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# Bioaccessibility of Difenconazole in Rice Following Industry Standard Processing and Preparation Procedures

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**ABSTRACT:** For pesticide registration a post application assessment is made on the safety of any residue remaining in the edible portion of the treated crop. This assessment does not typically consider the bioaccessibility of pesticide residues. The effects of this on potential exposure to incurred difenoconazole residues passing through the human gastrointestinal tract were studied, including the impact of commodity processing. It has previously been demonstrated that solvent extraction methods have the potential to overestimate the bioaccessible fraction, so in vitro simulated gut systems may offer a better approach to determine residue bioaccessibility to refine the risk assessment process. The bioaccessibility of difenoconazole residues associated with processed rice samples was assessed using in vitro intestinal extraction and colonic fermentation methods. The mean bioaccessibility following intestinal digestion was 33.3% with a range from 13% to 70.6%. Quantification of the colonic bioaccessible fraction was not possible due to compound metabolism. Mechanical processing methods generally increased the residue bioaccessibility, while chemical methods resulted in a decrease. Both mechanical and chemical processing methods reduced the total difenoconazole residue level by ca. 50%.

**KEYWORDS:** pesticide registration, risk assessment, human exposure, ingestion, microbial degradation

## INTRODUCTION

For plant commodities treated with pesticides it is crucial to ensure that any residues present do not result in adverse health effects when ingested. Accurate pesticide risk assessment involves hazard identification, dose–response assessment, risk characterization, and exposure assessment, i.e., residue levels present in the agricultural commodity and the quantity consumed.<sup>1</sup> Accurate measurement of residue levels in crops is critical for consumer risk assessment, which in turn is a function of exposure and hazard. Currently, the bioaccessibility, i.e., fraction of a pesticide residue released from a food matrix into the gastrointestinal tract that is available for absorption, is assumed to be 100%.<sup>2</sup> However, an accurate determination of this fraction may contribute to more accurate risk assessments. Bioaccessibility measures are also undertaken to determine the release of bioactive compounds.

There are many plant commodities where consumption can take place immediately once the food item has reached maturity, such as strawberries (*Fragaria* spp.) and carrots (*Daucus carota* subsp.). In these cases, the quantity of residue ingested does not have the potential to be attenuated by processing methods as is common for wheat (*Triticum* spp.) and rice (*Oryza* spp.). It has previously been shown that standard industrial methods of processing rice can alter the levels of inorganic<sup>3</sup> and organic nutrients.<sup>4,5</sup> It is therefore possible that these same processing techniques have the potential to alter not only the total pesticide residue level but also its bioaccessibility.

Difenconazole is a broad-spectrum fungicide used for disease control in vegetables, fruits, cereals, and other field

crops. It has a preventative and curative action through its ability to inhibit demethylation during ergosterol synthesis.<sup>6,7</sup> The mammalian acute oral LD<sub>50</sub> value is 1453 mg kg<sup>-1</sup> as determined by rat studies, and the ADI (acceptable daily intake) is 0.01 mg kg<sup>-1</sup> bw day<sup>-1</sup>.<sup>7</sup> No unacceptable risks to public bystanders have been identified assuming an acceptable safety margin or for occupational exposure when adequate safety apparatus is worn. Difenconazole has a log *P* of 4.36, a solubility in water of 15 mg L<sup>-1</sup> at 20 °C, a Henry's law constant (dimensionless) of 7.31 × 10<sup>-10</sup>, making it non-volatile, and a p*K*<sub>a</sub> of 1.07, and it is stable at pH 7.

