavin (analytical standard), Nicotinamide ($\geq 99.5\%$), Nicotinic acid ($\geq 95.0\%$), Kaempferol (analytical standard), Quercetin (analytical standard), Chryseriol (analytical standard), Tricin (analytical standard), Apigenin ($\geq 95.0\%$), Luteolin ($\geq 98\%$), Naringenin (analytical standard), Isoquercitrin (analytical standard), Rutin (analytical standard), p-Coumaric acid (analytical standard), Caffeic acid ($\geq 98\%$), Ferulic acid (USP reference standard), Chlorogenic acid (analytical standard), Neochlorogenic acid (analytical standard), and Cryptochlorogenic acid

(analytical standard).

2.8. Determination of total polyphenol (TPC) and flavonoid (TFC) content

The hydroalcoholic extract which was previously utilized for quantitative phytochemical analysis was also processed for measuring total polyphenols and flavonoids. The supernatants were treated with Folin-Ciocalteu reagent (Singleton et al., 1999). Absorbance was measured at 415 nm for total flavonoid content (TFC) and at 760 nm for total polyphenol content (TPC) using a UV/VIS spectrophotometer (Ultrospec 2100 pro, Amersham BioSciences, UK). The standard curve was obtained using varying concentrations of rutin and gallic acid. The TFC and TPC concentrations in the samples were displayed as milligrams of rutin equivalent (for TFC) and gallic acid equivalent (for TPC) per gram of dry weight.

2.9. Determination of antioxidant capacity

This original technique (Popov & Lewin, 1994) is provided by Analytik Jena AG (Jena, Germany) as a full system named Photochem. The principles outlined in the Photochemiluminescence Assay can be applied to separately measure hydrophilic and lipophilic antioxidants (Nemes et al., 2018). For the lipid-soluble antioxidant capacity, the measurement was done with the lipid-soluble antioxidant capacity (ACL) kit. For the water-soluble antioxidant capacity, the measurement was done with the water-soluble antioxidant capacity (ACW) kit.

2.10. Feeding experiment in the model organism *Drosophila melanogaster*

The nutritional value, potential toxicity and health-promoting effects of yellow leaf protein concentrate (YLPC), green leaf protein concentrate (MW-GLPC) and green pellet (GP) were investigated in an experimental model. *Drosophila melanogaster* possesses thoroughly studied genetics and a short life span, which makes it a suitable model organism. Its metabolic systems have similarities with those of humans, such as insulin signaling, fat metabolism, and interactions between the gut and brain, which makes it highly suitable for investigating physiological changes caused by diet (Neamtu et al., 2020).

The nutritional value (in zero nutrient media), the potential toxicity (in normal media) and the potential anti-inflammatory/antidiabetic effects (in high-sugar media) were evaluated in detail taking into account the survival rate of *Drosophila* in pupal and fly developmental stages.

2.10.1. Media preparation

Media was prepared in three different types of dietary conditions: zero nutrient media; normal media; and high-sugar media. The zero nutrient media was prepared by mixing 1 g of carbon powder with 1 g of agar (VWR, No. 20767.298) in 100 mL of water, then boiling the mixture. After cooling it down to 50 °C, 3 mL aliquots were dispensed into vials. In case of zero nutrient media, there is no nutrients. For the other dietary conditions, 70 g of yeast paste was blended with 1.2 L of water, followed by the addition of either 51.35 g (for normal media) or 513.45 g (for high-sugar media) sucrose and 30 g wheat flour. The mixture was boiled, then 10 g agar powder was inserted and stirred thoroughly. Boiling continued for over 30 min, yielding a final volume of 1 L. The media was cooled in a water bath to 50 °C, then 1 g NIPAGIN (Thermo Fisher, Belgium) was mixed in. Next, 4 mL of this media was aliquoted in each vial. The preparation rate was determined in accordance with the established methodologies reported in previous studies (Aleya et al., 2023; Neamtu et al., 2020).

In the experimental groups; freeze-dried and grounded MW-GLPC, YLPC and GP were added to the media with different concentrations (2.5% and 20%). In the control groups, no additions were made to the basic media. Each concentration application in the experimental groups



Fig. 2. A) Yield of wet fractionation to obtain fresh green leaf protein concentrate (MW-GLPC) and by-products B) Yield of wet fractionation to obtain fresh yellow leaf protein concentrate (YLPC) and by-products. Data are presented as mean \pm SD (n = 3).

and the control group were repeated three times.

2.10.2. Application

Viability assessments were done using flies of the same age and genotype, with all tests conducted in parallel to ensure the comparability of results. All experiments were achieved at 25 °C and constant humidity. The wm4h *Drosophila melanogaster* strain (Bloomington Stock Center, USA) was used. Approximately two hundred 5-day-old male and female wm4h strain *Drosophila melanogaster* flies were placed in an embryo collection cage positioned above a plate containing zero nutrient media with additional yeast paste. Egg collection plates replaced every 2 h. After 48 h, embryos aged between 0 and 2 h were obtained. Under a microscope, embryos were carefully collected using fine forceps and transferred into vials containing zero nutrient media, normal media, or high-sugar media with samples. 50 eggs were placed in each media. These experimental systems were kept in the incubator set at 25 °C and 40-60% relative humidity to complete their development. Every 24 h, the number of surviving pupae and flies was counted individually and the survival rate was reported. The experimental conditions were selected in accordance with the established methodologies reported in the previous study of [Aleya et al. \(2023\)](#).

2.10.3. Calculation of relative viability

Survival rates were used to calculate relative viability for graphical presentation. Relative viability was determined by normalizing each sample to the mean viability of its respective control group which was defined as 100%. All experimental groups are reported as percent change relative to the corresponding control for each experiment ([Aleya et al., 2023](#)).

2.11. Statistical evaluation of the experimental data

Data analysis was performed by using Microsoft Excel 2016 and the SPSS 25.0 software package (SPSS Inc., Chicago, IL, USA). The analysis of variance was performed by using one-way ANOVA among treatments and two-way ANOVA between treatments. Using Tukey's post hoc test, mean separation was done and significant differences were accepted at

$p < 0.05$.

3. Results and discussion

3.1. Wet fractionation of fresh triticale green biomass with yield

Perennial grasses are well known in the green biorefinery as they're environmentally sustainable, they produce a high yield of biomass for bioenergy production, and they reduce the need for costly inputs like fertilizers and water. Triticale, a hybrid of wheat and rye, is not perennial but its relatively low cultivation demands and adaptability provide a unique advantage as an annual crops in biorefining systems. It has emerged as a promising candidate for green biorefinery applications, demonstrating superior potential among annual grasses. Its robust growth and high biomass yield make it an attractive feedstock. Triticale's ability to grow in suboptimal soils and its lower fertilizer requirements compared to traditional cereal crops further reduce environmental impacts and input costs, aligning with the goals of green biorefining. As an alternative to more conventional crops, triticale holds considerable promise in advancing the sustainable agriculture ([Jørgensen et al., 2020](#)).

However, little information is available on the production of triticale leaf protein concentrate ([Pisulewska et al., 1989, 1991](#)). In this study, triticale green fodder was tested for the extraction of concentrated leaf protein. The quality and quantity of concentrated leaf protein obtained were evaluated. [Fig. 2](#) shows the yield of protein concentrates and other by-products per 1 kg compared between two processing methods. After mechanical pressing of triticale's green biomass, the proportions of fiber and green juice (GJ) were found to be 23% and 77%, respectively. This fiber proportion is considered relatively low compared to other crops such as broccoli ([Domokos-Szabolcsy et al., 2022](#)).

