Development and Application of a Rapid Immunoassay for Difenzoquat in Wheat and Barley Products

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A sensitive and simple enzyme-linked immunosorbent assay (ELISA) is described for the quantification of difenzoquat (DFQ) in foods using polyclonal antibodies. Two hapten analogues of DFQ with five-carbon spacer arms attached to one of the aromatic rings were synthesized. The resulting antiserum was specific to DFQ. The minimum detection limit was 0.8 ng/mL for beer and 16 ng/g for cereals with an IC_{50} (50% inhibition of binding) of 0.28 ng/mL in this assay. The recoveries of DFQ spiked at three levels into beer, cereal, and bread ranged from 72% to 101%. The mean intra-assay and inter-assay coefficients of variation in this procedure were 4.6% and 6.9% for five commodities spiked at 100 ng/g or ng/mL DFQ, respectively. The ELISA procedure was applied to a limited survey of 13 beers and 12 breads, but no detectable DFQ residue was found.

Keywords: *Difenzoquat; herbicide; ELISA; immunoassay; beer; cereal; food*

INTRODUCTION

Difenzoquat [1,2-dimethyl-3,5-diphenylpyrazolium methylsulfate (see Figure 1)], is a herbicide that is used principally to control wild oats in cereal crops. It is not metabolized by plants or animals and degrades only by photolysis. Although it is not a heavily used pesticide, the contribution of its residue in food products to the total amount of pesticide residues in the Canadian diet is unknown, in part, because of the inconvenience of its analysis. The polarity of the difenzoquat molecule means that it cannot be included in the general screening method used for the analysis of many pesticides. By using HPLC (Lawrence et al., 1981), DFQ could be quantified in wheat products down to 20 ng/g. However, peaks were broad and asymmetric. Difenzoquat has also been analyzed by thermospray mass spectrometry (Barcelo et al., 1993), but this method was not applied to food products. Most recently, a capillary electrophoresis method was developed (Carneiro et al., 1994; Galceran et al., 1994) for difenzoquat residues but again not applied to food substances.

Of all the quaternary nitrogen herbicides, paraquat is the only one for which antibodies have been developed (Van Emon et al., 1986; Bowles et al., 1992). Paraquat haptens were prepared by conjugating a pentanoic acid to one of the pyridinyl nitrogen atoms in the paraquat molecule. The detection limits reported for the paraquat immunoassays were 0.1−0.5 ng/mL. Unlike paraquat, which is dicationic, difenzoquat is monocationic and does not provide a simple functional group through which it can be linked to a carrier protein. Conjugation to one of the pyrazolium nitrogens would mask the cationic site, which is anticipated to be an important center with respect to antibody−antigen binding. Our intention, therefore, was to link a five-carbon spacer arm to one of the difenzoquat phenyl rings distal to the charged nitrogen and immunize the rabbits with the resulting hapten conjugate according to our established procedure (Newsome et al., 1993).

This paper concerns the development of an ELISA method for the rapid and convenient determination of difenzoquat residues in food products such as barley, wheat, and bread (≥16 ng/g) and beer (≥0.8 ng/mL). The requirement of the cationic site of the hapten for the recognition of the DFQ epitope was also examined.

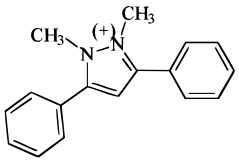

Figure 1. Structure of difenzoquat.

MATERIALS AND METHODS

Chemicals and Supplies. Pesticide standards were obtained from the pesticide repository of the Food Research Division and were stated to be at least 99% pure by the respective manufacturers. Cereal, bread, and beer were purchased in local stores.

