Towards the development of a new gluten reference material

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4th ICC Latin American Cereals Conference

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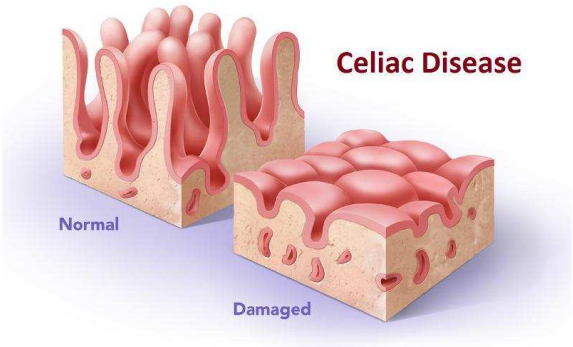
OUTLINE – TWO FACES OF WHEAT



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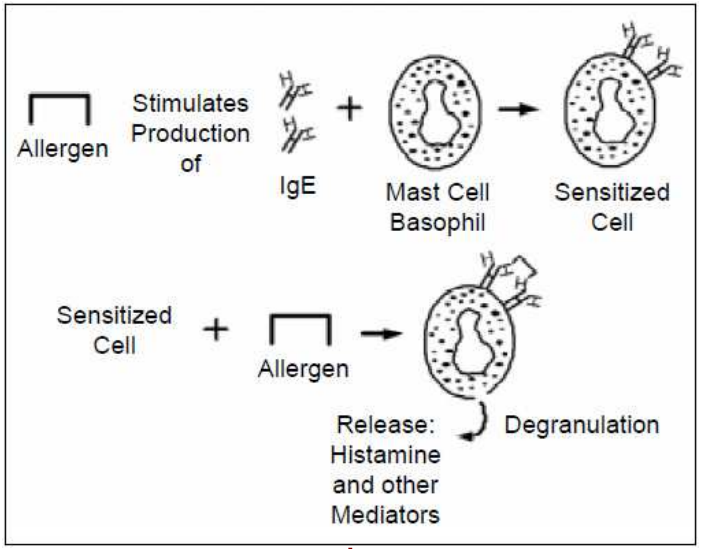
HYPERSENSITIVITY REACTIONS TRIGGERED BY CEREALS



NCGS??

Molina-Infante et al. 2015

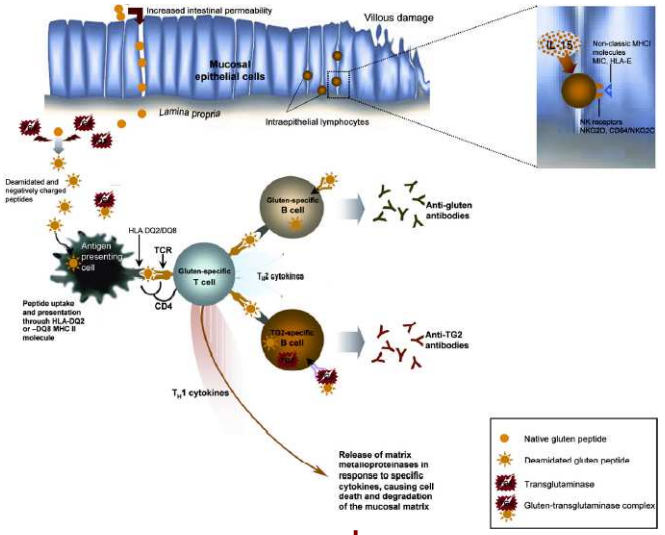
Food allergy



Taylor et al. 2001

Gliadins, glutenins, albumins, globulins

Celiac disease



Briani et al. 2008

Gliadins, glutenins

LEGISLATION AT PRESENT

1169/2011
2000/13/EC, 2003/89/EC, 2006/142/EC, 2007/68/EC

CEREALS CONTAINING GLUTEN

(crustaceans, egg, fish, peanut, soybean, milk, nuts, celery, mustard, sesame, lupine, mollusks sulphur-dioxide)

41/2009
(Codex Stan. 118-1979 rev. 2008)

- Gluten-free: <20 ppm gluten
- Very low gluten level: 20-100 ppm gluten

• Food allergy
• No thresholds
• Zero tolerance for WHEAT (or other allergen sources)

• Celiac disease
• Thresholds for GLUTEN

FURTHER GLUTEN-FREE LEGISLATION EXAMPLES FROM AROUND THE GLOBE

Canadian Food and Drug Act
Canadian Food and Drug Regulations
Div. 24, Section B.24.018
No threshold, but <20 mg/kg gluten level may be accepted

FALCPA
21 CFR
<20 mg/kg gluten

Food Standards Code
Standards 1.2.7, 2.9.5
Gluten-free: no detectable gluten (!)
Low-gluten: <20mg gluten/100 g food

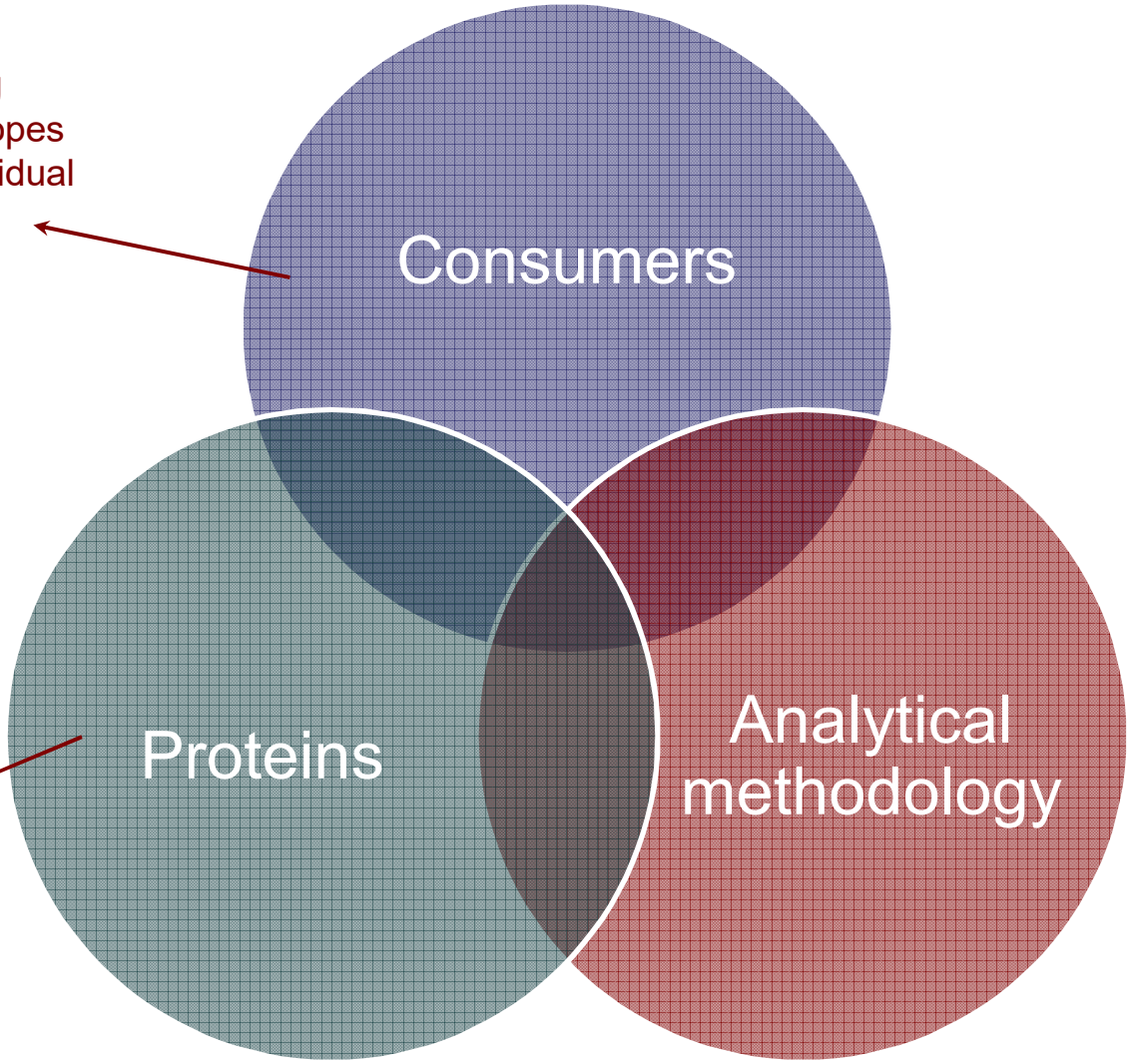
Chile
Decree 977/96,
article 518
<3 mg/kg gluten (!)

