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Proposal for C-hordein as reference material in gluten quantification

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Overestimation

Contaminant barley

Pure oat

Gluten quantification:
Gluten-free?

R5 sandwich ELISA

When using
gliadin as
reference material

True gluten content

Correct content when
using C-hordein as
reference material

1 **Proposal for C-hordein as reference material in gluten quantification**

2

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11

Abstract

The concentration of residual barley prolamins (hordeins) in gluten-free products is overestimated by the R5 ELISA method when calibrated against the wheat gliadin standard. The reason for this may be that the composition of the gliadin standard is different from the composition of hordeins. This study showed that the recognition of whole hordein by R5 antibody mainly came from C-hordein, which is more reactive than the other hordeins. The proportion of C-hordein in total hordein ranged from 16% to 33% of common Finnish barley cultivars used in this study, and was always higher than that of omega-gliadin, the homologous protein class in the gliadin standard, which may account for the overestimation. Thus, a hordein standard is needed for barley prolamins quantification instead of the gliadin standard. When gluten-free oat flour was spiked with barley flour, the prolamins concentration was overestimated 1.8–2.5 times with the gliadin standard, whereas estimates in the correct range were obtained when the standard was 40% C-hordein mixed with an inert protein. A preparative-scale method was developed to isolate and purify C-hordein, and C-hordein is proposed as a reference material to calibrate barley prolamins quantification in R5-based assays.

Key words: Prolamin, Gluten quantification, R5, Hordein, C-hordein, Gluten-free

34 Introduction

35 In order to predict the potential toxicity of cereal products for people with
36 celiac disease or gluten sensitivity, gluten content is measured directly, since
37 there is at present no assay that estimates all of the immunopathogenic
38 elements. The currently most widely accepted method for gluten quantification
39 is an enzyme-linked immunosorbent assay (ELISA) based on the R5
40 antibody. This is classified as a Type 1 method by Codex Alimentarius
41 (standard 118-1979). With appropriate extraction, this method performs well in
42 most food matrices when calibrated against wheat (*Triticum aestivum* L.
43 emend Thell.) gliadin. In the quantification of hordein in barley-containing
44 foods, however, the method leads to overestimation. Kanerva et al.¹ reported
45 7-30 times overestimation when barley (*Hordeum vulgare* L.) contamination in
46 oat (*Avena sativa* L.) was measured with a gliadin reference material. When
47 Hernando et al.² investigated the spiking of different barley cultivars in maize
48 (*Zea mays* L.) flour, the R5 ELISA overestimated its content by 1.2 times.
49 Later the same group³ reported that overestimation of barley in maize flour
50 was 1.8 times, when extracted with 'cocktail solution', a phosphate-buffered
51 saline with reducing and disaggregating agents.⁴ The K_d value, the
52 equilibrium dissociation constant between the antibody and its antigen, of total
53 hordein of cultivar Risø 56 against R5 antibody was 39 times greater than the
54 that of the reference gliadin.⁵ There is no data suggesting that barley has
55 greater toxicity to coeliacs than wheat, so it is important to have correct gluten
56 quantification of barley-containing products.

57

58 One reason for the overestimation may be that the composition of gliadin

reference material is substantially different from that of hordeins. The current gliadin reference material is a selection of 28 European wheat cultivars,⁶ with 86.4% gliadin content, including 41.7% α -gliadin, 47% γ -gliadin, and 11.3% ω -gliadin by HPLC-RP analysis. When hordein is extracted with reducing agents, its typical composition is 7-8% D-hordein (corresponding to HMW-glutenin), 10-20% C-hordein (corresponding to ω -gliadin), 70-80% B-hordein (LMW-glutenins), and a minor amount of γ -hordein (corresponding to γ -gliadin).^{7, 8, 9} Each of these protein groups has different reactivities against R5 antibody. The proportion of ω -gliadin, which is structurally similar to C-hordein, in total gliadin of common wheat and in the gliadin reference is less than the proportion of C-hordein in total hordein.

70

The R5 antibody recognizes mainly the epitope QQPFP, and homologous epitopes LQPFP, QLPYP, QQSFP, QQTFP, PQQPFP, QQPYP and PQQPFP to a weaker degree.¹⁰ C-hordein, the primary structure of which is almost entirely repeats of PQQPFPQQ, is strongly recognized by the R5 antibody and has 15-20 times more reactivity than the reference gliadin.¹¹ This explains the results of earlier barley overestimation.

