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New insights into wheat toxicity: Breeding did not seem to contribute to a prevalence of potential celiac disease's immunostimulatory epitopes

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ABSTRACT

Gluten proteins, namely gliadins, are the primary trigger of the abnormal immune response in celiac disease. It has been hypothesised that modern wheat breeding practices may have contributed to the increase in celiac disease prevalence during the latter half of the 20th century. Our results do not support this hypothesis as *Triticum aestivum* spp. *vulgare* landraces, which were not subjected to breeding practices, presented higher amounts of potential celiac disease's immunostimulatory epitopes when compared to modern varieties. Furthermore, high variation between wheat varieties concerning the toxic epitopes amount was observed. We carried out quantitative analysis of gliadin types by RP-HPLC to verify its correlation with the amount of toxic epitopes: ω-type gliadins content explain about 40% of the variation of toxic epitopes in tetraploid wheat varieties. This research provides new insights regarding wheat toxicity and into the controversial idea that human practices may have conducted to an increased exposure to toxic epitopes.

1. Introduction

Wheat is one of the most important crops in the world for its global impact on human nutrition (Shewry & Tatham, 2015). From wheat flour a large variety of food products can be made, such as leavened and unleavened breads, noodles, cookies, cakes, and pastries. The glutenins and gliadins are the two main protein components that determine in a complementary way the technological characteristics of wheat flour. The balance between glutenins and gliadins, the two gluten components, is crucial to the gluten network properties and considered as a major end-use determinant of wheat quality (Wrigley, Békés, & Bushuk, 2006).

Nevertheless, digestion-resistant gluten peptides are the primary trigger of the immune response in celiac disease, one of the most common immune based diseases in present-day society. This disease causes villous atrophy of intestinal mucosa, leading to poor nutrient absorption and the most common symptoms include malnutrition, diarrhoea, growth retardation, anaemia, and fatigue. To date the only effective treatment is a strict, life-long, gluten-free diet (Lebwohl, Ludvigsson, & Green, 2015).

Within gluten proteins, gliadins are considered to have the highest clinical relevance both regarding the innate and adaptive immune responses that lead to the development of celiac disease. Moreover, the different gliadin types (α/β-type, γ-type and ω-type gliadins) have been shown to have an important and variable role in the pathogenesis of the disease (Camarca et al., 2009; Tye-Din et al., 2010) and a hierarchy of T cell–stimulating peptides (immunodominance) was described (Anderson, Degano, Godkin, Jewell, & Hill, 2000; Anderson et al., 2005; Shan et al., 2002, 2005; Tye-Din et al., 2010).

Most gliadins are encoded at six main *loci*, *Gli-A1*, *Gli-B1*, *Gli-D1*, *Gli-A2*, *Gli-B2*, and *Gli-D2*, on the short arms of homeologous chromosome groups 1 and 6. Nevertheless, its genetic control is complex (Anderson & Greene, 1997; Anderson, Gu, Kong, Lazo, & Wu, 2009; Anderson, Huo, & Gu, 2013) and a genetic linkage between *Gli-1 loci* that encode some gliadins and *Glu-3 loci* (Low molecular weight-glutenin subunits, LMW-GS) was described (Branlard & Metakovsky, 2006). In terms of technological characteristics,

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gliadins are regarded as "lubricants" for aggregated glutenins being responsible for the viscosity of wheat dough (Belitz, Grosch, & Schieberle, 2004). Also, the amounts of specific gliadin types and total gliadins in wheat flour are associated with different rheological properties (Branlard & Dardevet, 1985; Khatkar, Fido, Tatham, & Schofield, 2002; Uthayakumaran et al., 2001; van Lonkhuijsen, Hamer, & Schreuder, 1992; Wieser, Seilmeier, & Belitz, 1994).

Regarding wheat breeding, it is well-known that within gluten proteins, glutenins have received the most attention of scientists and breeders as these proteins are the major genotypic determinants of dough strength by conferring viscoelasticity to the wheat dough which determines whether a particular wheat variety is suitable for bread making (Shewry et al., 2003). It has been suggested that breeding practices may have contributed to an increased prevalence of celiac disease (van den Broeck, de Jong et al., 2010). However, these authors studied a limited number of varieties and a few wheat grown species.

In order to develop a more comprehensive approach, we performed a comprehensive study involving modern *Triticum aestivum* spp. *vulgare* varieties and landraces, *T. aestivum* spp. *spelta* varieties, and modern *Triticum turgidum* spp. *durum* varieties and landraces from different countries, multiplied in the year 2014 in the same location. The amount of potential celiac disease immunostimulatory epitopes was measured with the R5 monoclonal antibody. In addition, we conducted quantitative analysis by Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) and Acid-Polyacrylamide Gel Electrophoresis (A-PAGE) of different gliadin types in order to evaluate the correlation between toxic epitopes and different gliadins' content. We conclude by proposing a fast model to discriminate wheat ploidy based on the content of the different gliadin types.

2. Material and methods

2.1. Plant materials

The wheat varieties studied include *T. aestivum* spp. *vulgare* modern varieties (53), *T. aestivum* spp. *vulgare* landraces (19), *T. aestivum* spp. *spelta* varieties (20), *T. turgidum* spp. *durum* modern varieties (15) and *T. turgidum* spp. *durum* landraces (19) (Table 1). The landraces and spelt wheat varieties used came from the Plant Genetic Resources Centre (CRF, INIA, Alcalá de Henares) and USDA-ARS National Small Grains Collection. All genotypes were grown in the Agronomy Engineers School Experimental Station (Madrid, Spain) (40°25′N, 3°42′W) during the 2013–2014 season, with conventional fertilization and full fungicide protection. Harvested kernels were maintained at constant moisture before milling. Wholemeal flour was used for protein extraction and fractionation. In order to simplify the references along the text in relation to the different wheat varieties analyzed in this study, we used the following associations: *T. aestivum* spp. *vulgare* varieties which were subjected to breeding practices as *T. aestivum* modern varieties, *T. aestivum* spp. *vulgare* landraces varieties as *T. aestivum* landraces, *T. aestivum* spp. *spelta* varieties as *Triticum spelta*, *T. turgidum* varieties which were subjected to breeding practices as *T. turgidum* modern varieties and *T. turgidum* landraces were referenced likewise.

2.2. R5 competitive ELISA immunoassay

Celiac-related toxic epitopes were quantified using the commercial product RIDASCREEN® Gliadin competitive (R-Biopharm AG, Darmstadt, Germany). This product is based on an enzyme-linked immunosorbent assay (ELISA) with competitive format and the Codex Alimentarius standard R5 monoclonal antibody, recognizing as core sequences the toxic following peptides: QQPFP, QQQFP, LQPFP, QLPFP, QLPYP, among others that occur repeatedly in the proteins of gluten. The format of this competitive assay has the advantage of detecting individual peptide fragments compared to the sandwich ELISA format. The detection limit is 1.36 mg/kg of gliadin and the quantification limit is 5 mg/kg of gliadin. All instructions of RIDASCREEN ® Gliadin competitive product were strictly followed. Several dilutions were performed for better quantification of the different samples. $n = 2$ replicates.

2.3. Protein content determination and RP-HPLC quantitative analysis

Wholemeal protein content, on a 14% moisture basis, was estimated by near-infrared reflectance analysis (NIR) using a Technicon Infralyzer 300 (Technicon Instrument Co. Ltd, Hants, U.K.) (AACC, 1983).

Gliadins were separated by Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) as described elsewhere (Wieser et al., 1994) and the extraction was carried out based on Singh, Shepherd, and Cornish (1991). For HPLC analysis, a RP-C8 column was used (25 cm, 4.5 mm i.d., 5 μm, Macherey-Nagel, Germany) maintained at 50 °C during the separation process, and an injection volume of 100 μL was used. A gradient elution was performed; eluent A consisting of 0.1% (v/v) aqueous trifluoroacetic acid and eluent B consisting of acetonitrile and trifluoroacetic acid (99.9/0.1%, v/v), with following elution program: 0 min 28% B, 30 min 56% B, flow rate of 1 mL/min. Detection was made by ultraviolet (UV) absorbance at 210 nm. After each analysis the column was cleaned by using 90% B for 5 min and equilibrated to 28% B over 10 min. Development of a standard curve for the RP-HPLC quantitative procedure was conducted through the use of increasing Bovine Serum Albumin (BSA) concentrations. The measurement repeatability was assayed and proved to be <3%.

2.4. Acid-Polyacrylamide Gel Electrophoresis (A-PAGE)

Gliadins were fractionated in acidic pH 3.1 by employing 7.5% polyacrylamide gel (A-PAGE) as described elsewhere (Lafiandra & Kasarda, 1985). Two replicates for each wheat variety were used and the varieties 'Chinese Spring' and 'Marquis' were used as standards to assign the different gliadin types, namely α, β, γ, and ω, in decreasing mobility in acid-PAGE. The relative amounts of the different gliadin types derived from the areas beneath the peaks in the different assigned regions of the density plot were calculated using ImageJ software (Schneider, Rasband, & Eliceiri, 2012).

