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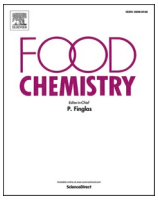

Rye secalin isolates to develop reference materials for gluten detection

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ABSTRACT

Gluten-free products must not contain more than 20 mg/kg of gluten to be safe for consumption by celiac disease patients. Almost all analytical methods are calibrated to wheat, wheat gluten or gliadin, and there is no rye-specific reference material available. The aim of this study was to assess the effect of the harvest year on rye gluten composition and to generate distinct rye isolates to serve as calibration standards. Four different extraction procedures of a specific rye cultivar mixture were tested yielding prolamins (PROL), glutelins (GLUT), gluten (G) and acetonitrile/water-extractable proteins (AWEP). The isolates were characterized using different methods such as RP-HPLC, GP-HPLC, SDS-PAGE and LC-MS/MS. The isolates were evaluated in the R5 ELISA which resulted in the following response order: $PROL_{iso}$ > $AWEP_{iso}$ > G_{iso} > $GLUT_{iso}$. This paper represents a significant step towards improving gluten analysis, particularly in the context of rye-contaminated gluten-free products.

1. Introduction

Rye grains are mainly used for food production or animal feed (Rani et al., 2021). They contain secalins as storage proteins, also known as gluten proteins. The proteins can be classified into different fractions based on their solubility in specific solvents such as the ethanol-soluble prolamins and the alkaline- or acid-soluble glutelins (Osborne, 1895). Another way to characterize the secalins is the classification into different types based on their molecular weight such as high-molecular-weight (HMW)-secalins (70–90 kDa), ω-secalins (40–70 kDa), γ-75 k-secalins (50 kDa) and γ-40 k-secalins (30–45 kDa) (Schalk et al., 2017).

The consumption of rye-based foods can cause hypersensitivity reactions in individuals who have celiac disease (CD) (Scherf et al., 2016). For those affected a lifelong gluten-free diet is essential based on naturally gluten-free foods or gluten-free labelled foods. Regulation (EU) No. 1169/2011 and Commission Implementing Regulation (EU) No. 828/2014 describe the labelling requirements for gluten-free products in the EU, including prepacked and non-prepacked items. The term “gluten-free foods” is defined as dietary foods consisting of ingredients which do not contain wheat, rye, barley, oats, or their crossbred varieties and the gluten level does not exceed 20 mg/kg overall. This threshold is important for the analysis of gluten in order to ensure food safety for CD patients.

ELISA is the most important immunochemical technique for detecting gluten in foods. The R5 sandwich ELISA test kit is widely used, because it has been endorsed by the Codex Alimentarius as a type 1

Abbreviations: ANOVA, analysis of variance; AWEP, acetonitrile/water-extractable proteins; $AWEP_{iso}$, acetonitrile/water-extractable protein isolate; CD, celiac disease; DTT, dithiothreitol; DTT-p, reduced with dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EU, European Union; FA, formic acid; G, gluten; G_{iso}, gluten isolate; GLUT, glutelins; $GLUT_{iso}$, glutelin isolate; GP-HPLC, gel permeation high-performance liquid chromatography; HMW, high-molecular-weight; iBAQ, intensity-based absolute quantitation; LC-MS/MS, liquid chromatography–tandem mass spectrometry; mAb, monoclonal antibody; Mw, molecular weight; OMA, AOAC International official method of analysis; PROL, prolamins; $PROL_{iso}$, prolamin isolate; PWG, Prolamin Working Group; RM, reference material; RP-HPLC, reversed-phase high-performance liquid chromatography; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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method and also by the AOAC International as an official method of analysis (OMA) for gluten in foods containing wheat, barley and rye (AOAC OMA 2012.01) (Lacorn et al., 2022). The R5 monoclonal antibody (mAb) has been raised against ω-secalins and primarily recognizes the epitope QQPFP and the related sequences QQQFP, LQPFP and QLPFP that are present in many CD-immunoreactive peptides (Kahlenberg et al., 2006; Koehler et al., 2013; Méndez et al., 2005). The reference material (RM) used for calibration of the kit is the so-called Prolamin Working Group (PWG)-gliadin, which is a prolamin isolate of a mixture of 28 different European wheat flours (van Eckert et al., 2006). Several studies have shown that the use of PWG-gliadin as a RM led to overestimation of gluten in rye flours because of the high response of the R5 mAb against secalins (Lexhaller et al., 2016; Rzychon et al., 2017). Another reason for the overestimation of rye gluten is the prolamin/glutelin ratio, which is commonly assumed to be 1, indicating an equal distribution of prolamins and glutelins (1:1). In the R5 ELISA, the prolamins are measured and the result is therefore multiplied by 2 to calculate the total gluten content. However, a recent study on 32 different rye cultivars showed that the average prolamin/glutelin ratio is 4.4:1. Accordingly, the conversion factor for samples contaminated with rye should rather be 1.2, since rye contains substantially more prolamins than glutelins (Xhaferaj et al., 2023).

Wheat-based RMs such as flours or isolated gluten protein types have been proposed (Hajas et al., 2018; Lexhaller et al., 2019; Schall et al., 2020) and the impact of RM type (flour or isolate) as well as genetic and environmental variability on wheat protein composition was investigated by Hajas et al., 2018 and Schall et al., 2020. They showed that a selection of five wheat cultivars is suitable for RM production. Furthermore, flour mixtures appeared to be a better alternative for reducing genetic and environmental effects than single cultivar flours. Recently, a study on 32 rye flours identified seven rye cultivars from six different countries (Austria, Canada, Finland, Germany, Hungary (2), Poland) that showed the highest variability in secalin composition. The mixture of these seven cultivars is the first representative rye flour reported to be suitable for RM production (Xhaferaj et al., 2023), but no further fundamental studies on rye secalin isolates are available so far. Moreover, despite the need for appropriate RMs to accurately quantitate the gluten content in foods contaminated with rye, there is no RM available specifically for rye, that would be comparable to the established PWG-gliadin RM for wheat.

Building on our work so far, our hypothesis is that the rye flour mixture will be suitable for the production of secalin isolates that are representative of the secalin composition in the flour. Compared to the flour, isolates have better long-term stability and they are highly enriched in protein, making them easier to use. In order to test this hypothesis, the seven rye cultivars selected by Xhaferaj et al., 2023 were procured from a second harvest year to assess the extent of environmental variability and ensure that the mixture remains representative in terms of secalin composition. Furthermore, our aim was to provide four different protein isolates produced by different extraction procedures from the flour mixture. These isolates were characterized in-depth using complementary analytical techniques and the responses against the R5 antibody were determined. Therefore, this is the first study to investigate the potential use of rye-specific isolates as RMs in gluten quantitation. Using these rye-specific protein isolates as RM is expected to result in a reduction of the overestimation of the gluten content in rye-contaminated gluten-free foods by R5 ELISA. Overall, these new fundamental insights into rye secalins will help to improve gluten analysis and enhance food safety for individuals with CD.

2. Materials and methods

2.1. Rye grains and flour preparation

Seven selected rye cultivars (Xhaferaj et al., 2023) were collected for the protein characterization and isolation procedure from the harvest year 2022 (Table S1). Equal amounts of grains (4 kg) of each cultivar were mixed prior to milling. Both the grains of the individual cultivars and the grain mixture were milled using a laboratory mill (Cyclotec Mill 991,093, Foss Tecator AB, Höganäs, Sweden). Wholemeal flours were stored in zip-lock bags at 22 °C until further use. Prior to the extraction, the flour mixture (100 g) was defatted by stirring with 250 mL n-pentane/ethanol (95/5, *v*/v) three times, followed by stirring once with 250 mL n-pentane at 22 °C for 30 min (Schalk et al., 2017). Further, the suspensions were filtered (Sartorius Stedim Biotech GmbH) under vacuum and the flour was spread evenly on a tray, dried overnight and carefully homogenized with a spatula.