77

Using a separate hordein reference material would make the quantification of barley prolamin more accurate, as previous studies have already shown.^{1, 3, 5}

The degree of overestimation ranges widely, because each research group has isolated its own hordein standard from different cultivars, so it is questionable whether a single whole hordein from one cultivar could be used to calibrate the hordein concentration in barley-containing products.

84

85 The aim of this study was to investigate the proportion of C-hordein in whole
86 barley hordein, in order to explain the hordein overestimation with gliadin
87 reference material in R5 antibody-based ELISA. An additional aim was to
88 determine whether a reference material using C-hordein could be used to
89 quantify hordein, for example, to determine the barley contamination in oats.

90

91 **Materials and Methods**

92 **Materials**

93 Twenty-nine barley cultivars for feed and malt purposes were selected for this
94 study. Cultivars Brage, Tocada, Vilde, Marthe, Fairytale, Aukusti, Wolmari,
95 Barke, Polartop, Einar, Jyv, Saana, Edvin, NFC Tipple, Streif, Harbinger,
96 Scarlett and Elmeri were kindly provided by Boreal Plant Breeding Ltd.
97 (Finland). The sample of Xanadu was from the Kesko Corporation (Finland),
98 and that of Propino from Syngenta (Finland). These were the top 20 cultivars
99 grown in Finland during 2012-2015. In addition, cultivars KWS Asta, Lacey,
100 Minttu, Toria, Overture, Voitto, Annabel and SW Mitja (Boreal Plant Breeding
101 Ltd.) were analyzed. Hull-less cv. Jorma was from Risto Laitinen (Villala,
102 Finland). The barley kernels were milled to flour with a Brabender Quadrumat
103 Junior (Duisburg, Germany) and passed through an 850 μm sieve.

104

105 All the chemicals used were analytical quality or better. Bovine serum albumin
106 (BSA) with purity $\geq 98\%$ was purchased from Santa Cruz Biotechnology
107 (Dallas, Texas).

108

109 **Hordein composition by reverse-phase high-pressure liquid**
110 **chromatography (RP-HPLC)**

111 The hordein composition was determined by RP-HPLC. Barley flour (0.1 g)
112 was extracted following the Osborne sequence of 1 ml of mQ-water, followed
113 by 1 ml of 0.5 M NaCl, and 1 ml of mQ-water to remove the albumin and
114 globulin proteins at ambient temperature. After each extraction, samples were
115 centrifuged for 10 min at $20000 \times g$. The pellet was treated either once or 3
116 times with 40% (v/v) aqueous 1-propanol with 5% (v/v) 2-mercaptoethanol at
117 50°C for 30 min to extract the whole hordein fraction. The supernatants from
118 the three sequential extractions were bulked together. Further samples of
119 barley flour were also directly extracted either once or 3 times with the same
120 solvent. After centrifugation, the supernatants from all four of these extraction
121 types (Osborne or direct, single or triple) were filtered through a 0.45 μm
122 membrane prior to RP-HPLC separation. An injection volume of 10 μl of
123 whole hordein was separated on a C8 column (4.6×100 mm, 300 Å, 5 μm ,
124 Discovery, Sigma-Aldrich Co. LLC), at 35°C. A linear acetonitrile gradient from
125 20% solvent B (0.1% TFA in acetonitrile) to 50% solvent A (0.1% TFA in milli-
126 Q water) was run over 40 min at a flow rate of 0.7 ml/min, and the elution was
127 monitored at 210 nm. The hordein peaks were integrated (Empower 2, Waters
128 Corporation) and the peak areas were compared to determine the C-hordein
129 proportion of total hordein in each cultivar. Because the proportion of γ -
130 hordein was reported to be very minor, and the HPLC profile did not
131 distinguish it, it was counted together with B-hordein.