Phenylethynyltributyltin, 4-bromobenzoyl chloride, palladium acetate, triphenylphosphine, methylhydrazine, 4-pentenoic acid, 5% palladium on carbon, platinum oxide monohydrate, and silica gel (200−425 mesh, grade 643, Davisil, 4% water) for flash chromatography were purchased from Aldrich Chemical Co. (Milwaukee, WI). Solvents were of distilled-in-glass grade from Caledon Laboratories (Georgetown, ON). Methyl iodide was purchased from B.D.H. (Poole, England) and stored over copper wire. Thin layer chromatography was done with Whatman MK6F silica plates, 1 in. × 3 in., from Chromatographic Specialties (Brockville, ON).

Bovine serum albumin (RIA grade), ovalbumin, human serum albumin, goat anti-rabbit IgG peroxidase conjugate (second antibody), Tween 20, *o*-phenylenediamine dihydrochloride, 1-ethyl-3-[(dimethylamino)propyl]carbodiimide hydrochloride (EDC), and glycerol were purchased from Sigma Chemical Co. (St. Louis, MO). Freund's complete and incomplete adjuvants were obtained from Gibco (Grand Island, NY). Dialysis tubing (10 mm in diameter with a 12 000−14 000 molecular weight cutoff) was purchased from Spectrum Medical Industries Inc. (Los Angeles, CA). Flat-bottom polystyrene microtiter plates were obtained from Dynatech Laboratories, Inc. (Chantilly, VA).

Buffers. Phosphate-buffered saline (PBS, pH 7.4), contained 20 mmol of NaH_2PO_4 and 140 mmol of NaCl per liter of deionized water. Washing buffer (PBS-T) consisted of 0.1% Tween 20 in PBS. Coating buffer (pH 9.6) contained 13 mmol of Na_2CO_3 and 35 mmol of $NaHCO_3$, while citric buffer (pH 5.0) consisted of 51 mmol of Na_2HPO_4 and 24 mmol of citric acid per liter of deionized water. The substrate consisted of

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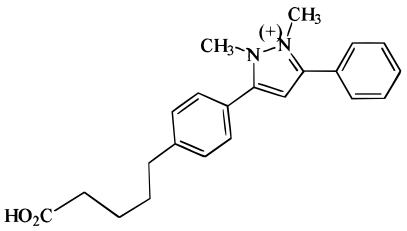

VII

Figure 2. Structure of the linked hapten **VII**.

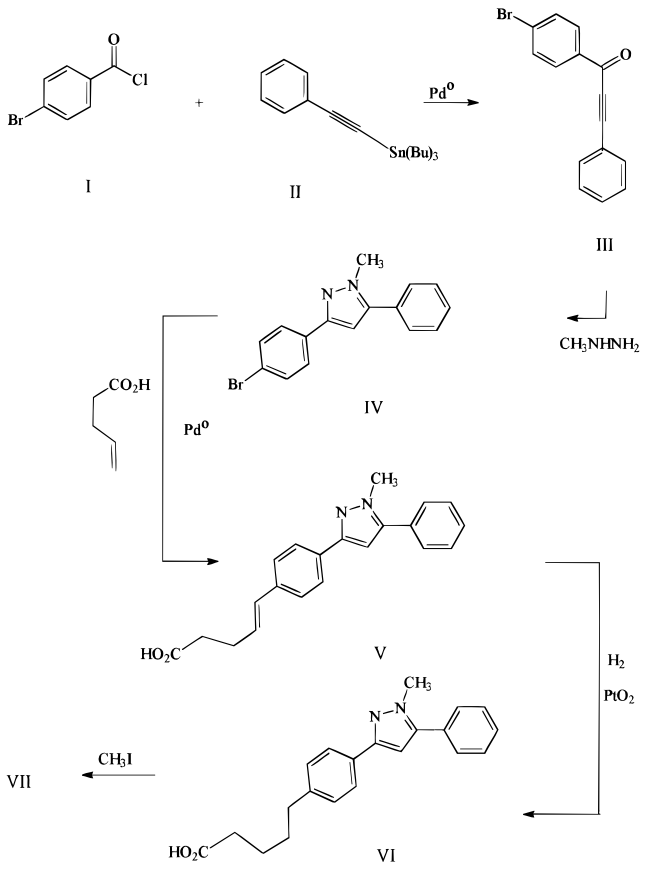

Figure 3. Synthetic scheme for the formation of the haptens **VI** and **VII**.