A variety of guidelines are available for testing residue chemistry.<sup>8–10</sup> These commonly make use of “vigorous” solvent-based extractions, which are designed to extract the maximum residue from the plant commodities irrespective of bioaccessibility based on human physiology. It has been suggested that such solvent extraction of pollutants may overestimate both the release of organics into the gastrointestinal tract chyme (bioaccessibility) and the bioavailable fraction (i.e., that entering the circulatory system).<sup>11–13</sup> In vitro gastrointestinal tract simulators are capable of simulating parameters such as the temperature, residence time, pH, O<sub>2</sub> concentration, bicarbonate and bile secretion, and biotic

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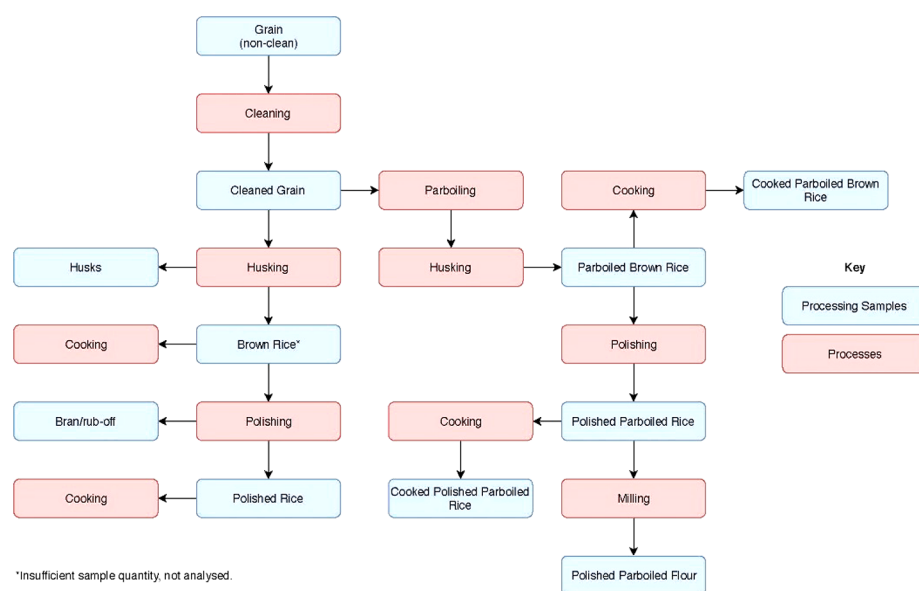
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**Table 1.** Total Difenconazole Residue Levels in Rice Samples Including Associated Processing Methods Employed to Generate the Sample and Associated Notes<sup>a</sup>

sample ID	sample	processing method	mean residue ( $\mu\text{g/g}$ )	standard deviation ( $\mu\text{g/g}$ )	notes
A	precleaned grain	N/A	1.77	0.27	initial sample state
B	clean grain	cleaning	1.16	0.13	cleaning A
C	husks	husking	8.30	0.36	byproduct, husking sample B
D	parboiled brown rice	parboiling	1.67	0.10	parboiling B
E	polished rice	polishing	0.12	0.01	polishing B
F	bran from polished rice	polishing	0.40	0.02	byproduct, polishing B
G	cooked parboiled brown rice	cooking	0.37	0.02	cooking D
H	parboiled polished rice	parboiling	0.40	0.01	polishing D
I	polished parboiled flour	milling	0.37	0.03	milling H
J	cooked polished parboiled rice	cooking	0.17	0.01	cooking H

<sup>a</sup>Increasing sample ID letter represents increased processing required to generate sample.



**Figure 1.** Relationship between rice-processing procedures and the consequent rice samples generated. Blue shading represents samples analyzed; red shading represents processing methods. Brown rice sample not analyzed due to insufficient sample quantity.

components of the large intestine.<sup>14–17</sup> Making use of *in vitro* gastrointestinal tract simulators, which are intrinsically designed to offer high levels of physiological relevance, may allow for the refinement of exposure data.

The aim of this investigation was to (A) assess the bioaccessibility of difenoconazole, associated with rice samples processed using industry standard machinery and techniques, from grain cleaning through to flour production and (B) investigate the differences in the bioaccessible fraction derived from solvent-based and *in vitro* gastrointestinal simulator-based extraction techniques.

## METHODS AND MATERIALS

**Ethics Statement.** This study was reviewed and approved by the University of Reading ethical committee. All samples were anonymized, stored, and treated along with the requirements of the UK Human Tissue Act. Verbal permission was obtained from all faecal sample volunteers. The University of Reading ethical committee waived the need for written informed consent from the participants as this faecal study did not involve dietary intervention.