Green leaf protein concentrate is typically a protein alternative for feed use, most commonly processed by thermal heat transfer or fermentation. The percentage of fresh MW-GLPC from the triticale green juice was 12%, while the percentage of brown juice (BJ) was 88% ([Fig. 2/A](#)). Compared to percentage of MW-GLPC from broccoli green juice according to the study of [Domokos-Szabolcsy et al. \(2022\)](#), the

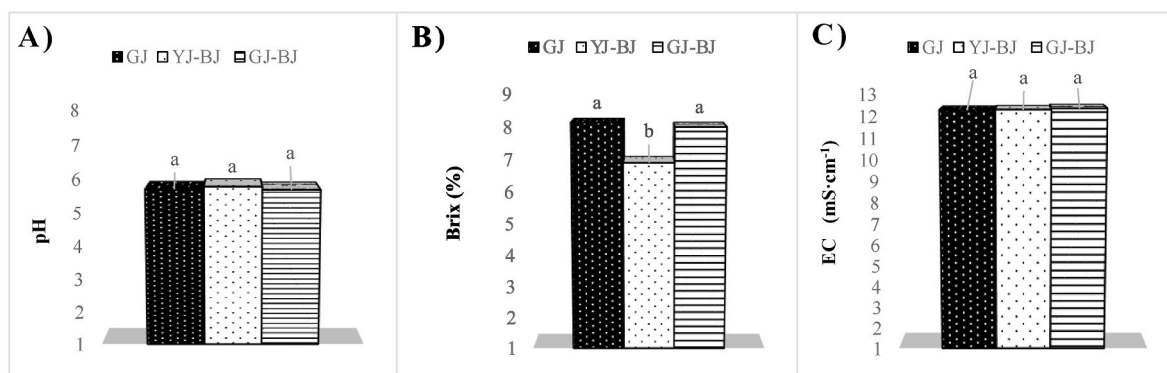


Fig. 3. Relevant physico-chemical parameters of liquid fractions from processed green biomass, including green juice (GJ); brown juice (GJ-BJ) and yellow juice (YJ-BJ): A) pH, B) BRIX, C) EC Data are presented as mean \pm SD ($n = 3$). Different letters in the same rows show significant differences according to Tukey's test at the level of ($p \leq 0.05$).

yield is lower. Alternatively, centrifugation of GJ resulted in green pellet and yellow juice fractions. Treating this yellow juice with microwave coagulation and vacuum filtration produced around a 4.5% yield of fresh yellow leaf protein concentrate (YLPC) from the yellow juice and around a 95.5% yield of yellow-brown juice (YJ-BJ) (Fig. 2/B). This microwave treatment combined with centrifugation was used to get rid of the green coloring and flavour, which is not attractive from a human food point of view. Since only the cytoplasmic proteins make up the LPC, significantly less of it can be collected than from MW-GLPC coagulated in one step. At the same time, membrane, pigment and cell wall bound proteins are retained in the green pellets left by centrifugation. This green fraction is also valuable, it can be suitable for animal feed. Thus, YLPC evaluated together with green pellets could be double purpose intended and higher yield could be realized (Hansen et al., 2023).

The yield of leaf protein concentrate (LPC) changed inversely with the liquid fraction which known as brown juice (BJ). For green biorefinery processes, BJ is typically one of the most abundant by-products, regardless of plant type. In detail, the single-step microwave heating process separated by vacuum filtration resulted in $\sim 5\%$ more initial brown juice (GJ-BJ) (681 g) than the yellow brown juice (YJ-BJ) resulting from combined processing (~ 650 g). Therefore, the biochemical assessment of brown juice as a by-product and the development of value-added products based on this knowledge is essential for the viability of green biorefining.

Among physical and biochemical parameters, pH is a critical issue. The initial pH of the GJ was 5.70, and this value remained stable during the microwave coagulation process (Fig. 3/A). The GJ-BJ obtained directly from microwave coagulated green juice had a pH of 5.68, whereas the YJ-BJ obtained from centrifuged green juice showed a slightly higher pH of 5.78. The results show that microwave heating applied in one step or even in combination did not significantly affect pH. In this pH range of 5.68 to 5.78, the brown juice is perishable. In order to ensure its long-term shelf-life, it is necessary to reduce the pH below 4, which can preferably be achieved by fermentation (Barna et al., 2022). According to research of Bákonyi et al. (2020), fermentation of brown juice by lactic acid bacteria under anaerobic conditions can cause a significant pH reduction.

The brix value of BJ was nearly the same as that of the untreated GJ (8.13% vs. 8.00%) suggesting no significant change in water-soluble sugar content (Fig. 3/B). However, the brix value of YJ-BJ decreased to 6.90%, indicating a decline in soluble-solid concentration. Meanwhile, electrical conductivity, which indicates the total concentration of dissolved salts, remained stable at around 12.30 mS cm^{-1} across all fractions, regardless of the processing method (Fig. 3/C).

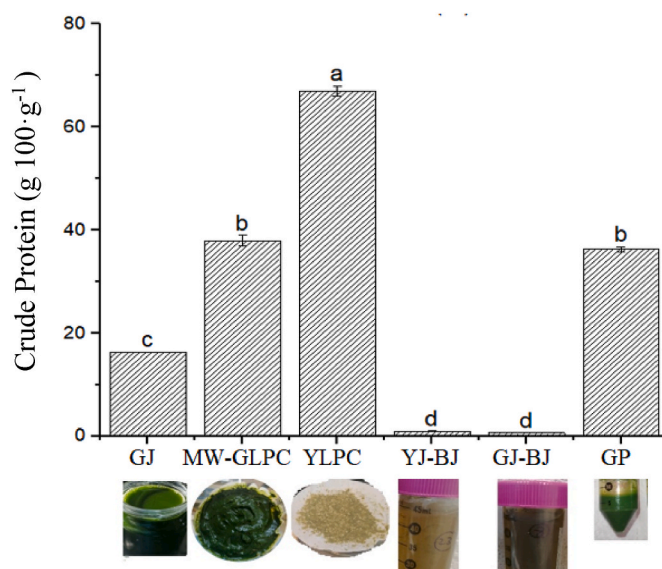


Fig. 4. Crude protein content of fractions that obtained by various processes: freeze-dried green juice (GJ); microwave coagulated green leaf protein concentrate (MW-GLPC); yellow leaf protein concentrate (YLPC); brown juice from yellow juice (YJ-BJ); brown juice from green juice (GJ-BJ); green pellet (GP). For freeze-dried samples, the crude protein content is given in $\text{g } 100 \text{ g}^{-1}$ DW, and for liquid samples, in $\text{g } 100 \text{ g}^{-1}$ FW. Data are presented as mean \pm SD ($n = 3$). Different letters above the columns show significant differences according to Tukey's test at the level of ($p \leq 0.05$).

3.2. Crude protein of fractionated triticale forage

The crude protein content analysis highlights key differences between the fractions obtained from triticale green biomass. After pressing, the raw protein content of freeze-dried green juice (GJ) was $16.38 \text{ g } 100 \text{ g}^{-1}$ DW. Taking into account our previous work with many plant species, it appears that depending on the species the protein content of freshly squeezed GJ varies between ~ 12 and $30 \text{ g } 100 \text{ g}^{-1}$ on dry matter basis in the fresh green juice. For instance, GJ obtained from green biomass of broccoli contained $27.43 \text{ g } 100 \text{ g}^{-1}$ DW, alfalfa contained $26.5 \text{ g } 100 \text{ g}^{-1}$ DW and lettuce contained $17.49 \text{ g } 100 \text{ g}^{-1}$ DW (Domokos-Szabolcsy et al., 2022). Compared to these, triticale showed lower values in the present experiment.

Confirming our results, Cui et al. (2025) measured $10.5\text{--}15.2 \text{ m/m}\%$ raw protein from different triticale genotypes under varying nutrient supply conditions. Microwave coagulation significantly increased the protein yield in the MW-GLPC fraction ($38.44 \text{ g } 100 \text{ g}^{-1}$ DW) compared

Table 2

Amino acid composition of fractions that obtained by various processes of Triticale leaves: freeze-dried green juice (GJ); microwave coagulated green leaf protein concentrate (MW-GLPC) yellow leaf protein concentrate (YLPC). AA means amino acid. DW means dry weight. BDL means below detection limit. Data are presented as mean \pm SD (n = 2). Tukey's test was performed at the level of ($p \leq 0.05$).