2.5. Statistical analysis

The results are expressed as mean ± standard deviation (SD). Differences among the different wheat groups concerning toxic epitopes amount were determined by one-way analysis of variance (ANOVA). Multiple comparisons were made using the Tukey's post hoc test, and the criterion for significance was $p < 0.05$. Bartlett's test was used to verify the homogeneity of variances and logarithmic transformation was applied whenever a variable did not pass the test. Pearson correlation produced a sample correlation coefficient, r, which measures the strength and direction of linear relationships between pairs of continuous variables ($p < 0.05$; Two-tailed). Multivariate analysis of variance (MANOVA) with Pillai's trace test was performed to assign differences between hexaploid and tetraploid wheat varieties using the multiple variables (total gliadins content and gliadin types content) and for controlling the correlation between the dependent variables, and planned contrast was used to access which dependent variables are different between the two groups. Canonical discriminant analysis (CDA) was performed to assign differences between hexaploid and

Table 1
General table gathering the results about origin, different gliadin types content and distribution, protein content and potential celiac disease toxic epitopes content by R5 monoclonal antibody.

T. aestivum modern varieties	Origin	Gliadin content[a]					Gliadin distribution[b]				Protein content (%)[c]	R5 reactivity (gliadins g/kg)
		ω5 g/100 g	ω1.2 g/100 g	α/β g/100 g	γ g/100 g	Total g/100 g	ω %	γ %	β %	α %		
'Abel'	France	0.32	1.32	4.98	2.84	9.46	18.70 ± 1.26	22.77 ± 2.46	25.23 ± 1.90	33.30 ± 3.10	14.13	149.46 ± 4.51
'Aca 601'	Argentina	0.25	0.83	2.57	2.17	5.82	21.32 ± 0.71	33.51 ± 0.55	27.89 ± 0.81	17.28 ± 2.07	10.58	122.24 ± 0.96
'Aca 801'	Argentina	0.33	1.10	3.74	2.74	7.91	29.10 ± 1.41	23.62 ± 0.11	21.42 ± 0.27	25.86 ± 1.04	11.85	124.56 ± 14.16
'Adagio'	France	0.47	1.19	3.00	2.51	7.17	16.52 ± 1.93	24.60 ± 2.24	29.25 ± 0.06	29.64 ± 0.37	11.60	61.40 ± 5.79
'Alcazaba'	ND	0.58	1.18	4.89	2.66	9.32	20.57 ± 3.39	23.23 ± 0.69	26.80 ± 0.11	29.40 ± 2.59	12.42	190.56 ± 5.46
'Alejo'	ND	0.13	1.33	3.85	2.47	7.78	26.98 ± 0.39	23.85 ± 0.67	22.45 ± 0.39	26.71 ± 1.46	12.35	368.22 ± 34.46
'Alfori'	ND	0.83	2.07	4.60	3.12	10.64	24.20 ± 0.05	25.33 ± 1.00	15.45 ± 0.67	35.02 ± 0.28	13.63	88.72 ± 10.98
'Almansor'	Portugal	0.18	2.01	4.61	3.55	10.35	26.71 ± 2.46	25.99 ± 1.16	22.05 ± 0.96	25.25 ± 2.65	10.92	86.28 ± 10.11
'Amadina'	Mexico	0.18	1.53	2.29	2.82	6.83	27.38 ± 0.90	33.68 ± 0.99	17.08 ± 0.89	21.86 ± 0.80	10.87	270.88 ± 27.91
'Amiro'	ND	0.79	1.90	6.12	2.87	11.68	21.32 ± 0.26	25.30 ± 1.52	27.01 ± 2.48	26.38 ± 0.69	13.30	154.53 ± 2.21
'Ampuero'	ND	0.41	1.39	4.39	2.65	8.85	20.64 ± 0.78	26.14 ± 0.31	26.28 ± 0.21	26.94 ± 0.68	13.28	63.26 ± 10.37
'Anza'	Mexico	0.17	2.80	4.66	2.75	10.38	23.53 ± 2.68	35.14 ± 0.60	19.62 ± 2.01	21.71 ± 0.07	11.77	133.39 ± 7.88
'Apache'	USA	0.36	1.47	2.59	2.21	6.63	25.88 ± 2.15	29.21 ± 4.89	20.73 ± 0.84	24.17 ± 6.19	10.24	68.65 ± 4.90
'Apuesto'	ND	0.47	2.18	3.87	2.82	9.33	29.90 ± 3.04	27.07 ± 0.96	24.63 ± 0.10	18.39 ± 2.18	12.94	225.90 ± 39.84
'Arcole'	France	0.61	1.93	3.66	2.52	8.72	25.14 ± 0.60	27.08 ± 0.65	19.32 ± 1.78	28.46 ± 1.82	12.96	73.96 ± 5.11
'Arganda'	Spain	0.17	2.34	3.20	2.82	8.53	26.79 ± 2.07	27.39 ± 1.97	24.57 ± 1.82	21.25 ± 2.23	12.37	141.97 ± 13.26
'Arminda'	Netherlands	0.35	1.67	4.15	2.69	8.86	25.31 ± 0.26	28.94 ± 1.97	22.80 ± 1.28	22.95 ± 0.43	12.60	211.39 ± 22.64
'Asteroide'	ND	0.54	2.52	4.85	3.15	11.06	24.31 ± 0.55	24.48 ± 2.10	24.26 ± 2.66	26.95 ± 0.01	13.31	114.57 ± 7.47
'Átomo'	Spain	0.19	1.05	2.44	2.69	6.37	25.84 ± 0.63	30.37 ± 3.17	24.55 ± 0.58	19.24 ± 3.12	11.74	127.41 ± 15.29
'Atrevido'	Spain	0.21	0.90	2.54	2.54	6.20	19.31 ± 0.27	30.07 ± 0.55	33.91 ± 0.43	16.71 ± 0.38	12.29	257.36 ± 26.15
'Babui'	Spain	0.30	1.24	2.99	1.96	6.50	20.38 ± 0.45	25.79 ± 0.65	28.73 ± 1.50	25.10 ± 1.70	11.01	141.70 ± 21.71
'Balthasar'	France	0.39	1.00	3.58	2.03	7.00	27.35 ± 0.24	23.17 ± 0.42	22.65 ± 0.96	26.83 ± 0.31	11.92	85.35 ± 5.40
'Bungulla'	Australia	0.24	0.72	2.37	2.49	5.82	26.69 ± 0.50	32.58 ± 1.40	21.02 ± 0.91	19.70 ± 0.02	10.47	110.36 ± 5.75
'Caia'	Portugal	0.24	0.65	2.05	1.81	4.75	20.20 ± 0.20	30.56 ± 1.12	27.25 ± 0.71	21.99 ± 0.61	10.58	90.32 ± 6.92
'Camargo'	France	0.37	1.38	2.74	2.00	6.48	25.46 ± 0.74	23.62 ± 1.22	24.41 ± 0.11	26.52 ± 0.59	10.75	150.92 ± 12.01
