

1 **A comparison of two methods for determining titanium dioxide marker content**
2 **in broiler digestibility studies**

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10 Short title: Titanium dioxide broiler digestibility methodology

11

12 **Abstract**

13 The use of inert markers in broiler diets eliminates the need to quantitatively evaluate
14 feed intake and excreta output to determine diet digestibility, and enables nutrient
15 uptake at specific points along the gastrointestinal tract to be examined. Titanium
16 dioxide (TiO₂) is commonly used for this purpose and measured using a UV
17 spectrophotometric assay. Two experiments were conducted to observe whether an
18 inductively coupled plasma optical emission spectrophotometer (ICP-OES) assay is
19 able to replace the UV-spectroscopy assay for rapid analysis of TiO₂ in broiler feed
20 and ileal digesta samples. In the first experiment, TiO₂ was added at 5g/kg to 19
21 broiler diets. Ross 308, male broilers (n=452) fed these diets were involved in a
22 series of digestion studies to determine ileal digesta recovery of TiO₂. In the second
23 experiment, defined amounts of TiO₂ were added to ileal digesta samples from Ross
24 308, male broilers (n=176) and TiO₂ recoveries were determined. The feed and ileal
25 samples from both experiments were analysed by both UV-spectroscopy and ICP-

26 OES, and relatedness of the findings from the two assays was determined. Overall
27 relatedness of the two assays was strong for determination of TiO₂ concentration in
28 both the broiler diets and ileal digesta samples ($r = 0.908$ and $r = 0.884$ respectively).
29 Overall recovery of supplemented TiO₂ was 97.62% by the UV-spectroscopy assay
30 and 98.77% by the ICP-OES assay. The ICP-OES assay in this study was as
31 accurate as spectrophotometric determination for quantification of TiO₂ content. The
32 ICP-OES method can also be used to analyse several elements within one assay,
33 with a single preparation step, so the measurement of TiO₂ may be incorporated into
34 the analysis of other minerals. Time and resources dedicated to determining diet
35 digestibility in broilers could be minimised by using the ICP-OES assay to replace the
36 UV-spectroscopy assay when measuring TiO₂ concentration.

37

38 **Keywords:** Broiler, Titanium Dioxide, Digestibility, Methodology

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40 **Implications**

41 Titanium dioxide (TiO₂) is commonly added as an inert marker to broiler diets to
42 enable diet digestibility to be determined. This study demonstrates that an ICP-OES
43 assay could replace the commonly used UV-spectroscopy assay for the
44 determination of TiO₂ concentration in poultry diets and ileal digesta. This is
45 advantageous because the ICP-OES assay used in this study has comparatively
46 greater detection limits and sensitivity than the UV-spectroscopy assay. Additionally
47 the ICP-OES assay enables TiO₂ determination to be incorporated into other mineral
48 concentration analyses.

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50

51 **Introduction**

52 Inert digestibility markers added to broiler diets eliminate the need to evaluate
53 quantitative feed intake and excreta output, and enable nutrient utilisation to be
54 examined along the gastrointestinal tract (Short *et al.*, 1996). Inert markers must
55 maintain digestive transit at the same speed as other dietary nutrients in the tract and
56 be physiologically inactive, as well as being non-toxic, easily analysed, able to be
57 homogenously mixed into a diet, indigestible and non-absorbed (Jagger *et al.*, 1992;
58 Titgemeyer *et al.*, 2001). Titanium dioxide (TiO₂) has some advantages over the
59 commonly used chromic oxide (Cr₂O₃), with studies showing improvements in
60 reproducibility and homogeneity (Jagger *et al.*, 1992). TiO₂ is also approved for use
61 as a feed additive by the Food and Drug Administration, unlike Cr₂O₃ (Titgemeyer *et al.*
62 *et al.*, 2001). Another commonly used marker is acid insoluble ash, but it has been
63 suggested that its digestive transit does not accurately reflect that of feed passage
64 (Cheng and Coon 1990).

65 The method most widely used to determine TiO₂ concentration is UV-
66 spectroscopy, primarily based around the method of Short *et al.* (1996). This method
67 involves the initial hydrolysis of the sample with sulphuric acid followed by a colour
68 reaction. An intense orange/yellow colour results from the addition of hydrogen
69 peroxide to an acidic titanium solution, and the colour intensity can be quantified by
70 UV-spectrometry. This method has been used successfully in several species
71 including poultry (Short *et al.*, 1996), cattle (Titgemeyer *et al.*, 2001) and pigs (Jagger
72 *et al.*, 1992), but some authors reported being unable to achieve reliable results
73 using this process (Myers *et al.*, 2004).