2.2. Protein isolation

Four different protein isolates were obtained from the flour mixture with distinct extraction methods. The extractions resulted in the production of prolamins ($PROL_{iso}$), glutelins ($GLUT_{iso}$), the combination of prolamins and glutelins extracted together (referred to as gluten, G_{iso}), and acetonitrile/water-extractable proteins ($AWEP_{iso}$) as these are commonly used procedures for plant protein isolation. The isolation procedure was in many parts adopted from Schalk et al., 2017 and Batey et al., 1991 with a few changes.

2.2.1. Production of the prolamin and glutelin isolates

Defatted flour (2 × 40 g) was extracted with 160 mL of salt solution (400 mmol/L NaCl +67 mmol/L Na_2PO_4/KH_2PO_4 (pH 7.6)) by homogenizing with an Ultra Turrax blender (12,000 rpm, IKA-Werke, Staufen, Germany) for 5 min at 22 °C and stirring for 10 min with a magnetic stirrer. The suspensions were centrifuged at 3750 rcf and 22 °C for 25 min (Heraeus Multifuge X1, Fisher Scientific, Waltham, USA) and the supernatant was discarded. The extraction was repeated two times to remove the albumin and globulin fraction. For the prolamin extraction, the sediments were extracted three times with 160 mL of aqueous ethanol (60/40, *v*/v) by homogenizing with an Ultra Turrax blender for 5 min at 22 °C and stirring for 10 min with a magnetic stirrer. The suspensions were then centrifuged at 3750 rcf and 22 °C for 25 min and the extraction was repeated two times. The supernatants were combined. Last, the residue was used for glutelin extraction using 160 mL of buffer solution (2-propanol/water (50/50, v/v) with 0.1 mol/L Tris-HCl, pH 7.5, containing 2 mol/L (*w*/*v*) urea and 0.06 mol/L (w/v) dithiothreitol (DTT)) by homogenizing under argon with an Ultra Turrax blender for 5 min and magnetic stirring for 30 min at 60 °C in a water bath. After centrifugation (3750 rcf, 22 °C, 25 min) the supernatant was collected and the extraction was repeated twice. The respective prolamin and glutelin supernatants were separately concentrated, dialyzed and lyophilized to yield $PROL_{iso}$ and $GLUT_{iso}$, respectively (see Section 2.2.4).

2.2.2. Production of the gluten isolate

The gluten isolation was carried out with 50 g of defatted flour. The albumins and globulins were removed according to Section 2.2.1. Then, 200 mL of buffer solution (see Section 2.2.1) was used for extraction under argon by homogenizing with an Ultra Turrax blender (12,000 rpm) for 5 min and magnetic stirring for 30 min at 60 °C in a water bath, followed by centrifugation (3750 rcf, 22 °C, 25 min). The extraction was conducted three times and the supernatants were combined, concentrated, dialyzed and lyophilized, resulting in G_{iso} (see Section 2.2.4).

2.2.3. Production of the AWEP isolate

For the AWEP isolation, 50 g of defatted flour was used and the albumins and globulins were removed as described in Section 2.2.1. Then, 200 mL of acetonitrile/water (50/50, *v*/v) containing 0.1 % formic acid (FA) (*v*/v) was used for extraction by homogenizing with an Ultra Turrax blender (12,000 rpm) for 5 min. Then, the samples were sonicated for 30 s and stirred for 10 min with a magnetic stirrer. After centrifugation (3750 rcf, 22 °C, 25 min) the extraction was repeated twice without

sonication and the supernatants were combined, concentrated, dialyzed and lyophilized, to yield $AWEP_{iso}$ (see Section 2.2.4).

2.2.4. Concentration, dialysis and lyophilization of the protein isolates

The combined supernatants of each fraction (prolamins, glutelins, gluten and AWEP) were concentrated (Rotavapor R II, BÜCHI Labortechnik, Flawil, Switzerland) to approximately 300 mL. For dialysis a membrane with a molecular weight cut-off of 12–14 kDa was used (Spectrum Labs Spectra/Por Dialysis Membrane, Thermo Fisher Scientific, Waltham, MA, USA). The protein solutions were dialyzed against 0.01 mol/L acetic acid for 72 h and against deionized water at 22 °C for 24 h. During dialysis, the 0.01 mol/L acetic acid was changed three times a day. The proteins were lyophilized for 48 h at −60 °C and 0.1 mbar (Beta 1–8 LSCplus, Martin Christ, Osterode am Harz, Germany). After drying, the isolates were stored at −20 °C until use.

2.3. Protein characterization

2.3.1. Crude protein content

The crude protein content of the flour samples was determined using a Leco FP 528 nitrogen analyzer (Leco Corporation, St. Joseph, MO, USA) in duplicate according to ICC Standard No. 167. Similarly, the crude protein content of the isolates was determined using a DUMATHERM N Pro nitrogen analyzer (C. Gerhardt, Königswinter, Germany) in triplicate according to ICC Standard No. 167. The nitrogen content was multiplied by 5.7 to obtain the crude protein content in both flours and isolates.

2.3.2. Modified Osborne fractionation

The protein extraction for the analytical characterization of the protein composition in the flours and isolates was done according to Wieser et al., 1998 and involved the steps outlined in Xhaferaj et al., 2023. Flour (100 mg) was extracted and subjected to magnetic stirring, vortexing, and subsequent centrifugation. The resulting solutions containing albumins and globulins, as well as prolamins and glutelins, were then diluted to a volume of 2 mL using the appropriate extraction solvents. These solutions were filtered through a 0.45 μm Whatman SPARTAN filter (Cytiva Europe GmbH, Freiburg im Breisgau, Germany). The prolamin fraction was additionally reduced with 1 % (*w/v*) DTT resulting in the reduced prolamin fraction (DTT-p). The solutions were used for the chromatographic analysis of the proteins.

2.3.3. Protein characterization by RP-HPLC

RP-HPLC was utilized to determine the protein content and distribution. The setup, column, mobile phase, and separation gradients were used as outlined in Xhaferaj et al., 2023. Detection of proteins was monitored at 210 nm, and their quantitation was based on the corresponding absorbance areas of PWG-gliadin (van Eckert et al., 2006). The sum of the DTT-p prolamins and the glutelins resulted in the total gluten content. The secalin types were quantitated based on their percentage of the total peak area. The evaluation of the chromatographic profiles and the classification of the protein fractions was based on the literature (Gellrich et al., 2003; Schalk et al., 2017).

2.3.4. Molecular weight distribution by GP-HPLC

The relative molecular weight (Mw) distribution of secalins was determined using GP-HPLC. The separation used the exact same apparatus, column, and mobile phase as described by Xhaferaj et al., 2023. The DAD was used for detection at 210 nm. The integration limitations for particular Mw ranges were established using proteins with known Mw values. The proteins employed were cytochrome C (12.4 kDa) from horse heart, carbonic anhydrase (29 kDa) from bovine erythrocytes, and albumin (66 kDa) from bovine serum (Sigma-Aldrich, Darmstadt, Germany). The following partitions were used: (1) >66 kDa, (2) 66–29 kDa, (3) 29–12.4 kDa, (4) <12.4.