'Cartaya'	Spain	0.20	1.94	3.40	2.90	8.44	25.86 ± 0.27	29.07 ± 0.51	21.63 ± 1.45	23.44 ± 0.68	11.47	110.93 ± 11.48
'Chinook'	Canada	0.39	1.37	3.90	3.31	8.97	25.46 ± 0.76	25.56 ± 0.66	23.94 ± 1.44	25.04 ± 1.34	11.26	98.48 ± 0.28
'Étoile de Choisy'	France	0.55	0.93	4.45	3.03	8.96	23.18 ± 0.85	27.54 ± 0.79	29.30 ± 0.36	19.98 ± 0.42	12.52	162.03 ± 13.33
'Exotic'	France	0.26	1.01	1.98	1.49	4.74	21.66 ± 0.91	31.24 ± 0.69	20.66 ± 1.32	26.44 ± 2.92	11.66	126.73 ± 0.77
'Fengmai 27'	China	0.52	1.34	4.18	2.59	8.63	27.46 ± 1.22	22.87 ± 1.35	30.38 ± 0.15	19.29 ± 0.01	12.07	103.53 ± 4.66
'Forby'	France	0.83	1.25	3.00	1.20	6.29	19.10 ± 1.30	28.93 ± 0.24	24.61 ± 0.83	27.36 ± 0.71	13.12	100.75 ± 25.58
'Gaillard'	France	0.74	1.39	5.33	2.27	9.73	32.87 ± 2.57	20.77 ± 0.99	25.25 ± 0.62	21.12 ± 2.19	12.30	115.60 ± 31.35
'Halberg'	Australia	0.29	1.15	3.08	3.16	7.68	16.83 ± 0.92	47.73 ± 1.75	23.31 ± 1.23	12.13 ± 1.45	11.28	147.46 ± 5.76
'Heilo'	Mexico	0.19	1.23	3.40	2.95	7.76	21.77 ± 1.24	28.68 ± 0.92	30.41 ± 0.00	19.14 ± 2.17	12.19	159.41 ± 7.62
'Jerezano'	Spain	0.27	1.09	2.93	2.50	6.78	24.77 ± 0.47	28.56 ± 0.12	28.57 ± 0.71	18.10 ± 1.06	12.42	75.79 ± 1.33
'Kharkov'	Ukraine	0.55	2.72	3.55	3.50	10.31	27.21 ± 0.65	22.30 ± 2.82	25.74 ± 2.05	24.75 ± 1.42	13.84	177.34 ± 5.42
'Klein Jabalí'	Argentina	0.23	1.26	2.97	1.83	6.30	25.34 ± 1.21	26.73 ± 0.32	31.45 ± 0.74	16.48 ± 0.15	11.54	88.07 ± 0.35
'Kumberri'	France	0.35	0.88	1.73	1.47	4.43	28.03 ± 2.99	29.29 ± 0.88	22.37 ± 0.48	20.31 ± 3.39	10.71	50.99 ± 5.81
'Leader'	Canada	0.40	1.36	2.59	3.48	7.84	26.83 ± 0.26	30.01 ± 1.67	29.00 ± 1.47	14.16 ± 2.87	12.68	240.42 ± 34.82
'Marius'	France	0.99	1.15	5.75	2.96	10.86	25.86 ± 0.27	27.37 ± 0.39	22.58 ± 0.15	24.19 ± 0.27	11.05	33.65 ± 0.16
'Montserrat'	ND	0.35	1.43	5.17	3.45	10.39	26.75 ± 0.49	26.91 ± 0.50	23.67 ± 1.03	22.67 ± 1.04	11.42	63.87 ± 4.74
'Mulhacén'	Spain	0.21	1.46	3.50	3.18	8.35	19.43 ± 0.31	28.41 ± 0.68	26.18 ± 0.02	25.98 ± 0.96	11.08	123.94 ± 26.54
'Nambukomogi'	Japan	0.66	1.48	4.95	2.45	9.54	26.08 ± 0.47	25.91 ± 0.17	25.19 ± 0.80	22.82 ± 0.16	12.05	191.37 ± 19.11
'Norin 61'	Japan	0.35	0.83	2.47	1.81	5.47	30.74 ± 0.98	26.78 ± 0.27	23.16 ± 0.18	19.33 ± 0.52	10.30	130.31 ± 14.29
'Paledor'	France	0.32	1.24	3.82	2.31	7.68	25.91 ± 0.65	27.14 ± 1.16	31.61 ± 1.89	15.33 ± 1.39	11.68	90.93 ± 10.59
'Pernel'	France	0.16	0.54	2.24	1.74	4.67	20.92 ± 0.17	31.31 ± 1.00	26.14 ± 0.29	21.64 ± 0.87	9.23	32.14 ± 0.88
'Pirana'	Portugal	0.15	0.77	2.72	2.21	5.85	20.25 ± 0.04	30.07 ± 0.15	30.99 ± 0.44	18.69 ± 0.55	11.52	152.60 ± 30.56
'Radja'	France	0.25	1.21	2.86	2.68	7.01	24.33 ± 0.54	28.53 ± 0.46	26.87 ± 0.44	20.27 ± 0.37	10.68	95.01 ± 28.53
'Rescue'	Canada	0.48	1.32	3.77	3.04	8.60	26.51 ± 1.13	26.98 ± 1.02	27.64 ± 0.61	18.88 ± 0.50	11.89	130.14 ± 1.96
'Sarina'	Netherlands	0.19	1.46	3.53	2.56	7.74	28.85 ± 0.10	24.48 ± 0.67	28.34 ± 1.73	18.33 ± 0.96	13.27	113.64 ± 12.20
'Soissons'	France	0.30	0.89	3.86	2.57	7.62	34.86 ± 2.04	21.39 ± 1.99	20.75 ± 1.41	23.01 ± 1.36	13.36	194.24 ± 18.45
'Tejada'	Spain	0.57	0.80	2.68	2.82	6.87	29.09 ± 0.91	26.17 ± 1.43	33.02 ± 0.45	11.73 ± 1.89	11.64	151.94 ± 6.77
'Timstein'	USA	0.33	1.18	3.67	2.72	7.89	22.11 ± 1.15	23.91 ± 1.13	29.38 ± 1.35	24.60 ± 1.33	13.97	263.51 ± 46.33
T. aestivum landraces	Origin	ω5	ω1.2	α/β	γ	Total	ω	γ	β	α	Protein	R5
'Barbilla rojiza de Huelva'	Spain	0.22	0.80	3.40	2.50	6.92	21.01 ± 0.42	27.45 ± 0.50	28.26 ± 0.18	23.28 ± 0.75	12.20	101.06 ± 12.40
'Basto'	Spain	0.78	1.85	5.58	4.36	12.57	26.19 ± 2.60	27.93 ± 1.67	28.17 ± 1.99	17.72 ± 2.92	16.10	324.98 ± 15.78
'Blanco de Segarra'	Spain	0.56	1.69	6.36	3.49	12.10	22.05 ± 2.63	26.36 ± 1.61	25.59 ± 0.98	26.00 ± 1.99	12.80	158.01 ± 39.99
'Canaleja'	Spain	0.37	1.94	6.11	3.00	11.42	23.72 ± 1.71	29.82 ± 0.70	26.08 ± 1.56	20.37 ± 0.55	12.80	214.29 ± 12.50
'Cañamaciza'	Spain	0.97	3.89	4.90	4.72	14.47	21.15 ± 0.26	25.89 ± 0.36	27.33 ± 0.43	25.63 ± 0.19	14.00	258.49 ± 12.14
'Candeal de Vellisca'	Spain	0.46	1.78	4.83	2.24	9.31	29.69 ± 0.95	22.59 ± 1.31	32.74 ± 0.56	14.99 ± 0.20	13.65	175.07 ± 1.80
'Caspino'	Spain	0.37	0.98	3.57	3.06	7.98	24.29 ± 1.80	23.99 ± 0.80	24.34 ± 0.10	27.39 ± 2.70	12.40	129.79 ± 14.01
'Catalán rojo'	Spain	0.34	1.34	4.93	3.08	9.68	20.89 ± 0.21	26.34 ± 0.82	29.48 ± 0.55	23.28 ± 0.06	12.20	176.98 ± 25.98
'Jeja blanca'	Spain	0.40	3.26	4.50	3.26	11.42	23.18 ± 1.91	25.05 ± 1.85	26.68 ± 0.93	25.09 ± 0.87	14.00	354.32 ± 31.66
'Jeja colorada de Albacete'	Spain	0.44	1.51	5.34	4.24	11.52	20.22 ± 0.59	28.49 ± 0.87	28.47 ± 0.69	22.83 ± 0.41	14.00	201.12 ± 9.99