Latin America
New regulation

Brazil Law 10,674 of 16/05/2003
The presence of gluten must be labeled
No threshold

SPECIFIC ISSUES OF GLUTEN ANALYSIS

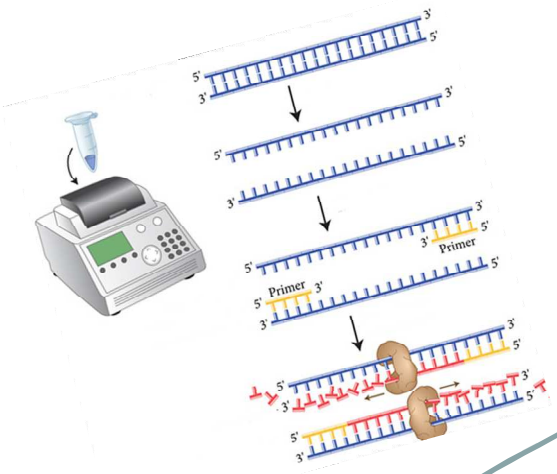
- Multiple triggering proteins and epitopes
- Variability of individual threshold levels



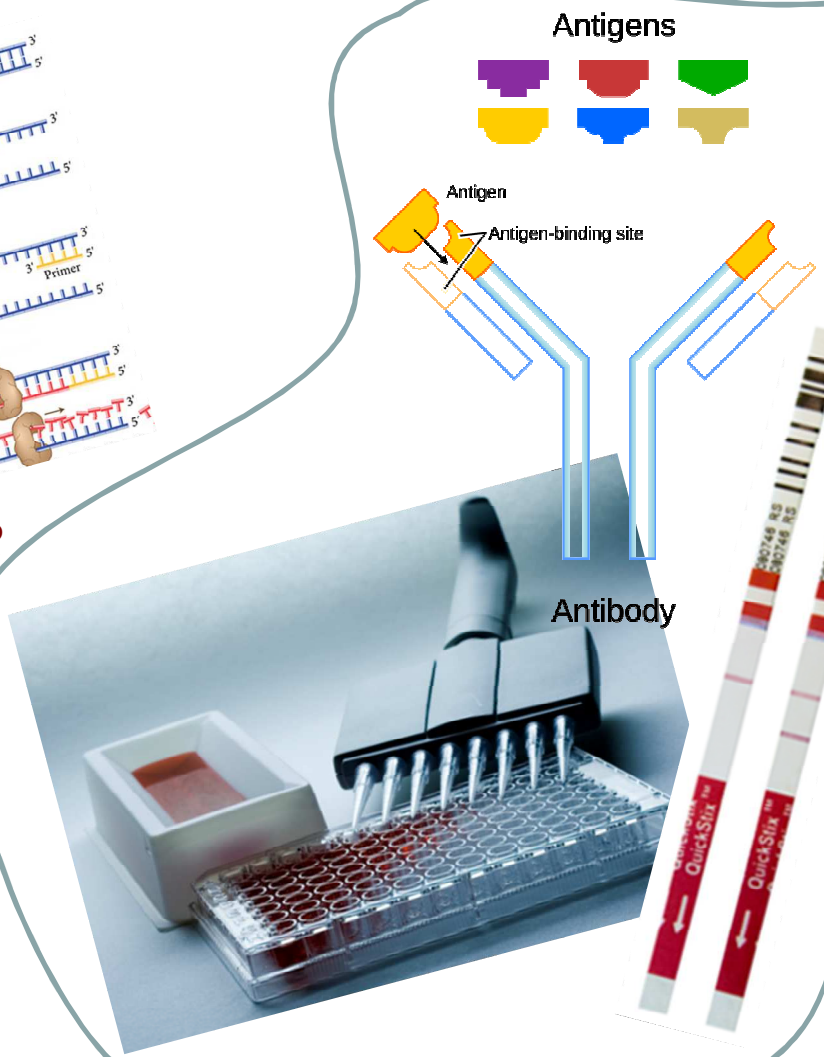
- Gluten is a complex mixture of proteins
- GxE variability
- Processing-induced protein modification

Denery-Papini et al. 1999, Wieser 2000, Valdés et al. 2003, Hishenhuber et al. 2006, Bermudo Redondo et al. 2005, Kerbach et al. 2009, Takács et al. 2010

COMPLIANCE AND MONITORING-ANALYTICAL POSSIBILITIES

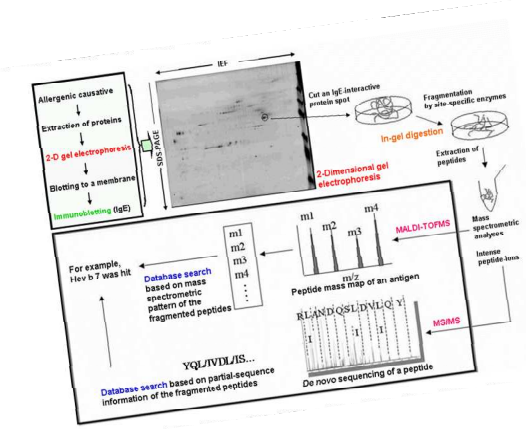


DNS based PCR



Immonanalytical solutions
ELISAs
Deepstick rapid tests

HPLC MS,
Proteomics
Allergenomics



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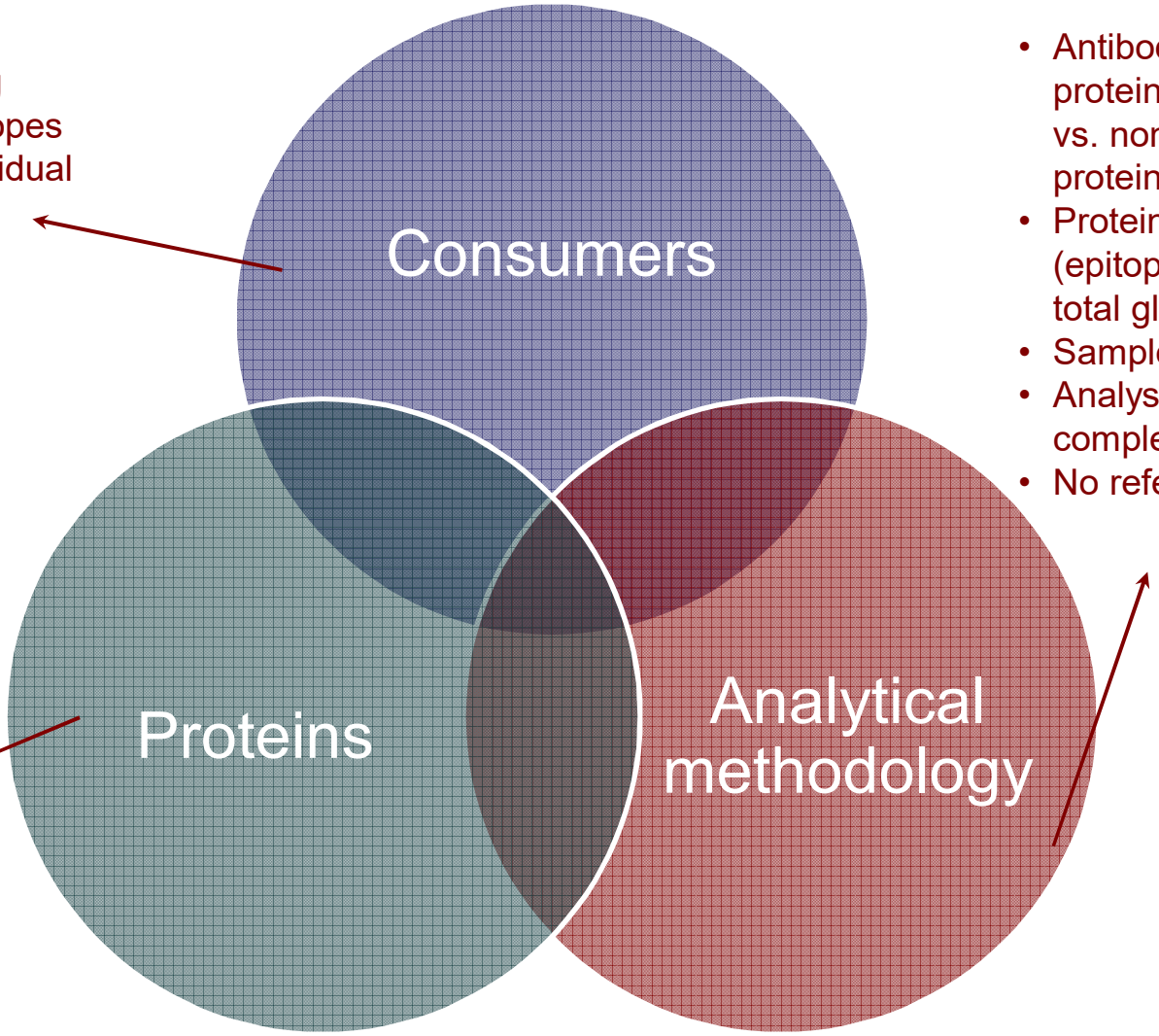
SPECIFIC ISSUES OF GLUTEN ANALYSIS

- Multiple triggering proteins and epitopes
- Variability of individual threshold levels



- Antibody and target protein selection (toxic vs. non-toxic target protein)
- Protein polymorphism (epitope amount vs. total gluten amount)
- Sample preparation
- Analysis in processed, complex matrices
- No reference materials

- Gluten is a complex mixture of proteins
- GxE variability
- Processing-induced protein modification

Denery-Papini et al. 1999, Wieser 2000, Valdés et al. 2003, Hishenhuber et al. 2006, Bermudo Redondo et al. 2005, Kerbach et al. 2009, Takács et al. 2010

INTENSIVE EFFORTS FOR DEVELOPMENTS OF METHODS, REFERENCE MATERIALS AND VALIDATION

EFFORTS FOR DEVELOPING REFERENCE GLIADIN MATERIAL

ELSEVIER

Journal of Cereal Science 43 (2006) 331–341

Journal of CEREAL SCIENCE

www.elsevier.com/locate/jnlabr/yjcrs

Towards a new gliadin reference material–isolation and characterisation

R. van Eckert [a,b,c], E. Berghofer [d], P.J. Ciclitira [e], F. Chirdo [f], S. Denery-Papini [g], H.J. Ellis [e], P. Ferranti [h], P. Goodwin [i,2], U. Immer [j], G. Mamone [h], E. Méndez [k], T. Mothes [l], S. Novalin [d], A. Osman [l,3], M. Rumbo [f], M. Stern [m], L. Thorell [n], A. Whim [i], H. Wieser [o,*]

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Extracted from a mixture of 28 European wheat cultivars

Abstract

Twenty-eight wheat cultivars representative of the three main European wheat producing countries, France, UK and Germany, were selec
a source for the preparation of a reference gliadin. One kilogram of kernels from each cultivar were mixed and milled. The resulting white ...

ISOLATED GLIADIN OF WORKING GROUP ON PROLAMIN ANALYSIS AND TOXICITY (PWG GLIADIN)

WG PAT Working Group on Prolamin Analysis and Toxicity

Aims History Executive Members Next Meeting WGPAT Meetings PWG Gliadin Handling of PWG Gliadin Links Contact

PWG-Gliadin

PWG-gliadin is a reference material that has been produced under the guidance of the Prolamin Working Group (PWG). Its isolation and characterization is described in detail in van Eckert et al. (2006). Briefly, PWG-gliadin has been extracted from a mixture of 28 European wheat cultivars. Albumins and globulins were eliminated by extraction using 0.4 M NaCl solution and gliadins were extracted with 60% ethanol. The gliadin extracts were concentrated, desalted by ultrafiltration, freeze-dried, and homogenized. The residual material after lyophilization is referred to as PWG-gliadin.