132

133 **Hordein fractions reactivity against R5 antibody**

134 The hordein fractions were collected, and their protein content was quantified
135 with a BSA standard (linear range 0-60 μg , $R^2 = 0.988$) by peak area. The
136 collected fractions were dried first under a nitrogen stream and then by
137 vacuum centrifugation (Savant SpeedVac SC110A concentrator, USA). The
138 dried fractions were completely solubilized in cocktail solution (R7006, R-
139 Biopharm, Darmstadt, Germany) and analyzed by R5 sandwich ELISA
140 (R7001, R-Biopharm, Darmstadt, Germany).

141

142 **Purification of C-hordein in a preparative scale**

143 The flour of cv. Jorma was first washed with acetone to remove phenolic
144 compounds and filtered, then extracted following the Osborne sequence
145 described earlier. The monomeric hordein was extracted with 40% (v/v) 1-
146 propanol at 50°C without reducing agent. After centrifugation at 18000 $\times g$,
147 the supernatant was left at 4°C overnight. The suspension was centrifuged
148 again to remove the precipitates before mixing with buffer A. Buffer A
149 consisted of 40% (v/v) aqueous 1-propanol, 10 mM glycine, and 50 mM citric
150 acid-sodium citrate buffer at pH 3.0, and buffer B consisted of 40% (v/v)
151 aqueous 1-propanol, 0.5 M NaCl, 10 mM glycine, and 50 mM citric acid-
152 sodium citrate buffer at pH 3.0. An ion exchange column (35 mm diameter $\times$
153 300 mm length) was packed with TOYOPEARL SP-650M (TOSOH
154 Bioscience LLC, Japan) and coupled with ÄKTAprime plus system (GE
155 Healthcare, Sweden). After the sample was loaded, the separation gradient
156 was 0-50% B from 0 ml to 600 ml at a flow rate of 15 ml/min. The eluate was
157 collected in 10 ml fractions, and monitored at 280 nm. The components of
158 fractions were analyzed by SDS-PAGE (NuPage Bis-Tris 10%, Invitrogen,

LifeTechnologies). The fractions containing C-hordein were combined and dialyzed against mQ-water with a cut-off MW 10 000 (Sigma-Aldrich D9527). The dialysed C-hordein was lyophilized (Heto-Holton DW8-85, Denmark). For the purpose of this study, this fraction was used as a benchmark of 'pure' C-hordein.

164

C-hordein as a reference material in sandwich and competitive ELISA

Lyophilized C-hordein and BSA were dissolved separately in 60% (v/v) aqueous ethanol at 0.3 mg/ml, and then mixed in ratios of 1:9 (C-hordein:BSA), 2:8, 3:7, 4:6 and 5:5, further referred as 10%, 20%, 30%, 40%, 50% C-hordein at a constant protein concentration of 0.3 mg/ml. Because BSA does not react with the R5 antibody, the mixture of C-hordein with BSA in different ratios was expected to mimic total hordein in ELISA. The samples were tested in sandwich and competitive ELISA assays (R7021, R-Biopharm, Darmstadt, Germany) to compare with the kit reference materials.

174

Prolamin quantification of oat flour spiked with barley flour

Flours of cultivars Elmeri, Einar and Marthe, with C-hordein contents of 33.1%, 25.6% and 17.4%, respectively, were selected for spiking in commercial gluten-free oat flour (Provena, Raisio Nutrition Ltd. Finland). An aliquot of 0.1 g of barley flour was added to 0.4 g whole grain oat flour and homogenized by vortexing. The mix was further diluted to 400 times by oat flour manually in steps ($\times 4$, $\times 10$, $\times 10$) to ensure homogeneity. The hordein concentration was also determined by R5 sandwich ELISA, and calculated with gliadin reference material and 40% C-hordein. The spiking was

184 conducted in three replicates and each replicate was measured in two
185 dilutions, and the means and standard errors were calculated. The prolamin
186 concentration of the three barley cultivars was also determined by RP-HPLC
187 using BSA as standard by peak area. Flour was extracted with 40% (v/v)
188 aqueous 1-propanol with 5% (v/v) 2-mercaptoethanol and injected on a C8
189 column as described earlier.
190

191 **Results**

192 **Hordein HPLC profile and C-hordein proportion**

193 When whole hordein was separated on a C8 column, D-hordein was eluted
194 first, followed by C-hordein and B-hordein (Figure 1).⁸ The 29 analyzed
195 cultivars could be divided into three C-hordein peak patterns, of which type
196 (b), for example cv. Barke, was the most common, present in 19 cultivars. The
197 C-hordein proportion in total hordein of cv. Elmeri after a single extraction was
198 26.0% and after three sequenced extracts was 24.7%; the corresponding
199 figures after Osborne extraction were 25.5% and 25.7%. There was no
200 significant difference between the single and triple extraction results, so the C-
201 hordein content of all cultivars was determined with a single direct extraction
202 (Table 1).