Table 1 (*continued*)

T. aestivum landraces	Origin	ω5	ω1.2	α/β	γ	Total	ω	γ	β	α	Protein	R5
'Jeja manchega'	Spain	0.40	2.15	5.70	4.16	12.40	20.37 ± 0.15	30.70 ± 0.21	25.30 ± 0.07	23.63 ± 0.29	14.50	212.09 ± 29.11
'Mocho Rojo'	Spain	0.30	1.30	3.76	2.39	7.74	22.11 ± 0.53	30.26 ± 1.17	27.21 ± 1.19	20.42 ± 0.51	13.27	350.19 ± 28.85
'Pichi'	Spain	0.27	0.92	3.38	2.86	7.43	21.69 ± 0.68	27.16 ± 0.47	27.74 ± 0.80	23.41 ± 0.60	12.60	174.74 ± 18.01
'Rabón de Hinojosa'	Spain	0.34	1.24	4.01	2.43	8.02	21.50 ± 0.81	26.68 ± 0.75	26.07 ± 1.21	25.74 ± 1.16	11.30	158.83 ± 15.25
'Rojo de Caravaca'	Spain	0.34	2.03	3.48	3.79	9.64	22.00 ± 0.32	27.04 ± 1.04	33.08 ± 0.33	17.89 ± 1.04	12.72	141.08 ± 11.19
'Somiedo'	Spain	0.44	0.95	4.43	2.39	8.21	23.10 ± 0.79	25.97 ± 0.57	29.43 ± 0.25	21.51 ± 0.46	13.40	71.43 ± 0.92
'Trigo de aruga'	Spain	0.46	1.07	3.54	2.58	7.65	22.78 ± 0.76	24.48 ± 1.56	28.91 ± 0.35	23.84 ± 1.97	12.60	140.80 ± 5.92
'Trigo tremesino'	Spain	0.21	0.96	2.75	2.45	6.38	22.80 ± 0.22	25.18 ± 1.03	29.42 ± 1.10	22.61 ± 0.29	13.10	420.50 ± 29.79
'Trujillo'	Spain	0.84	1.41	5.52	2.59	10.35	23.04 ± 2.06	25.01 ± 1.61	22.38 ± 0.95	29.57 ± 0.49	15.10	366.26 ± 2.76
T. spelta	Origin	ω5	ω1.2	α/β	γ	Total	ω	γ	β	α	Protein	R5
'1320'	Tajikistan	0.54	1.02	4.94	2.90	9.41	23.40 ± 1.58	27.30 ± 0.44	25.07 ± 0.59	24.23 ± 1.73	13.00	207.91 ± 35.94
'1334'	Spain	0.36	1.72	2.86	3.86	8.79	23.88 ± 0.00	28.02 ± 0.59	28.81 ± 0.48	19.28 ± 1.06	13.30	291.45 ± 27.72
'1338'	Spain	0.47	1.25	4.45	4.13	10.30	25.35 ± 0.07	22.61 ± 1.15	23.71 ± 0.52	28.33 ± 1.60	13.50	274.50 ± 43.73
'1339'	Spain	0.64	1.95	5.86	3.38	11.83	23.85 ± 0.64	24.67 ± 0.24	25.58 ± 1.01	25.90 ± 1.42	15.00	366.26 ± 43.24
'1344'	Spain	0.54	2.11	5.41	3.51	11.58	26.78 ± 0.77	22.59 ± 0.30	25.22 ± 0.01	25.41 ± 0.46	15.60	197.13 ± 24.47
'1345'	Spain	0.47	1.75	4.64	3.95	10.81	25.92 ± 0.27	25.02 ± 1.65	21.85 ± 0.11	27.21 ± 1.49	15.20	320.69 ± 12.22
'1348'	Spain	0.68	1.89	4.96	3.85	11.38	24.77 ± 0.44	27.35 ± 1.01	26.41 ± 0.51	21.47 ± 1.09	14.70	439.39 ± 33.66
'1349'	Spain	0.34	1.68	4.65	4.00	10.67	24.57 ± 1.55	25.48 ± 0.07	29.79 ± 0.43	20.16 ± 1.20	15.70	357.44 ± 18.46
'1373'	Spain	0.97	1.93	4.02	3.97	10.89	28.26 ± 0.31	24.58 ± 0.09	24.50 ± 0.64	22.66 ± 0.43	15.40	277.48 ± 39.45
'1375'	Spain	0.40	1.47	3.97	4.48	10.32	25.32 ± 2.05	28.60 ± 2.02	26.39 ± 0.24	19.69 ± 0.27	14.40	362.54 ± 28.88
'1376'	Spain	0.53	1.64	5.53	2.62	10.32	21.91 ± 1.00	24.92 ± 0.79	26.36 ± 1.00	26.80 ± 0.79	13.90	359.61 ± 29.52
'1381'	Spain	0.51	2.06	5.49	3.67	11.72	22.76 ± 0.08	26.20 ± 1.78	27.73 ± 0.29	23.31 ± 1.57	13.30	372.36 ± 30.57
'1385'	Tajikistan	0.55	0.92	4.23	2.47	8.18	22.37 ± 1.01	27.11 ± 0.09	25.15 ± 0.46	25.37 ± 0.46	13.30	222.16 ± 9.57
'1386'	Spain	0.57	1.72	5.17	4.76	12.23	22.54 ± 2.11	26.08 ± 0.43	25.28 ± 0.66	26.09 ± 3.20	14.80	546.13 ± 9.78
'1393'	Spain	0.38	1.09	3.46	3.21	8.13	20.97 ± 0.60	26.51 ± 0.11	30.63 ± 2.01	21.89 ± 1.29	12.40	226.47 ± 13.33
'1395'	Spain	0.64	1.73	4.50	3.51	10.38	30.15 ± 1.99	21.07 ± 0.60	30.54 ± 0.41	18.25 ± 0.98	15.60	202.14 ± 17.45
'1398'	Spain	0.74	1.23	4.76	3.87	10.60	30.43 ± 0.52	23.38 ± 0.24	25.16 ± 0.35	21.04 ± 1.11	14.40	252.97 ± 9.77
'1400'	Spain	0.41	1.48	3.41	3.20	8.50	23.38 ± 2.04	28.51 ± 0.96	26.31 ± 0.03	21.80 ± 1.11	14.40	209.39 ± 6.29
'1403'	Spain	0.31	1.32	4.23	3.85	9.71	20.73 ± 0.89	29.92 ± 1.76	31.81 ± 0.43	17.54 ± 1.29	13.10	323.09 ± 19.68
'1405'	Spain	0.40	1.39	4.39	3.87	10.06	23.77 ± 2.02	29.78 ± 1.42	27.98 ± 0.69	18.47 ± 1.29	14.30	413.90 ± 11.57
T. turgidum modern varieties	Origin	ω5	ω1.2	α/β	γ	Total	ω	γ	β	α	Protein	R5
'Amilcar'	Spain	0.36	0.54	4.88	2.29	8.08	20.00 ± 1.28	25.33 ± 0.35	27.66 ± 1.10	27.01 ± 0.53	15.33	57.10 ± 1.77
'Avispa'	Spain	0.23	0.49	5.78	2.96	9.45	22.03 ± 0.37	25.14 ± 0.06	28.87 ± 0.52	23.96 ± 0.82	15.03	80.34 ± 5.10
'Burgos'	Spain	0.15	0.53	5.13	2.67	8.48	20.49 ± 0.42	27.59 ± 0.03	26.41 ± 1.37	25.51 ± 0.91	14.87	90.64 ± 1.08
'Carioca'	Spain	0.33	0.88	5.58	2.63	9.42	22.86 ± 1.38	26.36 ± 1.16	27.02 ± 0.60	23.76 ± 0.83	13.81	74.64 ± 0.59
'Carpio'	Spain	0.28	1.46	4.54	3.90	10.18	20.99 ± 0.25	24.54 ± 1.04	27.78 ± 0.38	26.70 ± 0.41	15.23	144.74 ± 14.55
'Claudio'	Spain	0.43	1.08	6.58	3.03	11.12	20.14 ± 1.10	24.28 ± 0.84	29.72 ± 0.08	25.86 ± 0.18	16.29	149.68 ± 11.37
'Core'	Spain	0.31	1.03	5.92	2.81	10.07	19.48 ± 1.16	25.72 ± 0.33	26.67 ± 0.10	28.13 ± 1.59	14.74	106.75 ± 7.32
'Don Jaime'	Spain	0.34	1.19	7.61	2.99	12.13	19.94 ± 0.28	24.88 ± 0.45	29.25 ± 0.00	25.93 ± 0.73	12.62	65.73 ± 8.61
'Don Pedro'	Spain	0.46	1.06	4.88	2.58	8.97	23.60 ± 0.34	24.83 ± 0.22	26.17 ± 0.09	25.39 ± 0.21	14.30	149.05 ± 6.47
'Don Ricardo'	Spain	0.29	1.29	3.49	2.86	7.93	22.86 ± 1.01	26.72 ± 1.52	24.96 ± 0.46	25.46 ± 0.04	13.30	91.33 ± 10.55
'Don Sebastián'	Spain	0.32	0.79	5.57	2.62	9.29	22.09 ± 1.59	26.02 ± 0.09	28.25 ± 2.35	23.64 ± 0.85	14.76	205.38 ± 15.33
'Gallareta'	Spain	0.34	0.77	4.45	2.22	7.77	19.33 ± 0.91	27.96 ± 0.17	25.92 ± 1.43	26.79 ± 0.69	13.65	119.99 ± 4.78
'Italo'	Spain	0.35	1.27	6.80	2.79	11.21	18.98 ± 1.28	24.35 ± 1.08	25.93 ± 1.63	30.74 ± 1.44	14.51	111.98 ± 9.65
'Regallo'	Spain	0.36	0.90	5.26	2.41	8.93	19.65 ± 0.51	24.91 ± 0.05	25.41 ± 0.03	30.03 ± 0.49	16.14	97.83 ± 3.20
'Simeto'	Spain	0.45	0.91	5.73	4.05	11.13	21.85 ± 1.29	23.46 ± 1.20	24.60 ± 0.26	30.10 ± 0.18	15.76	108.08 ± 7.20
T. turgidum landraces	Origin	ω5	ω1.2	α/β	γ	Total	ω	γ	β	α	Protein	R5
'Amorós Blanco'	Spain	0.05	0.91	6.59	3.33	10.87	24.69 ± 0.10	25.36 ± 0.20	24.10 ± 0.24	25.85 ± 0.06	16.00	97.46 ± 5.26
'Arisnegro'	Spain	0.36	1.34	5.47	2.65	9.81	26.85 ± 0.81	25.05 ± 0.18	26.20 ± 0.18	21.89 ± 0.81	16.53	206.74 ± 29.11
'Arisnegro Velloso Grano Rojo'	Spain	0.69	1.20	5.92	2.55	10.36	20.85 ± 0.32	24.04 ± 0.56	29.07 ± 0.18	26.03 ± 1.06	16.15	67.88 ± 7.33
'Azul de Carmona'	Spain	0.34	1.30	6.68	3.33	11.65	22.60 ± 0.35	25.39 ± 2.59	25.74 ± 0.97	26.27 ± 1.28	16.46	152.21 ± 19.64
'Basto Duro'	Spain	0.06	0.33	5.78	2.95	9.12	15.83 ± 0.47	24.62 ± 0.86	27.15 ± 0.10	32.39 ± 0.49	15.77	26.40 ± 1.65
'Baza'	Spain	0.20	0.74	4.73	3.93	9.61	14.94 ± 0.13	27.42 ± 0.71	29.21 ± 1.21	28.43 ± 1.79	15.89	117.91 ± 0.98
'Bisbal Fort'	Spain	0.49	0.98	5.48	3.47	10.42	26.09 ± 0.76	27.18 ± 2.17	23.11 ± 0.16	23.62 ± 1.25	14.73	87.01 ± 4.22
'Blanco Velloso de Vegadeo'	Spain	0.35	0.74	5.68	3.84	10.61	20.43 ± 1.35	24.10 ± 0.55	24.52 ± 0.63	30.95 ± 0.18	16.08	121.17 ± 12.67
'Candeal'	Spain	0.21	0.80	5.06	2.92	8.99	20.31 ± 0.67	25.99 ± 0.55	28.68 ± 1.20	25.02 ± 1.32	14.08	77.87 ± 4.98
'Colorado de Cabra'	Spain	0.43	1.23	6.47	3.03	11.16	23.13 ± 0.31	25.80 ± 0.41	23.92 ± 0.92	27.16 ± 0.20	16.06	212.70 ± 47.64
'Don Benito'	Spain	0.50	2.35	6.23	2.42	11.50	28.47 ± 2.05	22.49 ± 0.35	24.96 ± 2.33	24.08 ± 0.63	16.08	212.39 ± 5.77
'Las Palmas 7'	Spain	0.50	1.81	4.66	3.42	10.39	25.68 ± 0.66	23.00 ± 0.64	19.21 ± 0.43	32.11 ± 0.45	16.17	164.43 ± 3.67