2.3.5. Molecular weight distribution by SDS-PAGE

The samples (20 mg for flours, and 2 mg for isolates) were extracted with 1 mL of the extraction buffer (293.3 mmol/L sucrose, 246.4 mmol/L Tris, 69.4 mmol/L SDS, 0.51 mmol/L EDTA, 0.22 mmol/L Brilliant Blue G-250, 0.177 mmol/L phenol red, 0.105 mmol/L HCl, pH 8.5) overnight under reducing conditions (DTT, 50 mmol/L). The suspensions were shaken for 10 min at 60 °C and centrifuged at 2370 rcf for 5 min at 20 °C. For the electrophoresis, 10 μL of the samples were applied on a NuPAGE 4–12 % Bis-Tris protein gradient gel (1.0 mm, 10-well, Invitrogen, Carlsbad, CA, USA) with MOPS running buffer (50 mmol/L MOPS, 50 mmol/L Tris, 3.5 mmol/L SDS, 1 mmol/L EDTA, pH 7.7) including DTT (5 mmol/L) as reducing agent (Lagrain et al., 2012; Geisslitz et al., 2020). The gels were run at 200 V and 115 mA according to the manufacturer's guidelines (Thermo Scientific) with a run time of 30 min. Staining, destaining and fixing was conducted according to Xhaferaj et al., 2023. The gels were scanned (LAS-3000, Fujifilm, Minato, Tokyo, Japan) and the Mw of the bands were estimated based on the marker proteins by the AIDA Image Analysis software V3.27.001 (Elysia-raytest, Angleur, Belgium).

2.4. Characterization of the secalin isolates by untargeted LC-MS/MS

2.4.1. Sample preparation

For reduction and alkylation, the protein isolates were dissolved in 500 μL Tris-HCl (0.5 mol/L, pH 8.5) and 500 μL 1-propanol and mixed with 100 μL tris(2-carboxyethyl)phosphine (0.05 mol/L, pH 8.5), followed by incubation for 30 min at 60 °C in a thermoshaker. For each sample, 100 μL 2-chloroacetamide solution (0.5 mol/L, pH 8.5) was added and the samples were shaken for 45 min at 37 °C in the dark. Finally, the samples were dried for approximately 3 h in the vacuum centrifuge (40 °C, 6 h, 8 mbar) (Martin Christ). Prior to the enzymatic digestion with trypsin, the samples were diluted in 1 mL of urea solution (0.1 mol/L Tris-HCl and 0.04 mol/L urea, pH 7.8). Trypsin (1:50, enzyme:protein, *w*/w) was added and the samples were further incubated for 18 h at 37 °C in the dark. The digestion was stopped by adding 10 μL of trifluoroacetic acid and the samples were evaporated to dryness. The resulting peptide mixtures were subjected to solid phase extraction on Discovery DSC_{18} columns (100 mg, Sigma Aldrich, MO, USA) according to the manufacturer's instructions and peptides were eluted with 40 % acetonitrile containing 0.1 % FA. The solvent was evaporated and the samples were stored at −20 °C until LC-MS/MS analysis.

2.4.2. LC-MS/MS measurements and data analysis

The peptide samples were dissolved in 1 mL of 2 % acetonitrile, 0.1 % FA in water and filtered (0.45 μm) prior to measurement. The samples were analyzed on a Vanquish UHPLC system coupled to a Q-Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). The peptides were separated on an Aeris PEPTIDE XB-C_{18} column (150 × 2.1 mm, 1.7 μm, Phenomenex, Aschaffenburg, Germany) with water/FA (999/1, *v*/v) (A) and acetonitrile/FA (999/1, v/v) (B) as solvents with a flow rate of 0.2 mL/min, a column temperature of 30 °C, an injection volume of 10 μL and a linear gradient: 0 min 5 % B, 0.4 min 10 % B, 32 min 32 % B, 34–37 min 80 % B, 38–45 min 5 % B. The peptides and proteins were identified and relatively quantitated using MaxQuant (version 2.2.0.0). The MS data were searched against the *Triticeae* protein reference database taken from UniprotKB (*Triticeae*, download February 23, 2023, protein entries: 557,477) using the search engine Andromeda. The method used in MaxQuant was performed in accordance with the procedure outlined in Tyanova et al., 2016. Methionine oxidation and protein acetylation at the N-terminus were selected as variable modifications. Trypsin was designated as proteolytic enzyme with a maximum of two permitted missed cleavage sites and carbamidomethylation on cysteines was set as a fixed modification. With match-between runs enabled (matching time window 0.7 min, alignment time window 20 min), the data were filtered for a minimum

length of seven amino acids and a 1 % peptide and protein false discovery rate. A total sum normalization of intensity-based absolute quantitation (iBAQ) protein intensities between samples was performed to correct for different total protein injection amounts. The identification of the proteins was carried out with the Andromeda algorithm, which uses a probability-based approach, assigning scores to protein groups in MaxQuant, with those scoring 20 or higher considered for relative quantitation due to their increased likelihood of presence in the sample. The iBAQ algorithm was used to calculate the relative abundance of the protein groups. A total sum normalization of iBAQ protein group intensities between samples was conducted for comparison.

2.5. Gluten quantitation by ELISA

The responses of the gluten isolates towards the R5 mAb were tested using the RIDASCREEN Gliadin Assay (R7001, R-Biopharm, Darmstadt, Germany). The methodological approach used in the study was based on Lexhaller et al., 2016. The isolates were diluted using the appropriate solvents and sample dilution buffers. The prolamin isolates were diluted with 60 % ethanol, the glutelins and the gluten isolates were diluted using the buffer solution and the AWEP isolates were diluted in acetonitrile/water (1:1, *v*/v) containing 0.1 % FA (*v*/v). The final dilution step was carried out in accordance with the manufacturer's instructions using the sample dilution buffer included in the kit to make sure that all solutions contained the standard amount of the kit buffer (1:12.5). At least four serial dilutions of each extract in the target range from 5 to 40 ng/mL were applied at least twice ($n = 2$) and the ELISA procedure was carried out strictly as described by the manufacturer. The ELISA absorbances were plotted against the protein concentrations of the isolate solutions, which were quantitated by RP-HPLC while taking dilution factors into account. To compare the responses of the isolates against the R5 mAb, the RP-HPLC concentrations were plotted against the ELISA concentrations using the kit standard for calculation. Calibration curves were constructed using the cubic spline function of the RidaSoft Win Software (R-Biopharm).

2.6. Statistics

All analytical measurements were carried out as triplicates. In case the relative standard deviations of HPLC analyses were above 5 %, another triplicate was analyzed. Mean values ($n = 3$) and absolute standard deviations (SD) were calculated for all quantitative values. To assess variances between the means of the Mw distribution (GP-HPLC) and protein composition (RP-HPLC), one-way and two-way ANOVA (Tukey's post hoc test, $p < 0.05$) were used. Student's *t*-tests were performed with a significance level of $p = 0.05$ to compare the same cultivar from two different harvest years. All statistical analyses were performed using Origin 2021b software (OriginLab Cooperation, Northampton, MA, USA).