17.5 mg of *o*-phenylenediamine dihydrochloride (OPD) and 10 μL of 30% H_2O_2 in 25 mL of citric buffer.

Instrumentation. Microtiter plates were washed with PBS-T using a Bio-Rad microplate washer with five wash and soak cycles for 8 s each. A 12 channel pipetter was used for dispensing liquids. Optical densities of microtiter wells were measured on a dual-beam Titertek Multiscan MCC with a 492 nm sample filter and 620 nm reference filters. Data were transmitted to a spreadsheet program for analysis. The instrument was checked periodically by a Spectrocheck plate and software (QC Technology, New York).

^{1}H and ^{13}C NMR spectra were run on a Bruker 200 MHz instrument at 22 °C in $CDCl_3$ or acetone-d_6 referenced to TMS. Mass spectra were recorded on a VG Analytical ZAB 2F instrument in low resolution using a direct inlet probe and electron impact mode at 70 eV.

SYNTHETIC PROCEDURE

To functionalize DFQ to couple to a protein, a five-carbon spacer arm was attached to one of the phenyl rings distal to the pyrazole center (see Figure 2). The synthetic scheme is outlined in Figure 3.

Synthesis of Pyrazole IV. A solution of 4-bromobenzoyl chloride (1.24 g, 5.6 mmol), phenylethynyltributyltin (2.21 g, 5.6 mmol), palladium acetate (4.8 mg, 0.021 mmol) and triphenylphosphine (13.1 mg, 0.05 mmol) in chloroform (ethanol-free) was left at room temperature for 24 h. The reaction mixture was then diluted with diethyl ether (20 mL) and stirred for 30 min with potassium fluoride (5 g) in water (10 mL). The precipitated tributyltin fluoride was removed by filtration and the aqueous phase removed in a separatory funnel. The remaining organic phase was washed with NaCl-saturated phosphate buffer (pH 7) before being dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The yellow-brown solid (1.7 g) was dissolved in toluene (12 mL) with pyridinium *p*-toluenesulfonate (20 mg) and methylhydrazine (0.37 mL, 7.0 mmol) in an Erlenmeyer flask (25 mL) with a stirring bar and heated on a hot plate (≈100 °C). Beads of an immiscible liquid (presumably water) were rapidly formed on the sides of the flask, and TLC analysis (15% EtOAc−hexane) showed that the reaction was complete within 10 min. The reaction mixture was cooled, diluted with ethyl acetate, and washed first with water and then with NaCl-saturated phosphate buffer (pH 7). The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness to yield a yellow-brown solid (1.68 g). The crude product was recrystallized from 5% toluene-hexane to yield light yellow needles, mp 136−137 °C (0.98 g, 57%). TLC (15% EtOAc−hexane) showed a single spot at R_f 0.3. MS *m*/*z* 312, 314 (100%); ^{1}H NMR ($CDCl_3$) δ 3.92 (s, 3H), 6.58 (s, 1H), 7.46 (br s, 5H), 7.52 (d, 2H), 7.70 (d, 2H); ^{13}C NMR ($CDCl_3$) δ 37.61, 103.17, 121.44, 127.05, 128.66, 128.73, 130.46, 131.71, 132.41, 145.27, 149.37.