	AA g 100 g ⁻¹ DW				AA g·mL ⁻¹	
	GJ	MW-GLPC	YLPC	GP	YJ-BJ	GJ-BJ
NH3	0.275 \pm 0.014	0.458 \pm 0.009	0.878 \pm 0.179	0.426 \pm 0.023	BDL	BDL
His	0.311 \pm 0.013	0.757 \pm 0.001	1.074 \pm 0.067	0.587 \pm 0.005	BDL	BDL
Asn	0.122 \pm 0.024	0.372 \pm 0.008	0.426 \pm 0.230	0.271 \pm 0.062	BDL	BDL
Ser	0.867 \pm 0.026	2.092 \pm 0.036	2.944 \pm 0.001	1.694 \pm 0.056	0.00032 \pm 0.00003	0.00029 \pm 0.00001
Arg	1.432 \pm 0.001	3.271 \pm 0.065	4.320 \pm 0.235	2.851 \pm 0.017	0.00032 \pm 0.00002	0.00023 \pm 0.00002
Gly	0.969 \pm 0.010	2.345 \pm 0.070	3.241 \pm 0.133	1.943 \pm 0.032	0.00036 \pm 0.00001	0.00031 \pm 0.00004
Asp	1.724 \pm 0.044	3.549 \pm 0.083	6.163 \pm 0.693	2.951 \pm 0.192	0.00068 \pm 0.00002	0.00065 \pm 0.00004
Glu	2.089 \pm 0.025	3.562 \pm 0.068	6.147 \pm 0.667	3.036 \pm 0.157	0.00071 \pm 0.00003	0.00069 \pm 0.00007
Thr	0.783 \pm 0.003	1.810 \pm 0.011	2.924 \pm 0.089	1.502 \pm 0.041	0.00028 \pm 0.00001	0.00025 \pm 0.00002
Ala	1.138 \pm 0.017	2.450 \pm 0.046	3.951 \pm 0.365	2.067 \pm 0.145	0.00044 \pm 0.00002	0.00039 \pm 0.00004
Pro	0.819 \pm 0.015	1.912 \pm 0.056	2.877 \pm 0.189	1.676 \pm 0.094	0.00028 \pm 0.00001	0.00026 \pm 0.00002
Cys	0.096 \pm 0.008	0.209 \pm 0.001	0.245 \pm 0.015	0.138 \pm 0.002	BDL	BDL
Lys	1.117 \pm 0.014	1.841 \pm 0.045	4.179 \pm 0.952	1.553 \pm 0.115	0.00042 \pm 0.00002	0.00029 \pm 0.00006
Tyr	0.669 \pm 0.030	1.649 \pm 0.011	2.727 \pm 0.071	1.392 \pm 0.012	0.00022 \pm 0.00001	0.00019 \pm 0.00002
Met	0.192 \pm 0.001	0.733 \pm 0.015	1.250 \pm 0.030	0.564 \pm 0.019	BDL	BDL
Val	0.923 \pm 0.106	2.009 \pm 0.008	4.110 \pm 0.407	1.738 \pm 0.060	0.00037 \pm 0	0.00031 \pm 0.00005
Ile	0.657 \pm 0.081	1.391 \pm 0.002	3.019 \pm 0.406	1.224 \pm 0.041	0.00024 \pm 0	0.00019 \pm 0.00003
Leu	1.340 \pm 0.027	3.181 \pm 0.058	5.212 \pm 0.615	2.834 \pm 0.130	0.00044 \pm 0.00002	0.00038 \pm 0.00004
Phe	0.834 \pm 0.034	2.239 \pm 0.023	2.997 \pm 0.091	1.868 \pm 0.016	0.00026 \pm 0.00001	0.00022 \pm 0.00002
SUM AA	14.805	35.827	60.183	30.315	0.0056	0.0049

to the raw green juice (16.38 g 100 g⁻¹ DW), demonstrating its effectiveness as a thermal coagulation method (Fig. 4). GJ is a heterodisperse system containing partly dissolved proteins and partly proteins that have remained bound to membrane/cell wall fragments that have passed through the squeeze plate during pressing. Heat coagulation, including conventional heat transfer or the use of microwaves, is effective in concentrating these dissolved and membrane/cell wall-bound proteins. Depending on the plant species, raw protein in LPC can reach up to 46 g 100 g⁻¹ DW (alfalfa) or 50 g 100 g⁻¹ DW (ryegrass) as compiled by Domokos-Szabolcsy et al., 2023. Another fractionation of green juice, in which a centrifugation step was inserted before the microwave coagulation, resulted in a significantly higher (67.22 g 100 g⁻¹ DW) yellowish concentrated leaf protein (YLPC) fraction. During centrifugation, the supernatant contained only dissolved proteins, from which significantly less coagulum could be obtained. However, YLPC is a more concentrated, purer protein fraction, which is desirable for food purpose utilization. In addition, during centrifugation, a significant amount of proteins bound to photosynthetic pigments, membranes, and cell wall debris remained in the pellet. This was confirmed by the measurement of 36.51 g 100 g⁻¹ DW crude protein in the GP, which is

similar to the value obtained with MW-GLPC coagulated in one step. Even though its green color and green taste may be unfavourable by human, GP can be used as feed due to its high protein content. The detailed protein recovery data is presented in Supplementary Table 1.

The liquid by-products YJ-BJ (1.06 g 100 g⁻¹ FW) and GJ-BJ (0.73 g 100 g⁻¹ FW) retained minimal protein, confirming successful extraction into solid fractions but yielding nutritionally dilute residues. These results emphasize microwave coagulation's role in protein recovery, while the superior protein content in YLPC points to the effect of combining centrifugation with microwave treatment. The centrifugation process effectively pre-concentrate the proteins before microwave coagulation further aggregates them.

3.3. Amino acid composition

The amino acid composition of fractions derived from the green biomass of triticale are shown in Table 2. The total amino acid concentration varied proportionally with the crude protein content of each fraction, showing slightly lower values. This indicates that most of the nitrogen measured by the Dumas method was incorporated into true protein. The true protein content, which represents the summarized amino acid content, was 14.82 g·100 g⁻¹ in the case of GJ (Table 2.). In comparison, we found more than two fold higher values in MW-GLPC (35.827 g·100 g⁻¹). The highest crude protein content according to Dumas was 67.218 g·100 g⁻¹ in YLPC (Fig. 4), correlating to the true protein content of 60.21 g·100 g⁻¹. The results show that microwave coagulation combined with centrifugation was able to achieve a 4-fold concentration of protein. In addition, in the GP side fraction remaining during the extraction of YLPC, 30.315 g·100 g⁻¹ total sum AA is concentrated, which is similar to the value measured in MW-GLPC. Essential amino acids represents 48–51% of the total sum amino acids in green juice and various LPCs. Some of these amino acids originate from the Rubisco enzyme protein, while others come from membrane and pigment-bound proteins. The relatively high proportion of essential amino acids found in protein concentrates indicates an adequate essential amino acid supply for monogastric animals and is comparable to the ratio of essential amino acids in soybeans (47–48%) as mentioned Santamaría-Fernández and Lübeck (2020). Overall, the product candidates from green biomass show a similar essential amino acid profile despite the method of protein extraction and concentration, which is advantageous for the commercialization of the product, whether for feed or food purposes.

The amount of each amino acid varied proportionally in relation to the total amino acids. Among the essential amino acids, Lys, as a limiting AA, is of particular importance in monogastric animals. In MW-GLPC made from triticale, 1.8 g 100 g⁻¹ Lys could be measured, while in YLPC it was 5.179 g·100 g⁻¹ the most abundant EAA after Leu. Our results are comparable to those of Santamaría-Fernández et al. (2019), measured 2.2 g·100 g⁻¹ Lys in Trifolium LPC and 2.6 g·100 g⁻¹ in soybean. Moreover, the Lys content of YLPC intended for food use, is almost two fold higher that of soybean. This is beneficial because grass grains are generally considered to be low in lysine (Leinonen et al., 2019). Methionine is the other limiting amino acid in monogastric animals, such as poultry, due to its involvement in one-carbon (1C) metabolism, feather production, and muscle development (Conde-Aguilera et al., 2025). Met also plays a vital role in various human cellular processes (Navik et al., 2021). In case of triticale the two-step protein concentration process resulted in a 6.5-fold increase in the YLPC (1.25 g·100 g⁻¹) Met concentration compared to the pressed GJ (0.192 g·100 g⁻¹). A study by Kaszás et al. (2022) showed that the Met content of soybeans is relatively low, g 100 g⁻¹. Thus, the Met level in YLPC is noteworthy, as it is a nutritionally limiting amino acid (Lam & De Lumen, 2003). Microwaves applied in one step or in combination with centrifugation preferentially coagulated the green juice proteins, as little actual protein was transferred in the brown juice remaining after vacuum filtration. For instance, GJ-BJ and YJ-BJ values of 0.0049 and

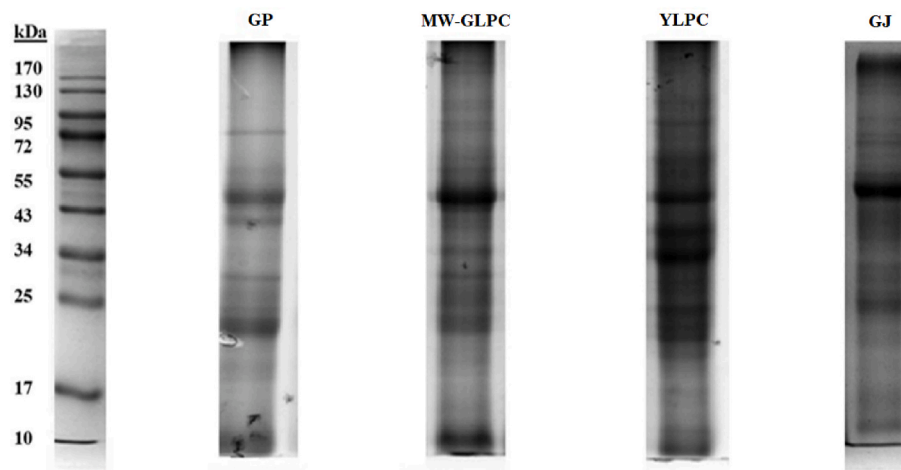


Fig. 5. Protein pattern as SDS-PAGE of the fractions: green pellet (GP); microwave coagulated leaf protein concentrate (GLPC); yellow leaf protein concentrate (YLPC); freeze-dried green juice (GJ).