In 2005 PWG-gliadin was handed over to the Institute for Reference Material and Measurements of the European Commission (IRMM), Geel, Belgium to getting approved as a certified reference material. IRMM decided that PWG-gliadin would not be approved and returned it to PWG in 2006. Information on its composition is provided in the following sections.

PWG-gliadin can be obtained from the chairman of the Working Group for Prolamin Analysis and Toxicity:

Prof. Dr. Peter Koehler
German Research Center for Food Chemistry
Lise-Meitner-Straße 34
85354 Freising
GERMANY
Phone: +49 8161 712928
Fax: +49 81861 712970
E-mail: peter.koehler@tum.de

- *García E, Llorente M, Hernando A, Kieffer R, Wieser H, Méndez E. Development of a general procedure for complete extraction of gliadins for heat processed and unheated foods. Eur J Gastroenterol Hepatol 2005; 17: 529-539.*

- *Méndez E, Llorente M, García E, Vela C, Immer U, Janssen FW. Report of a collaborative trial to investigate the performance of the R5 enzyme-linked immuno assay to determine gliadin and gluten-free food. Eur J Gastroenterol Hepatol 2005; 17: 1053-1063.*

- *Stern M, Ciclitira P, van Eckert R, Feighery C, Janssen FW, Méndez E, Mothes T, Troncone R, Wieser H. Analysis and clinical effects of gluten in coeliac disease. Eur J Gastroenterol Hepatol 2001; 13: 741-747.*

- *Stern M. Current Therapy. In: Fasano A, Troncone R, Branski D (eds). Frontiers in Celiac Disease. Pediatric Adolesc Med. Basel: Karger 2006; 12: 114-122.*

- *Valdés I, García E, Llorente M, Méndez E. Innovative approach to low-level gluten determination in foods using a novel sandwich ELISA protocol. Eur J Gastroenterol 2003; 15: 465-474.*

- *Van Eckert R, Berghofer E, Ciclitira PJ, Chirdo F, Denery-Papini S, Ellis HJ, Ferranti P, Goodwin P, Immer U, Mamone G, Méndez E, Mothes T, Novalin S, Osman A, Rumbo M, Stern M, Thorell L, Whitt - A, Wieser H. Towards a new gliadin reference material - Isolation and characterisation. J Cer Sci 2006; 43: 331-341.*

- *Wieser H, Koehler P. The biochemical basis of celiac disease. Cer Chem 2008; 85: 1-13.*

- *Gessendorfer B, Koehler P, Wieser H. Preparation and characterization of enzymatically hydrolyzed prolamins from wheat, rye, and barley as references for the immunochemical quantitation of partially hydrolyzed gluten. Anal Bioanal Chem 2009; 395:1721-1728.*

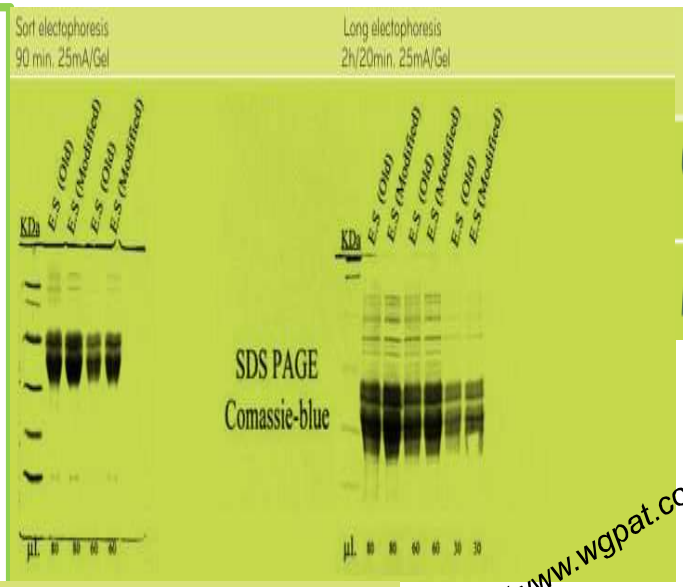
- *Gessendorfer B, Wieser H, Koehler P. Optimisation of a solvent for the complete extraction of prolamins from heated foods. J Cereal Sci. 2010; 52: 331-333.*

Source: http://www.wgpat.com/pwggliadin.html

ADVANTAGES AND DISADVANTAGES OF PWG-GLIADIN

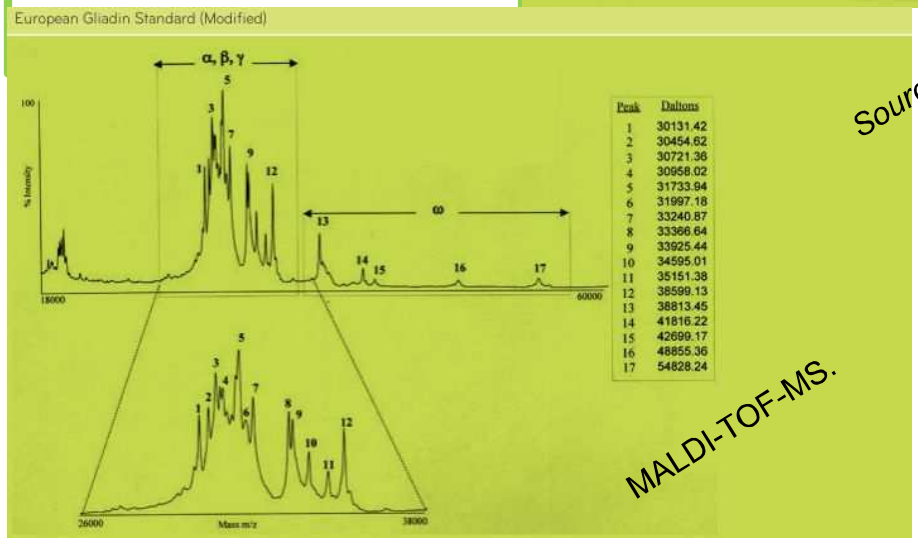
Advantages

- ✓ high purity
- ✓ good solubility
- ✓ homogenous
- ✓ well-characterized
- ✓ stability study
- ✓ storage study
- ✓ …



	Date of testing	Raw protein content (Dumas, N x 5,7)
Old PWG-gliadin*	2005 2006 (repeated)	89.4 → 0.8% (n = 10) 94.6 → 0.7% (n = 3)
Modified PWG-gliadin	2006	96.9 → 0.4% (n = 3)

Source: http://www.wgpat.com/pwggliadin.html



Peak	Daltons
1	30131.42
2	30454.62
3	30721.36
4	30958.02
5	31733.94
6	31997.18
7	33240.87
8	33366.64
9	33925.44
10	34595.01
11	35151.38
12	38599.13
13	38813.45
14	41816.22
15	42699.17
16	48855.36
17	54828.24

Disadvantages

- ➢ reproduction (wheat varieties and process)
- ➢ limited amount
- ➢ represents only a part of gluten proteins
- ➢ calculation of gluten content

Towards development of reference materials for gluten analysis – QUESTIONS MEAN GOALS

as representative as possible for the multitude of wheat cultivars grown around the world

- ? One cultivar or a mixture of several cultivars (genetic effects, G)
- ? Importance of geographic origin, harvest years, etc (environmental effects, E)
- ? Isolates (gliadin/gluten) or
- ? White flour or whole wheat flour or
- ? Processed incurred model food matrices

Effects of genetic and environmental variability

Effects of food processing (dough formation and baking)

RELATIVE MAGNITUDE OF THE EFFECTS OF THESE FACTORS ON THE RESULTS OF GLUTEN QUANTITATION USING COMMERCIAL ELISA KITS?

MATERIALS AND METHODS 1

Wheat samples from all over the world

CDC Go Sara Weigum	Canada
CDC Go Wes Froese	
CDC Go Pen West Seeds	
Carberry	
Cardale	
Glenlea	
Akteur	Germany
Winnetou	
Dekan	
MV-Magvas	Hungary
MV-Mazurka	
MV-Verbunkos	
Yumai-34	China
Bezostaja-1	Russia
Hereward	England
Soissons	France
Mulan	Austria
Capo	
Westonia	Australia
Frame	
Chara	
Yitpi	
Wyalkatchem	

Studied factors

GxE

- Variety (n=23)
- Harvest year (2011, 2012, 2014; n=4)

Others

- Processing
- Different ELISA methods

Characterization of wheat flours

- Crude protein (Dumas)
- Wet and dry gluten
- Composition (NIR/NIT)
- Size exclusion HPLC
- Reverse phaes HPLC
- Different ELISA kits

MATERIALS AND METHODS 2

APPLIED ELISA KITS

Parameter	RIDASCREEN® Gliadin	AgraQuant® ELISA Gluten G12
Antibody	**R5 monoclonal**	**G12 monoclonal**
Target molecule	**ω-secalin**	**33mer gliadin peptide**
Target sequence	**QQPFP**[a]	**QPQLPY**[b]
Calibrator	PWG-gliadin	Vital wheat gluten extract
Kit reporting unit	gliadin in µg/kg (ppb)	gluten in mg/kg (ppm)

- two commercial kits
- different antibodies
- different target epitopes

[a] QQPFP pentapeptide occurs repetitively in prolamins (α-, γ- and ω-subfractions) and is conserved in different wheat varieties (Valdés et al., 2003).

[b] QPQLPY sequence is repeated three times within the immunogenic 33-mer of α-gliadin (Morón et al., 2008).