Synthesis of Linked Pyrazole VI. In each of two 4 mL Reactivials equipped with a magnetic stirring bar were placed palladium acetate (0.5 mg, 0.002 mmol), triphenylphosphine (2.5 mg, 0.01 mmol), sodium acetate (79 mg, 1 mmol), 4-pentenoic acid (103 mg, 1 mmol), pyrazole IV (300 mg, 0.96 mmol), and dimethylformamide (1.5 mL). The Reactivials were then heated with stirring in an aluminum block at 130 °C for 20 h. On cooling, the combined mixtures were poured into 1 N NaOH (15 mL) and extracted with EtOAc (10 mL, 2×). The aqueous layer was then acidified and extracted with CH_2Cl_2 (10 mL, 2×). The combined organic layers were dried over sodium sulfate, filtered, and evaporated to dryness to yield an amber solid (405 mg). TLC (5% MeOH−CH_2Cl_2) showed a single major spot at R_f 0.4, which fluoresced under long UV wavelength. The crude product was dissolved in 95% EtOH (7 mL) with PtO_2·H_2O (10 mg) and stirred vigorously under a slight pressure of hydrogen for 3 h (no fluorescent spot remained on TLC). The reaction mixture was filtered through a 0.45 μm filter disk and evaporated to dryness to yield a light yellow solid (399 mg) which showed two or three minor traces on TLC (5% EtOH−CH_2Cl_2) besides the major spot at R_f 0.5. Recrystallization from 95% EtOH gave a white solid, mp 124−137 °C. MS *m*/*z* 334 (100%), 247 (60%); ^{1}H NMR ($CDCl_3$) δ 1.71 (m, 4H), 2.38 (m, 2H), 2.66 (m, 2H), 3.93 (s, 3H), 6.58 (s, 1H), 7.22 (d, 2H) 7.47 (br s, 5H), 7.73 (d, 2H); ^{13}C NMR ($CDCl_3$) δ 24.37, 30.67, 33.87, 35.29, 37.42, 103.14, 125.60, 128.52, 128.64, 128.68, 128.74, 130.65, 130.93, 141.57, 145.03, 150.56, 178.94.

Synthesis of Pyrazolium Salt VII. A solution of the pyrazole **VI** (151 mg, 0.45 mmol) and methyl iodide (0.3 mL, 4.5 mmol) in acetonitrile (2.5 mL) was heated at 100 °C in a 4 mL Reactivial under nitrogen for 5 days. The reaction mixture was then evaporated to a viscous syrup which was flash chromatographed on silica gel with acetone to yield an off-white solid (135 mg). This was recrystallized from acetone−hexane to give water-soluble, white crystals (102 mg), mp 167−168 °C (turns yellow on melting). ^{1}H NMR (acetone-d_6) δ 1.80 (m, 4H), 2.48 (m, 2H), 2.90 (m, 2H), 4.47 (s, 3H), 4.48 (s, 3H) 7.24 (s, 1H), 7.64 (d, 2H), 7.75−7.94 (br m, 7H); ^{13}C NMR (acetone-d_6) δ 25.20, 31.33, 33.98, 35.92, 36.96, 36.99, 108.43, 124.92, 127.57, 130.21, 130.24, 130.39, 130.42, 132.00, 146.98, 149.37, 149.54, 174.44.

Immunogen. The DFQ haptens (**VI** and **VII**) were conjugated to human serum albumin in the following manner. To prevent oxidation of the iodide ion, DFQ derivative **VII** (40 μmol) was dissolved in 0.5 mL of peroxide-free 1,4-dioxane and 0.2 mL of $Na_2S_2O_3$ (40 μmol) in water. One milliliter of cold

0.05 M sodium phosphate (pH 7.0) and 100 mg of EDC were added to the mixture. After mixing, the reaction mixture was frozen. Human serum albumin (28 mg) was dissolved in 1.5 mL of the same phosphate buffer and added to the frozen DFQ mixture. The reaction mixture was placed on an orbital mixer for 18 h at 4 °C. The resultant conjugate was dialyzed against running water for 48 h and stored frozen in 1 mL aliquots.