Table 3

Molecular weight (M.W.) distribution of polypeptides/oligopeptides in: freeze-dried green juice (GJ); microwave coagulated green leaf protein concentrate (MW-GLPC); yellow leaf protein concentrate (YLPC); green pellet (GP); brown juice from yellow juice (YJ-BJ); brown juice from green juice (GJ-BJ).

GJ		MW-GLPC		YLPC		GP	
M.W. (Da)	Conc. (mg·g ⁻¹)	M.W. (Da)	Conc. (mg·g ⁻¹)	M.W. (Da)	Conc. (mg·g ⁻¹)	M.W. (Da)	Conc. (mg·g ⁻¹)
810.52	5.42	811.46	2.04	808.02	3.15	811.14	1.92
416.64	1.46			350.51	0.81	347.14	0.67
348.49	1.56			180.03	0.44	181.29	0.51
183.40	0.25						

GJ-BJ		YJ-BJ	
M.W. (Da)	Conc. (mg·mL ⁻¹)	M.W. (Da)	Conc. (mg·mL ⁻¹)
789.84	46.54	709.01	43.66
710.88	54.29	699.19	51.38
534.96	18.27	451.27	26.59
465.95	21.69	292.50	19.05
300.97	16.03	259.43	20.47
267.56	16.05	181.43	7.34
185.46	7.10		

0.00567 g mL⁻¹ were obtained, respectively.

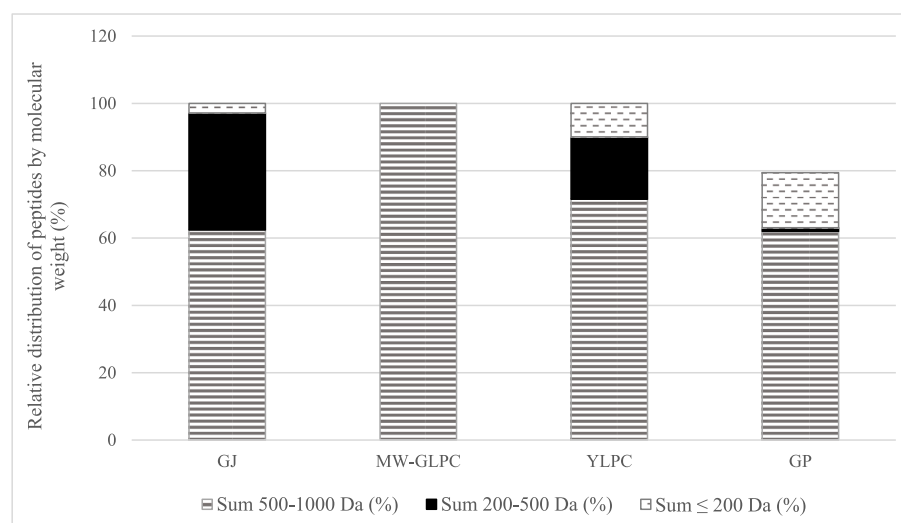


Fig. 6. Relative distribution of peptides by molecular weight in: freeze-dried green juice (GJ); microwave coagulated green leaf protein concentrate (MW-GLPC); yellow leaf protein concentrate (YLPC); green pellet (GP). Tukey's test was performed at the level of ($p \leq 0.05$).

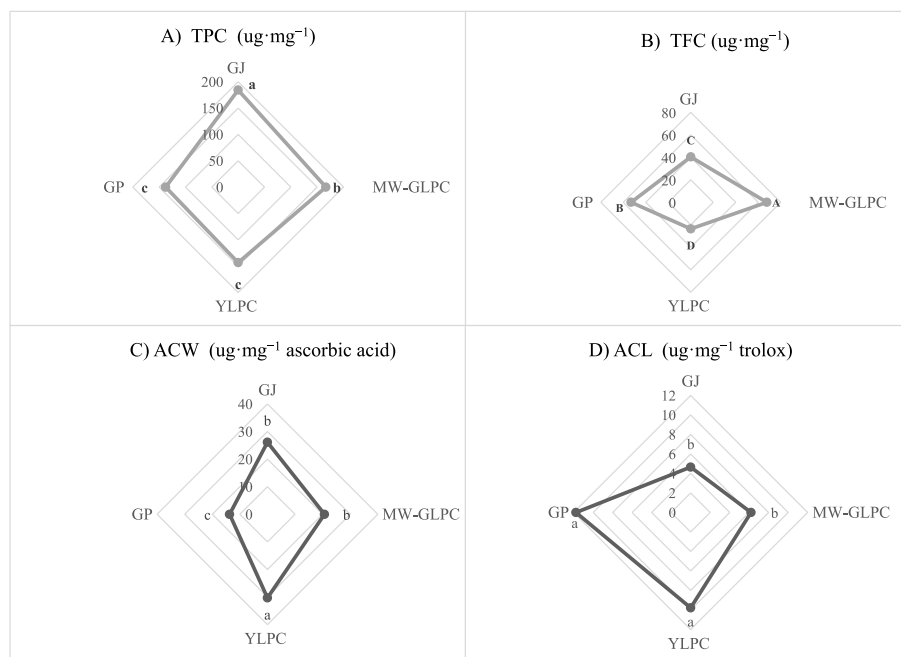


Fig. 7. Quantification of phenolics, flavonoids, and antioxidant capacity: A) total polyphenol content (TPC), B) total flavonoid content (TFC) C) Water-soluble antioxidant capacity (ACW), D) Lipid-soluble antioxidant capacity (ACL). freeze-dried green juice (GJ); microwave coagulated green leaf protein concentrate (MW-GLPC); yellow leaf protein concentrate (YLPC); green pellet (GP). Different letters on points are significant according to the Tukey's test at ($p \leq 0.05$).

3.4. Protein pattern by SDS-PAGE

The soluble protein profile in a fraction is significantly affected by the subsequent protein concentration process. SDS PAGE analysis clearly shows differences in the protein expression patterns between various LPC and GJ and GP (Fig. 5). GJ exhibited the highest number of bands, reflecting its high soluble protein content. In contrast, GP displayed the fewest visible bands. The number of visible bands in MW-GLPC was a result of coagulated protein caused by the heat treatment (Domokos-Szabolcsy et al., 2022).

In C3 plants, highly abundant proteins like the Rubisco large subunit (55 kDa) and small subunit (14 kDa) are more prone to being solubilised. In a study, it's been reported that the thermal stability of Rubisco subunits differs among plant species, likely due to variations in their protein environment (Nynäs et al., 2021). Comparing results of this study, YLPC obtained from tritikale exhibited more visible bands than white protein obtained from broccoli within the Rubisco large subunit and small subunit range. On the other hand among all fractions, GJ showed the most visible bands within the ~ 43 kDa to ~ 55 kDa range.

3.5. Peptides separation by SEC-HPLC

Below, the molecular weight distribution of polypeptides/oligopeptides smaller than 10 kDa in the different triticale fractions was demonstrated in Table 3. In untreated freshly pressed and lyophilized green juice, peptides were detected at a concentration of 8.69 mg g^{-1} . Of these, 810 Da oligopeptides were the most abundant, accounting for 62.4% (Fig. 6). Among the leaf protein concentrates obtained by microwave coagulation, we could not exceed 4.4 mg g^{-1} of total peptides in yellow LPC, while only 2.2 mg g^{-1} in green LPC with a maximum of three peaks. By contrast, in the aqueous fraction remaining after coagulation, such as brown juice and yellow juice, 6-7 oligopeptide peaks could be obtained with concentrations between 168 and $180 \mu\text{g mL}^{-1}$. At least 56.41% of these peptides belong to the 500-1000 Da molecular weight range, with the rest (29.88 - 39.23%) in the 200-500 Da range (Fig. 6). According to our standard mixtures, oligopeptides in the 200-1000 Da molecular weight range contain 2-7 amino acid units. Fractions

from the green biomass of triticale, mainly brown and yellow juice, contain such oligopeptides. The presence of oligopeptides in brown juice as by-product has been reported in several studies (Barna et al., 2022; Kaszás et al., 2024), but to our knowledge, we confirm it for the first time by SEC-HPLC in the present work. In addition, a peak molecular weight of ~ 180 Da was observed in all fractions except green LPC, which, considering the average molecular weight of amino acids (110 Da), is likely to be a free amino acid or dipeptide.