(*continued on next page*)

Table 1 (*continued*)

T. turgidum landraces	Origin	ω5	ω1.2	α/β	γ	Total	ω	γ	β	α	Protein	R5
'Mazachón de Balazote'	Spain	0.46	1.55	6.00	4.17	12.18	25.92 ± 0.37	25.46 ± 0.43	26.23 ± 0.68	22.39 ± 0.12	15.51	151.85 ± 9.40
'Recio'	Spain	0.39	1.84	5.97	2.54	10.74	22.11 ± 0.25	24.16 ± 0.57	30.47 ± 1.01	23.27 ± 0.69	15.51	81.06 ± 8.55
'Recio Cañihueco'	Spain	0.31	0.47	6.39	3.74	10.91	14.14 ± 1.82	28.51 ± 1.06	29.07 ± 0.23	28.28 ± 2.65	15.09	51.24 ± 3.97
'Rubio de Montijo'	Spain	0.50	0.90	4.59	5.36	11.35	21.21 ± 0.80	26.39 ± 0.60	22.79 ± 0.93	29.61 ± 0.73	16.14	131.43 ± 4.99
'Trigo'	Spain	0.39	1.33	7.34	3.28	12.34	19.96 ± 0.73	24.72 ± 0.28	26.10 ± 0.16	29.22 ± 0.85	15.16	98.87 ± 0.09
'Trigo Fuerte'	Spain	0.23	0.61	4.11	3.76	8.71	22.80 ± 0.31	22.78 ± 0.08	28.34 ± 0.08	26.08 ± 0.31	13.98	77.04 ± 1.16
'Valenciano'	Spain	0.63	2.32	6.51	2.78	12.23	25.64 ± 0.01	22.12 ± 0.61	24.90 ± 0.90	27.33 ± 0.28	16.21	223.14 ± 30.04

ND, not determined.
[a] Gliadin content as is basis by RP-HPLC (g/100 g flour).
[b] Gliadin distribution by A-PAGE (%).
[c] 14% humidity.

tetraploid wheat varieties, and build a potential ploidy diagnostic model applicable to real samples. CDA is the most frequently used supervised pattern recognition technique, that is, the class membership has to be known for the analysis. In the method of CDA a linear function of the original variables is calculated which maximises the ratio of between-class variance and minimized the ratio of within-class variance. The latent variable obtained in this way, called canonical variate, is a linear combination of the original variables, and its values are the roots. If we have k classes, k-1 canonical variates can be determined. Finally, a percentage of correct classification is calculated using the discriminant function. Statistics were computed using the statistical package STATISTICA from Statsoft (v10; Tulsa, Oklahoma, USA). Box plot figure was generated by GraphPad software (GraphPad Prism v6.03, GraphPad Software, La Jolla, California, USA) and correlograms were generated in R (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

The different gliadin types content measured by RP-HPLC, relative content of gliadin types obtained by A-PAGE, as well as the general protein content and celiac disease toxic epitopes's content by R5 monoclonal antibody are shown in Table 1.

3.1. Wheat breeding did not contribute to a prevalence of potential celiac disease's immunostimulatory epitopes

In order to evaluate the presence of celiac disease-related T cell stimulatory peptides in a large population of wheat varieties (126) composed by *T. aestivum* ssp. *vulgare* modern varieties (53), *T. aestivum* ssp. *vulgare* landraces (19), *T. aestivum* ssp. *spelta* landraces (20), *T. turgidum* modern varieties (15) and *T. turgidum* landraces (19), we used the Codex Alimentarius standard R5 monoclonal antibody, known to recognize the potential celiac-toxic repetitive pentapeptide epitopes (Kahlenberg et al., 2006; Osman et al., 2001). Moreover, the R5-based assay showed a good correlation with the data derived from T-cell lines made from biopsies of HLA-DQ2–and HLA-DQ8–positive celiac disease patients (Gil-Humanes, Piston, Tollefsen, Sollid, & Barro, 2010).

Our results indicate that wheat varieties differ significantly in the level of the analyzed T-cell–stimulatory epitopes (Table 1). On the one hand, it means that there is a good potential of selection of varieties with low content of toxic epitopes. For example the *T. aestivum* modern variety 'Pernel' presented more than 11-fold less toxic epitopes than the variety 'Alejo'. This natural variation in toxicity of wheat was confirmed by other authors (Spaenij-Dekking et al., 2005; van den Broeck, de Jong et al., 2010; van den Broeck, Hongbing et al., 2010). On the other hand, in response to the suggestion that wheat breeding may have contributed to wheat varieties with higher toxicity for celiac patients, as *T. aestivum* landraces, which are not subjected to breeding practices, at least in a level compared to modern varieties, showed higher content of toxic epitopes than modern varieties, we can infer that breeding practices did not contribute to a prevalence of celiac disease immunostimulatory epitopes (Fig. 1).

The opposite scenario was suggested by other authors (van den Broeck, de Jong et al., 2010) who classified the toxicity of hexaploid wheat varieties by immunoblotting with Glia-α9 and Glia-α20 antibodies. Despite this, the presence of the Glia-α9 epitope was higher in the modern varieties, whereas the presence of the Glia-α20 epitope was lower, as compared to the landraces. Our results even suggest the contrary regarding the significant difference of means. Moreover, it should be noted that the 75th percentile of *T. aestivum* landraces is clearly greater than the 25th, emphasizing the unequal distribution in this group (Fig. 1). Although, the R5-based assay does not contemplate all the toxic epitopes and further complementary assays are necessary as T-cell lines made from biopsies of HLA-DQ2– and HLA-DQ8–positive celiac disease patients and ultimately *in vivo* gluten challenge and subsequent

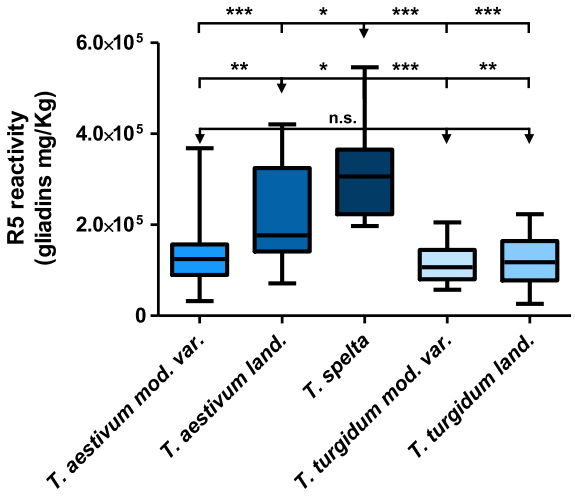

Fig. 1. Box plot representation of R5 reactivity values for the different groups of wheat analyzed. Lower and upper boundaries of each box indicate the 25th and the 75th percentile, respectively. Ranges are represented as bars (whiskers) below and above the box and indicate the minimum and maximum value, respectively. The horizontal line in each box represents median. n.s. not significant, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

analysis of bioptically obtained small intestinal tissue, the gold standard for celiac disease toxicity testing, fundamental and immunodominant stimulatory epitopes (Anderson et al., 2000, 2005; Shan et al., 2002, 2005; Tye-Din et al., 2010) are monitored by this method.

In fact, considering that breeding programs with focus on gluten proteins/quality have most often the glutenins and the glutenin macropolymer as target (Shewry et al., 2003), gliadins which have the highest clinical relevance (Camarca et al., 2009), remain somewhat unchanged. Also, the content of gliadins could be negatively affected by the growing content of glutenins as compensation behaviour of storage protein synthesis in wheat was observed in aneuploid lines (Dumur et al., 2004) or after inhibition of the expression of α-gliadins by RNA interference (RNAi) (Wieser et al., 2007). Furthermore, a recent work did not find any clear evidence that wheat breeding for higher gluten content in United States may have contributed to the increase in celiac disease prevalence during the latter half of the 20th century (Kasarda, 2013). In this sense, although the reason for this rise is still unknown, it seems that wheat breeding is not a cause. Probably, the better diagnosis of symptomatic subjects by serological tests and asymptomatic subjects belonging to at-risk groups by the detection of HLA DQ2/DQ8 alleles (Lebwohl et al., 2015) contributed to the knowledge of the actual prevalence of the disease.