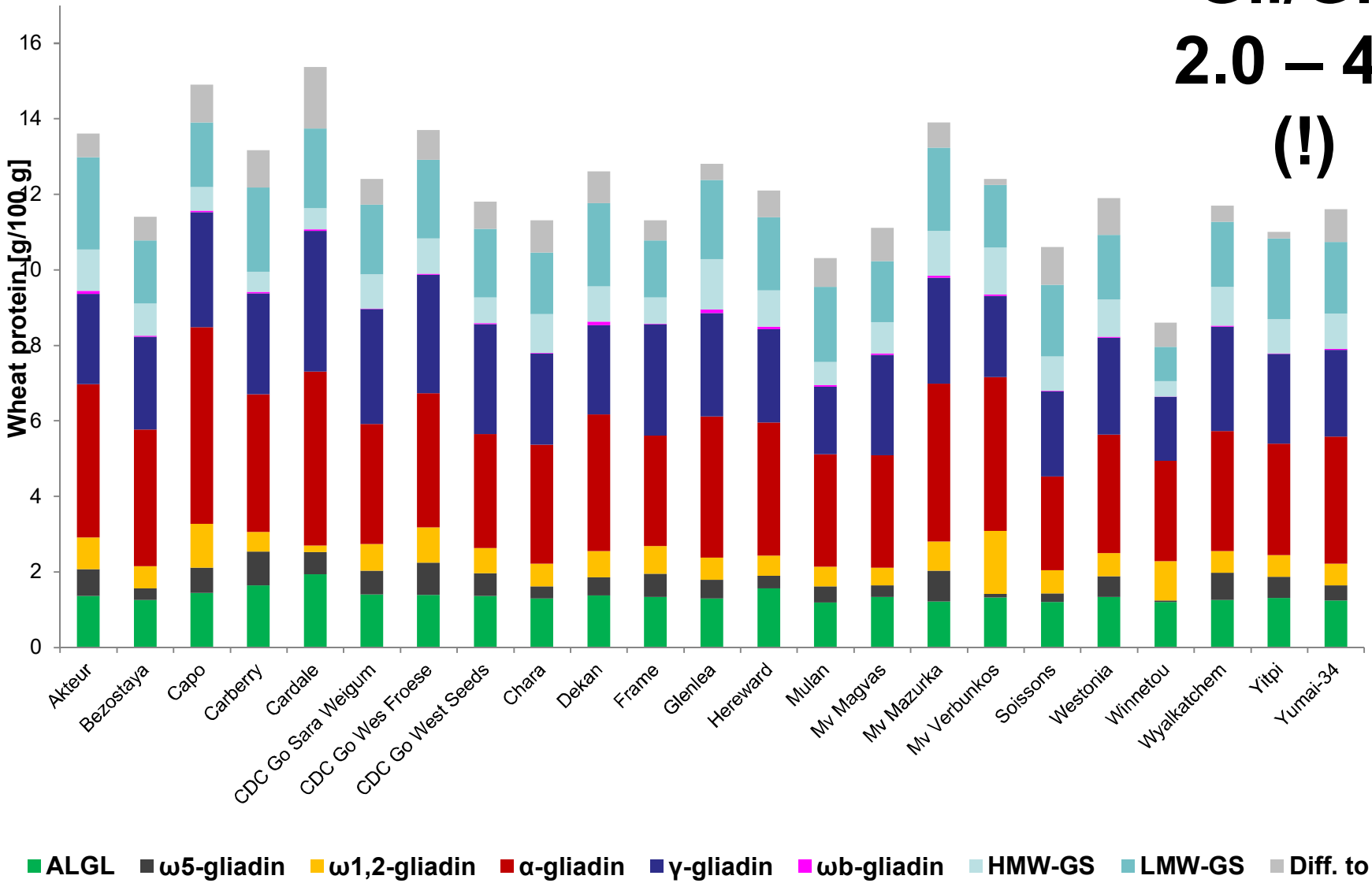
RESULTS: VARIABILITY OF WHEAT VARIETIES COMPOSITION

Parameter (%)	Varieties	Mean (n=21)	Flour mixture (contains 21 varieties)
Protein content (Dumas method)	8.6 - 14.9	12.0	12.7
Ash content	0.41 - 0.91	0.55	0.58
Wet gluten content (Glutomatic)	27.1 - 42.7	34.5	34.7
Dry gluten content	9.7 - 16.1	12.5	12.3

RESULTS: VARIABILITY OF WHEAT VARIETIES

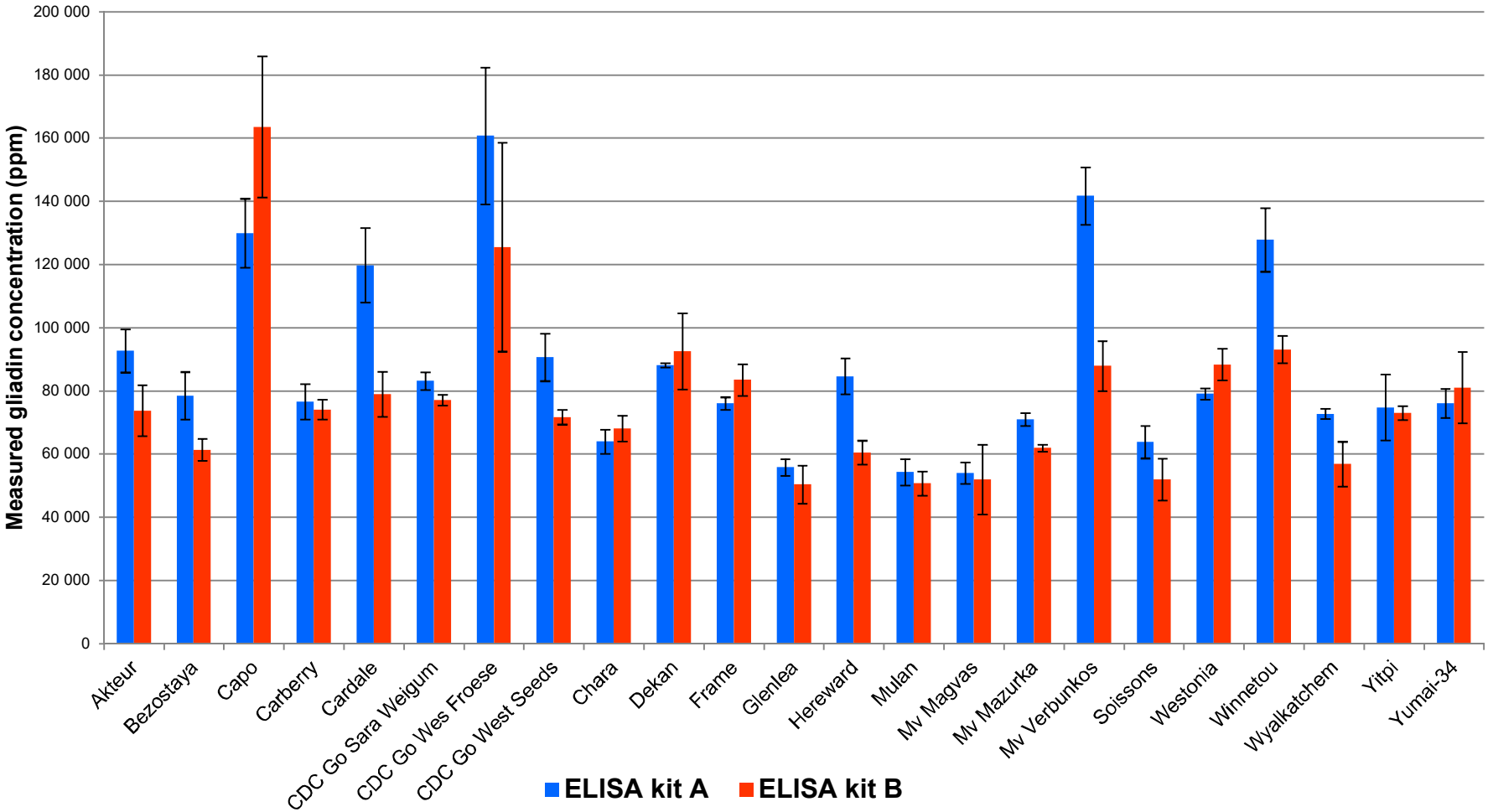
PROTEIN PROFILING BY RP-HPLC

Gli/Glu
2.0 – 4.2
(!)



RESULTS: VARIABILITY OF WHEAT VARIETIES

GLIADIN CONCENTRATION DETERMINED BY ELISA KITS



Lívia Hajas[a], Katharina A. Scherf[b], Kitti Török[a], Zsuzsanna Bugyi[a], Eszter Schall[a], Roland E. Poms[c], Peter Koehler[b] and Sándor Tömösközi[a]Variation in protein composition among wheat (Triticum aestivum L.) cultivars to identify cultivars suitable as reference material for wheat gluten analysis, Food Chemistry, https://doi.org/10.1016/j.foodchem.2017.05.005