3.6. Quantification of phenolics, flavonoids, and antioxidant capacity

GJ exhibited the highest polyphenol concentration $184.26 \mu\text{g g}^{-1}$, indicating a superior presence of bioactive phenolic compounds compared to the others (Fig. 7/A). This suggests that GJ may possess enhanced antioxidant properties. MW-GLPC also demonstrated a relatively high TPC value $166.52 \mu\text{g g}^{-1}$; supporting its potential as a valuable source of polyphenols. Conversely, samples YLPC and GP showed comparatively lower polyphenol levels.

MW-GLPC showed the highest flavonoid concentration at $67.72 \mu\text{g g}^{-1}$, suggesting a richer presence of these bioactive compounds relative to the others (Fig. 7/B). Flavonoids are well-known for their antioxidant, anti-inflammatory, and antimicrobial activities, thus MW-GLPC may hold greater potential due to its flavonoid concentration (Zehiroglu & Ozturk-Sarikaya, 2019).

YLPC exhibits the highest ACW value at $30.28 \mu\text{g}\cdot\text{mg}^{-1}$ ascorbic acid, indicating the strongest water-soluble antioxidant potential among the samples (Fig. 7/C). Close behind is GJ with an ACW of $26.11 \mu\text{g}\cdot\text{mg}^{-1}$, which still shows substantial antioxidant. MW-GLPC follows representing a mid-level antioxidant capacity. In contrast, GP has the lowest ACW at $13.72 \mu\text{g}\cdot\text{mg}^{-1}$, less than half that of YLPC, indicating a significant amount of water-soluble antioxidants stayed in the yellow juice after the centrifuge process.

GP showing the highest capacity at $11.76 \mu\text{g}\cdot\text{mg}^{-1}$ trolox, indicating the strongest presence of lipid-soluble antioxidants (Fig. 7/D). This considerable value suggests that, compared to others, GP is particularly rich in compounds that protect cell membranes and lipids from oxidative damage (Zehiroglu & Ozturk-Sarikaya, 2019). YLPC follows with a

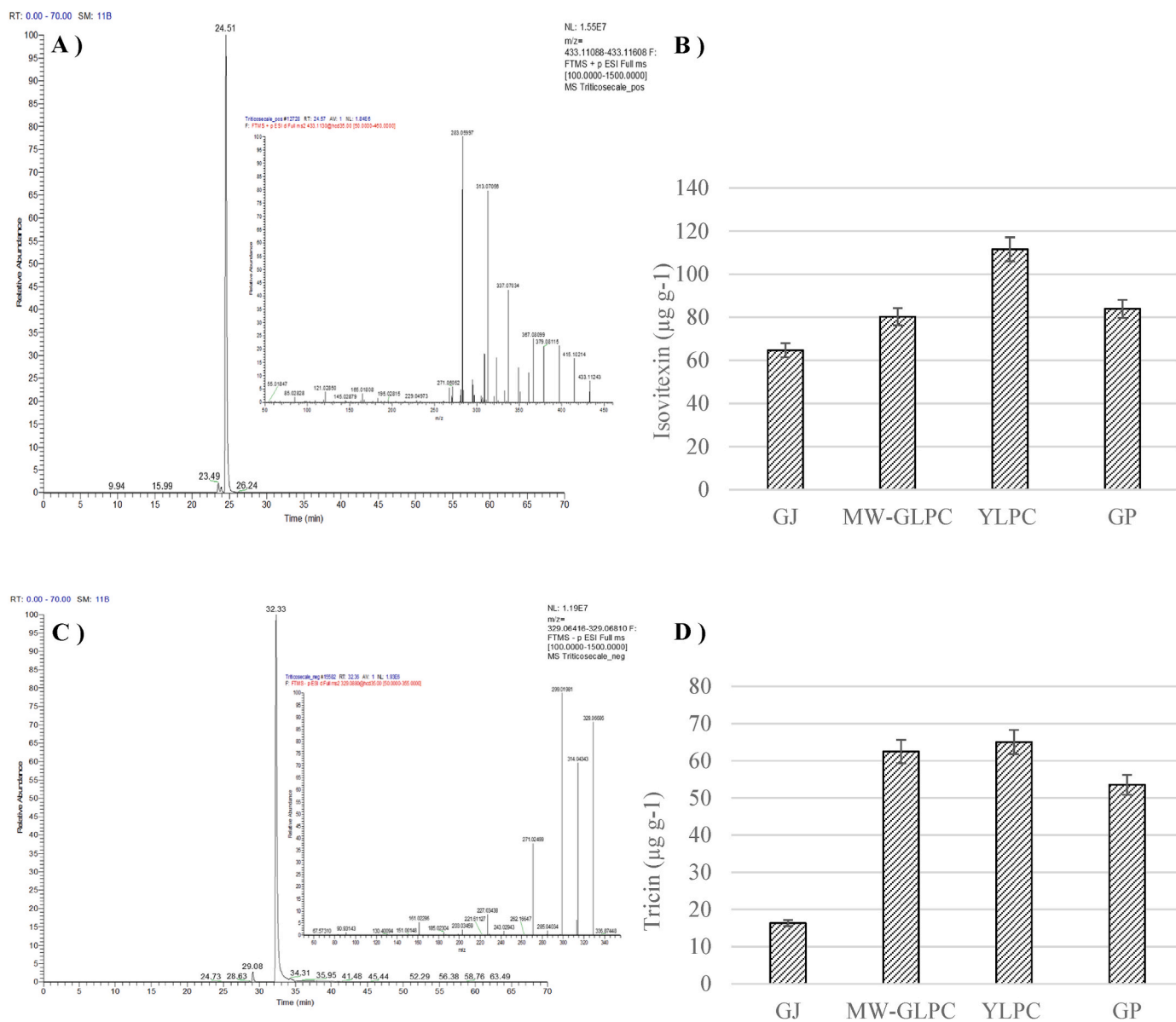


Fig. 8. Identification and quantitative changes of flavonoids in fractions from green biomass of triticale; A) Ion chromatogram and fragmentation spectrum of Isovitexin; B) Isovitexin in triticale green juice and leaf protein concentrates processed from it; C) Ion chromatogram and fragmentation spectrum of Tricin; D) Tricin in triticale green juice and leaf protein concentrates processed from it; E) Ion chromatogram and fragmentation spectrum of Chrysoeriol; F) Chrysoeriol in triticale green juice and leaf protein concentrates processed from it; G) Ion chromatogram and fragmentation spectrum of Luteolin; H) Luteolin in triticale green juice and leaf protein concentrates processed from it. Data are presented as mean \pm SD ($n = 3$). Tukey's test was performed at the level of ($p \leq 0.05$).

notable ACL of $9.78 \mu\text{g}\cdot\text{mg}^{-1}$, also reflecting substantial lipid antioxidant potential. MW-GLPC and GJ have lower ACL values, 6.19 and $4.66 \mu\text{g}\cdot\text{mg}^{-1}$ respectively.

3.7. Evaluation of bioactive components

The green juice (GJ) pressed from the triticale is a rich source of flavonoids. UHPLC-ESI-ORBITRAP-MS/MS was used to identify 39 flavonoid compounds based on specific retention time, accurate mass, isotopic distribution and fragmentation pattern MS (data not shown). Among these, the quantitative changes of 11 flavonoid aglycone and sugar derivatives were monitored, taking into account the processing method of green juice (Fig. 8).

In the GJ, apigenin aglycone was found at concentration of only 0,2

$\mu\text{g g}^{-1}$. Interestingly isovitexin (6-C glucoside derivative of apigenin), was present in the highest amount at $64.7 \mu\text{g g}^{-1}$ (Fig. 8/A; B). Isovitexin is known in wheatgrass and barley (Wang et al., 2020), but to our knowledge this is the first report of its presence in triticale. Our results showed that the concentration of isovitexin in GJ could be further increased along with protein extraction processes. For instance, the concentration of isovitexin showed the highest increase of more than 1.7-fold over YLPC ($111.6 \mu\text{g g}^{-1}$) compared to GJ, but significant increase was also found in the GP ($83.9 \mu\text{g g}^{-1}$) remaining after centrifugation of GJ (Fig. 8/B).