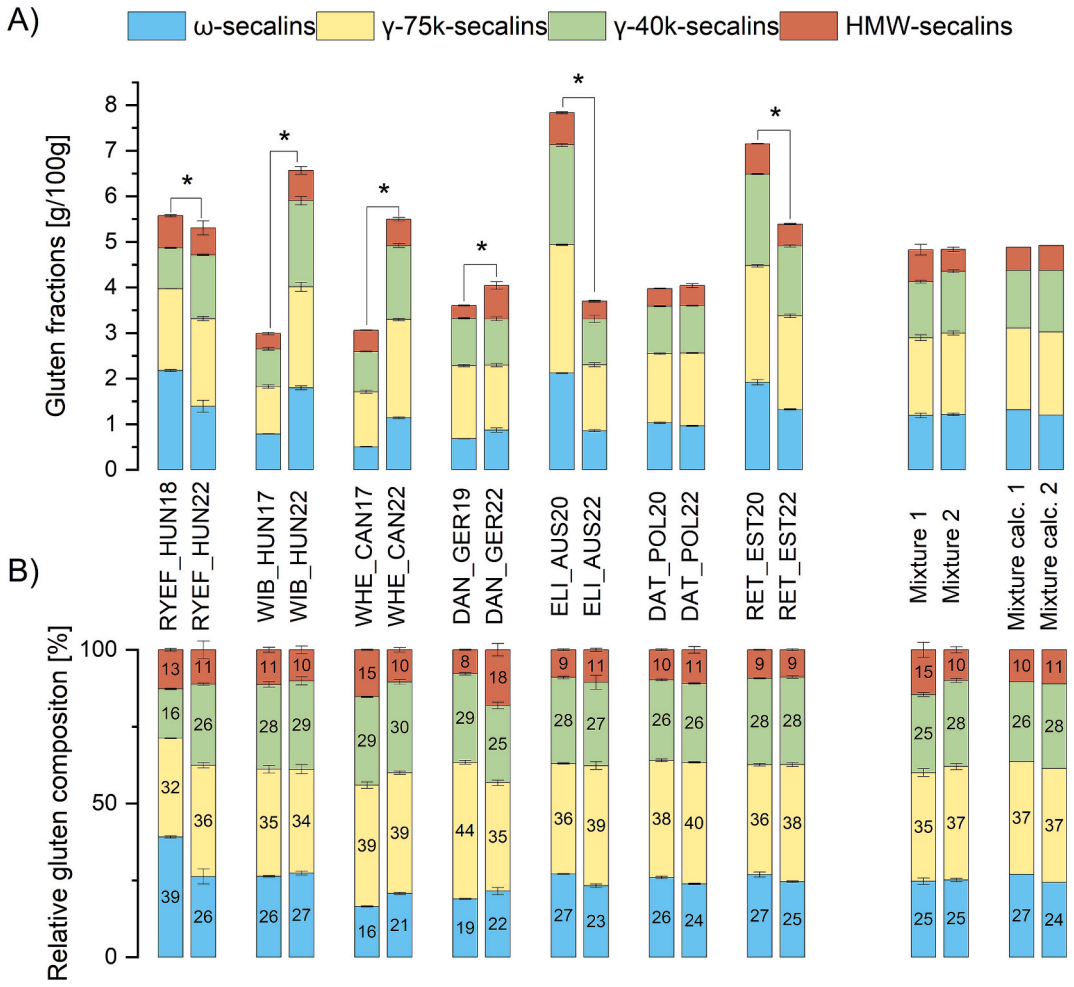

Fig. 1. Secalin composition of the seven selected rye cultivars and their mixture from two different harvest years. A: Secalin content (g/100 g of flour), B: secalin distribution (%) relative to total secalins. The analysis was performed by RP-HPLC. The mixture consists of the flours of the selected seven cultivars in equal proportions. Mixture calc. is the calculated composition resulting from the mean values of the seven single flours. Error bars indicate the standard deviation of the mean ($n = 3$). The asterisk indicates a significant difference between the gluten content of both years (two sample *t*-test, $p < 0.05$).

3. Results and discussion

3.1. Influence of different harvest years on the secalin distribution

The gluten composition of the seven selected rye cultivars and the respective flour mixtures from two harvest years was analyzed utilizing stepwise fractionation and RP-HPLC to assess the extent of environmental variability (Fig. 1, Table S2). Depending on the harvest year, all samples showed significant differences in total gluten content except for DAT_POL (2020 and 2022) and the flour mixture (absolute values) (Fig. 1A, Table S3, *t*-test, $p < 0.05$). The cultivar ELI_AUS showed the highest differences, with 7.8 g secalin/100 g flour for ELI_AUS20 and 3.7 g secalin/100 g flour for ELI_AUS22. Regarding the absolute values, the flour mixtures (first and second collection) showed no significant differences within the γ-75 k-, ω- and HMW-secalins, only for the γ-40 k-secalins (*t*-test, $p = 0.009$). When comparing the relative secalin distribution, the HMW-secalins had the lowest and the γ-75 k-secalins the highest percentage for most of the samples except RYEF_HUN18 that had the highest percentage of ω-secalins (Fig. 1B, Table S4). Since most samples had comparable proportions of ω-secalins and γ-40 k-secalins, the distribution order resulted in the following: γ-75 k-secalins > γ-40 k-secalins ≈ ω-secalins > HMW-secalins. The relative secalin distribution within the flour mixtures from the first collection (25 % ω-secalins, 35 % γ-75 k-secalins, 25 % γ-40 k-secalins and 15 % HMW-secalins) and the second collection (25 % ω-secalins, 37 % γ-75 k-secalins, 28 % γ-40 k-secalins and 10 % HMW-secalins) was largely comparable. The composition of secalins showed no differences between the fractions besides within γ-75 k-secalins and ω-secalins (*t*-test, $p > 0.05$, Table S4).

The differences in molecular weight were also compared among each sample with GP-HPLC. The analysis was conducted within four Mw ranges for each fraction (prolamins, DTT-p prolamins and glutelins): (1) >66 kDa, (2) 66–29 kDa, (3) 29–12.4 kDa and (4) <12.4 kDa. In all samples, range (2) within the prolamin fraction had a consistent percentage of 10.1–12.0 % (Table S5). The samples DAT_POL, DAN_GER, and ELI_AUS from the year 2022 contained larger percentages of Mw range (4) compared to the same samples from the years 2019/2020. For RYEF_HUN, WHE_CAN, and WIB_HUN there were no significant differences and RET_FIN had a lower percentage of range (4) when the harvest years were examined. Higher molecular weight proteins in range (1) decreased as a result of the reduction of prolamins with DTT. Over all samples, the proportion of (1) varied from 2.1 % to 9.5 %. In comparison to the first collection, all samples from the second collection contained a lower percentage of (3) within the DTT-p prolamins, resulting in an increase of (4). Significant differences within the Mw ranges were found by ANOVA when comparing the harvest years, but there were no trends (Table S5).

Overall, the results show significant differences in gluten composition and Mw between the samples of different harvest years. The agronomic techniques employed during cultivation, such as fertilization and pest control, have an impact on the gluten composition, as do environmental factors during the growth season, such as temperature, rainfall, and sunlight (Johansson et al., 2003). The relative contributions of the cultivar and the harvest year (3 years) to the overall variation in grain characteristics, chemical composition, and functional aspects relevant to the baking quality of rye were investigated by Hansen et al., 2004. They demonstrated that the cultivar had the highest impact on the protein content, while the harvest year had a noticeably smaller impact (Hansen et al., 2004). Our results revealed a statistically significant interaction between cultivar and harvest year on protein content, with the harvest year demonstrating a higher effect size (two-way ANOVA, $p < 0.05$). This different result may be due to greater unknown variations in weather conditions between the harvest years investigated in the present study compared to Hansen et al., 2004. The effects of genetics and environment on a variety of flours from different harvest years, as well as on the variability of gluten and ELISA responses were investigated (Schall et al., 2020). They discovered that utilizing blended flour resulted in higher gliadin recovery in ELISA compared to when using individual cultivars. However, the recovery values were not significantly influenced solely by the harvest year, but there were notable interactions between the ELISA kit, the protein source, and the harvest year. The results of our study show that there was variability in the protein composition depending on the harvest year, mostly in the absolute values, less so in the relative values. Nevertheless, the gluten composition in the mixed flour remained the same despite the harvest year, except for minor deviations. The findings support the hypothesis that the use of a rye flour mixture will reduce the impact of genetic and environmental effects compared to one single cultivar. The use of blended flours is therefore advantageous as a basis for RM production compared to single cultivars for rye flours.