Concerning tetraploid varieties, the *T. turgidum* modern varieties and *T. turgidum* landraces did not present significant differences for the toxic epitopes content (Fig. 1). Moreover, likewise hexaploid varieties, we verified a high heterogeneity for the toxic epitopes content in the tetraploid population with values ranging from 26.40 ± 1.65 ('Basto duro') to 223.14 ± 30.04 ('Valenciano') g/kg (Table 1). This type of heterogeneity is comparable to that verified by other authors (Salentijn et al., 2013; Spaenij-Dekking et al., 2005; van den Broeck, Hongbing et al., 2010).

There is some evidence that wheat's D genome has more celiac disease toxic epitopes than A and B genomes (Salentijn et al., 2009; van Herpen et al., 2006). We verified that, although tetraploid varieties presented the lowest content of toxic epitopes, they are not significantly different from modern hexaploid varieties (Fig. 1). Comparable results were obtained by other authors (Spaenij-Dekking et al., 2005) who reported that large differences exist with regard to the presence of T-cell–stimulatory gluten peptides in several wheat accessions, independent of the ploidy level or genome background. Even so, when *T. aestivum*, modern and landraces wheat varieties, and all the hexaploid wheat varieties (including *T. spelta*) are compared with tetraploid wheat varieties for the toxic epitopes amount, both cases are significantly different ($p = 0.02010$ and $p = 0.00024$, respectively).

In relation to breeding practices, once again, it does not seem to have a negative effect on the content of potential celiac-toxic peptides as tetraploid varieties presented a balanced content for modern and landraces varieties.

Finally, *T. spelta* varieties proved to have the higher amount of toxic epitopes when compared to the analyzed groups of wheat, with a mean of approximately 311.15 g/kg. It is well known that the toxicity of spelt wheat remained elusive for some time, to the point of having created a myth that spelt wheat could be ingested by celiac patients. Studies about amino acid sequences of α-gliadins from bread wheat and spelt demonstrate only minor differences, concluding that the amino acid sequences in spelt include the same sequences known to be harmful to persons with celiac disease (Kasarda & D'Ovidio, 1999). Later, peptic-tryptic digests of gliadins from spelt wheat were found to exert toxic effects on Caco-2/TC7 cells and to agglutinate K562(S) cells (Vincentini et al., 2007). Our results, beyond the agreement with the cited studies, emphasizes that spelt wheat must be looked at with extra caution by celiac patients.

3.2. Quantitative analysis of gliadins

The knowledge of protein composition in wheat allows breeders to explore desirable genotypes based on technological and marketing parameters. The amounts of certain gliadin types are associated with different rheological properties (Branlard & Dardevet, 1985; Khatkar et al., 2002; Uthayakumaran et al., 2001; van Lonkhuijsen et al., 1992; Wieser et al., 1994) and considering the substantial heterogeneity in intestinal T cell responses to different gliadin types (Camarca et al., 2009), improving knowledge of gliadin composition does not only benefit breeders for end-use product quality purposes, but also in making informed selections to achieve potential celiac-safe wheat varieties by manipulating gliadin/gluten composition through reorganization of gliadin alleles.

In this sense, we carried out RP-HPLC analysis of all wheat varieties for quantitative purposes. The detailed results are shown in Table 1 and ranging values in Table 2.

In agreement with previous studies (Wieser et al., 1994), as we can see in Table 1, both total gliadin and the proportions of the different gliadin types varied considerably among the wheat varieties analyzed, revealing inter-varieties heterogeneity. Within the gliadin types, the α-type gliadins were generally present in greatest amount, followed by the γ-type gliadins. The ω-type gliadins were present at lower levels and specifically the ω1,2-type was dominant (Tables 1 and 2).

Gliadins are regarded as a "lubricant" for aggregated glutenins, being responsible for the viscosity of wheat dough (Belitz et al., 2004). Individually, γ-type gliadins have been shown to be positively correlated with dough strength (Branlard & Dardevet, 1985) and also with loaf volume, suggesting a positive contribution to bread making (van Lonkhuijsen et al., 1992). On the other hand, when compared to the other gliadin types, ω1,2-type produce the least positive effects on mixing tolerance and loaf volume (Khatkar et al., 2002), suggesting a lower importance to bread making. Regarding our results in terms of γ-type content and considering that modern varieties were subjected to breeding practices, we did not find any evidence of an increased amount of γ-type gliadins. Nevertheless, ω5-type gliadins were generally present in higher quantities in modern varieties than spelt and tetraploid varieties. A positive correlation between these proteins and baking volume, SDS-sedimentation value, dough resistance, dough extensibility and extensigram area has been reported (Wieser et al., 1994).

This is a complex issue as some specific gliadin alleles are known to have different effects on wheat quality and not being significantly influenced by the amount of the corresponding gliadin type (Branlard & Metakovsky, 2006). Also, the fact that obviously some wheat varieties are of good quality level and others are not, indicate that these results must be looked in more individual terms than group for breeding purposes.

Table 2
Minimum and maximum values of the content of different gliadin types in the three wheat groups analyzed.

Gliadins		*Triticum aestivum*	*Triticum spelta*	*Triticum turgidum*
ω5	g/100 g	0.13–0.99	0.31–0.97	0.05–0.69
	%	1.65–13.28	3.16–8.91	0.42–6.71
ω1,2	g/100 g	0.54–3.89	0.92–2.11	0.33–2.35
	%	10.42–28.54	10.86–19.52	3.65–20.41
α/β	g/100 g	1.73–6.36	2.86–5.86	3.49–7.61
	%	33.10–54.79	32.53–53.56	40.46–63.39
γ	g/100 g	1.20–4.72	2.47–4.76	2.22–5.36
	%	19.17–44.36	25.37–43.90	21.07–47.25
Total	g/100 g	4.43–14.47	8.13–12.23	7.77–12.34

3.3. Correlation studies

In order to understand if total gliadins content, different gliadin types content and protein content are correlated with the content of the analyzed toxic epitopes, we performed a correlation study and the results are shown in Fig. 2.

A positive correlation of ω-gliadins content, γ-gliadins content, total gliadins content and protein content, with R5 reactivity was observed when all wheat varieties are analyzed (Fig. 2a). The highest correlation was found between α/β-type gliadins content and total gliadins content ($r = 0.8495$; $p < 0.0001$). Interestingly, the variation of α/β-type gliadins content does not explain the variation verified in R5 reactivity. Despite this, it should be noted that α/β-type gliadins, historically the focus of efforts to map the gluten peptides that are toxic in celiac disease, have important immunostimulatory epitopes (Camarca et al., 2009; Tye-Din et al., 2010) and we only report, in this work, that the variation of its content and the variation of R5 reactivity did not present any correlation.

Regarding *T. aestivum* varieties (Fig. 2b), some correlations were maintained with the exception of ω5-type gliadins content: ω1,2-type gliadins content, total ω-gliadins content, γ-gliadins content, total gliadins content and protein content showed a positive correlation with R5 reactivity. Also, α/β-type gliadins content did not show any correlation with the toxic epitopes amount measured by R5. The strongest correlations were between ω1,2-type and ω-total gliadins, and between the different gliadin types and total gliadins content. On the other hand, the fact that protein content shows a stronger correlation with the measured toxic epitopes amount when compared to gliadins content, explaining almost 30% of the variation, probably indicates the contribution of other gluten proteins in addition to gliadins (Camarca et al., 2009;