Coating Protein. Coating proteins were prepared from ovalbumin by a mixed anhydride coupling reaction rather than by reaction with EDC to avoid recognition of EDC reaction by products. The two DFQ haptens, **VI** and **VII** (40 μmol), were each dissolved in 0.5 mL of 1,4-dioxane and 0.2 mL of $Na_2S_2O_3$ (40 μmol) in water. Fifteen microliters of tri-*n*-butylamine and 8 μL of isobutyl chloroformate were added. After 60 min in the dark, the reaction mixtures were frozen. Forty milligrams of ovalbumin in 3 mL of cold 0.2 M $NaHCO_3$ (pH 9.3) were added to each of the frozen immunogen mixtures. After 18 h at 4 °C with agitation, the solutions were dialyzed against running water for 48 h and aliquots were stored at −20 °C. The conjugate of immunogen **VII** coating protein at 1 μg/mL plus 10 μg/mL ovalbumin was used to sensitize the 96 well plates, in a manner previously reported (Newsome et al., 1993). These sensitized plates were used for all of the DFQ analyses.

Immunization. Two groups of three, male, New Zealand White rabbits were used to obtain antibodies against the DFQ hapten−human serum albumin conjugates. The immunogen was diluted in PBS and emulsified in Freund's complete or incomplete adjuvant to give a concentration of 1 mg/mL. Rabbits were injected subcutaneously with 0.5 mL of the complete adjuvant emulsion at four sites. Booster injections were given at 4 week intervals, substituting incomplete adjuvant for complete adjuvant.

Serum titers were monitored. Animals having the highest titers and most sensitive inhibition curves were exsanguinated under anaesthesia from 4 to 6 months after the initial immunization. For long-term storage, the serum was kept frozen at −20 °C in 100 μL aliquots. Once thawed, an equal volume of 50% glycerol was added and the solution was stored at −20 °C. The glycerolated antibodies can be used for at least 6 months with no deleterious effect.

Sample Preparation. The commodity to be analyzed (100−500 g) was thoroughly mixed in a Waring blender. A 10 g subsample of blended commodity was homogenized in 100 mL of 2 N HCl using a Polytron for 30 s and then sonicated in an ultrasonic bath for 30 min. Samples were centrifuged at 1500*g* for 30 min. The solids were removed by filtration through Whatman No. 1 paper, and about 5−10 mL of filtrate was collected in polypropylene tubes. Just prior to analysis, an equal volume of cold 2 N NaOH was added to a 1 mL aliquot of the acidic extract while vortexing, and an aliquot (25 μL) of the neutralized sample was taken for ELISA. The unused acidic extract was stored in a freezer.

Beer samples were first degassed under vacuum in a sonicator and then diluted 1:10 with PBS. A 25 μL aliquot was taken for ELISA.

In the recovery studies, samples were artificially contaminated with DFQ at 3 levels (5, 100, and 200 ng/g or ng/mL). These fortified samples were incubated for 30 min at room temperature prior to Polytron and ultrasonic extraction.

Immunoassay. The ELISA procedure was similar to the one previously reported (Newsome et al., 1993). Briefly, a 1 mL aliquot of antiserum diluted 1:80000 with 0.1% BSA in PBS diluent was added to 25 μL of sample or standard. After mixing and incubation at 4 °C for 60 min, 200 μL was added to the wells of the sensitized plate in triplicate. After a further 30 min of incubation at 4 °C and washing, a second antibody horseradish peroxidase conjugate was added. Following further incubation at room temperature for 30 min and washing, the substrate OPD and H_2O_2 were added. Thirty minutes later, the color reaction was stopped and the optical densities were read at 492 nm. The DFQ was determined by a least-squares plot of the logit of the OD against the log of the concentration of the standards. The standard curve consisted of eight concentrations of DFQ (0, 0.02−1.25 ng/mL) in H_2O except for beer. DFQ standards were dissolved in a similarly diluted (1:10) beer in PBS to provide a matrix-modified standard curve for beer. The same beer was used for all of the beer studies.