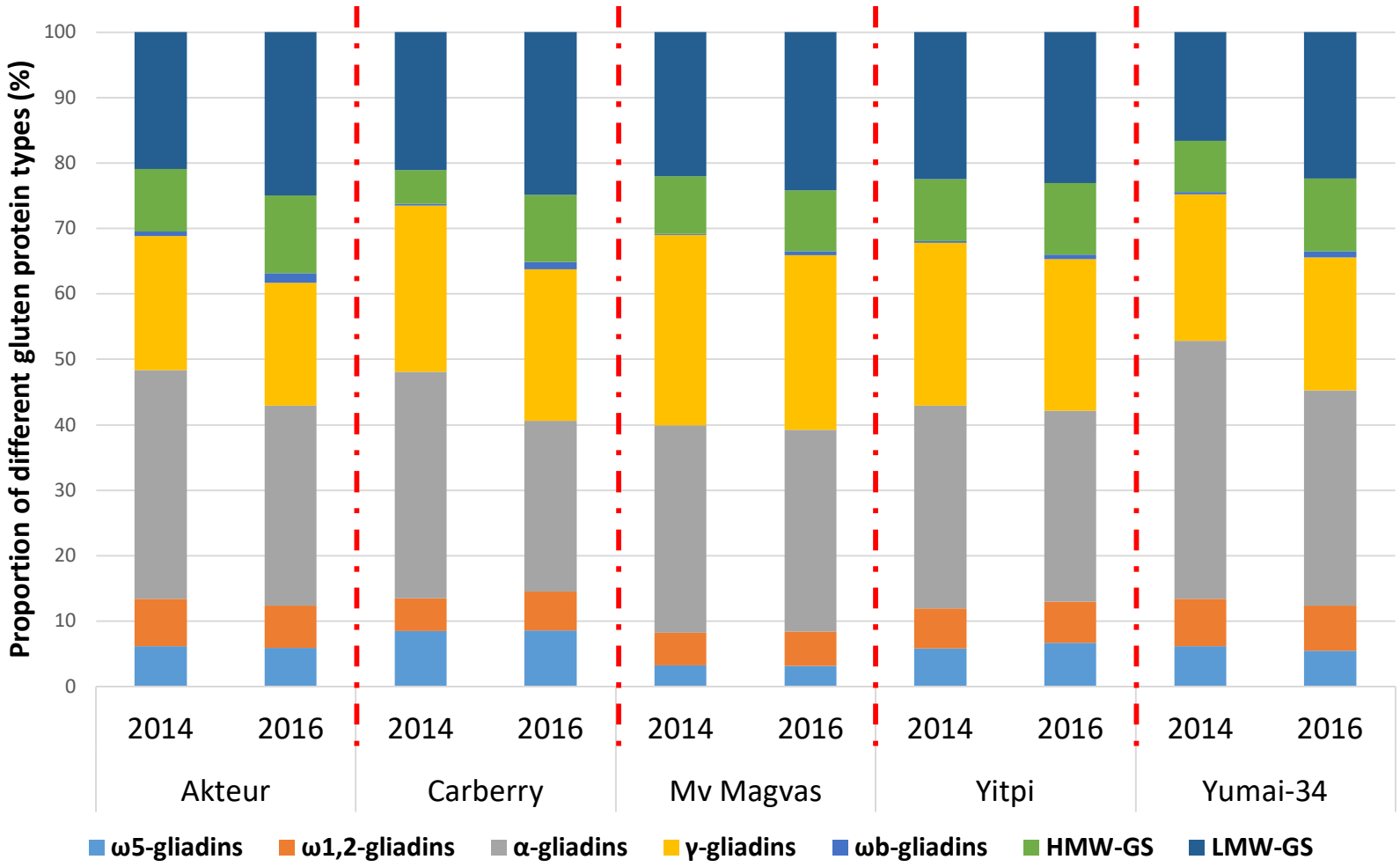
SELECTION CRITERIA FOR A PROPER REFERENCE MATERIAL

- **Qualitative** criteria: based on RP-HPLC profiles of the gliadin fraction and SDS-PAGE profiles of the proteins
- **Quantitative** criteria: derived from Dumas, RP-HPLC and ELISA results

CHOSEN CLUTIVARS FOR SECOND PHASE OF DEVELOPMENT:

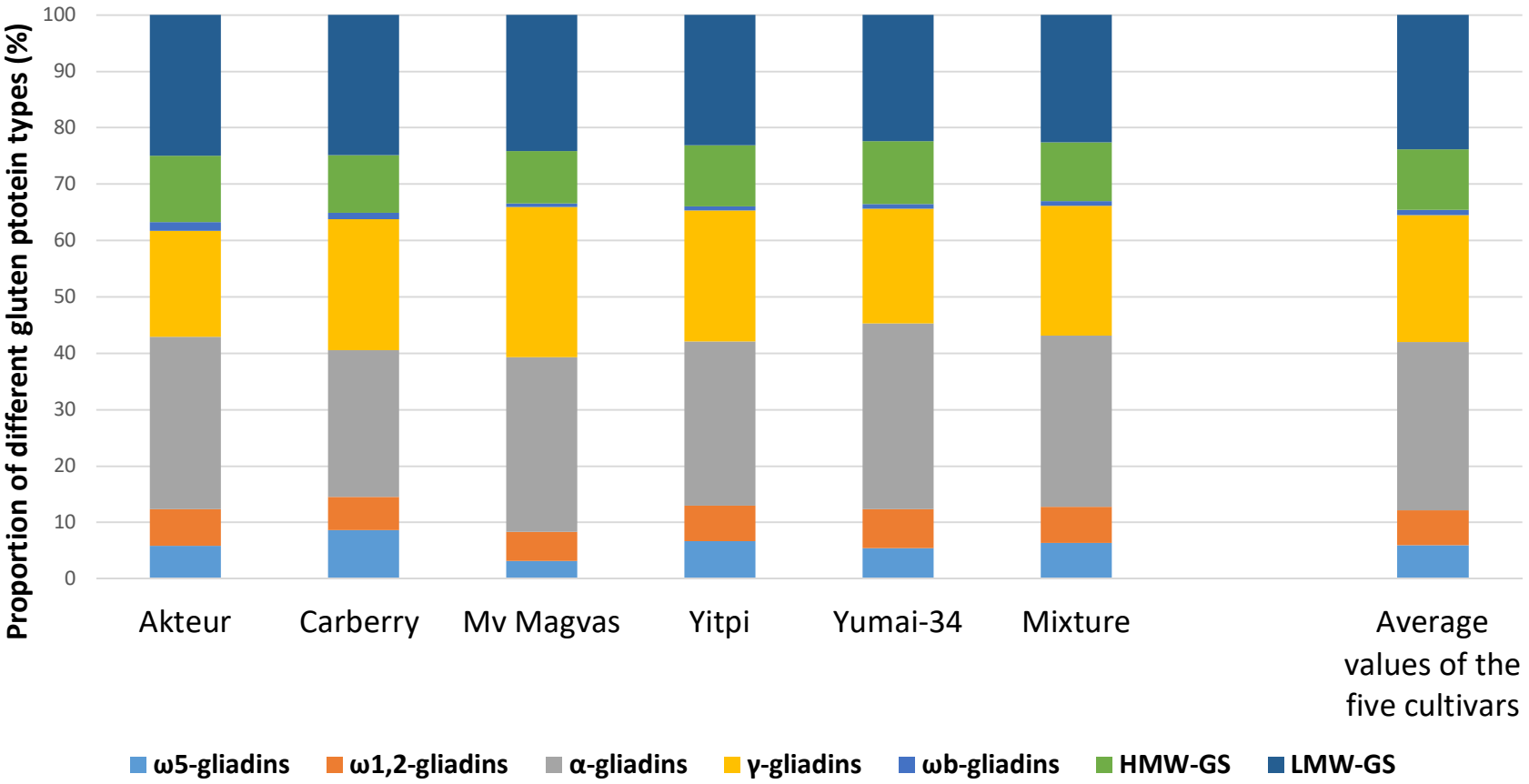
NAME	ORIGIN
AKTEUR	GERMANY
CARBERRY	CANADA
MV MAGVAS	HUNGARY
YITPI	AUSTRALIA
YUMAI-34	CHINA