74 In poultry research TiO₂ as a dietary marker has been used successfully to
75 determine calcium and phosphorus utilisation (Walk *et al.*, 2012). Mineral digestibility

76 and utilisation in poultry is frequently analysed by induced coupled plasma optical
77 emission spectrophotometer (ICP-OES) in preference to UV methods as the ICP-
78 OES assay can be used to analyse many elements in one preparation. Titanium
79 concentration can be detected by ICP-OES, which suggests that there is potential for
80 TiO₂ measurement to be made concurrently with mineral content, thus reducing
81 analysis time and resource use.

82 A comparison between a UV-spectroscopy assay and ICP-OES assay for
83 determination of TiO₂ has previously been investigated by Boguhn *et al.* (2009) in
84 turkey diets and digesta. In this paper it was suggested that there was incomplete
85 recovery of TiO₂ for both assays used, and hence values read to be lower than
86 expected. However, detailed inspection of the results of the turkey data presented by
87 Boguhn *et al.* (2009) confirms that for some of the samples the readings were higher
88 than expected when the UV-spectroscopy assay was used, and lower than expected
89 when the ICP-OES assay was used. This suggests that potentially that neither, or
90 just one, of the assays is producing values that are representative of the TiO₂
91 concentration in the sample. It is possible that the UV-spectroscopy assay is
92 amplifying the value, and the ICP-OES assay is not detecting all the TiO₂ in the
93 sample. The conclusion made by Boguhn *et al.* (2009) that both assays can be used
94 to determine TiO₂ may therefore be questionable. Rodehutschord *et al.* (2012) have
95 subsequently used ICP-OES to analyse TiO₂ concentration in broiler ileal digesta
96 indicating that the new ICP methodology is an attractive prospect to workers in the
97 field, but highlighting that this is an area that requires further validation. The aim of
98 this study therefore was to investigate consistency of TiO₂ recovery from an ICP-OES
99 and a UV-spectroscopy assay, and evaluate if the ICP-OES assay can be used as an

100 alternative to the UV-spectroscopy assay for the determination of TiO₂ as a marker in
101 poultry digestibility studies.

102

103 **Material and methods**

104 *Birds and Husbandry*

105 For experiment 1, Ross 308, male broilers (n=452) were involved in a series of
106 digestion studies to determine ileal digesta recovery of TiO₂ either by UV-
107 spectroscopy by the method of Short *et al.* (1996), or by an inductively coupled
108 plasma optical emission spectrophotometer (ICP-OES) assay. Birds were fed one of
109 19 experimental diets in mash form, each with TiO₂ added at 5g/kg; 6 semi-synthetic
110 starch dextrose based diets, and 13 more commercial style diets based on cereals
111 including wheat, rapeseed, maize and rye and soya bean meal. All 19 diets were
112 analysed for TiO₂ concentration. Each diet was fed to a minimum of 20 birds. All birds
113 were from breeder flocks aged 42-45 weeks old and were obtained from a
114 commercial hatchery at day of hatch. Chicks were randomised by weight and placed
115 in 0.64 m² floor pens in groups of four, bedded on clean wood shavings. Birds were
116 allowed ad libitum access to the treatment diets and water for the duration of the
117 trials; which spanned between two and four weeks. The room was thermostatically
118 controlled to produce an initial temperature of 32°C and reduced to 21°C by day 21.
119 The lighting regimen used was 24 hours light on day 1, with darkness increasing by 1
120 hour per day until 6 hours of darkness was reached and this was maintained
121 throughout the remainder of the study. Birds were euthanised by cervical dislocation.
122 Digesta sample collection was carried out on a total of 144 14 day-old birds, 144 21
123 day-old birds and 164 28 day-old birds. At each bird age, digesta was pooled per pen
124 of four birds, and averaged across diet. Digesta content was removed from the

125 intestinal section distal to the Meckel's diverticulum and proximal to the ileo-ceco-
126 colonic junction of each bird. The digesta samples were then freeze-dried and ground
127 through a 1mm screen.

128

129 For experiment 2, Ross 308 male broilers (n=176) were fed a diet that contained no
130 TiO₂ from d0-42. The birds were from a breeder flock age of 43 weeks old, and were
131 obtained from a commercial hatchery at day of hatch. Chick placing, room
132 temperature and lighting regime were as previously described. Birds were allowed ad
133 libitum access to the treatment diets and water for the duration of the trial. Digesta
134 content was removed from the intestinal section distal to the Meckel's diverticulum
135 and proximal to the ileo-ceco-colonic junction of each bird. The samples were freeze-
136 dried and ground through a 1mm screen. TiO₂ was subsequently added to the
137 digesta samples at 0, 5, 10, 15 and 20g/kg to encompass the range found in poultry
138 digestibility studies.

