

Demonstration of the utility of biomarkers for dietary intake assessment; proline betaine as an example

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Abbreviations: NANS, National Adult Nutrition Survey

1 **Abstract (no more than 200 words)**

2 **Scope:** There is a dearth of studies demonstrating the use of dietary biomarkers for
3 determination of food intake. The objective of this study was to develop calibration curves for
4 use in quantifying citrus intakes in an independent cohort.

5 **Methods and results:** Participants (n=50) from the NutriTech food-intake study consumed
6 standardized breakfasts for three consecutive days over three consecutive weeks. Orange juice
7 intake decreased over the weeks. Urine samples were analyzed by NMR-spectroscopy and
8 proline betaine was quantified and normalized to osmolality. Calibration curves were
9 developed and used to predict citrus intake in an independent cohort; the Irish National Adult
10 Nutrition Survey (NANS) (n=565). Proline betaine displayed a dose-response relationship to
11 orange juice intake in 24h and fasting samples ($p < 0.001$). In a test set, predicted orange juice
12 intakes displayed excellent agreement with true intake. There were significant associations
13 between predicted intake measured in 24h and fasting samples and true intake ($r = 0.710$ -
14 0.919). Citrus intakes predicted for the NANS cohort demonstrated good agreement with self-
15 reported intake and this agreement improved following normalization to osmolality.

16 **Conclusion:** The developed calibration curves successfully predicted citrus intakes in an
17 independent cohort. Expansion of this approach to other foods will be important for the
18 development of objective intake measurements.

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26 **1 Introduction**

27 In an endeavor to overcome some of the methodological issues associated with current dietary
28 assessment methods, dietary biomarkers are being utilized. Dietary biomarkers, for example
29 urinary nitrogen a marker of protein intake, provide unbiased estimates of intake and can
30 therefore be used to validate classical self-reporting approaches [1, 2]. More recently
31 metabolomics has emerged as a valuable tool in the discovery of dietary biomarkers. A
32 number of dietary biomarkers have been successfully identified including biomarkers of fish
33 [3-5], red meat [6-8], cruciferous vegetables [9, 10], whole-grain cereals [11, 12] and coffee
34 [13, 14]. However, the majority of these studies with the exception of alkylresorcinols,
35 biomarkers of wholegrain intake, have not demonstrated dose-response relationships between
36 dietary biomarkers and food intake. Furthermore, clear examples of how such biomarkers can
37 be used for assessment of intake are lacking.

38
39 To date one of the most studied dietary biomarkers identified using a metabolomics approach
40 is proline betaine [15, 16]. A number of acute and medium term interventions and cohort
41 studies have identified proline betaine as a robust biomarker of citrus fruit intake [15-20].
42 Proline betaine was originally identified as a potential citrus fruit biomarker by Atkinson et al.
43 [21]. Following this Heinzmann and colleagues performed an acute intervention study [16]. In
44 this acute study participants consumed a mixed-fruit meal and urine samples were collected
45 and analyzed by ¹H Nuclear Magnetic Resonance (NMR) [16]. Multivariate analysis
46 identified proline betaine as a potential biomarker of citrus fruit intake. Furthermore, proline
47 betaine was assessed in a number of fruit and commercially available fruit juices and was
48 found in higher concentrations in citrus fruit. The urinary excretion profile of proline betaine
49 was measured following orange juice consumption in six participants. This biomarker was
50 then confirmed using data from participants in the INTERMAP UK cohort, demonstrating a

51 high sensitivity and specificity for citrus fruit consumption (90.6 % and 86.3 %, respectively)
52 and a significant correlation with citrus consumption ($R^2 = 0.80$) [16]. Furthermore, following
53 consumption of 200 ml of orange juice as part of a standardized test breakfast Lloyd et al.
54 identified proline betaine and a number of biotransformed products in postprandial urine [17].
55 Urinary proline betaine measurements also distinguished between low, medium and high
56 habitual intakes of citrus foods (estimated by food frequency questionnaire (FFQ)) with
57 sensitivities and specificities of 80.8 – 92.2 % and 74.2 – 94.1 %, respectively, for elevated
58 proline betaine in high reporters of citrus fruit consumption [17]. In another study, urinary
59 metabolomes were profiled for volunteers that had consumed an acute dose of orange or
60 grapefruit juice, volunteers that had consumed orange juice regularly for one month as part of
61 their habitual diet and also volunteers whom had reported high or low consumption of citrus
62 products in a large cohort study [15]. Proline betaine was identified as a biomarker of citrus
63 fruit intake in all research designs [15]. Considering that independent metabolomics studies
64 with different population groups, different analytical methods and exposures consistently
65 reported proline betaine as a marker of citrus fruit intake, the evidence base is therefore
66 substantial to support its use. However, a clear demonstration of the utility of this biomarker
67 in predicting citrus intake is lacking.