According to Lam et al. (2023), soluble flavone aglycones and their O-conjugates, such as apigenin, luteolin, chrysoeriol, and triclin are constitutive metabolites in grass green leafy shoots. In line with this, triclin, luteolin and chrysoeriol were identified in relatively higher

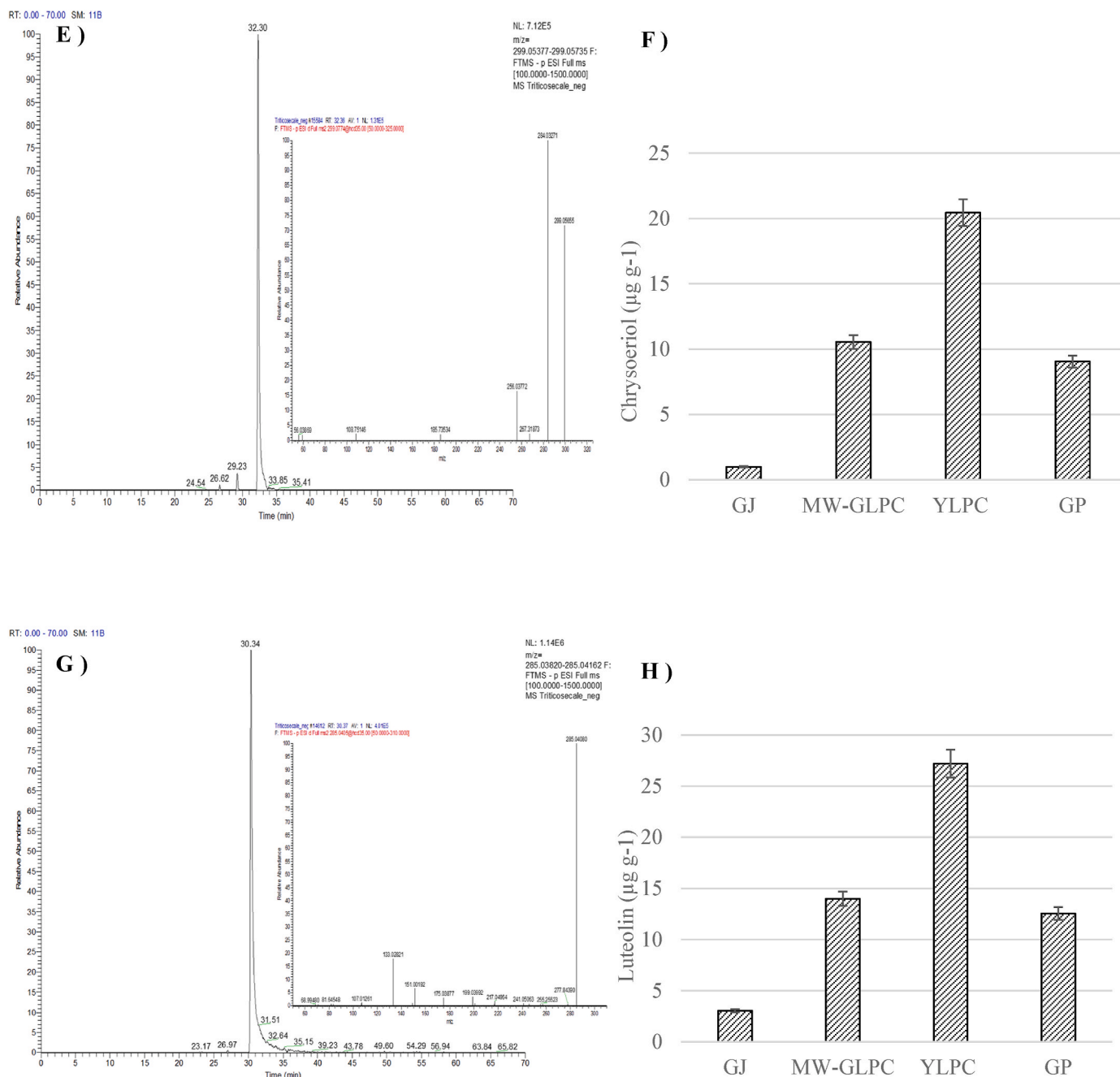


Fig. 8. (continued).

concentration in GJ (Fig. 8/C; E; G).

Tricin (5,7,4'-trihydroxy-3',5'-dimethoxyflavone) and its conjugates are known to accumulate in most cereal crops, in varying concentrations depending on the species (Zhou & Ibrahim, 2010). Indeed, Salcolin A (Tricin-4'-O-(erythro- β -guaiacylglyceryl)ether); Tricin-7-O-glucuronyl glucoside; Salcolin B (Tricin-4'-O-(threo- β -guaiacylglyceryl)ether); Tricin-O-hexosylhexoside and an unknown Tricin-O-hexoside were identified from the pressed GJ of triticale in addition to tricrin aglycone (data not shown). Of these, tricrin aglycone was detected at 16.35 $\mu\text{g g}^{-1}$ in GJ (Fig. 8/D), but increased more (3.8-fold; 4-fold) in MW-GLPC (62.5 $\mu\text{g g}^{-1}$) and YLPC (65.05 $\mu\text{g g}^{-1}$) after microwave coagulation and after combined treatment of green juice with centrifugation and microwave coagulation.

A similar trend was observed for changes in chrysoeriol and luteolin concentration in line with the protein concentration methods used, with

max values of 20.45 $\mu\text{g g}^{-1}$ and 27.2 $\mu\text{g g}^{-1}$ in YLPC, respectively (Fig. 8/F; H). The variation in the concentrations of these flavonoid components also shows a clear correlation with the crude protein content. The different sampling techniques and various treatments of the raw materials might explain these differences.

In terms of minor flavonoids, MW-GLPC has the highest amount of apigenin (5.25 $\mu\text{g g}^{-1}$) compared to other fractions (Fig. 8/I). MW-GLPC's apigenin content highlights its nutritional potential since apigenin is a health-promoting flavone which may offer antioxidant properties (Lam et al., 2023). The highest concentration of rutin, which is a flavonol glycoside, observed in YLPC (4.25 $\mu\text{g g}^{-1}$). According to the findings of Ganguly et al. (2025), rutin demonstrated anti-diabetic effects in *Drosophila melanogaster*, which is consistent with results of the present study.

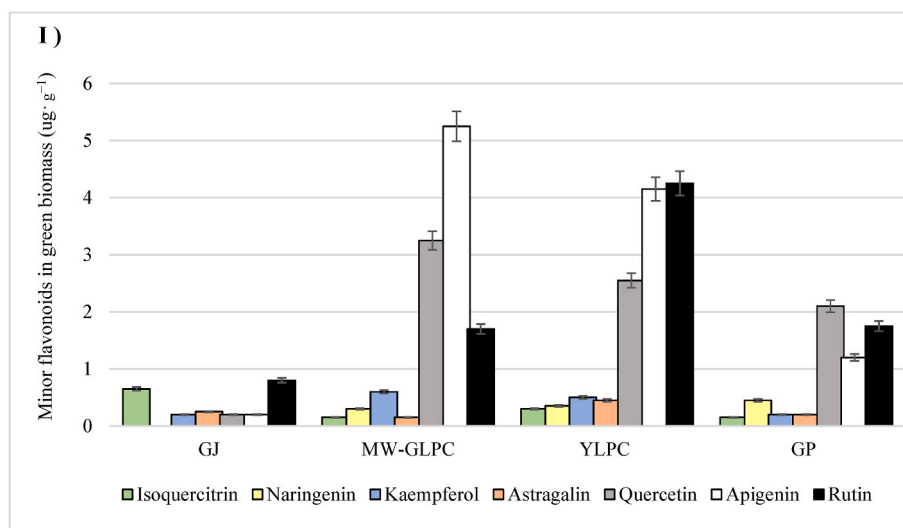


Fig. 8. (continued).

3.8. Nutritional and biofunctional effects in *Drosophila melanogaster*

Drosophila melanogaster has well-characterized genetics and a short life cycle which makes it a good model organism. Its metabolic pathways share remarkable common points with humans, including insulin signaling, lipid metabolism, and gut-brain communication, making it a relevant model for studying diet-induced physiological changes. The survival rates of *Drosophila melanogaster* pupae and flies were assessed under three different nutrient media — zero, normal, and high-sugar — each supplemented with varying concentrations (2.5% and 20%) of the processed triticale biomass fractions (MW-GLPC, YLPC, GP). Relative viabilities of the experimental groups were expressed as percent change relative to the control group which was defined as 100% (Aleya et al., 2023).

Survival rate measurement in zero nutrient media was carried out to investigate nutritive potentials of the samples. The survival rate in normal media was measured to determine whether the samples possess any level of toxic effects (Alaraby et al., 2020).

By measuring the survival rate of *Drosophila* in high-sugar media, we aimed to investigate whether YLPC (for food purposes) and MW-GLPC and GP fractions (for feed purposes) have a positive effect in the high-sugar containing environments (Musselman et al., 2019).