RESULTS: VARIABILITY - PROTEIN COMPOSITION OF THE CHOSEN CULTIVARS IN TWO HARVEST YEARS BY RP-HPLC



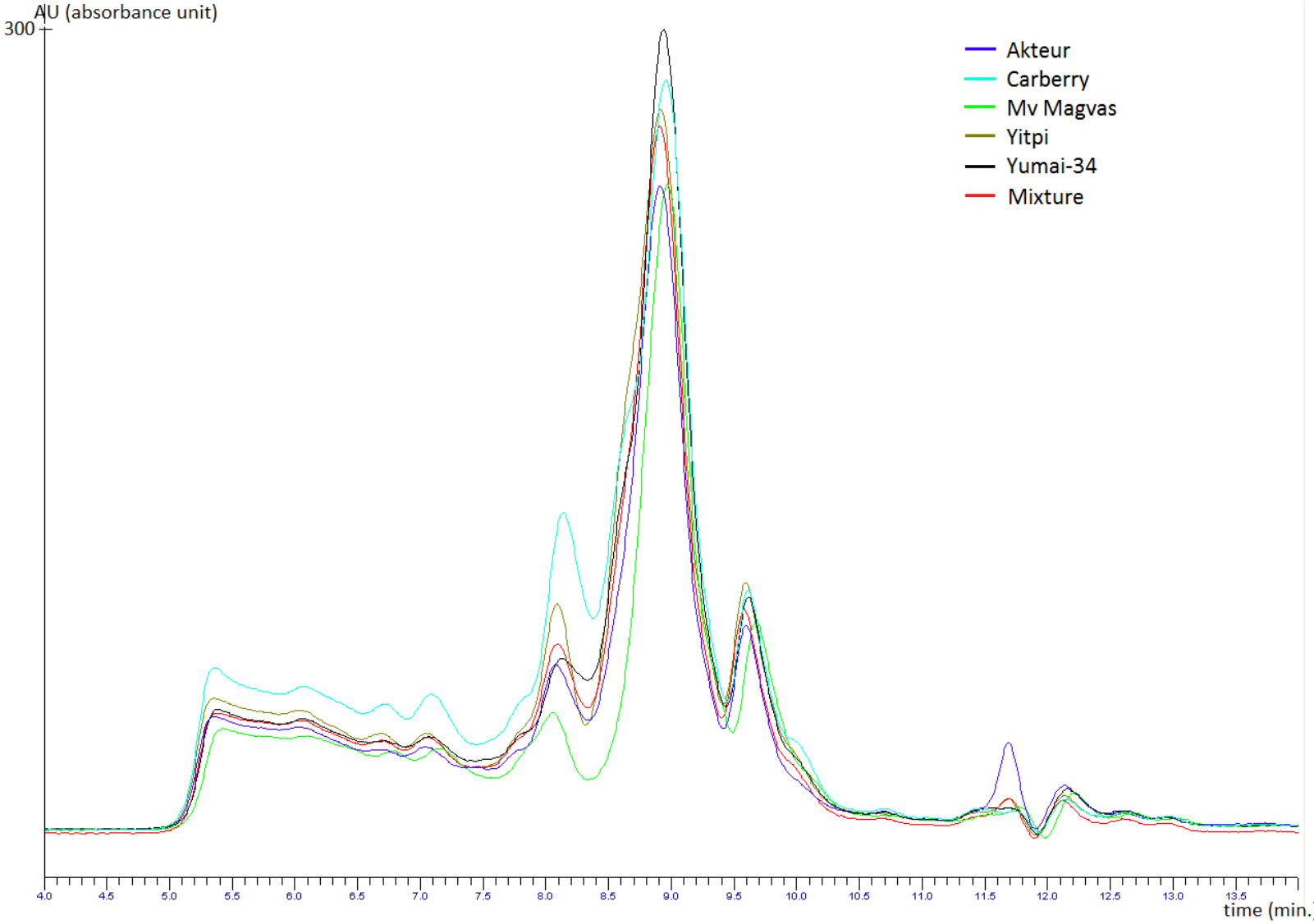
RESULTS: SINGLE CULTIVAR VS MIXTURE OF THE CHOSEN ONES

PROTEIN COMPOSITION OF THE FLOURS BY RP-HPLC



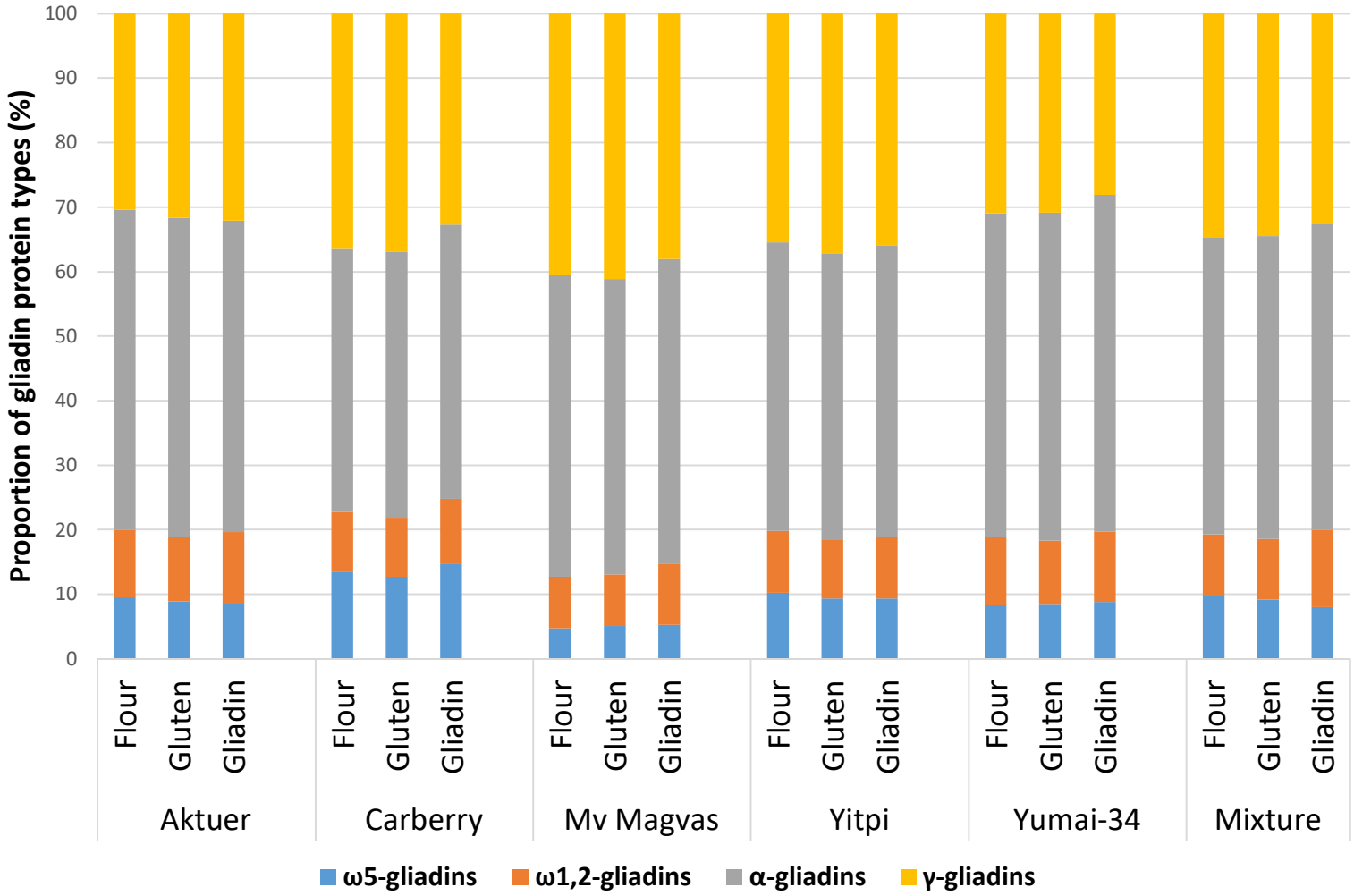
RESULTS: SINGLE CULTIVAR VS MIXTURE OF THE CHOSEN ONES

CHROMATOGRAMS OF THE SOLUBLE PROTEINS BY SE-HPLC



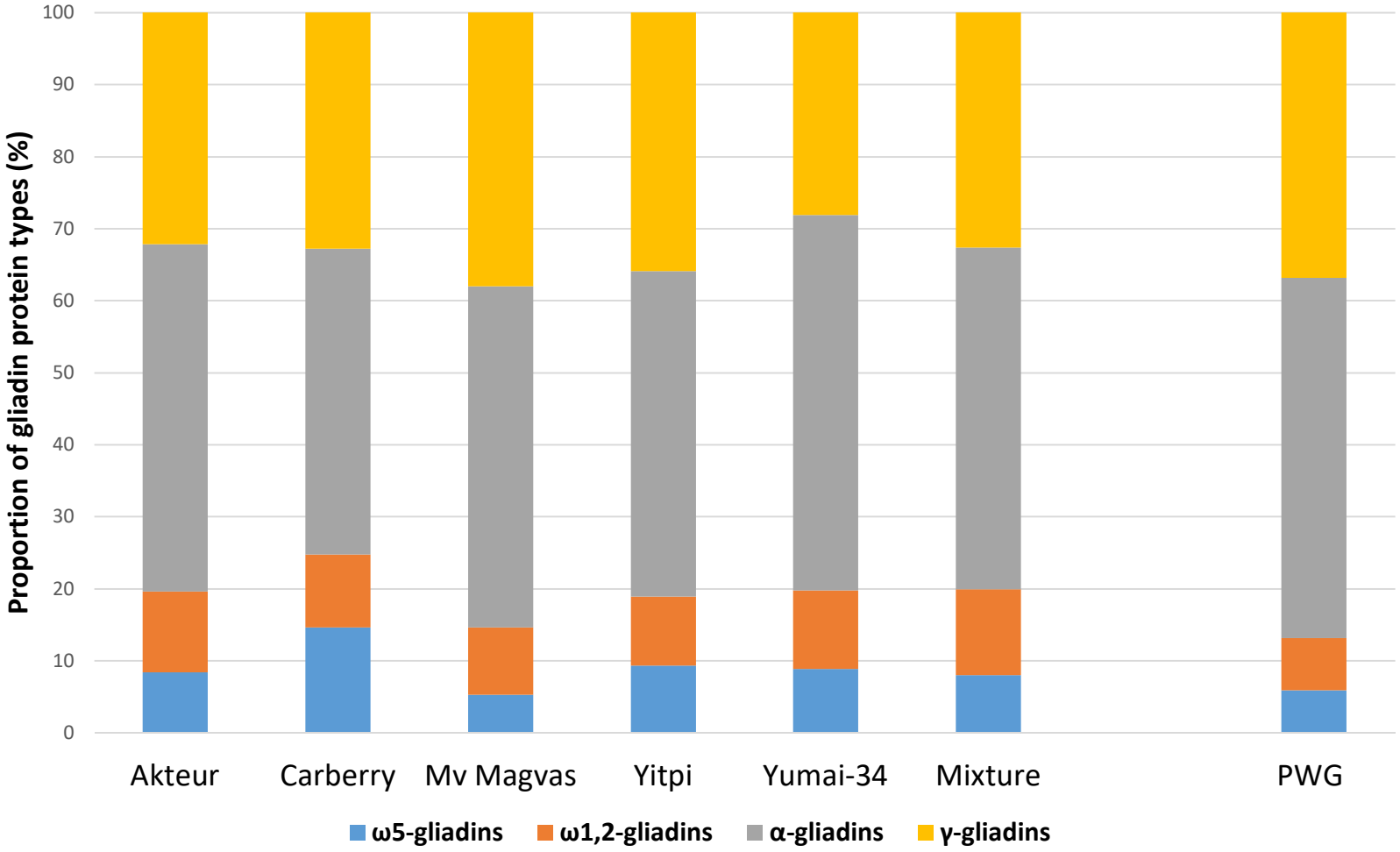
RESULTS: WHEAT FLOUR VERSUS ISOLATES

GLIADIN COMPOSITION OF THE FLOURS, GLUTEN AND GLIADIN ISOLATES BY RP-HPLC

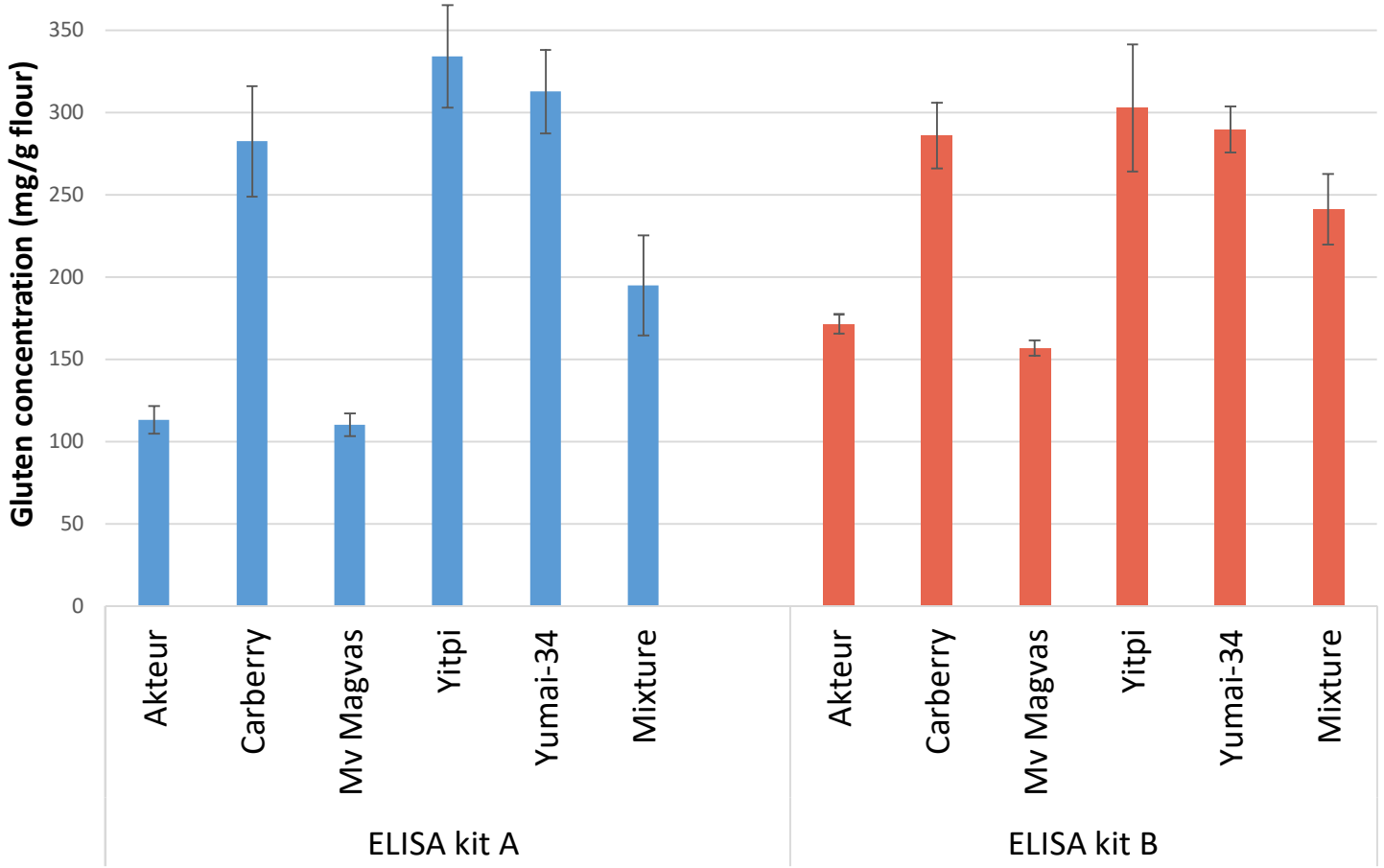


RESULTS: COMPARISON WITH EXISTING PWG GLIADIN

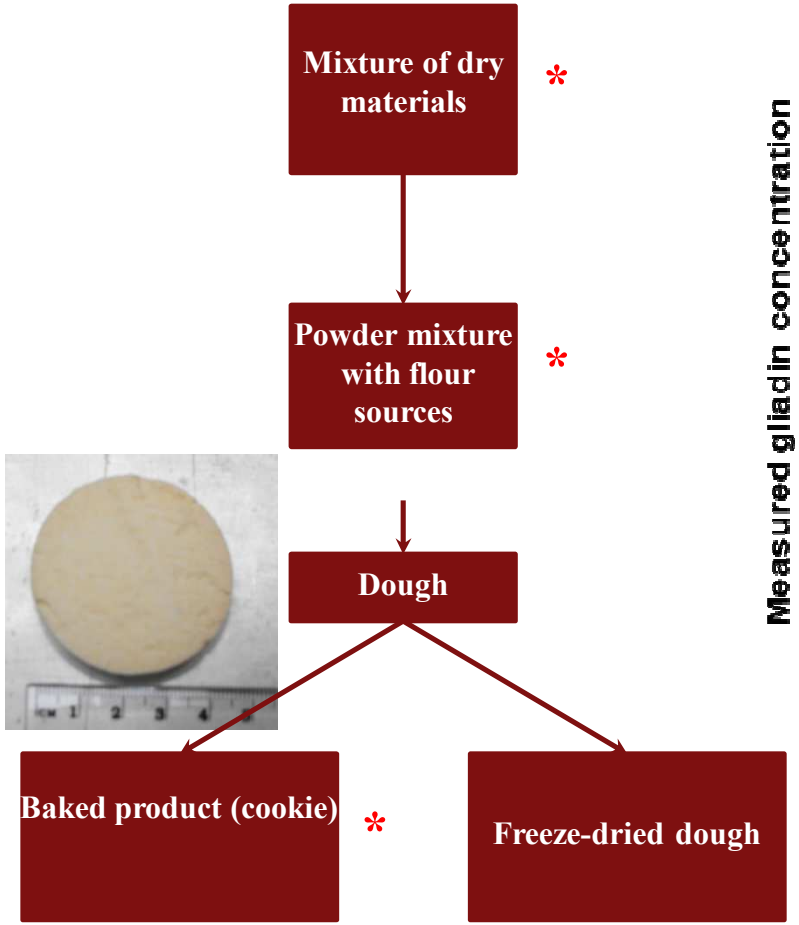
GLIADIN COMPOSITION OF PWG GLIADIN AND THE GLIADIN ISOLATES FROM THE SELECTED CULTIVARS

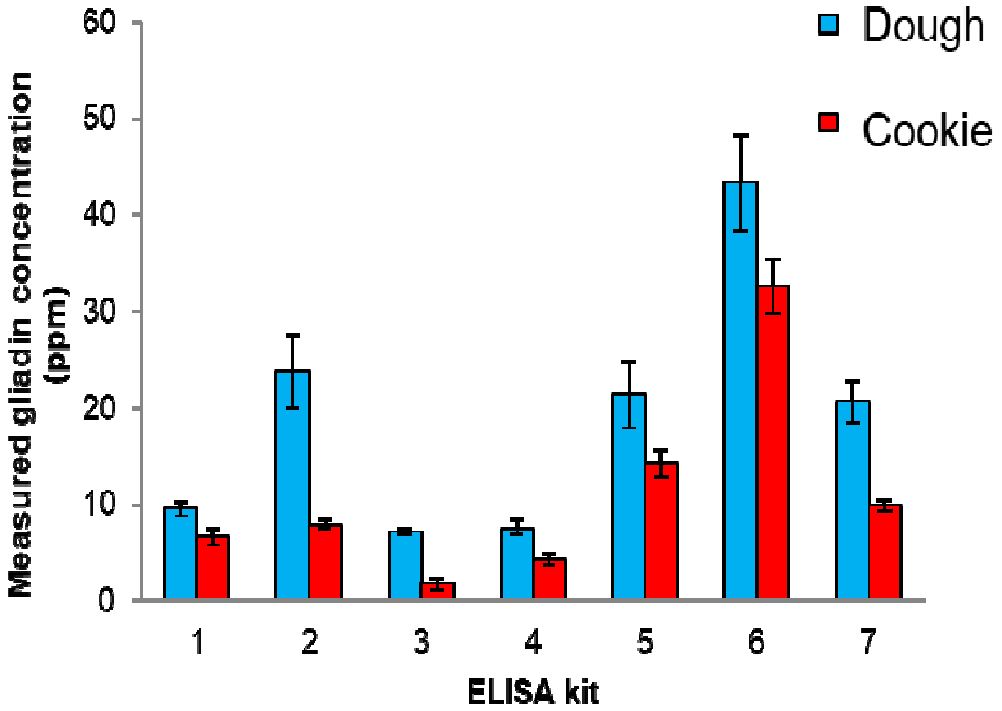


RESULTS: COMPARISON OF ELISA RESULTS - *GLUTEN CONCENTRATION OF SINGLE CULTIVARS AND THEIR MIXTURES DETERMINED BY TWO ELISA KITS*



RESULTS: INVESTIGATION THE EFFECT OF HEAT TREATMENT ON ELISA RESULT APPLYING INCURRED DOUGH MATRIX AS MORE REALISTIC FOOD MODEL





Bugyi Zsuzsanna, Török Kitti, Hajas Lívia, Adonyi Zsanett, Popping Bert, Tömösközi Sándor. Comparative study of commercially available gluten ELISA kits using an incurred reference material. QUALITY ASSURANCE AND SAFETY OF CROPS & FOODS 5:(1) pp. 79-87. (2013)

Bugyi Zs, Nagy J, Török K, Hajas L, Tömösközi S. Towards development of incurred materials for quality assurance purposes in the analysis of food allergens. ANALYTICA CHIMICA ACTA 672:(1-2) pp. 25-29. (2010)

CONCLUSION

- **GxE effects:** Naturally, varieties (G) and harvest years (E) have significant effects on the protein composition and also on the ELISA results

- **Mixture of varieties or individual variety**
  - Technically and from the viewpoint of standardization, the utilization of one variety is much simplier
  - Blending of varieties can compensate very well the differences between individual varieties

- **Isolates or wheat flours:**
  - The production of standardized wheat flour is much simplier and they represents the reality better
  - Isolates are much stable and better characterizable but there production is much more complicated
- **Native proteins vs processed food matrices:** Processed food matrices incurred with wheat flour or isolates can be a more realistic modell – **but** analytical method-dependent processing effects are measurable wtih different ELISA kits.