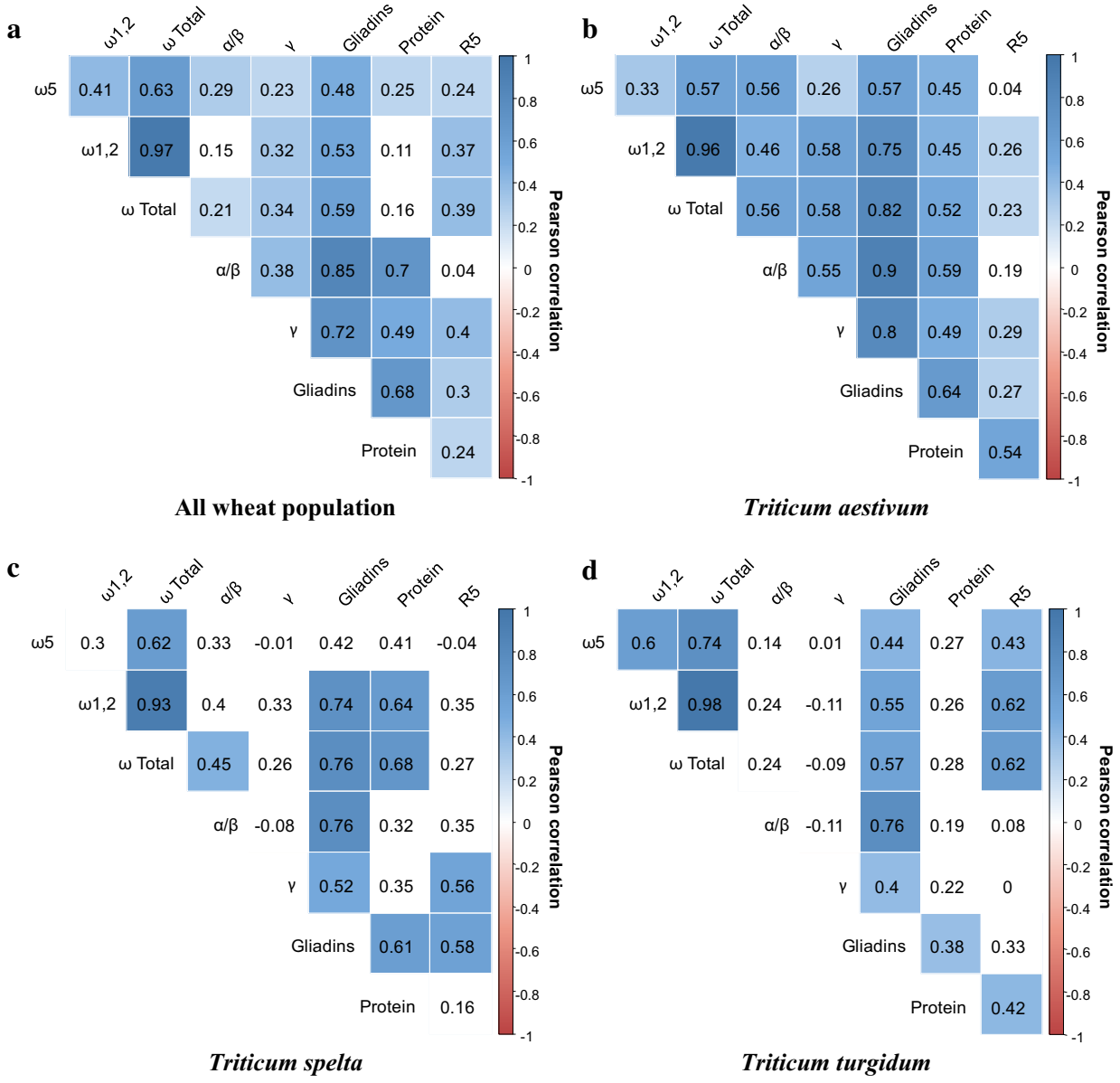

Fig. 2. Correlogram of all wheat population, *T. aestivum* varieties, *Triticum spelta* varieties and *T. turgidum* varieties. Positive correlations are displayed in blue and negative correlations in red colour. Colour intensity is proportional to the correlation coefficient. In the right side of the correlogram, the legend colour shows the Pearson correlation coefficient and the corresponding colours. In the above figures, correlations with *p*-value > 0.05 are considered as insignificant and in this case the correlation coefficient values are leaved blank. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Tye-Din et al., 2010) or even non-gluten proteins (Huebener et al., 2015) for the toxic epitopes amount and in last case for the pathogenesis of celiac disease.

In the cases of *Triticum spelta* and *T. turgidum* varieties, as can be observed in Fig. 2c and d, fewer significant correlations were found comparing to *T. aestivum* varieties. For example, in spelt wheat, only γ-type gliadins content and total gliadins content present positive significant correlation with the toxic epitopes amount. Concerning the tetraploid varieties, the same trend was observed, as only ω-gliadins (ω5-type, ω1,2-type and ω total) content and protein content are correlated with the toxic epitopes amount. This suggests a differentiated role of the different gliadin types in the variation of the content in toxic epitopes and potentially in the toxicity of *Triticum spelta* and *T. turgidum* varieties: γ-type gliadins in *Triticum spelta* varieties and ω-type gliadins in *T. turgidum* varieties. Nevertheless, further studies are necessary to elucidate this possibility.

In order to evaluate if there is any "hidden" correlation between α-type gliadins content and β-type gliadins content with R5 reactivity, we carried out A-PAGE analysis as this technique can separate the four gliadin types and in this sense to overcome the limitation of RP-HPLC to distinguish these types (Wieser et al., 1994). The relative amounts of the different gliadin types, and specifically α-type and β-type gliadins, derived from the areas beneath the peaks in the different regions of the density plot shown in Fig. 3 are presented in Table 1.

No correlation was found between the extrapolated values for the α-type and β-type gliadins and R5 reactivity, as had already occurred when the group composed by two types was analyzed. Thus, the variation of the α-type and β-type gliadins content does not allow to explain the verified variation in the toxic epitopes amount for all of the wheat varieties analyzed.

In general, the gliadins content and R5 reactivity did not present a high correlation coefficient (0.27–0.58). The different gliadin proteins have a different amino acid composition and sequence, even within the same gliadin type (van Herpen et al., 2006), and R5 detects sequences that must be present to ensure reaction. Thus, R5 only quantifies the reactive gliadins and not the total gliadins, demonstrating a limitation of this method for quantifying gluten as it only detects reactive proteins.

3.4. Highlighting gliadin content pattern differences by discriminant analysis

Multivariate analysis of variance (MANOVA) with Pillai's trace test was used to determine the significant differences between the hexaploid and tetraploid wheat varieties for the content of gliadins and different gliadin types (ω5-type, ω1,2-type, α/β-type and γ-type gliadins). Univariate tests with contrasts were then performed to verify in which gliadin types there was an effect of the hexaploid and tetraploid wheat varieties on its abundance. Canonical discriminant analysis (CDA) was subsequently performed in order to identify the gliadin types which underlie the differences between wheat ploidy, and build a potential diagnostic model applicable to real samples.

For the total gliadins content and individual gliadin types content, the MANOVA with Pillai's trace test revealed that wheat ploidy (F 28.127, df 5, $p < 0.01$), have a significant influence on the combined dependent variables. Planned contrast (Supplementary Table S1) showed that, inside each group (hexaploid and tetraploid varieties), the abundance of total gliadins and different gliadin types, with the exception of the ω5-type and γ-type gliadins, were significantly different. Finally, CDA allowed to achieve a function capable of discriminating between the two groups (eigenvalue = 1.169144, canonical correlation = 0.734159, Wilks λ = 0.461011, $\chi^2 = 94.46860$, $p < 0.01$), as shown in Fig. 4.

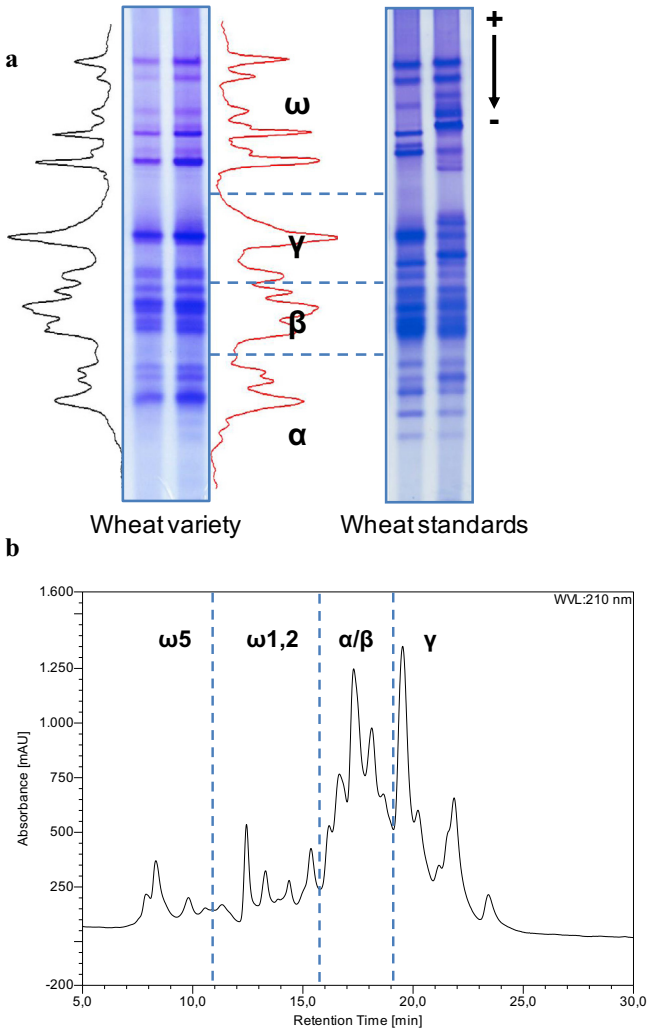

Fig. 3. Acid-PAGE pattern of wheat variety 'Rescue' (a) showing the different gliadin types, namely α, β, γ, and ω in decreasing mobility in acid-PAGE, the density plots of the two replicates and the standard varieties 'Chinese Spring' and 'Marquis', from the left to the right. Reversed-phase HPLC results for 'Rescue' gliadins (b). ω5, ω1,2, α/β and γ represent the different identified gliadin types. Absorbance was registered at 210 nm.