139

140 All feed and digesta samples from both experiment 1 and experiment 2 were
141 analysed for TiO₂ concentration by both the UV-spectroscopy and ICP-OES assays
142 described below.

143

144 *Calibration Standards*

145 250mg titanium dioxide was dissolved in 100ml of 7.4M sulphuric acid (H₂SO₄) and
146 diluted to 500ml with distilled water to produce a standard titanium solution of
147 0.5mg/ml. This standard solution was used to prepare the calibration curve for both
148 the UV-spectroscopy and ICP-OES assays. For the ICP-OES assay, the TiO₂
149 standard solution was diluted with ultra-pure water in varying increments to produce

150 standards between 0 and 10ppm. These standards were measured on an ICP-OES
151 (Optima 2100 DV ICP-OES, model PQ Excell VG Elemental, Perkin-Elmer, USA) set
152 to detect Ti at wavelength 334.936nm, and a calibration curve was derived from the
153 readings. For the UV-spectroscopy assay, graded volumes of TiO₂ standard solution
154 was pipetted into individual 100ml volumetric flasks and made up to 10ml with 7.4M
155 H₂SO₄. 10ml 30% hydrogen peroxide (H₂O₂) was then added to the solutions and the
156 contents were made up to 100ml with distilled water before measurement on a
157 spectrophotometer (Unicam Helios, Berkshire, UK) set at 410 nm.

158

159 *UV-Spectroscopy Assay*

160 The UV-spectroscopy assay was based on that of Short *et al.* (1996). Briefly,
161 triplicate aliquots (approximately 0.3g) of each digesta sample and 5 replicates of
162 each of the 19 feed samples were ashed in porcelain crucibles for 16 hours at 650°C.
163 Once cooled, 10ml H₂SO₄ (7.4 M) was added to each crucible and the samples were
164 heated for approximately 1 hour until completely dissolved. The contents were then
165 transferred quantitatively into 100ml volumetric flasks via filter papers (Whatman 541)
166 using distilled water. 10ml of 30% H₂O₂ was then added to each flask and the flasks
167 made to volume with distilled water. Solutions were thoroughly mixed prior to reading
168 on a spectrophotometer set at 410nm. Sample analysis was repeated if the Z-value
169 between the same samples exceeded 5%.

170

171 *ICP-OES Assay*

172 For the ICP-OES assay an aqua regia digestion step was carried out according to
173 AOAC 985.01. Briefly, 10ml of aqua regia (35.5-37.5% hydrochloric acid (HCl) and
174 68-72% nitric acid (HNO₃) at a ratio of 3:1) was added to 50ml glass conical flasks

175 containing triplicate aliquots (approximately 0.5g) of each digesta sample and 5
176 replicates of each feed sample, and left at room temperature (14.4°C +/- 0.15 SEM)
177 for a minimum of 12 hours. The samples were then boiled until completely dissolved,
178 for approximately 1 hour. The contents were then filtered through Whatman 541 filter
179 papers into 50ml volumetric flasks and made to volume with ultra-pure water, before
180 transferral into 15ml tubes. The samples were assayed on an ICP-OES set to detect
181 Ti at wavelength 334.936. Sample analysis was repeated if the Z-value between the
182 same samples exceeded 5%. Four digesta samples were repeated using a reduced
183 sample size (approximately 0.2g) with 8 replicates to assess whether smaller
184 quantities of material were viable for the assay.

185

186 *Statistical Analysis*

187 All data was analysed using IBM SPSS statistics version 21. T-Tests were conducted
188 to differentiate between means. The relatedness of the readings from each assay
189 was investigated using Pearson product-moment correlation coefficient and
190 interpretations of the strength of the relationship between the two methods was
191 based on guidelines by Cohen (1988); weak relationship $r = 0.10$ to 0.29 , medium
192 relationship $r = 0.30$ to 0.49 and strong relationship $r = 0.50$ to 1.0 . Linear
193 regressions were calculated using the true and measured titanium concentrations.
194 Significance was accepted at $P < 0.05$.

195

196 **Results and Discussion**

197 There were no significant differences between any TiO₂ concentrations
198 measured by the UV-spectroscopy assay and the ICP-OES assay. There were
199 consistently strong relationships between the two methods for analysis of TiO₂

200 concentration in the diets (Table 1) and ileal digesta (Table 2). This suggests that the
201 ICP-OES assay used in this study is successful at identifying diet and ileal digesta
202 TiO₂ concentration, and hence has the potential to replace the widely used UV-
203 spectroscopy assay.