3.2. Isolate characterization

3.2.1. Protein content and yield of the isolates

The flour mixture of the seven chosen samples (collection 2022, Table S1) was used for protein isolation and yielded four different isolates including prolamins ($PROL_{iso}$), glutelins ($GLUT_{iso}$) as well as gluten (G_{iso}) and acetonitrile/water-extractable proteins ($AWEP_{iso}$). The protein isolation of the flour mixture yielded in 2.2 g/100 g flour of $AWEP_{iso}$, 1.8 g/100 g flour of $GLUT_{iso}$, 5.2 g/100 g flour of G_{iso}, and 8.1 g/100 g flour of $PROL_{iso}$ (Table S6). RP-HPLC and the Dumas method were each used to measure and compare the protein content. $AWEP_{iso}$ and $PROL_{iso}$ had the highest crude protein content (Dumas method) with 84.6 % and 83.7 %, respectively. The crude protein content for G_{iso} and $GLUT_{iso}$ was 77.3 % and 52.3 %, respectively. Gessendorfer et al., 2009 published research on the production of hydrolyzed prolamins from rye. They produced 2.2 g of secalins from 100 g of flour with a protein content of 83.8 %. Their yield was lower than that of $PROL_{iso}$, but the protein content (83.7 %) is in line with that of $PROL_{iso}$. In general, little is known regarding gluten or glutelin yields from rye flours.

3.2.2. Molecular weight distribution by SDS-PAGE

SDS-PAGE was used to determine the Mw distribution of the protein isolates and the flour mixture (Fig. 2). All four isolates had similar band patterns in the ranges of 60 to 120 kDa (γ-75 k- and HMW-secalins) and 30 to 40 kDa (γ-40 k-secalins). However, ω-secalins in the Mw range 45 to 55 kDa only displayed weak or no bands for the isolates. This outcome was unexpected, since the content of ω-secalins measured by RP-HPLC

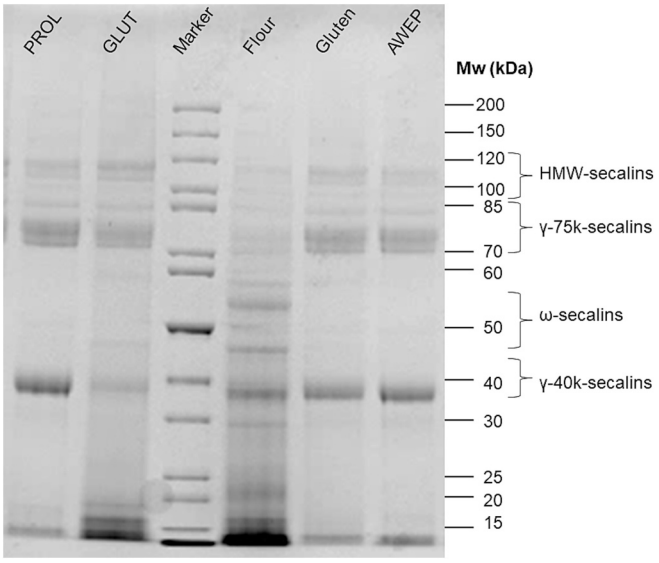

Fig. 2. SDS-PAGE of the four isolates produced from the rye flour mixture. AWEP: acetonitrile/water-extractable proteins; GLUT: glutelin isolate; Mw: molecular weight; PROL: prolamin isolate.

was similar to that of γ-40 k-secalins for the isolates and the flour mixture. The less intense staining of ω-secalins could be attributed to the Coomassie dye, which yields different staining results based on the amino acid composition of the proteins. Coomassie dye primarily attaches to amino acids containing basic groups through its sulfonyl anions (Tal et al., 1985). Since ω-secalins have a lower concentration of key basic amino acids (such as lysine, arginine, and histidine), it is possible that the bands appear less intense when compared to other proteins. $GLUT_{iso}$ differed from $PROL_{iso}$, G_{iso}, and $AWEP_{iso}$ in its band pattern, with a substantially weaker band at 30–40 kDa (γ-40 k-secalins) and a more intense band at 100–120 kDa (HMW-secalins). The band intensities for $PROL_{iso}$, G_{iso}, and $AWEP_{iso}$ revealed a similar secalin composition. Compared to previous studies on rye flours the resulting secalin band pattern was consistent, except for the weak staining of ω-secalins (Gellrich et al., 2003; Gessendorfer et al., 2009; Schalk et al., 2017).

3.2.3. *Molecular weight distribution by GP-HPLC*

The Mw distribution of the four distinct isolates ($PROL_{iso}$, $GLUT_{iso}$, G_{iso} and $AWEP_{iso}$) was compared to the respective fractions extracted directly on analytical scale from the flour mixture from which they originated ($PROL_{flour}$, $GLUT_{flour}$) using GP-HPLC (Fig. 3A, Table S7). Additionally, the prolamin fraction was compared after reduction of disulfide bonds (DTT-p $PROL_{iso}$ and DTT-p $PROL_{flour}$). For the majority of the data, the Mw of each range ((1) >66 kDa, (2) 29–66 kDa, (3) 12.4–29 kDa, and (4) <12.4 kDa) varied significantly (one-way ANOVA, Tukey's post hoc test, $p < 0.05$). With small standard deviations (mean SD 3.2 %), the ANOVA quickly identifies compositional changes in the

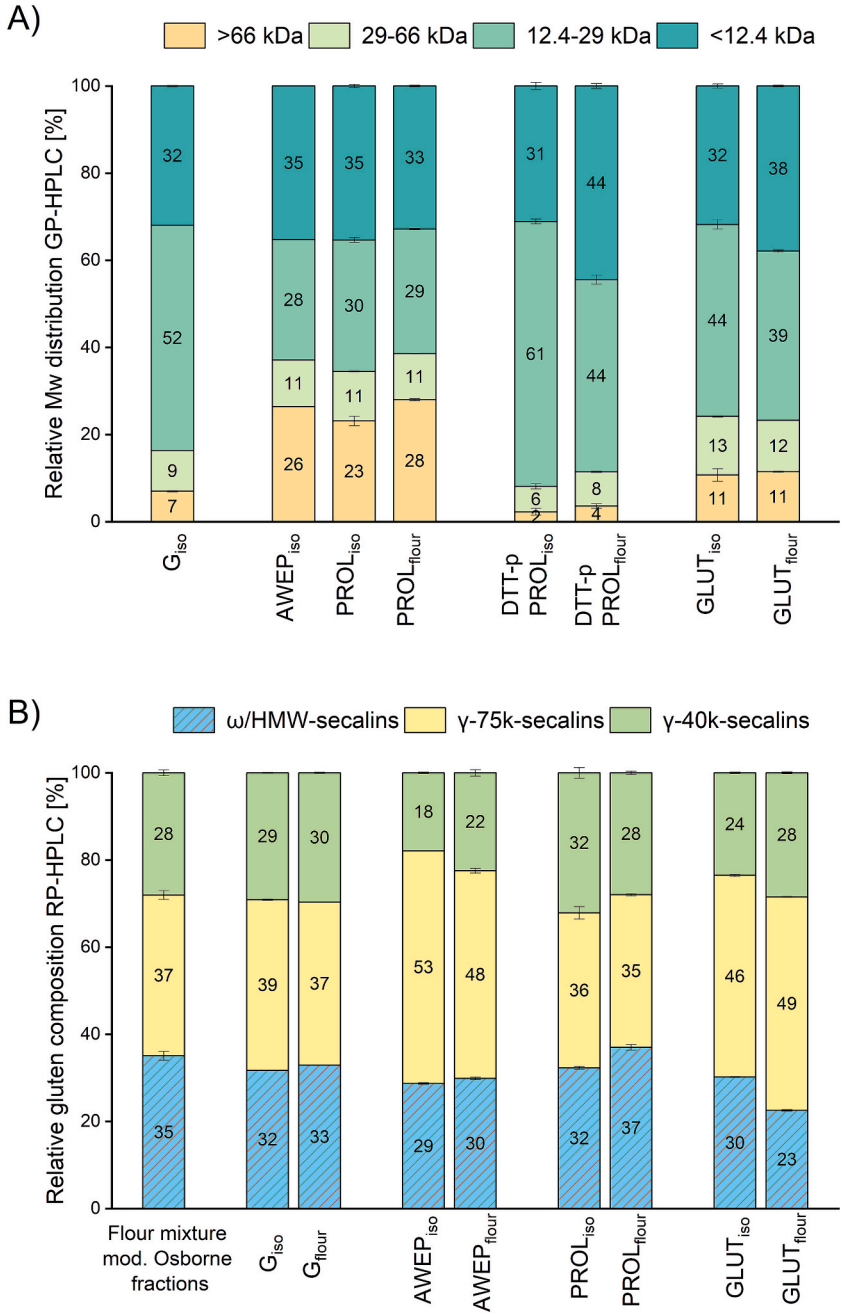

Fig. 3. Secalin composition of the four isolates and the flour mixture extracted with the respective solvents. A: Relative molecular weight distribution analyzed by GP-HPLC, B: secalin distribution (%) relative to total secalins analyzed by RP-HPLC. Error bars indicate the standard deviation of the mean (n = 3). AWEP: acetonitrile/water-extractable proteins; DTT-p: reduced prolamins; G: gluten; GLUT: glutelins; iso: isolate; PROL: prolamins.