3.8.1. Effect of MW-GLPC on *Drosophila* survival under different nutrient supply conditions

Due to its mild green color, odor, and taste, MW-GLPC is primarily intended as a protein alternative for feed applications (Santamaría-Fernández & Lübeck, 2020). The nutritional value of MW-GLPC was assessed in zero nutrient media (data not shown). No surviving individual was observed in the control group of zero nutrient media. This was not changed by MW-GLPC which was added to the media at a concentration of 2.5%. However, at concentration of 20% MW-GLPC, the survival rate increased markedly, an average of 25.33 pupa individuals and 18.33 fly individuals survived. This indicates that MW-GLPC, at high concentrations, can partially compensate for the lack of nutrients.

In normal media with balanced nutrient supply, the potential toxicity of MW-GLPC can be inferred by varying its supplementation rates. Adding MW-GLPC in a concentration of 2.5%, slightly improved both pupa and fly metrics with a rise of 1.85% and 10.42%, respectively (Fig. 9/A). At 20% concentration, MW-GLPC resulted in a higher survival rate increasing by 20.37% for pupae and 22.92% for flies. This suggests that MW-GLPC is not toxic in standard conditions and may even have supportive effects at higher concentrations which aligns with the

results of zero nutrient media.

To observe the diabetic complications in *Drosophila*, a high-sugar diet is applied to individuals; which has several adverse effects on growth, metabolism, glucose homeostasis, cardiac function and lifespan (Van Dam et al., 2020). At 2.5% concentration of MW-GLPC, survival rate increased slightly by 4.34% for pupa and 12.28% for fly, showing a mild protective effect (Fig. 9/B). However, at 20% concentration, both pupa and fly survival rates dropped below control group levels.

This finding aligns with literature on *Drosophila* metabolic responses to high-sugar diets, where increased-sugar disrupts insulin signaling, shortens lifespan and decrease survival rate (Neamtu et al., 2020).

3.8.2. Effect of YLPC on *Drosophila* survival under different nutrient supply conditions

YLPC fraction, obtained by centrifugation (that eliminates undesirable green color, odor, and taste) and microwave coagulation, may serve as a protein-rich food supplement. It may also be beneficial due to its various phenolic components, which was present at higher levels in YLPC compared with the other fractions. In zero nutrient media with 2.5% concentration of YLPC, neither the pupae nor the flies survived (data not shown). Nonetheless, the 20% concentration of YLPC enabled a significant increase in both pupa and fly survival rates, with average numbers of 18 and 15.67 individuals, respectively. This indicates that YLPC at higher concentrations may provide nutritional support partially in the absence of standard nutrients.

In case of normal media, viability varied with YLPC concentration. At 2.5% concentration of YLPC, number of pupae and flies didn't increase compared to the control (Fig. 9/C). However, 20% concentration of YLPC led to a remarkable increase in survival, rising by 42.59% pupae and 25% flies. The initial decrease in survival rate at a low YLPC concentration (2.5%) compared to the control may indicate suboptimal nutrient interactions that cause inhibitory effects in the organismal physiology at this dose (Aleya et al., 2023). However, at a higher concentration (20%) it promotes survival rate, potentially by providing beneficial components such as isovitexin. Besides its high protein content, YLPC has a remarkable concentration of isovitexin. According to the findings of Tao et al. (2023), isovitexin promotes healthspan upregulating antioxidant genes and enhancing anti-stress capacity.

In case of high-sugar media, survival rates of pupae were consistently higher in all YLPC-supplemented groups compared to the control. The relative viability at 2.5% concentration of YLPC increased by 8.69% for pupae and 8.77% for flies (Fig. 9/D). At 20% concentration of YLPC, the viability increased by 11.59% and 10.52%, respectively. The results indicate a positive effect of YLPC on survival of *Drosophila* in case of a

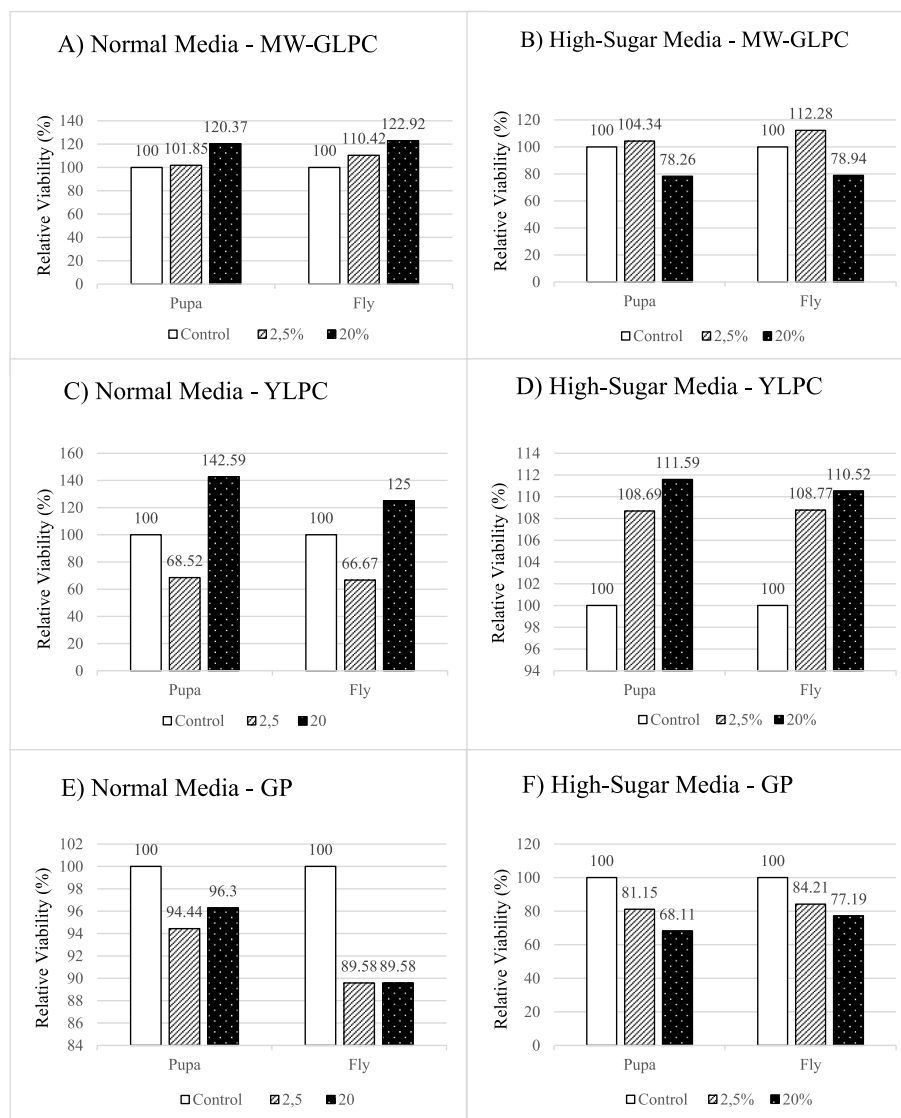


Fig. 9. A) Relative viability in Normal media with MW-GLPC, B) Relative viability in Normal media with YLPC, C) Relative viability in Normal media with GP, D) Relative viability in High-sugar media with MW-GLPC, E) Relative viability in High-sugar media with YLPC, F) Relative viability in High-sugar media with GP. Data are presented as mean \pm SD ($n = 3$). Tukey's test was performed at the level of ($p \leq 0.05$). The control groups were used as the reference value and all results were expressed relative to the control groups which were assigned as 100%.

high-sugar diet, which might be related to its high flavonoid content, such as luteolin. Luteolin has been reported to exhibit anti-inflammatory and anti-diabetic effects in humans (Maryam et al., 2024).

The highest concentration of rutin among the fractions, was found in YLPC as well. Ganguly et al. (2025), demonstrated that rutin administered with a high-sugar diet reduces oxidative-stress and alleviates the adverse health effects induced by excessive sugar intake in *Drosophila*.

Our results are consistent with these prior findings by demonstrating that YLPC improved the survival under high-sugar diet conditions, potentially due to its bioactive contents. While our findings highlight its potential as an antidiabetic dietary supplement, further investigation is required to confirm it. Comprehensive metabolic analyses including assessment of glucose or trehalose concentrations, triglyceride levels, and insulin signaling activity are necessary to determine whether YLPC directly affects glucose homeostasis and metabolic pathways (Ben El Caid et al., 2025; Van Dam et al., 2020).

3.8.3. Effect of GP on *Drosophila* survival under different nutrient supply conditions

GP is a pellet formed during the centrifugation of GJ which contains

solid particles that have passed through the press jacket during pressing. This fraction contains high amounts of proteins bound to pigments and membranes as confirmed by protein quantitative measurements. Due to its strong green color and taste, GP is not attractive as food. But it may be acceptable as feed, so it is important to investigate its nutritional value. In case of zero nutrient media, with 2.5% GP concentration, pupa and fly survival were nearly absent. Minimal survival was observed at the highest concentration (20%) of GP, indicating that GP provides limited nutritional value, only apparent at high concentrations (data not shown). These results suggest that although GP contains macronutrients and phytochemical compounds, it is not sufficient as a stand-alone nutrient source unless provided in large quantities.