204 The ICP-OES assay had to be modified to analyse ileal digesta samples in
205 experiment 1 as some of the samples contained TiO₂ levels that saturated the ICP-
206 OES detector, which compromised the sensitivity of the measurement. When a
207 smaller sample size (0.2g) was analysed, the samples all read in the optimum
208 necessary range for detection by the ICP-OES, so smaller quantities can be
209 universally used to avoid any need to dilute the samples with ultrapure water.
210 Coefficients of variation for the smaller sample size were less than 5%.

211 Relatedness between the two methods in determination of ileal digesta TiO₂
212 was numerically greater when phytase was included in the diets (Table 2). Phytase
213 improves digestibility and therefore increases TiO₂ digesta content (Rutherford *et al.*,
214 2004). The sensitivity of the UV-spectroscopy assay decreases as TiO₂ concentration
215 decreases (Boghurn *et al.*, 2009), whereas the sensitivity of the ICP-OES assay is
216 consistent and not dictated by concentration in the sample. This suggests that in the
217 presence of high TiO₂ concentration, such as in the digesta samples from birds fed
218 phytase, the two assays were similar in sensitivity, but in the samples with lower TiO₂
219 concentration the similarity in sensitivity between the two assays reduced, and the
220 UV-spectroscopy assay was comparatively less reliable. This also potentially
221 explains why observed deviances in TiO₂ level in the diet away from the
222 supplemented 5g/kg were greater when analysed by UV-spectroscopy than by ICP-
223 OES. The observed deviances are likely because dietary TiO₂ levels were measured
224 per kg feed.

225 In this study there were no significant differences between the measured
226 values, or between the calculated slopes determined by the two assays for the
227 analytical recoveries of TiO₂, whereas previous research has shown marked
228 differences between the two assays (Boguhn *et al.*, 2009). Also, Boguhn *et al.* (2009)
229 found that values from the ICP-OES assay were lower than the expected values,
230 which was not the case in this study (Table 1 and 2). This may be due to the shorter
231 digestion time used (25 minutes in contrast to 60 minutes), so there may have been
232 incomplete dissolution of the samples. Further verification of full Ti recovery was
233 made in the second study where known amounts of Ti were added to digesta before
234 quantification analysis via both methods. This found consistently strong relationships
235 between the two methods at the different TiO₂ supplementation levels in the digesta
236 samples (Table 3) and that the slopes produced by both methods were almost
237 identical. The observed recovery of supplemented TiO₂ was 97.62% by the UV-
238 spectroscopy assay and 98.77% by the ICP-OES assay in this study.

239 The main advantage of the ICP-OES assay when compared to the UV-
240 spectroscopy is that the former has been shown to be more sensitive at quantitative
241 analysis with improved detection limits. The ICP-OES assay is also less time-
242 consuming, and the ICP-OES enables several elements to be detected in parallel
243 which reduces preparation time and the amount of sample, and hence potentially the
244 number of birds, required.

245 There are however, some advantages to the UV-spectroscopy assay
246 compared with the ICP-OES assay. The ICP-OES assay is more expensive due to
247 the cost to run the ICP-OES and to maintain the argon gas supplies, although this is
248 mitigated by the potential for concurrent mineral analysis. The ICP-OES assay is also
249 more hazardous as involves the use of aqua regia which is moderately more

250 corrosive than sulphuric acid. Furthermore the detection range is greater in the UV-
251 spectroscopy method which reduces any potential need for dilution of samples, but in
252 this study, a reduced sample weight (0.2g) was shown to overcome any requirement
253 for dilution with the ICP-OES method.

254 In conclusion, the ICP-OES assay used in this study was successful at
255 determining TiO₂ added as an inert marker in broiler digestibility studies, and could
256 replace the widely used UV-spectroscopy assay. The ICP-OES assay is more
257 sensitive at quantitatively analysing TiO₂ concentration, consumes less time than the
258 UV-spectroscopy assay, and allows the TiO₂ determination to be carried out
259 concurrently with other mineral analysis by ICP-OES. However it is essential that the
260 current sample weight (0.2g digesta) is used for detection.