data. Despite the statistical differences between the flour mixture and the resulting isolates, the Mw range distributions were similar. Comparing the results for both $AWEP_{iso}$ and $PROL_{iso}$ to $PROL_{flour}$, the Mw distribution was comparable for all ranges showing the following pattern with the ranges: (4) > (3) > (1) > (2) (Fig. 3A). When comparing DTT-p $PROL_{iso}$ to DTT-p $PROL_{flour}$, a similar Mw distribution pattern was observed with range (1) being the lowest followed by range (2) and higher values for range (3) and (4). Similarly, a comparable Mw distribution was found in comparison of $GLUT_{iso}$ to $GLUT_{flour}$. The data show that after protein isolation, the Mw slightly changed. One factor contributing to the shift in Mw distribution is the distinct behaviour of proteins during the isolation process, including aggregation, as well as the impact of steps like freeze-drying and re-dissolving for analysis. These factors result in modifications to the protein properties when compared to the analysis of fresh extracts.

3.2.4. Protein distribution by RP-HPLC

RP-HPLC was used for an individual comparison of the secalin distribution between the four isolates to the flour mixture using the corresponding extraction method (Fig. 3B, Table S8). For comparison, the HMW- and ω-secalins were merged into a single fraction, because they cannot be differentiated by retention time and the glutelin isolate is not expected to contain ω-secalins. The pairwise comparison between $PROL_{iso}$ and $PROL_{flour}$, $GLUT_{iso}$ and $GLUT_{flour}$, $AWEP_{iso}$ and $AWEP_{flour}$, and G_{iso} and G_{flour} revealed similarities in their secalin distribution, despite statistical differences (*t*-test, $p < 0.05$, Table S9). The differences in relative values between single secalin fractions were not more than ±5 %, except for $GLUT_{iso}$ showing 7 % more ω/HMW-secalins compared to $GLUT_{flour}$. $PROL_{iso}$ had 5 % less ω/HMW-secalins compared to $PROL_{flour}$. Since the GLUT fraction is the second extraction step after extraction of the PROL fraction, minor shifts in composition can be expected. When extracting with 60 % ethanol, similar secalin compositions were obtained for $PROL_{iso}$ and $PROL_{flour}$. Most similarities in secalin distribution occurred between G_{iso} and G_{flour} (ω/HMW: 32 % and 33 %, γ-75 k: 39 % and 37 %, γ-40 k: 29 % and 30 %) and it closely resembled the distribution of the flour mixture which was extracted in several steps using the modified Osborne fractionation (ω/HMW: 35 %, γ-75 k: 37 % and γ-40 k: 28 %) (Fig. 3B). Overall, the results demonstrate some variances in composition, but generally match the composition of the source flour mixture. Similarly, van Eckert et al., 2006 found no significant changes between the protein fractions between the flour and the isolate during the production of PWG-gliadin.

3.2.5. Characterization by untargeted LC-MS/MS

The four isolates were reduced, alkylated, and digested with trypsin before measurement in order to determine the specific proteins and peptides by LC-MS/MS. Following the identification of 314 protein groups in total, a fasta header search for the terms "gluten", "gliadin", "glutenin", "prolamin", "glutelin", "hordein" and "secalin" revealed that 27 of these were designated as gluten proteins. Among the 314 protein groups, 13 uncharacterized proteins, 847 peptides, and 21 fragments were found. The fasta header and the homology of the amino acid sequence were used to assign the protein groups to the secalin fractions (ω-, γ-75 k-, γ-40 k-, and HMW-secalins). Only protein groups originating from *Secale cereale*, *Secale strictum*, and *Triticum aestivum* were considered suitable for selection. Furthermore, an analysis of proteins derived from *Triticum aestivum* was conducted using the BLAST tool, accessible via the UniProtKB homepage, to identify related protein groups. This approach was employed due to potential discrepancies in database entry designations (e.g., ω-secalins from *Triticum aestivum*) and the high sequence similarity among wheat and rye proteins. The UniProtKB accessions in brackets correspond to the highest scoring identification as suggested by the software.

Based on the selection criteria, only one protein group (C4NFP2) with two identified peptides could be assigned to the ω-secalins (C4NFP2) (Table 1). Two protein groups (K7WF86 and H6ULI9) were assigned to the γ-75 k-secalins. K7WF86 had the greatest score (179.4) and six identified peptides, while H6ULI9 had three identified peptides and the highest iBAQ value. For γ-40 k-secalins and HMW-secalins, three protein groups (H8Y0N7, F4ZL28, and A0A5B9Y471) and (Q94IJ7, W6AW92, and W6AW98) were assigned, respectively (Table 1). Within the HMW-secalins, all selected proteins were from the *Triticum aestivum*

Table 1
List of the selected protein groups and peptides identified in the four secalin isolates by LC-MS/MS. The immunoactive peptides were identified based on the epitope search. The search procedure to identify immunoactive epitopes within peptides identified by LC-MS/MS uses the following strategies: R5 mAb: The epitopes recognized by the R5 mAb ELISA (Amnuaycheewa et al., 2022; Kahlenberg et al., 2006; Osman et al., 2001), immunoactive peptides recognized by CD4+ T cells (Sollid et al., 2020) and potentially harmful epitopes selected based on the EFSA search strategy (Q-X1-P-X2; X1 = L, Q, F, S, E; X2 = Y, F, A, V, Q) (Naegeli et al., 2017). The relevant epitopes are written in bold. iBAQ: intensity based quantification.

UniProt KB	Species	UniProt KB Name	Score	iBAQ	Peptides	Amino acid sequence found using the search procedure	Characteristics
ω-Secalins							
C4NFP2	*Secale cereale x Triticum turgidum* subsp. *durum*	Omega secalin	44.94	55,578,000	2	QLNPSEQEL**QSPQQPV**PK	Potentially immunoactive
γ-75 k-Secalins							
K7WF86	*Secale cereale*	75 k gamma secalin	179.38	2,631,800,000	6	S**QEPF**PQVHQP**QQPS**PQQ**QQPS**IQLSLQQQLNPCK	Potentially immunoactive
H6ULI9	*Secale strictum*	75 k gamma secalin	76.16	4,422,500,000	3		
γ-40 k-Secalins							
H8Y0N7	*Secale cereale* subsp. *afghanicum*	Gamma prolamin (fragment)	108.93	1,707,100,000	1		
A0A5B9Y471	*Triticum aestivum*	Gamma-gliadin	39.55	2,731,000,000	5	PLFQIVQGQSIIQ**QQPA**QLEVIR	Potentially immunoactive
F4ZL28	*Secale strictum* subsp. *africanum*	Alpha-gliadin storage protein	38.07	229,180,000	6	LQV**QPQQSFP**HQPQQQTLQSFLEQQLISCR	R5 ELISA epitope
HMW-Secalins							
W6AW92	*Triticum aestivum*	HMW glutenin subunit	151.56	1,706,200,000	10	AQQLAA**QLPA**MCR	Potentially immunoactive
W6AW98	*Triticum aestivum*	HMW glutenin subunit x	114.06	1,357,800,000	9		
Q94IJ7	*Triticum aestivum*	HMW glutenin subunit x	22.57	76,470,000	7	QPGQGQ**QGYYPTSPQ**HPGQGQQPGQGQQPGQGK	Recognized by $CD4^+$ T cells