**Rice Samples.** All samples were sourced from Syngenta (Bracknell, UK). Rice was selected from a field trial where difenoconazole was applied as an emulsifiable concentrate formulation containing 250 g L<sup>-1</sup> difenoconazole. Two applications, separated by a 15-day interval, were made at 250 g ai/ha. Samples were collected at

21–24 days after the final application. Rice (grain) was processed into polished rice, parboiled rice, cooked rice, and rice flour. Relevant practices and standardized procedures were applied to simulate the practices used by industry. Prior to processing, total solvent-extractable difenoconazole was measured for use in the bioaccessibility calculation as well as recovery confirmation. Table 1 reports the sample types, associated processing relationships, and total extractable difenoconazole. No residues were detected in a control rice sample (data not shown).

**Rice Processing.** Samples were kept separated at all times and processed in equivalent conditions. Remaining sample material was disposed of in compliance with local regulations. Figure 1 provides an overview of the processing techniques used to generate the relevant samples. **Cleaning:** The “Nonclean Grain” specimen was collected at this stage prior to the cleaning process. Grains were cleaned using a Rationel Kornservice sample cleaner SLN3. Shrivelled (undeveloped and broken) grain was removed (<1.9 mm). The specimen “Clean Grain” was taken. **Husking:** A portion of the “Cleaned Grain” was husked with a rubber husker. The specimens “Husks” and “Brown Rice” were taken. The latter sample was unfortunately not available in sufficient quantity for bioaccessibility testing; however, its downstream processing products were. **Polishing (Decortication):** The “Brown Rice” was polished using a vertical shelling machine (Vertikalschäler, Schule) through the process of abrasive decortication. The specimens “Bran/Rub-Off” and “Polished Rice” were taken. **Parboiling:** “Cleaned Grain” was steeped in excess water (ratio 1 g/5

mL) and heated to 76–85 °C. The steeped grain was stored in a closed container at room temperature (duration 3 h). The steeped grain was then transferred to an autoclave and steamed at 115 °C for 15 min. The steamed grain was then transferred to the drying oven. The grain was dried for 16 h until a final moisture content of 7.6–14.9% was achieved. The now dry parboiled rice was husked using a rubber husker. The specimen “Parboiled Brown Rice” was taken. The husked parboiled brown rice was polished using a vertical shelling machine. The specimen “Parboiled Polished Rice” was taken. **Cooking:** The “Parboiled Brown Rice” was cooked for 80 min in excess water (99–102 °C). The specimen “Cooked Parboiled Brown Rice” was taken. The “Parboiled Polished Rice” was cooked for 60 min in boiling water (98–104 °C). The specimen “Cooked Polished Parboiled Rice” was taken. **Milling:** The “Parboiled Polished Rice” sample was milled using a cross beater mill (Perten 3100). The specimen “Polished Parboiled Flour” was taken.

**Total Residue Analysis.** The solvent extraction method was designed to generate total recoverable residue. Briefly, plant material was electronically macerated (Stuart Homogenizer with SHM/10 probe, Bibby Scientific) for 5 min in the presence of solvent to ensure the breakdown of cell membranes. The total volume of solvent was adjusted to compensate for the water content of the plant matrix with a target volume ratio of 1:10. A representative subsample is removed and centrifuged (16 000 × g, 10 min) to remove plant particulates. The liquid fraction was then passed through SPE cleanup tubes (Oasis HLB, 60 mg, 3 mL, Waters Corp.) to reduce interference during measurement. Nonpolar compounds are typically removed with hexane, and the AI (active ingredient) is recovered with a combination of polar solvents. Where necessary, the SPE recovered sample is blown to dryness under a stream of N<sub>2</sub> and resuspended in 1 mL of MeCN.

At each stage of the experiment the residual fraction (material that has passed through the *in vitro* systems) was extracted with the above solvent method to determine total residues left in the rice matrices. A 10 g amount was used for the total difenoconazole residue analysis, 1 g was used for the intestinal digest solid analysis, and 1.3 g was used for the colonic solid analysis.