In case of normal media, all GP concentrations resulted in slightly lower survival rates than the control group (Fig. 9/E). Supplementation with 2.5% and 20% GP produced slightly reduced number of pupae and flies. These findings indicate that GP does not enhance the survival. The mild reduction at low and high concentrations suggests a neutral or slightly inhibitory effect, which is potentially due to interference with normal dietary balance or metabolic processing (Aleya et al., 2023).

In case of high-sugar conditions GP did not confer a beneficial effect,

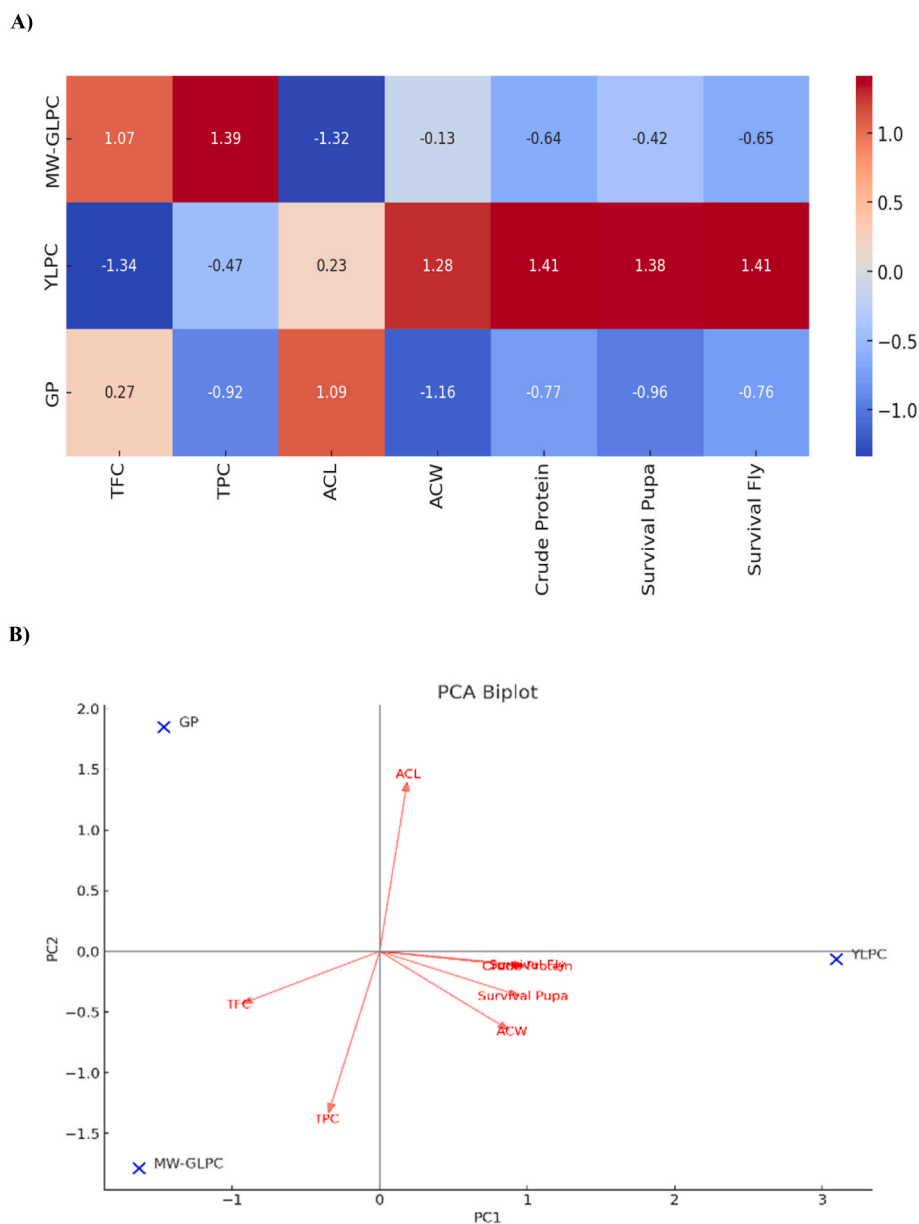


Fig. 10. A) Heatmap of principal component analysis. TFC: Total Flavonoid Content, TPC: Total Phenolic Content, ACL: Lipid-soluble Antioxidant Capacity, ACW: Water-soluble Antioxidant Capacity (Hydrophilic), Survival (H.S.): Survival rate in high-sugar media with 20% concentrate of sample. MW-GLPC: Microwaved green leaf protein concentrate. YLPC: Yellow leaf protein concentrate, . GP: Green pellet. B) Biplot of principal component analysis. PC1: component indicating nutritional/biological strength driven by Crude protein and Survival rate (biological value) vs. TFC/TPC (bioactive load) PC2: antioxidant capacity based on ACW and ACL.

contrary to YLPC. In fact, survival decreased in a dose-dependent manner with increasing GP concentration (Fig. 9/F). This result suggests that some substances in GP may exacerbate the negative physiological effects of a high-sugar diet, particularly at higher concentrations (Neamtu et al., 2020).

Overall survival results showed that YLPC in high concentrations, such as 20%, had the most beneficial effects compared to MW-GLPC and GP in nutrition. While MW-GLPC and GP have no positive impacts in high-sugar environment, positive impacts of YLPC propound it as a promising food ingredient with health-promoting properties, particularly in case of high-sugar systems. Nevertheless, further metabolic analyses are required to clarify whether YLPC improves metabolic regulation rather than exerts a general stress-protective effect.

3.9. Principal component analysis

PC1 primarily represents a gradient of nutritional and biological strength, with positive loadings dominated by variables such as crude protein content and survival rate of pupa, reflecting overall nutritional value; while the negative loadings on PC1 are mainly associated with TFC and TPC, which indicate higher bioactive compound load (Fig. 10/A). On the other hand, PC2 reflects variation in antioxidant capacity, largely driven by ACL and ACW (Fig. 10/B).

The positioning of the samples shows clear differentiation among treatments. YLPC is positioned at the right along PC1, suggesting superior nutritional and biological attributes, particularly in protein content and survival rates. In contrast, MW-GLPC and GP cluster toward the negative side of PC1, indicating higher levels of bioactive components but comparatively lower nutritional value indicators, particularly in survival metrics of model organism.

The positive correlations along PC1 shows that YLPC has a high biological value. This trend suggests that YLPC contributes effectively to viability of the model organism, supporting its potential as a valuable food ingredient. The feeding experiment is consistent with this result as the YLPC group showed the highest survival rate in high-sugar conditions.

4. Conclusion

This study investigates the transformation of triticale green biomass, which in its native form possesses limited nutritional value, into value-added extraction products through different processing steps involving centrifugation and microwave coagulation. These processes significantly enhanced the concentration and availability of protein and bioactive compounds.

The centrifugation step facilitated the separation of soluble and insoluble fractions, while the microwave coagulation step enhanced protein denaturation and aggregation. As a result, yellow leaf protein concentrate (YLPC) was obtained to evaluate as a promising food ingredient; whereas microwave coagulated green leaf protein concentrate (MW-GLPC) and green pellet (GP) showed a higher potential as a nutrient-rich animal feed rather than food, due to their green color and odor.

Specifically YLPC demonstrated higher levels of some components, such as flavonoids and phenolic compounds which possess antioxidant and anti-inflammatory activities. Moreover, YLPC contained a high amount of protein which is an essential macronutrient involved in tissue repair, enzymatic function, and metabolic regulation. These characteristics of YLPC strengthen its potential for functional food applications.

In conclusion, combined processing methods can enhance the protein yield of green biomass by transforming it into nutritionally valuable, flavonoid-rich food and feed candidates while promoting sustainability through the utilization of green biorefineries.

CRedit authorship contribution statement

S. Reyhan Yavuz: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation. **Wildan Suhartini:** Investigation, Formal analysis. **Nóra Bákovyi:** Validation, Investigation. **Zoltán Kovács:** Investigation. **László Kaszás:** Investigation. **Áron Béni:** Investigation. **Andrea Balláné Kovács:** Investigation. **Endre Máthé:** Investigation. **Nevien Elhawat:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Miklós G. Fári:** Resources, Investigation. **Szilvia Veres:** Investigation. **Tarek Alshaal:** Writing – review & editing, Writing – original draft, Validation, Investigation, Conceptualization. **Éva Domokos-Szabolcsy:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Data curation, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fbio.2026.108763>.

Data availability

Data will be made available on request.

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