species as well as one protein group in the γ-40 k-secalins. The protein group W6AW92 contained most identified peptides (10). The iBAQ value (sum of all isolates) was used to calculate the relative secalin distributions in the isolates using the identified protein groups. The percentage of ω-secalins resulted in less than 0.5 % in all samples, hence they were not considered for the following order of distribution. The low ω-secalin content can be attributed to the use of a suboptimal cleavage enzyme. The secalin distribution differed between all isolates (Fig. 4) and within G_{iso} the distribution was as follows: 63 % γ-75 k-, 24 % γ-40 k- and 13 % HMW-secalins. The composition of $AWEP_{iso}$ was 39 % γ-75 k-, 45 % γ-40 k-, and 15 % HMW-secalins. $PROL_{iso}$ was composed of out of 43 % γ-75 k-, 35 % γ-40 k-, and 21 % HMW-secalins. When compared to all isolates, $GLUT_{iso}$ demonstrated the lowest proportion of 8 % for γ-40 k-secalins and the highest percentage of 36 % for HMW-secalins, as well as a percentage of 56 % for γ-75 k-secalins. Because HMW proteins make up a higher fraction of glutelins in general, the largest HMW-secalin percentage in $GLUT_{iso}$ of 36 % was expected. It should be noted that the focus of this experiment was on the qualitative identification of secalins rather than on quantitative analysis. The analytical characterization by LC-MS/MS provides the following order: γ-75 k > γ-40 k ≥ HMW. Lexhaller et al., 2019 used LC-MS/MS to conduct a study on rye gluten protein types. In contrast to our work, their investigation of gluten protein types purified by preparative RP-HPLC revealed more gluten protein groups for each protein type. The protein groups, W6AW92 (HMW) and F4ZL28 (γ-40 k), were also identified in our study in comparison to Lexhaller et al., 2019. Compared to the other protein types, their analysis revealed more protein groups for HMW- and ω-secalins. Since our isolates were not separated into single secalin fractions and purified the number of protein groups is not quite comparable. The reliability of the reference sequences in the databases used in this investigation has a significant impact on the data presented. There are 29 and 145 UniProtKB entries for "secalin" in Triticeae and Secale species, respectively, however none of them have been reviewed as of March 2023. The study of rye proteins using the UniProtKB database may be insufficient for a variety of reasons. Since some secalins have not yet been defined or are not listed in the database, it does not entirely cover all rye proteins. Another cause of improper designations is the wrong naming of protein entries that are highly similar in sequence. Since the quality of the database determines the findings, the interpretation of the data should generally be examined carefully (Colgrave et al., 2013).

3.2.6. *Identification of potentially immunoactive peptides by LC-MS/MS*

In order to identify immunoactive peptides present in the isolates, the peptides identified by MaxQuant were searched using three search procedures for different purposes. The initial search involved the search for epitopes recognized by the mAb R5 listed in Table 1 (Kahlenberg et al., 2006; Osman et al., 2001). Thus, the specific protein fraction carrying these epitopes can be identified by R5 ELISA. The second search consisted of using the list of CD-relevant epitopes recognized by CD4+ T cells (Sollid et al., 2020). Additionally, using the European Food Safety Authority (EFSA) search strategy, the available peptide sequences were checked for complete and partial matching sequence overlaps with previously identified epitopes (Naegeli et al., 2017). For this purpose, the peptide sequences were explored, following the pattern Q-X1-P-X2, where X1 may be represented by the amino acids leucine (L), glutamine (Q), phenylalanine (F), serine (S), or glutamic acid (E), and X2 may be represented by tyrosine (Y), phenylalanine, alanine (A), valine (V), or glutamine. These peptide sequences are considered to be potentially immunoactive. Among the 14 peptides identified during the search, six were located within the specified protein categories (Section 3.2.5, Table 1). The remaining eight peptides identified were not derived from storage proteins. Four of these sequences were flagged as potentially harmful, with one being recognized by CD4+ T cells and one by the R5 mAb (Table 1). Furthermore, within the K7WF86 protein group (γ-75 k-secalins), three tetrapeptides (**SQEPF**PQVHQP**QQPS**PQQ**QQPS**IQLSLQQQLNPCK) showed potential immunoactivity. These sequences were also present in four other protein groups (C4NFP2, A0A5B9Y471, W6AW92, and W6AW98), each containing one tetrapeptide. The peptide identified within the protein group Q94IJ7 has the sequence QPGQG**QQGYYPTSPQ**HPGQGQQPGQGQQPGQGK. This sequence is recognized by CD4+ T lymphocytes because it is a component of the DQ8.5-glut-H1 limited epitopes and contains the 9-mer core region QGYYPTSPQ, according to Sollid, 2002. This epitope was uncovered through experiments with T-cell clones derived from HLA-DQ2/8 CD patients and was determined to be associated with HMW-glutenins, as reported by Kooy-Winkelaar et al., 2011. The protein group Q94IJ7 was assigned to the HMW-secalin fraction. This decision was made based on the similarity in amino acid composition between HMW-secalins and HMW-glutenins, suggesting that this protein group likely belongs to HMW-secalins. A BLAST search within UniProtKB on the sequence resulted in 9 protein groups (5 of them reviewed, March 2023) with 100 % coverage of the sequence, which are part of the glutenin subunits of *Triticum aestivum* and *Triticum urartu*. However, peptide sequences discovered in the isolates using our search method did not generally indicate a large amount of peptides. The enzyme selected for digestion has a significant impact on the quantity of immunoreactive peptides to be found (Manfredi et al., 2015). Trypsin and chymotrypsin are the two enzymes that are most frequently utilized in proteomics to break down gluten proteins (Martínez-Esteso et al., 2017; Pasquali et al., 2019; Schalk et al., 2017). Gluten proteins in general are rich in proline and glutamine residues, which are resistant to digestion by many enzymes. Because gluten proteins have few tryptic cleavage sites, the resulting peptides are longer and less abundant. Therefore, in order to establish more accurate predictions regarding the possible immunoreactivity, it is important to examine the isolates with several enzymes, and this should be taken into account in next studies.

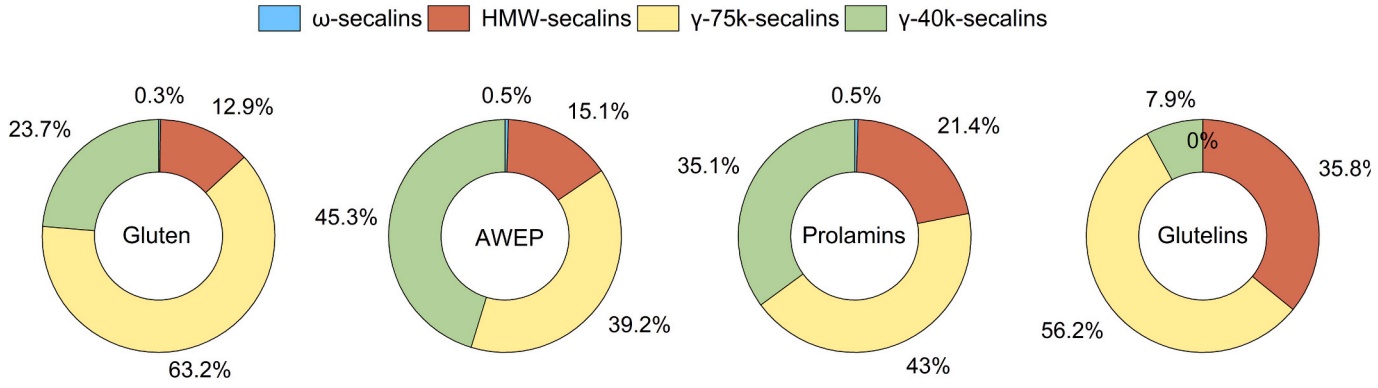

Fig. 4. Secalin distribution of the four isolates analyzed by LC-MS/MS. AWEP: acetonitrile/water-extractable proteins.