**Intestinal Digestion for Bioaccessibility.** Digestion of the samples was performed according to ref 14 with slight alterations. Sample (60 g) was added to 150 mL of distilled water, and the mixture was shaken for 5 min. **Oral Phase:** The sample solution was transferred to a glass screw-topped bottle, mixed with  $\alpha$ -amylase (20 mg, Sigma) in CaCl<sub>2</sub> (0.001 mol L<sup>-1</sup>, pH 7.0, 6.25 mL), and incubated at 37 °C for 30 min on a rotary shaker. **Gastric Phase:** The pH was decreased to 2.0 with 6 mol L<sup>-1</sup> HCl, and pepsin (2.7 g, Sigma) dissolved in HCl (0.1 mol L<sup>-1</sup>, 25 mL) was added. The sample was incubated at 37 °C for 2 h on a rotary shaker. **Small Intestinal Phase:** Pancreatin (560 mg, Sigma) and bile (3.5 g, Sigma) were dissolved in sodium bicarbonate (0.5 mol L<sup>-1</sup>, 125 mL), which was added to the digestion vessel. The pH was converted to 7.0 with either 6 mol L<sup>-1</sup> NaOH or 6 mL L<sup>-1</sup> HCl. The sample was incubated at 37 °C for 3 h on a rotary shaker. The sample solution was then transferred to 1 kDa MWCO (molecular weight cutoff) dialysis tubing and dialyzed overnight against NaCl (ca. 2 L, 10 mmol L<sup>-1</sup>) at 37 °C to remove low molecular mass digestion products. The NaCl was changed, and dialysis was continued for an additional 2 h. Finally, the sample was freeze dried for at least 7 days. The total end digestion volumes prior to dialysis were recorded (240–340 mL), and a subsample (50 mL) was removed for bioaccessible fraction analysis. A subsample of the freeze-dried solids (mean 1.08 g) was also analyzed to determine the nonbioaccessible fraction. The mean recovery of difenoconazole from the samples was 98.7% ± 5.1% over a range from 87.9% to 109.2%.

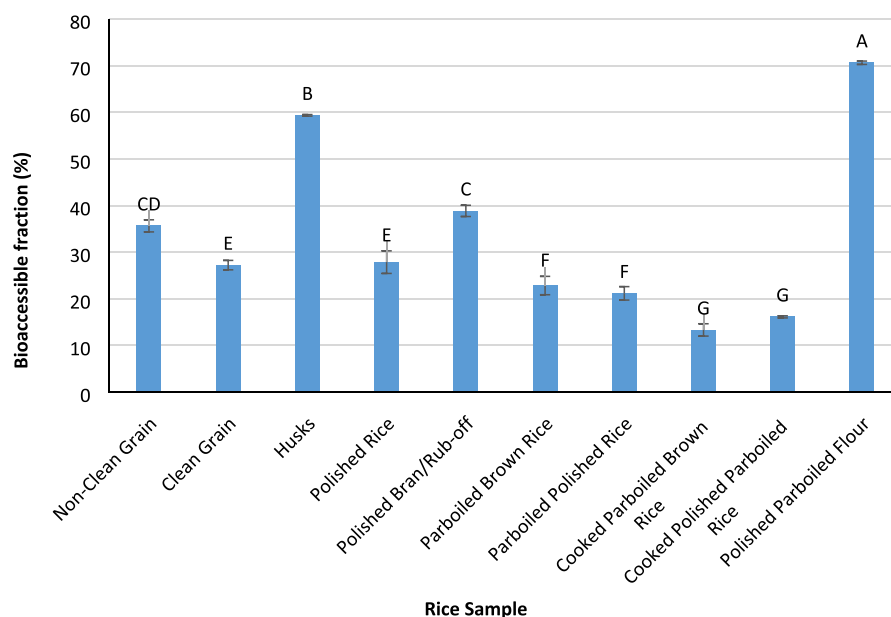
**Colonic Fermentation.** Gut model cultures were performed according to ref 18 with alterations to allow for detection of low difenoconazole concentrations. The faecal slurry preparations were obtained from a single healthy volunteer free of known gastrointestinal diseases. Volunteers did not consume pre- or probiotic supplements and had not taken antibiotics or medicines known to affect the intestinal environment in the 3 months prior to providing a sample. Each sample was run in triplicate and contained 0.5 g of rice