RESULTS AND DISCUSSION

As it was anticipated that the development of selective DFQ antibodies would be enhanced if the linkage to the albumin protein was most distant from the pyrazolium charged nitrogen, the synthesis of hapten **VII** was pursued (Figure 2). Retrosynthesis of this molecule suggested that its basic structure could be formed by the combination of a methylhydrazine moiety and an appropriately substituted 1,3-diarylpropane unit. The overall approach is summarized in Figure 3. Logue and Teng (1982) have demonstrated the construction of acetylenic ketones from acid chlorides and tributyltin acetylides, and the formation of the pyrazole ring from methylhydrazine and an acetylenic ketone is well-known (Coispeau et al., 1970). Addition of the linker chain used the convenient conditions of the Heck reaction (Patel et al., 1977) followed by mild hydrogenation. Although the hydrogenated pyrazole **VI** had a wide melting point, neither the ^1H nor the ^{13}C NMR showed any impurity peaks. The possibility of a zwitterionic structure in **VI** in equilibrium with the non-protonated pyrazole ring may account for the apparent impurity indicated by the melting point. The most difficult step was the final methylation reaction which proved to be very slow.

To test whether the cationic site is critical for the recognition of the epitope of DFQ, both hapten conjugates of **VI** and **VII** were used as immunogens. Animals immunized with the immunogen that lacked the quaternary nitrogen **VI** produced low titers even after six booster doses. The IC_{50}, which is the concentration of the inhibitor that inhibits 50% of the antibody−antigen binding, ranged from 4 to 226 ng/mL in plates sensitized with heterologous coating protein **VII** and required much higher concentrations of the first antibodies. High titer but no inhibition by DFQ up to 500 ng/mL was noted if homologous coating protein **VI** was used, suggesting only a small proportion of the IgG would recognize the epitope of the cationic DFQ. The data clearly showed that inclusion of the cationic site is highly preferable but not essential.

The quaternary nitrogen DFQ immunogen **VII** elicited high titers as early as 1 month which plateaued after four booster doses. At the dilution of 1:80000, antiserum showed an IC_{50} of 0.28 ± 0.06 ng/mL of DFQ (mean ± SD, $n = 56$). The antibodies did not recognize non-charged DFQ if heterologous coating protein **VI** was used. This DFQ specific antiserum also did not cross-react with any other related pesticides as shown in Table 1. It is interesting to note that the antibody does, to a certain extent (0.01%), recognize the common phenylpropylammonium cationic epitope of cyperquat, *N*-methyl-4-phenylpyridinium ion. Examination of the molecular structure of cyperquat (see Figure 4) shows that its pendant phenyl group is similarly distant from its quaternary ammonium center as the phenyl group in the difenzoquat molecule is from its pyrazolium centre.

The acid extraction method employed here, for cereals and breads, was similar to that of Lawrence et al. (1981) and Van Emon et al. (1987). Unlike the Van Emon method, we neutralized instead of evaporated the acid. For consistent results, it is critical to neutralize the acid with cold, equimolar base prior to the ELISA assay. Extraction of DFQ in cereal samples with PBS afforded only 35−45% yield.

Table 1. Specificity of Antisera toward Difenzoquat and Other Related Compounds

compound	IC_{50}[a] (ng/mL)
difenzoquat	0.275
cyperquat	2093[b]
paraquat	no[c]
diquat	no
dodemorph acetate	no
alachlor	no
atrazine	no
2,4-D	no
metalaxyl	no
triadimefon	no
captan	no

[a] IC_{50} is the concentration of the inhibitor that inhibits 50% of the antibody−antigen binding. [b] At 500 ng/mL cyperquat inhibits 25% binding. [c] No inhibition at concentrations up to 500 ng/mL.

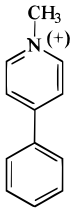

Figure 4. Structure of cyperquat.

To evaluate effectively the sensitivity, reproducibility, and applicability of the antiserum to different matrices, it is essential that the analyte in the solution used to generate the standard curve behaves the same as the analyte in the sample. Standard curves prepared in blank bread, barley, or wheat cereals were superimposable on those prepared in water. However, the beer standard curve was slightly off or tilted from the water standard curve. The slopes of the standard curves were not statistically different by Student's paired *t* test at $p < 0.01$, and the IC_{50} values were essentially the same. Bigger daily variations of recoveries were obtained if water, diluted methanol, or PBS standard curves were used for beers. As we found that standard curves generated by either lager or ale were the same, we chose one light beer to be the matrix modifier for all of the beer analyses and water for other samples in constructing the standard curves.