203

204 From 2012 to 2015, cv. Barke and NFC Tipple were the two most popular
205 malting cultivars in Finland, accounting for over 50% of the hectareage for this
206 crop. Feed cultivars were far more diverse, with cv. Elmeri, Wolmari, Brage
207 and Aukusti accounting for over 40% of the feed barley hectareage (National
208 Land Survey of Finland, 2012-2015; <http://www.maanmittauslaitos.fi/en>). The
209 C-hordein content of the 29 cultivars ranged two-fold, from 16.5% to 33.1%
210 (Table 1). There was slight variation in the C-hordein content of cv. Elmeri
211 from 2010, 2014 and 2015. According to this data set, the weighted average
212 C-hordein content of whole hordein in Finland 2012-2015 was 25-26%.

213

214 **Hordein subunit reactivity against R5 antibody**

215 The reactivity of D-, C- and B-hordeins against the R5 antibody varied widely
216 in sandwich ELISA (Figure 2). C-hordein was 10-20 times more reactive than
217 gliadin standard, which in turn was 8-25 times more reactive than B-hordein.
218 The slope of the curve indicated that C-hordein and gliadin standard had
219 similar affinity with the R5 antibody, while B-hordein had less, and D-hordein
220 had almost none. The three types of C-hordein and B-hordein reacted
221 similarly with R5 antibody, although their HPLC patterns were different.

222

223 **Preparation of C-hordein by FPLC**

224 When hordein was extracted with 40% (v/v) aqueous 1-propanol without
225 reducing agents and kept at 4°C overnight, precipitates appeared that were
226 mainly composed of B-hordein. At pH 3, C-hordein and B-hordein were clearly
227 separated in cation-exchange chromatography (Figure 3a). Fractions 11-15
228 (Peak 1) absorbed at 280 nm but did not contain prolamins, indicating that
229 they were other compounds. Fractions 17-25 (Peak 2) contained C-hordein
230 and were collected and combined in their native form. Following increased
231 NaCl concentration, B-hordein eluted in fractions 54-57 (Peak 3) (0.08-0.11 M
232 NaCl). HPLC of lyophilized C-hordein showed its high purity in comparison
233 with the whole hordein (Figure 3b).

234

235 **Purified C-hordein as reference material in ELISA**

236 In sandwich ELISA, the affinity (the slope) of C-hordein with R5 antibody
237 behaved similarly with gliadin standard, and at a ratio of 3 C-hordein: 7 BSA
238 (30% C-hordein), the reaction almost matched that of the gliadin standard
239 (Figure 4a). In competitive ELISA, the affinity of C-hordein with R5 antibody

was similar to that of the peptide standard, being closest at a ratio of 1 C-hordein: 9 BSA (10% C-hordein) (Figure 4b). Using the C-hordein and BSA in different ratios could replace both the gliadin standard in R5 sandwich ELISA, and the peptide standard in R5 competitive ELISA. In sandwich ELISA, the curves of purified whole hordein of common cultivars, such as cvs. Barke and NFC Tipple (C-hordein proportions 24.5% and 28.1% respectively), were above that of the gliadin standard and between that of the 30% and 50% C-hordein standard. The curve of cv. KWS Asta, with its low C-hordein content (16.5%), was close to the gliadin standard curve and that of 30% C-hordein (Figure 4c).

250

251 **Measuring barley contamination in oat**

252 With the same spiking, the determined prolamin concentrations of three barley
253 flours were different by HPLC, R5 sandwich with gliadin standard, and R5
254 sandwich with 40% C-hordein standard, but in the same order of Elmeri >
255 Einar > Marthe (Figure 5). The prolamin concentration calibrated by gliadin
256 standard was 2.5 times (cv. Elmeri), 1.8 times (cv. Einar), and 1.2 times (cv.
257 Marthe) the HPLC results. However, calibrated with the 40% C-hordein
258 standard, the estimated prolamin concentration was 1.2 times (cv. Elmeri),
259 0.85 times (cv. Einar) and 0.63 times (cv. Marthe) the HPLC results. For cvs.
260 Elmeri and Einar, the estimated value by the 40% C-hordein standard were
261 not significantly different from those determined by HPLC, but for cv. Marthe
262 the estimate was significantly lower, until the standard was changed to 30%
263 C-hordein.