sample with 5 mL of 10% faecal slurry to 45 mL of media, giving a 50 mL total system volume. A negative control containing no sample/difenoconazole and a positive control containing 0.65  $\mu$ g of difenoconazole with no sample (0.1  $\mu$ g/mL assuming end volume of 65 mL after automated pH adjustment) was also run. The cultures were maintained for 24 h, after which samples were taken to simulate transit time in the colon, which is ca. 30 h for a healthy person. These were separated into solid and liquid phases and stored at -20 °C prior to analysis. These solutions were also fortified with penconazole for use as an internal standard; however, it was heavily metabolized and therefore undetectable. Derived concentrations and recovery values were based on the total extractable compound and external standards, respectively.

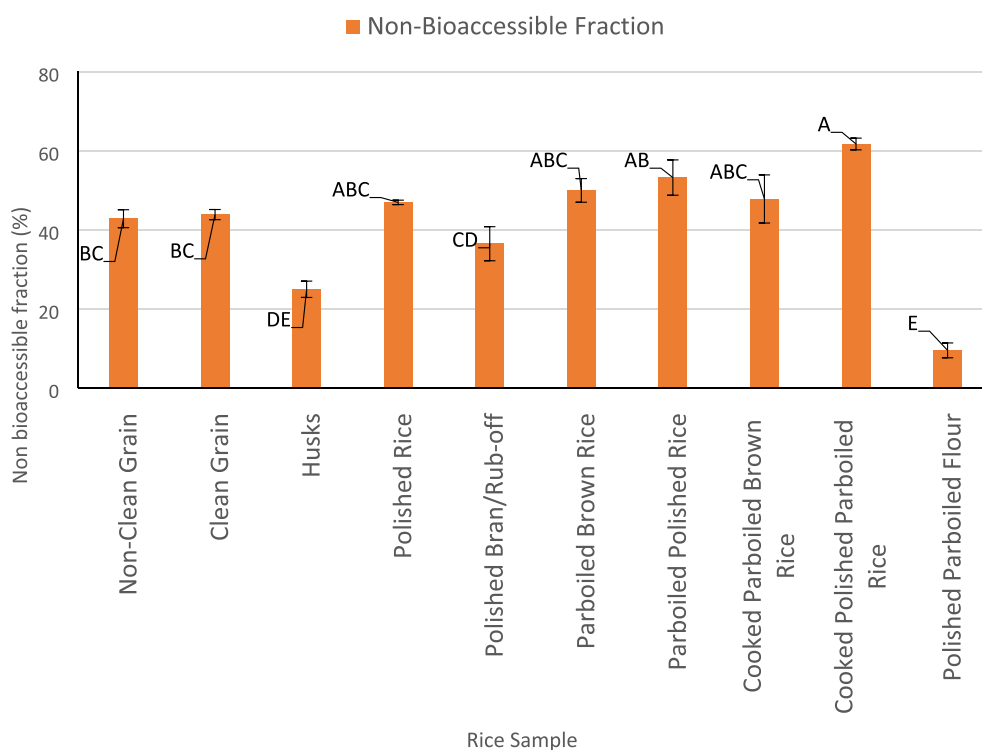
**Compound Abiotic Degradation Analysis.** Three 20 mL samples (22 mL screw cap vials, Sigma) sourced from each of the negative control singlets were spiked with 0.2  $\mu$ g of difenoconazole and penconazole to give a concentration of 0.01  $\mu$ g/mL. As part of the 20 mL, sodium hypochlorite (15%) solution was also added to give a final concentration of 0.5% (v/v) to suppress the effects of the colonic flora. The samples were incubated at 37 °C for 24 h. One milliliter aliquots were nondestructively sampled at 0, 30, 60, 120, 240, 480, 960, and 1440 min. These samples were ultracentrifuged (16 000 × g, 10 min) prior to analysis as described above.