The use of matrix modifier in recent immunoassays is not uncommon (Silverlight and Jackman, 1994; Ibrahim et al., 1994a,b; Mountfort et al., 1994). Although we did not demonstrate that the beer matrix used was DFQ-free, since chromatographic methods were unable to detect it at our detection range, we did not use the same beer that was used to construct the standard curve to do our recovery and inter-assay coefficients of variation studies. Thus, this approach indirectly supports the assumption that the beer used for the standard curve was DFQ-free.

The current tolerance limit for DFQ in wheat and barley is 0.1 μg/g. Our ELISA was capable of detecting 20 pg/mL DFQ in water based on the lower datum point, which is at least 10% inhibition, in the standard curve. Recoveries ranged from 88% to 101% with standard deviations (SD) <9 at 50, 100, and 200 ng/mL levels of fortification in beer. However, recoveries for cereals had a wider spread and ranged from 72% to 98% with SD ≤14 at 50, 100, and 200 ng/g levels of spike. Results are given in Table 2. The minimum detection limit in the cereal was 16 ng/g. Blank samples were also assessed, and no false positives were detected. The recoveries and reproducibilities of the assay were comparable with the other paraquat immunoassays (Fatori and Hunter, 1980; Van Emon et al., 1987; Bowles et al., 1992; Selisker et al., 1995).

Table 2. Percent Recovery[a] of Difenzoquat from Various Commodities

		difenzoquat added (ng/mL or ng/g)		
	n	10	100	200
beer[b] (lager)	5	87.8 ± 6.3	94.2 ± 5.0	87.6 ± 8.2
beer (ale)	5	90.9 ± 4.8	100.9 ± 7.7	93.7 ± 8.5
cereal (barley)	3	86.1 ± 1.4	93.4 ± 10.7	76.8 ± 3.8
cereal (wheat)	3	79.9 ± 8.5	94.2 ± 14.2	93.9 ± 13.2
bread[c]	3	80.0 ± 4.7	98.3 ± 5.1	72.1 ± 1.4

[a] Values are expressed in mean ± SD. [b] Five brands of lager and eight brands of ale were tested for DFQ. No detectable DFQ was found. [c] Twelve brands of commercial breads were sampled; no DFQ was detected.

Table 3. Intra-assay and Inter-assay Coefficients of Variation of the Procedure

	intra-assay ($n = 3$)		inter-assay ($n = 4$)	
	mean ± SD	CV (%)	mean ± SD	CV (%)
beer (lager)	71.4 ± 0.4	0.6	98.6 ± 8.9	9.1
beer (ale)	88.5 ± 5.2	5.9	79.8 ± 5.1	6.3
cereal (barley)	76.4 ± 6.2	8.1	80.1 ± 4.8	6.0
cereal (wheat)	79.6 ± 2.6	3.2	82.6 ± 10.1	12.2
bread	90.3 ± 7.4	8.2	94.1 ± 1.0	1.0

[a] Values are expressed in percent recovery when samples were fortified at 100 ng/g or ng/mL of DFQ.

The precision of the ELISA was determined by running the extracts of the samples that were fortified at 100 ng/g for cereals and bread or 100 ng/mL for beer three times a day and for 4 days. The intra-assay and the inter-assay coefficient of variations were 0.6−8.2% and 1−12.2%, respectively. Results are shown in Table 3. We have applied our procedure for DFQ residues to 13 beer and 12 bread samples purchased in local stores. No DFQ residues were found, even when beer samples were run without dilution.

This study demonstrates the feasibility of applying the ELISA method for the analysis of DFQ residues in barley and wheat products. The simplicity, sensitivity, and selectivity of the assay make it suitable for routine surveillance.

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