264

265 Discussion

266 This study investigated the reasons for the overestimation of barley prolamin
267 when measured by the R5 antibody ELISA with the gliadin standard. C-
268 hordein was 80-200 times more reactive with R5 antibody than B-hordein,
269 whereas D-hordein did not react significantly with R5 antibody. When
270 detecting whole hordein with R5, the main recognition was from C-hordein, so
271 the proportion of C-hordein in whole hordein was crucial for correct prolamin
272 quantification. The C-hordein proportion varied from 16.5% to 33.1% in
273 common barley cultivars in Finland, with an average C-hordein around 26%.
274 The composition of the gliadin standard was not comparable to that of
275 hordein. Using the gliadin standard caused a 1.8-2.5 times overestimation of
276 barley flour spiked in gluten-free oat flour, but use of a 40% C-hordein
277 standard allowed calibration to give the correct concentration. A preparative-
278 scale method was developed to isolate and purify C-hordein by cation-
279 exchange chromatography, and C-hordein is proposed as a reference
280 material to calibrate barley prolamin quantification in the R5 ELISA.

281

282 The varying reactivity of hordein subunits against the R5 antibody is
283 attributable to the number of epitopes. The main R5 epitope, QQPFP, has
284 been shown to occur 13 times in C-hordein (Uniprot Q40055), and minor
285 epitopes QQPYP, QQTFP, PQPFP and QLPFP each occur once. One main
286 QQPFP epitope and 7 minor epitopes were found in B3 hordein (Uniprot
287 I6TEV5), and 5 QQPFP epitopes in B1 hordein (Uniprot P06470). Only one
288 QQPFP epitope was found in λ 3-hordein (Uniprot P80198) and no R5 epitope
289 was found in D-hordein (Uniprot Q84LE9). Our results agreed with those of

290 Tanner et al.⁵ who measured hordein subunits of cultivars Sloop and Risø 56
291 against R5 antibody and calculated their dissociation constant (K_d) value
292 according to the best fit. In their study, the K_d value of B-hordein was 132
293 times than that of C-hordein in cv. Sloop, which means that it is much less
294 sensitive to R5 antibody. In 'Risø 56', a B-hordein null cultivar, the K_d value of
295 γ -hordein was 74 times greater than C-hordein. The K_d value of C-hordein
296 differed two-fold in these two cultivars. The current study showed that the C-
297 hordein of common European cultivars Harbinger, NFC Tipple and Barke
298 behaved similarly in the recognition of the R5 antibody, although their
299 peptide/protein composition as demonstrated by the HPLC profiles differed.
300 C-hordein of a single cultivar can consist of up to 20 polypeptides, thus
301 forming different HPLC profiles. The primary structures of these prolamins
302 polypeptides are highly homologous, but their molecular weights differ.¹² In
303 this study, three groups of cultivars were gathered according to the RP-HPLC
304 profiles of their C-hordein, and an even more detailed classification is
305 available based on the gel patterns.¹³

306

307 The ratio of B-hordein to C-hordein in this study ranged from 1.89 for cv.
308 Elmeri to 4.68 for cv. KWS Asta. This range is comparable to that of Scottish
309 cultivars, from 1.79 to 4.15, determined by a similar HPLC method.¹⁴ The
310 corresponding protein group to C-hordein in wheat is ω -gliadin, which shows
311 about 70% sequence homology, with a similar repetitive sequence in the
312 central domain of PFPQQPQQ.¹⁵ The ω -gliadin content of total gliadin has
313 been reported to range from 6.2% to 20.0%,¹⁶ or from 10% to 19%.¹⁷ High ω -
314 gliadin proportions (17-20%) were found in feed wheat cultivars carrying