3.3. R5 ELISA responses of the isolates

The response of the R5 mAb to different rye isolates was evaluated by the comparison of the resulting absorbances using the R5 sandwich ELISA (Fig. 5). The estimated prolamin, glutelin, and gluten contents were determined using linear approximations (Table S10). The slope of the linear equation provides information about the strength of the response of the isolates to the R5 mAb. $PROL_{iso}$ (slope = 6.719, R^2 = 0.997) showed the highest response against the R5 mAb followed by $AWEP_{iso}$ (slope = 4.306, R^2 = 0.999), G_{iso}, (slope = 2.872, R^2 = 0.995) and $GLUT_{iso}$ (slope = 1.331, R^2 = 0.999). This order is in accordance with the findings by Lexhaller et al., 2016 where the prolamins showed the highest response against the R5 mAb compared to glutelins. The epitope QQPFP is repetitively present in several gluten proteins including secalins (Méndez et al., 2005). The R5 mAb was developed using rye secalins as antigen, leading to the expectation that the antibody would strongly bind to rye prolamins, as demonstrated in previous studies (Lexhaller et al., 2016; Wieser & Koehler, 2009). The rye glutelins showed a high sensitivity too, which can be attributed to the fact that the epitope QQPFP is also prominently found in the γ-75 k-secalins. Using UniProtKB, the sequences of the secalins obtained from the untargeted LC-MS/MS experiment were examined in terms of presence of the QQPFP epitope. This epitope is present in C4NFP2 13 times (as ω-secalins), K7WF86 ten times (as γ-75 k-secalins), H6ULI9 twelve times (as γ-75 k-secalins), H8Y0N7 only once (as γ-40 k-secalins), and A0A5B9Y471 three times (as γ-40 k-secalins). The other protein groups found by LC-MS/MS (γ-40 k (F4ZL28), HMW (W6AW92, W6AW98, Q94IJ7) did not contain the QQPFP epitope. The R5 mAb response to $PROL_{iso}$ and $AWEP_{iso}$ was comparable, which may be because both extraction methods mostly extracted prolamins, resulting in a similar protein composition in the two samples. G_{iso} is composed of prolamins and glutelins and showed a response between $PROL_{iso}$ and $GLUT_{iso}$. Further studies in relation to validation parameters such as accuracy, recovery and repeatability should be carried out.

3.4. Comparison of the isolates

The four isolates differed significantly in yields showing the highest yields for $PROL_{iso}$ followed by G_{iso} and $GLUT_{iso}$ and the smallest yield for $AWEP_{iso}$. In terms of protein content, the lowest was that of $GLUT_{iso}$, indicating the lower extractability of the glutelins. In terms of protein composition compared to the original flours, minor differences in secalin distribution were observed in RP- and GP-HPLC analyses. The overall distribution pattern of the isolates measured by RP-HPLC was as follows: γ-75 k > ω/HMW ≥ γ-40 k. All four isolates demonstrated similar secalin composition and molecular weight distribution to the corresponding flours. Through the separation of proteins using SDS-PAGE, consistent band patterns were observed within the γ-75 k-, HMW-, and γ-40 k-secalins when comparing isolates and flours, with the exception of ω-secalins. Notably, all isolates exhibited less intense bands of ω-secalins in the SDS-PAGE gel. It is worth emphasizing the reliability of RP-HPLC results, as protein staining discrepancies may occur with the Coomassie stain. Comparing the RP-HPLC with the untargeted MS/MS results on the secalin distribution, differences were observed especially in the relative distribution of ω-secalins. Within the MS/MS experiments the ω-secalins were represented only with relatively small percentages. For all isolates besides $AWEP_{iso}$, the γ-75 k-secalins were present in the highest percentage in both the RP-HPLC (Section 3.2.4) and LC-MS/MS (Section 3.2.5) experiments. All four isolates were investigated in different concentrations in terms of the evaluation of the responsiveness towards the R5 mAb showing the following order: $PROL_{iso}$ > $AWEP_{iso}$ > G_{iso} > $GLUT_{iso}$ > PWG-gliadin. All four isolates were completely soluble using the R5 mAb sandwich ELISA protocol.

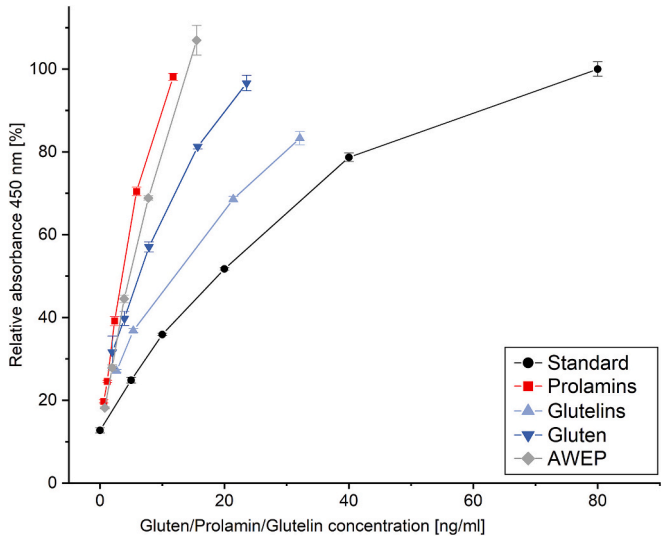

Fig. 5. ELISA responses to the four isolates. R5 sandwich ELISA absorbances (λ = 450 nm) of the test kit standard (PWG-gliadin), the prolamin, glutelin, gluten and acetonitrile/water-extractable protein (AWEP) fractions of rye as a function of the concentrations quantitated by RP-HPLC. Error bars indicate the minimum and the maximum value.

4. Conclusion

The first objective of this work was to investigate the influence of the harvest year on the protein variability of selected rye cultivars and the corresponding flour mixture. Mixing of cultivars led to a secalin distribution similar to the calculated mean reducing the genetic and environmental effects and hence the variability in the protein composition. The study further aimed to produce four different secalin isolates using the flour mixture. Depending on the extraction method, changes were observed in the secalin composition, which need to be considered in future RM production. Overall, we demonstrated the successful production of secalin isolates that are representative of the secalin composition in the flour. The LC-MS/MS characterization of the isolates revealed discrepancies in terms of the quality of the database. Some of the entries for rye proteins are incomplete or inaccurately named and not reviewed. Therefore, the interpretation of the data should be viewed critically. Moreover, further investigations using additional digestive enzymes are suggested for future research. The produced and characterized isolates have the potential to be used in a variety of tests such as the production of antibodies for ELISA test systems, RM for analytical methods such as ELISA, RP-HPLC, LC-MS/MS and the development of T cell assays. The use of a grain-specific RM on ELISA test systems is recommended when the source of contamination is known. More research in relation to validation parameters such as accuracy, recovery and repeatability should be carried out using the produced isolates. Furthermore, this study contributes significantly to the understanding of rye secalins.

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CRediT authorship contribution statement

Majlinda Xhaferaj: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gabriella Muskovics:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Zsuzsanna Bugyi:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Sándor Tömösközi:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Katharina A. Scherf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.142691.

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