The hexaploid and tetraploid wheat varieties form two defined groups with a 91.27% classification rate, with the samples from hexaploid group being more efficiently classified (97.83%) when compared to the samples from tetraploid group (73.53%). The calculated unstandardized linear discriminant coefficients (Supplementary Table S2) can be used to assign cases in each group. The variables (content of gliadin types) with the largest standardized regression coefficients (Supplementary Table S2) are the ones that contribute most to the prediction of group membership: α/β-type gliadins > ω1,2-type gliadins > ω5-type gliadins. The model was validated by leave-one-out cross validation and showed good recognition and prediction abilities, the prediction rates remain unchanged.

We found significant differences in hexaploid and tetraploid sets of wheat varieties for the content of gliadins and specific gliadin types as ω1,2-type and α/β-type gliadins (Supplementary Table S1 and Supplementary Fig. S1): tetraploid wheat varieties presented higher amount of gliadins and α/β-type gliadins, whereas hexaploid wheat varieties presented more quantity of ω1,2-type gliadins. The genetic control of gliadins is complex and gliadin *loci* are clearly multigenic (Anderson & Greene, 1997;

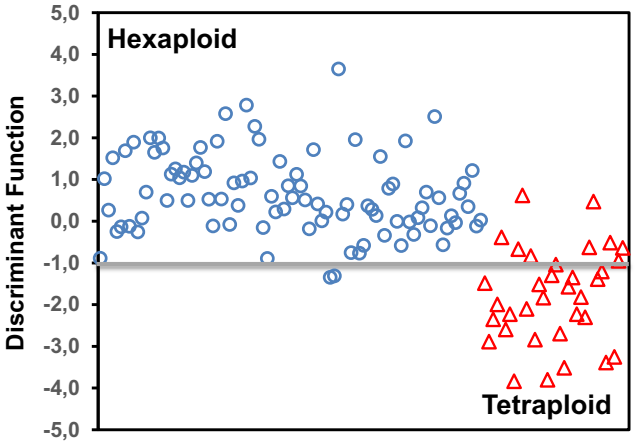

Fig. 4. Discriminant analysis: projections of the observation set using the determined canonical function. ○ denotes hexaploid wheat varieties and Δ denotes tetraploid wheat varieties.

Anderson et al., 2009, 2013). Furthermore, large differences in amount of α-gliadins from homoeologous *Gli-2 loci* in hexaploid and tetraploid wheat varieties were shown. For example, the relative *Gli-A2* amount level in a tetraploid wheat variety ('Probstdorfer Pandur') was 41% and in hexaploid bread wheat varieties this amount varied between 13% and 19% and in landraces between 12% and 58% (Salentijn et al., 2009). Therefore, we did not find any clear genetic evidence that explain these differences in amount of gliadins of hexaploid and tetraploid wheat varieties.

4. Discussion

Our findings point out to an important wheat genetic pool that can be further exploited for the development of celiac-safe wheat products. First, it means that there is a great potential for conventional breeding practices. Moreover, as the genetic diversity alone would hardly make possible to obtain a wheat variety without toxicity and retaining the unique viscoelastic properties of gluten, this natural potential can be highly and further exploited using cutting edge molecular breeding techniques encompassing mutagenesis, transgenesis and genome editing (Shewry & Tatham, 2015).

Also, these results constitute a firm basis and optimal start point for the detoxification technologies based on, for example, the selective modification (transamidation) of glutamine residues present in toxic epitopes, the hydrolysis of immunodominant gluten peptides by exogenous endopeptidases or the polymeric binders developed to hamper the recognition of stimulatory epitopes (Schuppan, Junker, & Barisani, 2009) as the previous selection of wheat material with low content in toxic epitopes will favour these detoxification strategies.

On the other hand, there is some evidence that early exposure to gluten and a double HLA-DQ2 gene dose both promote celiac disease development. In the 1980s, the addition of gluten to infant food led to a 5-fold increase in the occurrence of celiac disease (Ivarsson et al., 2000). Thus, the use of wheat varieties with low amount of toxic epitopes can be used in a prophylactic trend to reduce the putatively high risk that the wheat varieties with high amount of toxic epitopes represent to the development of celiac disease and in last case to the disease prevalence.

Anyway, it should be noted that gluten toxicity in celiac patients involves both innate and adaptive immunity and the immunoresponse varies widely between celiac patients (Lebwohl et al., 2015). Considering this complexity, demonstrating gluten safety is a complex endeavour and depends on *in vivo* gluten challenge studies. In this study, we analyzed the content of toxic epitopes by employing R5 monoclonal antibody, known to recognize the potential celiac-toxic repetitive pentapeptide epitopes in gluten proteins (Kahlenberg et al., 2006; Osman et al., 2001). Nevertheless, considering that there is a hierarchy of immunodominance and consistency of recognition of T cell epitopes *in vivo* as just three highly active peptides were responsible for most of the immune response seen in patients with celiac disease after eating wheat or barley or rye (Tye-Din et al., 2010) and are potentially recognized by R5, it is expected that a decrease in toxic epitopes will potentially decrease the toxicity for celiac patients. In this sense, as landraces present equal (*T. turgidum*) or even higher (*T. aestivum*) amount of toxic epitopes than the corresponding modern varieties, we can infer that breeding practices did not negatively contribute to celiac disease-related toxicity and ultimately did not contribute to the increased prevalence of the disease during the latter half of the 20th century.

Interestingly, the variation of total gliadin and different gliadin types content allowed to explain approximately 5–40% of the toxic epitopes content variation. Specific gliadin peptides have a fundamental role in the pathogenesis of celiac disease (Anderson et al., 2000, 2005; Shan et al., 2002, 2005; Tye-Din et al., 2010) and our results underline the importance of these glutamine-rich sequences rather than the amount of gliadins or even specific types of gliadins when the aim is to achieve potential celiac-safe wheat. Also, tetraploid wheat varieties showed higher amounts of total and α/β-type gliadins than hexaploid varieties. It was shown that the D genome is important for celiac-related toxicity (Salentijn et al., 2009; van Herpen et al., 2006) and as 33-mer, an α-gliadin derived peptide containing a cluster of epitopes and with a central role in the pathogenesis of celiac disease (Shan et al., 2002), appear to be exclusively encoded by genes located in the *Gli-2 locus* on chromosome 6D (Molberg et al., 2005), its absence in tetraploid wheat varieties appear to be the main reason explaining the reduced potential toxicity of these varieties when compared to hexaploid varieties. On the other hand, this suggestion reiterates the importance of the presence of specific sequences rather than the gliadins quantity *per se* as stated above.

In particular, the variation of α/β-type gliadins content was not correlated with the variation in the studied toxic epitopes in all of the groups (Fig. 2) and the importance of these proteins in celiac disease is well known (Camarca et al., 2009; Tye-Din et al., 2010). This suggests that the toxicity from α/β-type gliadins is highly sequence-dependent and represents an added problem to breeders as the quantity alone does not necessarily means toxicity. In fact, α/β-type gliadins are unusual because they have a single, polymorphic region with potent T cell–stimulatory activity (Tye-Din et al., 2010). Also, large differences in expression of α-gliadins from homoeologous *Gli-2 loci* were demonstrated in tetraploid and hexaploid wheat varieties (Salentijn et al., 2009). Considering the reduced effect of the environment in this study because the varieties were grown in the same conditions, location and year, these results show a very typical toxicity of the different wheat varieties and elucidate the potential danger of a straightforward breeding aimed to decrease the content of α/β-type gliadins. Nonetheless, positive significant correlations were observed for γ-type and ω-type gliadins, and R5 reactivity, in spelt and tetraploid wheat varieties, respectively, explaining approximately 40% of the variation in the case of tetraploid varieties. This is of particular interest because it was described that a ω-gliadin/C-hordein–derived peptide may be considered the canonical dominant T cell–stimulatory peptide in HLA-DQ2–associated celiac disease (Tye-Din et al., 2010).

In the case of *T. aestivum* varieties, with exception of α/β-type gliadins, all of the gliadin types were positively correlated with R5 reactivity and the fact that protein content shows a stronger

correlation with the measured toxic epitopes amount when compared to gliadins content, probably indicates the contribution of other gluten proteins (Camarca et al., 2009; Tye-Din et al., 2010) or even non-gluten proteins (Huebener et al., 2015) for the total amount of immunogenic peptides.

The content of α/β-type, ω1,2-type and ω5-type gliadins showed a good discriminant potential for hexaploid and tetraploid wheat varieties with a 91.27% classification rate (Fig. 4). The hexaploid group was more efficiently classified (97.83%) and reveals the potential of a simple, rapid and consistent approach for ploidy analysis.

The improvement of wheat is based on the identification of genetic variation in traits of interest. In the case of celiac disease toxicity, the trait of interest is the amount and distribution of toxic epitopes (Shewry & Tatham, 2015). Therefore, we hope that the knowledge acquired and provided by this work allows the development of celiac-safe wheat-based products with appropriate technological properties by conventional and molecular breeding or detoxified by chemo-enzymatic processes, overcoming the poor technological, organoleptic and nutritional characteristics of gluten-free products and contributing to a better quality of life of patients of one of the most common immune based diseases of our societies.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.06.043.

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