315 chromosome arm 1RS from rye.¹⁶ The proportion of C-hordein in total hordein
316 is generally higher than the proportion of ω -gliadin in total gliadin. The
317 composition of prolamins is affected by cultivar, environment and nitrogen
318 nutrition.^{18, 19} In a set of barley cultivars grown in Spain and Scotland, the C-
319 hordein proportion was highest in young endosperm, but at harvest ripeness,
320 the B to C hordein ratio decreased slightly in Scotland while dropping much
321 lower in Spain.²⁰ The C-hordein and ω -gliadin proportion was enhanced in
322 low-sulphur growing conditions.^{21, 22} The sulphur distribution varies in Europe,
323 for example, in central and northern Germany, Poland, most of Sweden, and
324 northern Finland, the subsoil sulphur value was lower than in northern
325 Scotland, the Netherlands, north-eastern Germany and south-eastern
326 Spain.²³ Increasing nitrogen fertilization increases both the hordein fraction of
327 whole protein and the C-hordein proportion of whole hordein.¹⁸ The proportion
328 of ω -gliadin in total gliadin increases with nitrogen nutrition and
329 temperature.^{17, 24, 25}

330

331 A C-hordein standard could be used to determine the prolamins content in, for
332 example, barley-contaminated oat, or purified barley starch, malt and malt
333 extracts. Cross-contamination of oat can occur during growing, harvest,
334 processing, storage and transportation, and the contamination of commercial
335 oat should be no more than 4%. Barley is the most common contaminant in
336 oat, since they are often part of the same cropping sequence.^{2, 26} If the
337 contaminant is suspected to be wheat, either a gliadin standard or 30% C-
338 hordein could be used for calibration. If the contaminant is suspected to be
339 barley, which is more likely, a C-hordein standard, based on the average C-

340 hordein proportion of the region, can be used for calibration. The process of
341 barley starch extraction is under low pH so that acid hydrolysis of hordein may
342 occur, hence competitive ELISA should be utilized to detect small fragments
343 of prolamin, along with the sandwich assay. The prolamin of barley malt is
344 partially hydrolyzed by its endogenous enzymes, so it should be quantified in
345 both sandwich and competitive assays. In the sandwich assay, the current
346 commercial reference material is the gliadin standard, while in the competitive
347 assay it is a hydrolysate mixture of the gliadin standard, one barley cultivar
348 and one rye cultivar.²⁷ Clearly, these two reference materials are not
349 comparable. In addition, the commercial peptide standard was hydrolyzed by
350 the digestive enzymes pepsin and trypsin, which is very different from the
351 malting or the starch separation process. Our results showed that the 30% C-
352 hordein and BSA mixture could represent gliadin standard, while the 10% C-
353 hordein mixture can represent the peptide standard. Thus, the use of a C-
354 hordein standard could not only allow correct determination of prolamin
355 content, but also make the results from sandwich and competitive assays
356 comparable.

357

358 Nevertheless, the validity of gliadin peptide standard for hydrolyzed products
359 has not yet been confirmed. In quantification of prolamin in spiked beer by
360 competitive R5 ELISA, calibration using gliadin peptide standard resulted in
361 overestimation by 180%.²⁸ The difficulty of prolamin quantification in beer is
362 that the raw material, type and level of hydrolysis vary widely among products.
363 The peptides in the standard may not be representative of the prolamin
364 hydrolysates in beer. Competitive R5 detects peptides longer than five amino

365 acids, but small peptides with 5-9 amino acids are considered non-toxic to
366 coeliac patients.²⁹ New methods are under development to detect prolamin
367 peptides in beer, such as LC-MS/MS,^{30, 31, 32, 33} and an ELISA method based
368 on the G12 antibody.³⁴ Further investigations should include comparison of
369 the R5 reactivity of hordein with that of hordein peptides hydrolyzed by
370 different enzymes, and how C-hordein as reference material can represent
371 the hydrolysates in hydrolyzed products.