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292 **Table 1** Relatedness of an ICP-OES assay and UV-spectroscopy assay for
 293 determination of TiO₂ concentration in broiler diets^a (Experiment 1)

Diet	Method of TiO ₂ Determination (g/kg)		
	ICP-OES	UV-spectroscopy	Relatedness ^b
Semi-synthetic starch dextrose ^c	6.03	6.29	0.684
Wheat Soyabean ^d	5.93	5.69	0.794
Wheat Soyabean 0FTU/kg phytase	5.85	5.97	0.778
Wheat Soyabean 500FTU/kg phytase	5.71	6.08	0.759
Wheat Soyabean 5000FTU/kg phytase	6.64	6.97	0.708
Wheat Rapeseed 0FTU/kg phytase	6.11	6.53	0.886
Wheat Rapeseed 500FTU/kg phytase	4.90	5.08	0.866
Wheat Rapeseed 5000FTU/kg phytase	6.49	6.53	0.963
Maize Rapeseed	6.87	6.98	0.995
Maize Soyabean	4.99	4.88	0.956
Maize, Rye, Wheat, Soyabean	4.87	5.16	0.758
Maize, Rye, Soyabean	5.75	5.47	0.689
SEM	0.14	0.23	

294 ^a Represent the average of a minimum of 5 replicates per diet, measured as per kg feed.

295 ^b Strength of the relationship between the ICP-OES and UV-Spectroscopy method for Ti
 296 measured in each diet where confidence in the result is P<0.05.

297 ^c Represents the average measured TiO₂ content of 6 semi-synthetic starch dextrose based diets

298 ^d Represents the average measured TiO₂ content of 3 wheat soyabean meal based diets

299 **Table 2** Relatedness of an ICP-OES assay and UV-spectroscopy
 300 assay for determination of TiO₂ concentration in broiler ileal digesta^a
 301 (Experiment 1)

	Method of TiO ₂ Determination (g/kg)		
	ICP-OES	UV-spectroscopy	Relatedness ^b
Semi-synthetic starch dextrose ^c	13.58	13.40	0.776
Wheat Soyabean ^d	13.99	13.53	0.550
Wheat Soyabean 0FTU/kg phytase	13.43	13.65	0.512
Wheat Soyabean 500FTU/kg phytase	15.63	15.87	0.822
Wheat Soyabean 5000FTU/kg phytase	13.32	12.42	0.887
Wheat Rapeseed 0FTU/kg phytase	13.16	12.48	0.529
Wheat Rapeseed 500FTU/kg phytase	14.19	14.95	0.613
Wheat Rapeseed 5000FTU/kg phytase	12.92	12.71	0.858
Maize Rapeseed	12.23	12.01	0.584
Maize Soyabean	12.49	12.99	0.726
Maize, Rye, Wheat, Soyabean	12.33	12.04	0.563
Maize, Rye, Soyabean	12.19	12.06	0.646
SEM	0.20	0.26	

302 ^a Represent the average response of a minimum of 20 birds per diet, 452 birds in total, with digesta
 303 samples collected at age 14, 21 or 28 days post-hatch. Analysis was replicated a minimum of 3 times
 304 per digesta sample.

305 ^b Strength of the relationship between the ICP-OES and UV-Spectroscopy method for Ti
 306 measured in each digesta sample where confidence in the result is P<0.05.

307 ^c Represents the average measured TiO₂ content of ileal digesta from birds fed one of 6 semi-
 308 synthetic starch dextrose based diets, from 32 birds per diet, 192 birds in total, fed as 8 pens of
 309 4 birds per diet

310 ^d Represents the average measured TiO₂ content of ileal digesta from birds fed one of 3 wheat
 311 soyabean meal based diets, from 64 birds per diet, 192 birds in total, fed as 16 pens of 4 birds
 312 per diet

313 **Table 3** Calculated slopes of linear regressions and relatedness of
 314 an ICP-OES assay and UV-spectroscopy assay for determination of
 315 TiO₂ recovery at different levels in broiler ileal digesta^a (+/- SEM)
 316 (Experiment 2)

TiO ₂ added to sample (g/kg)	Method of TiO ₂ Determination (g/kg)		Relatedness ^b
	ICP-OES	UV- spectroscopy	
0	0.13 (+/- 0.01)	0.15 (+/- 0.03)	0.952
5	4.94 (+/- 0.24)	4.79 (+/- 0.32)	0.745
10	10.06 (+/- 0.29)	9.84 (+/- 0.21)	0.868
15	14.80 (+/- 0.23)	14.63 (+/- 0.27)	0.918
20	20.04 (+/- 0.20)	19.74 (+/- 0.44)	0.734
Slope ^c	0.999	0.998	

317 ^a Represents the average response of spiked digesta pooled from 176 birds aged 42 days post-
 318 hatch. Analysis was replicated 10 times per sample.

319 ^b Strength of the relationship between the ICP-OES and UV-Spectroscopy method for Ti
 320 measured in each digesta sample where confidence in the result is P<0.05.

321 ^c Linear regressions where y was the measured titanium concentration and x was the
 322 true titanium concentration.