**HPLC MS/MS Analysis.** Samples were analyzed using a CTC-PAL autosampler connected to an Agilent 1100 series binary pump, degasser, and column oven with an Applied Biosystems API 4000 as the detector with Hichrom ACE C18 column (5  $\mu$ m 150 mm 4.0 mm i.d.). A Peak Scientific NM20ZA gas station was used for gas supply. Calibration standards were generated in acetonitrile (0.001, 0.005, 0.01, 0.05, and 0.1  $\mu$ g mL<sup>-1</sup> difenoconazole). These were used for total residue analysis as well as determining the remaining solvent extracted from the residual rice fraction. For the intestinal digestion and colonic fermentation analysis, the same standard concentrations were used; however, the standards were matrix matched, i.e., they were made up in either intestinal digestion or colonic solutions identical to that of the sample. Chromatography and mass spectrometer conditions are based on residue method REM 147.08 (Syngenta, UK). Briefly, the liquid colonic and predigestion samples were ultracentrifuged (16 000 × g, 10 min) prior to analysis by LC-MS/MS. The solid colonic and predigestion samples were refluxed for 2 h in methanol:concentrated ammonium hydroxide (80:20 v/v, 50 mL) followed by SPE cartridge cleanup (Oasis HLB, 60 mg, 3 mL, Waters Corp.). Residues were eluted in the dichloromethane:ethyl acetate (80:20 v/v) fraction, blown to dryness, and resuspended in acetonitrile. Samples were then transferred to autosampler vials ready for final determination by LC-MS/MS. The method (REM 147.08) has LOD = 0.002 mg kg<sup>-1</sup>, LOQ = 0.01 mg kg<sup>-1</sup>, and RSD ≤ 20%.

**Gut Model Compartment and Relationship to Bioaccessibility.** Two different *in vitro* systems were in use during this investigation. The intestinal digestion system is composed of oral, gastric, and small intestinal phases. Its purpose was to prepare the rice samples for colonic fermentation. Without this stage, maintaining a stable microbial community in the colonic model would not have been possible. Where bioaccessibility is represented as a percentage, the values for the intestinal digestion and colonic phases are discrete. Intestinal bioaccessibility (eq 1, bioaccessibility calculation for the intestinal digestion process) is calculated against the total residue mass in the untouched rice matrix, while colonic bioaccessibility (eq 2, bioaccessibility calculation for the colonic fermentation process) is calculated against the total residue mass remaining in the intestinally digested rice prior to colonic fermentation. This effectively accounts for the loss of compound (residue that was made bioaccessible during the intestinal digestion process) from the rice between the intestinal digestion and the colonic fermentation steps. Consequently, the total bioaccessibility (eq 3, total sample bioaccessibility calculation for rice samples) of a rice sample is a function of the residue mass made bioaccessible within the intestinal and colonic fermentation procedures against the total mass of the residue within the sample prior to analysis. Finally, the recovery (eq 4, residue recovery calculation for rice samples) was calculated as a function of the



**Figure 2.** Bioaccessible difenoconazole fraction after intestinal digestion (%). Means that do not share a letter are significantly different (A–G, 95% CI). Overall processing increases from left to right. Y error bars represent standard deviation.



**Figure 3.** Nonbioaccessible difenoconazole fractions after colonic fermentation (%). Means that do not share a letter are significantly different (95% CI). Y error bars represent standard deviation.

bioaccessible and residual fractions from both intestinal digestion and colonic fermentation procedures against the initial residue level within the rice sample (Table 1).

$$\left[ \frac{\text{total difenoconazole mass in intestinal digest fluid}}{\text{total solvent extracted in difenoconazole in nondigested sample}} \right]$$

× 100

(1)

$$\left[ \frac{\text{total difenoconazole mass in colonic fluid}}{\text{total solvent extracted difenoconazole from predigested sample}} \right] \times 100$$

(2)

$$\left\{ \left[ \left( \text{total difenoconazole mass in predigest fluid} \right) + \left( \text{total difenoconazole mass in colonic fluid} \right) \right] \div \left( \text{total solvent extracted difenoconazole from nondigested equivalent sample} \right) \right\} \times 100$$

(3)

$$\begin{aligned} & \{[(\text{total difenoconazole mass in predigest fluid and predigest residual}) \\ & + (\text{total difenoconazole mass in colonic fluid and colonic residual})] \\ & \div (\text{total solvent extracted difenoconazole from residual plant material})\} \\ & \times 100 \end{aligned} \quad (4)$$

**Statistical Analysis.** All statistical analyses were conducted using Minitab 17.0 (Minitab, LLC, Chicago). Once tested for normality and equal variance, percentage data were converted. One-way analysis of variance (ANOVA) was conducted to examine the effect of processing method with Tukeys posthoc testing.