372

373 In analytical work, a reference material or standard is necessary. For R5-
374 based gluten analysis, the PWG gliadin standard is available, but it is not
375 reproducible, and it is not accepted as a certified reference material in the
376 Institute of Reference Material and Measurements of the European
377 Commission due to its high glutenin content (Working Group on Prolamin
378 Analysis and Toxicity, 2016; <http://www.wgpat.com/pwggliadin.html>).
379 Validated and certified reference material is still missing in Codex 118-1979
380 standard. C-hordein as reference material is a group of homologues of
381 proteins, rather than a mixture of gliadins from different wheat cultivars. C-
382 hordein is a better substrate for R5 antibody because its repetitive sequences
383 correspond to R5 epitopes. Additionally, the use of C-hordein as calibrant has
384 the possibility to adjust by the percentage of C-hordein. Other antibody-based
385 (G12, Skerritt, α -20) methods have their own reference materials.^{34, 35, 36}
386 Researchers are trying to develop wheat-based reference materials
387 applicable to specific food products.^{37, 38}

388

In conclusion, this study determined that the high proportion of C-hordein in total hordein is the reason for the consistent overestimation of hordein by the R5 ELISA assay which uses gliadin as reference material in gluten-free analysis. We isolated C-hordein and propose it as the reference material for quantifying hordein concentration in barley-containing foods, including those that may have been contaminated with barley.

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545 **Figure 1.** Reverse-phase liquid chromatogram of total hordein extract
546 separated on a C8 column. (a) cv. Harbinger; (b) cv. Barke; (c) cv. NFC
547 Tipple. The total hordein was directly extracted with 40% (v/v) 1-propanol with
548 5% (v/v) 2-mercaptoethanol from barley flour.

549

550 **Figure 2** Reaction of isolated hordeins against R5 antibody in sandwich
551 ELISA. Three types of C-hordein and B-hordein were from cultivars
552 Harbinger, Barke and NFC Tipple. D-hordein was from cv. Harbinger.

553

554 **Figure 3** (a) Cation-exchange chromatogram of hordein. The black line shows
555 the absorbance at 280 nm, fractions from peaks 1, 2 and 3 were collected,
556 and the red line shows the gradient concentration of NaCl. The inset shows
557 SDS-PAGE of collected fractions; (b) Reverse-phase chromatogram of
558 purified C-hordein and whole hordein from cv. Jorma.

559

560 **Figure 4** Reaction of C-hordein mixed with bovine serum albumin (BSA) in
561 different ratios against R5 antibody (a) in sandwich ELISA; and (b) in
562 competitive ELISA. (c) Reaction of purified whole hordein of 6 cultivars in R5
563 sandwich ELISA compared with 30%, 40% and 50% C-hordein standards and
564 gliadin standard.

565

566 **Figure 5** Prolamin concentration of gluten-free oat flour spiked with three
567 barley flours, determined by HPLC, R5 sandwich ELISA with 40% C-hordein
568 standard, and R5 sandwich ELISA with gliadin standard. Error bars show
569 standard error.

570 **Table 1** C-hordein content as percentage of whole hordein of selected barley
571 cultivars.

Cultivar	Year	Origin	Content (%)	Cultivar	Year	Origin	Content (%)
KWS Asta	2013	CE	16.5	Saana	2014	Nordic	27.5
Marthe	2009	CE	17.4	Edvin	2010	Nordic	28.0
Tocada	2013	CE	18.6	Elmeri	2015	Nordic	28.1
Vilde	2010	Nordic+CE	18.6	NFC Tipple	2013	CE	28.1
Propino	2014	UK	20.3	Brage	2010	Nordic	28.3
Fairytale	2013	CE	20.7	Streif	2014	CE	28.5
Toria	2010	Nordic	22.6	Minttu	2013	Nordic	28.8
Overture	2013	CE	23.0	Elmeri	2014	Nordic	29.2
Aukusti	2010	Nordic	23.1	Harbinger	2014	Nordic	29.6
Wolmari	2010	Nordic	23.3	Scarlett	2013	CE	30.0
Barke	2013	CE	24.5	Jorma	2011	Nordic	30.5
Polartop	2010	Nordic	24.5	Voitto	2010	Nordic	31.1
Einar	2010	Nordic	25.6	Xanadu	2014	CE	31.1
Jyv�	2013	Nordic	25.6	Lacey	2010	NA	32.8
SW Mitja	2014	Nordic	25.7	Elmeri	2010	Nordic	33.1
Annabel	20	CE	25.9				

572 CE = central Europe

573 NA = North America

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575 Figure 1

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578 Figure 2

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581 Figure 3

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585 Figure 4

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590 Figure 5

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