CONCLUSION

Many question remained open but we made some decesion on the bases of our results:

- **We continue the RM development work**
  - **with two flours: one selected variety and blend of five varieties**
  - **on laboratory** (BME/Leibnitz) **and on pilot scales** (BOKU/AGES)

- **We continue the related research work** with isolates and incurred materials for clarifying the background of identified phenomena

ACKNOWLEDGMENTS

All students, colleagues at the Cereal Research Group at BME

Dr. Gergely Szilveszter (NIR/NIT, BME)

Wheat samples

- Dr. Roland Poms (MoniQA Association)
- Dr. Peter Köhler, Dr. Katharina Scherf (DFA)
- Dr. Terry Koerner, Health Canada
- Dr. Ferenc Békés, FBFD PTY Ltd, Australia
- Dr. Zoltán Bedő, Dr. László Láng, Dr. Mariann Rakszegi (Centre for Agricultural Research, Hungarian Academy of Sciences, Martonvásár, Hungary)

ELISA kits

- R-Biopharm AG
- Romer Labs

- MoniQA NoE (FOOD-CT-2006-036337)
- MoniQA Association Allergen Working Group (www.moniqa.org)
- „Characterization of the quality of old wheat varieties and their utilization in market-oriented wheat breeding" project (AGR_PIAC_13-1-2013-0074)
- „New aspects in wheat breeding: improvement of bioactive component composition and its effects" (OTKA K 112179 and 112169)
- „Improving gluten-free dough by a novel hemicellulose network" project (OTKA-ANN 114554)

THANK YOU FOR YOUR ATTENTION!

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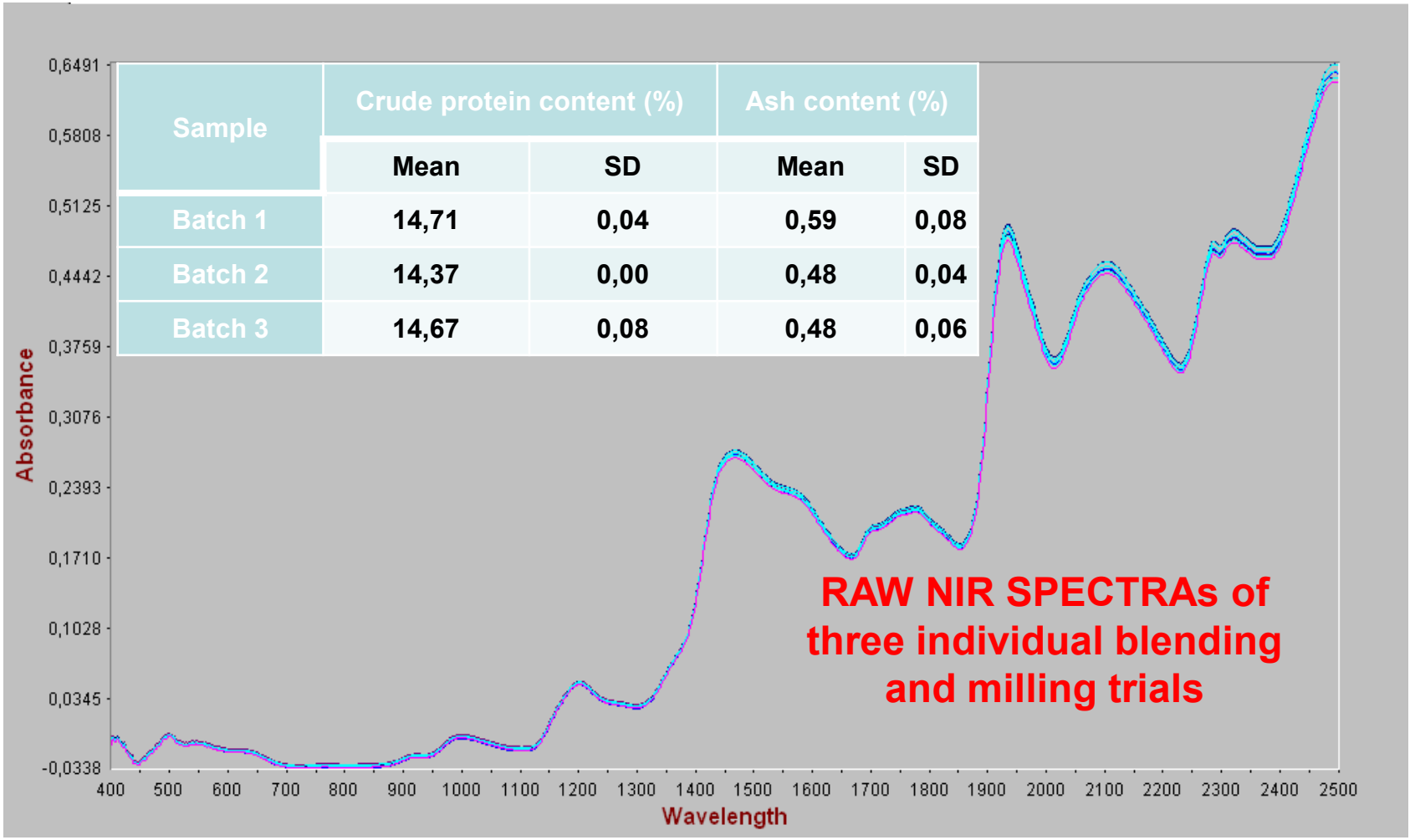
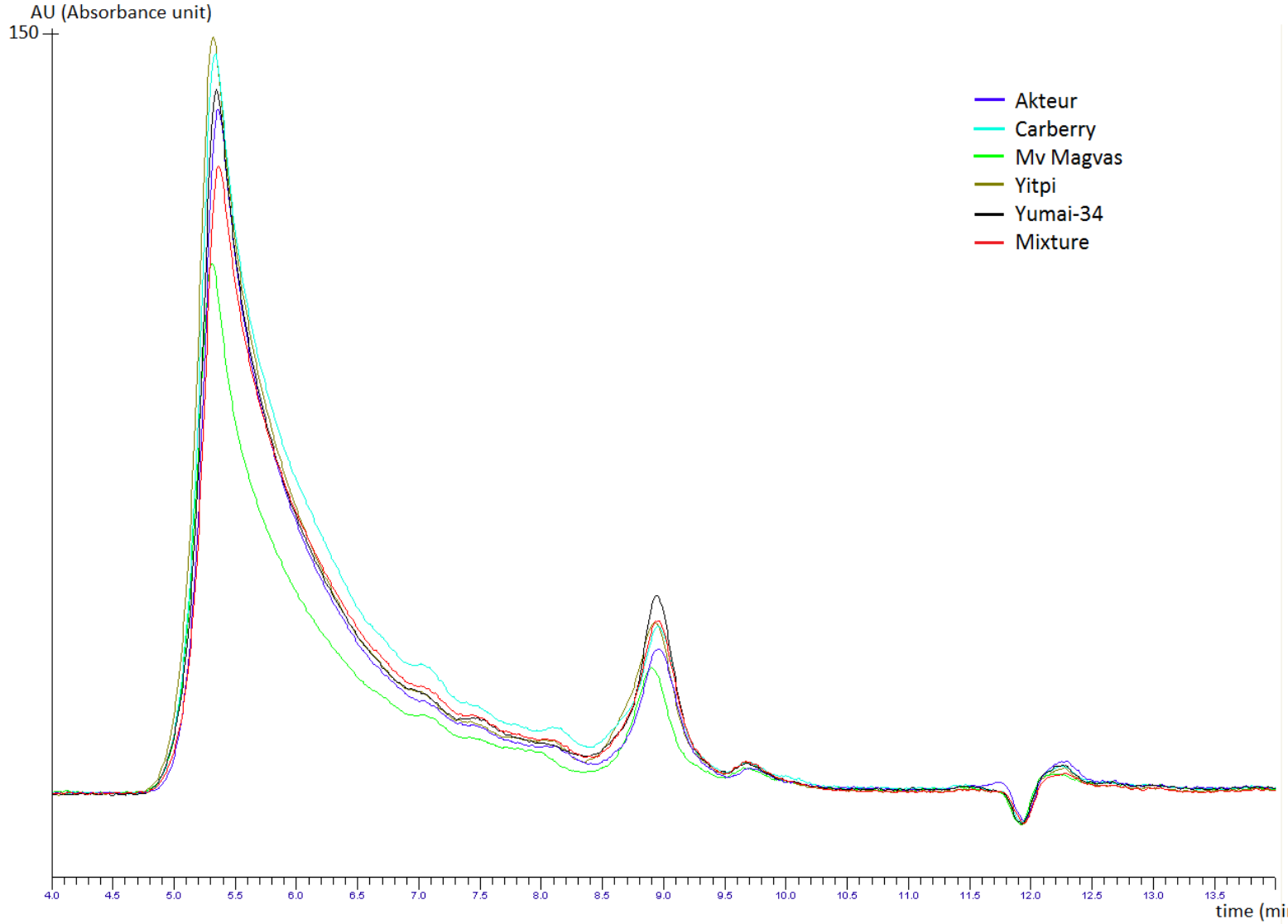
STEP FORWARD: PILOT-SCALE EXPERIMENTS WITH THE MIXTURE OF SELECTED WHEAT VARIETIES (BOKU/AGES, AUSTRIA)

Sample	Crude protein content (%)		Ash content (%)	
	Mean	SD	Mean	SD
Batch 1	14,71	0,04	0,59	0,08
Batch 2	14,37	0,00	0,48	0,04
Batch 3	14,67	0,08	0,48	0,06



RESULTS: SINGLE CULTIVAR VS MIXTURE OF THE CHOSEN ONES

CHROMATOGRAMS OF THE INSOLUBLE PROTEINS BY SE-HPLC



RESULTS: SINGLE CULTIVAR VS MIXTURE OF THE CHOSEN ONES

TOTAL PROTEIN AND GLUTEN CONTENT OF THE MIXTURE

	measured value of the flour mixture	average value calculated from the results of five cultivars
Protein content by Dumas (%)	15,44	15,66
Protein content by RP-HPLC (%)	12,67	12,90
Gluten content RP-HPLC (%)	11,26	11,49
Dry gluten content (%)	12,69	12,38

- The mixture of five cultivars seems to be homogenous, therefore
- The method we used for homogenization seems to be suitable

RESULTS: CORRELATIONS BETWEEN THE ELISA AND THE RP-HPLC RESULTS

	RP-HPLC				
	total gliadin	ω5 gliadins	ω1,2 gliadins	α gliadins	γ gliadins
ELISA kit A	0,90	0,67	0,84	0,87	0,74
ELISA kit B	0,95	0,75	0,89	0,89	0,78