## RESULTS

**Intestinal Digestion.** Overall there was strong evidence ( $p < 0.001$ ) to suggest rice processing techniques resulted in significantly different bioaccessible fractions (Figure 2). Parboiled Flour and Husks had the highest bioaccessibility,  $70.6\% \pm 1.3\%$  and  $59.3\% \pm 2.4\%$  respectively; the least bioaccessible samples were the cooked parboiled brown and white rice ( $13.3\% \pm 0.2\%$  and  $16.1\% \pm 0.2\%$ , respectively), followed by the parboiled white rice and parboiled polished white rice ( $21.2\% \pm 1.3$  and  $22.8\% \pm 1.4\%$ ). When considering the general processing technique applied to the rice samples (cleaning and husking, parboiling, polishing, and cooking), cleaning/husking and polishing result in higher bioaccessible fractions relative to samples that were cooked ( $p < 0.05$ ). The extraction efficiency of the solids generated by the digestion process (used to determine the nonbioaccessible fraction) was  $95.7\% \pm 3.5\%$ .

**Sample Colonic Fermentation.** Detection of the liquid bioaccessible difenoconazole fraction obtained from the colonic fermentation procedure was generally not possible even with the use of a 25 $\times$  concentration step, method tuning, and matrix-matched standards. Furthermore, the positive control (0.1  $\mu\text{g/mL}$  expected), although detected chromatographically (but at concentrations below the methods LOQ), showed significant difenoconazole degradation with only <1% recovery of the originally spiked difenoconazole. For this reason, difenoconazole mass balance was not achieved for the colonic fermentation procedure. Furthermore, the internal standard (penconazole) used in the investigation was not detected (no peak) in either the bioaccessible or the nonbioaccessible colonic fractions.

Detection of difenoconazole in the nonbioaccessible solid fraction of the colonic fermentation did not suffer from any of the bioaccessible liquid fraction quantification issues. Polished parboiled flour was the only sample to show a significantly ( $p < 0.05$ ) different (lower) nonbioaccessible fraction relative to 6 of the other samples tested with a mean nonbioaccessibility of  $9.5\% \pm 1.9\%$  (Figure 3). The recovery of difenoconazole from the samples was poor with a mean of  $41.6\% \pm 15.4\%$ , likely due to the unquantifiable nature of the bioaccessible fraction. The extraction efficiency of the solids generated by the fermentation process (used to determine the nonbioaccessible fraction) was  $92.3\% \pm 4.2\%$ .

**Difenoconazole and Penconazole Degradation Analysis.** Following the detection issues presented during the analysis of the bioaccessible liquid fraction generated during the colonic fermentation process, difenoconazole and penconazole were refermented at a concentration of 0.01  $\mu\text{g mL}^{-1}$ . This time the colonic suspension was autoclaved to remove any degradative effects associated with the colonic microbiota. Over the 24-h investigation there was no evidence to suggest the measured compound concentration was

significantly different from the initial concentration applied at any time point ( $p < 0.05$ ).

## DISCUSSION

The mean bioaccessibility of difenoconazole after intestinal digestion was 33.3% with a range from 70.6% to 13.3% as determined for polished parboiled flour and cooked parboiled brown rice. Colonic fermentation bioaccessibility ranged from 59.4% to 27.1% with a mean of 37.7%. These colonic values are not based of bioaccessibility measurements; instead, they assume mass balance and use the nonbioaccessible fraction to produce the upper and lower range boundaries for the bioaccessible fraction prior to bacterial degradation. For the majority of the samples analyzed, the difenoconazole bioaccessible fraction was significantly lower relative to the nonbioaccessible fraction in both the intestinal digestion and the colonic fermentation processes. This is an important finding as most pesticide risk assessment models make use of solvent extraction methods such as those employed to determine the total difenoconazole concentration in this experiment. When considering only the intestinal digestion results, this could potentially lead to the overestimation of risk by a factor of 1.4–7.5 (parboiled flour and cooked parboiled brown rice respectively) when only considering the total compound residue level, as opposed to the bioaccessibility of difenoconazole in these samples.

The low bioaccessibility of difenoconazole in the intestinal digestion step observed with the cooked and parboiled samples, arguably the most important samples with respect to human consumption, would suggest that the cooking and partial cooking processes significantly ( $p < 0.05$ ) reduce the bioaccessible fraction relative to the clean grain. This is particularly prominent when comparing parboiled polished rice and cooked polished parboiled rice with each subsequent stage, resulting in significantly lower difenoconazole bioaccessibility during the intestinal digestion process. No previous studies have investigated the effect of rice processing methods on the bioaccessibility of this compound. However, similar decreases in bioaccessibility have been observed with arsenic when parboiling<sup>19</sup> and cooking rice:<sup>20</sup> total phosphorus and phytate when soaking<sup>21</sup> and zinc when cooking.<sup>22</sup>

The greater bioaccessible fraction encountered in the parboiled flour samples is most likely a surface area effect because of milling. Given that the specific surface area of rice flour is 344  $\text{m}^2/\text{kg}$  dry matter, it is unlikely any other samples analyzed approached this value (Shimiya and Yano, 1987).<sup>23</sup> Increased surface area results in faster digestion or increased bacterial/enzymatic attack.<sup>24–26</sup> With respect to rice husks, although the bioaccessible fraction was greater than the nonbioaccessible fraction, the risk posed by this finding is mitigated as husks are considered a byproduct of grain processing and as such are not normally consumed. Husks contain high percentages of human indigestible compounds<sup>27</sup> including cellulose (31%), hemicellulose (22%), and lignin (22%) as well as amorphous silica<sup>28</sup> and offer little nutritional benefit.

The detection issues present in the bioaccessible fraction of the colonic fermentation process appear to indicate significant degradation of the parent compound. This is reinforced as degradation was also observed in the positive control. Unfortunately, there is no peer reviewed published data on the metabolism of difenoconazole by colonic microbiota or any other bacteria for comparative purposes. An unpublished

animal metabolism study provided to the FAO<sup>6</sup> suggested that difenoconazole is rapidly metabolized in rats, lactating goats, and laying hens. Critically, the rapid and almost complete metabolism of difenoconazole is an important factor to consider given that nutrient and water uptake occurs in the colon. With respect to risk assessment, the rapid metabolism of difenoconazole would result in a shift away from the parent compound toward the potential risks associated with the metabolites. This coupled with the high nonbioaccessible fractions detected in the colon would further reduce the human exposure risk as there would be significantly lower concentrations of difenoconazole crossing the colonic membrane. Future work would require the use of multiple <sup>14</sup>C labels on the difenoconazole chlorophenoxy, phenyl, and triazole rings to accurately confirm the metabolism products generated by the colonic microbiota.

Published data relating to the bioaccessibility of pesticides residues, both current and legacy formulations, specifically from plant commodities is limited. The data that is available is typically concerned with organochlorine pesticides (OCP)-based compounds on matrices such as soil, fish, and dust.<sup>29,30</sup> Critically, OCPs are structurally very different from the more polar compounds, which are representative of modern pesticides such as difenoconazole, which make these findings tenuous to apply to this investigation.<sup>31</sup> Regardless of this significant knowledge gap, these nonplant commodity investigations have equivalent outcomes, specifically that solvent-based extraction methods overestimate the bioaccessible fraction.<sup>32,33</sup> The results of this investigation suggest the same occurs in plant commodities and once again reinforces the potential for overestimation of risk if using solvent-based methods. Using in vitro gastrointestinal tract simulators as part of a “body of evidence” during pesticide registration has clear advantages, and methods like this should be employed as a toolkit to help refine risk assessments for pesticides and other organic compounds.

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### Notes

The authors declare no competing financial interest.

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