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69 The objective of the present work was to develop calibration curves for use in quantifying
70 citrus intakes in an independent cohort. This study investigated the dose-response relationship
71 of proline betaine with orange juice intake in a controlled intervention study and subsequently
72 developed calibration curves for use in quantifying citrus intakes.

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76 **2 Materials and methods**

77 **2.1 Study design and population; the NutriTech study**

78 Ethical approval was received from London Brent Ethics Committee (reference number:
79 12/LO/0139). The NutriTech study is a randomized control trial comprised of two parts; the
80 food intake study which aimed to investigate the use of metabolomic profiling as a method of
81 independent food quantification and secondly the weight loss intervention that aimed to
82 quantify the effect of diet on ‘phenotypic flexibility’ (adaptation of biological and
83 physiological processes in the state of challenged homeostasis) (NCT01684917) (Supporting
84 Information Figure S1). For the present study data from the NutriTech food intake study is
85 included. Recruitment took place between June 2012 and April 2014. Participants attended
86 the NIHR/Wellcome Trust Imperial Clinical Research Facility for three days over three
87 consecutive weeks. Eligibility criteria included males and females of all ethnicities, aged
88 between 18 and 65 y with a BMI of 18.5-35 kg/m² and free from any chronic medical
89 condition. Participants (n = 50) were randomly assigned (using nested randomization based
90 on sex, age and BMI) to one of five different diets; red meat, fish, poultry, processed meat or
91 a supplement and vegetarian option (Supporting Information Table S1). An independent
92 researcher, not linked to the study, performed the randomization by sealed envelopes. On each
93 day of each intervention week participants consumed a standardized breakfast at 8 am and
94 their test meals at midday (12 pm) and evening (7 pm). All test meals were designed to
95 provide similar intakes of dietary energy and fiber but macronutrient composition varied over
96 the intervention weeks with carbohydrate decreasing from week one to week three and protein
97 and fat intake increasing from week one to week three (Supporting Information Figure S2).
98 Leftovers were measured and recorded where appropriate. Urine and plasma samples were
99 collected (Supporting Information Figure S3). On day one of each intervention week no
100 biofluids were collected. A 24 h urine sample was collected on day two of each intervention

101 week. Participants began the 24 h urine collection at 8 am (or at the time of the first, fasting,
102 morning urine void of day two). All voids throughout the day were collected in a single 5 L
103 container. 24 h urine samples were kept chilled at 4°C until all urine had been collected and
104 the final volume was recorded (at 8 am day three or at the time of the first, fasting, urine void
105 of day three). On day three of each intervention week participants were only allowed void at
106 the designated times: 0 h (11.55 am), 2 h (2 pm or 2 h after their midday meal) and 6 h (6 pm
107 or 6 h after they eat their midday meal). Blood samples were also collected at 0 h, 2 h and 6
108 h. On day four of each intervention week, before participants left the clinical investigation
109 unit, fasting blood and urine samples were collected. Participants returned to their normal
110 dietary habits until returning to the study the subsequent week. The present study focused on
111 the breakfast meal. Each diet received the same breakfast which included white bread, eggs,
112 butter, yoghurt and orange juice. The amount of orange juice provided decreased from week
113 one to week three. In weeks one, two and three the breakfast was designed for females to
114 receive 250 g/d, 220 g/d and 50 g/d of orange juice respectively and males to receive 520 g/d,
115 450 g/d and 30 g/d respectively. Participants did not consume any other citrus fruits or juices
116 during the three day intervention. The 24 h urine samples and the fasting urine samples are
117 used in this present analysis.

118

119 **2.2 Validation study; the NANS study**

120 Dietary and urinary metabolomic data from the National Adult Nutrition Survey (NANS) was
121 used to demonstrate further the utility of dietary biomarkers in predicting intake. Ethical
122 approval was obtained from the University College Cork Clinical Research Ethics Committee
123 of the Cork Teaching Hospitals (ECM 3 (p) 4 September 2008) and recruitment began in May
124 2008. NANS investigated habitual food and beverage consumption, lifestyle, health indicators
125 and attitudes to food and health in a representative sample of 1500 adults aged 18 - 90 y in the

126 Republic of Ireland during 2008 - 2010 [22]. For the present study dietary and urinary
127 metabolomic data from 565 NANS participants are included in the analysis. The 565
128 participants were randomly selected from the main NANS database ensuring equal numbers
129 of men and women across the age range. Dietary data was collected, over four consecutive
130 days, using a four day semi-weighed food diary. Participants recorded detailed information on
131 the amount and type of all foods, drinks and nutritional supplements consumed over four
132 consecutive days in the food diary. Each of the 2552 food codes consumed during the survey
133 were assigned to one of 68 food groups. For the purpose of this analysis citrus containing
134 food groups (fruit squashes, cordials and fruit juice drinks, fruit juices and citrus fruit) were
135 combined to form the total citrus food group. Mean daily citrus intake (average citrus intake
136 based on the four days of recording) was computed for the total citrus food group. Under-
137 reporters of energy intake were identified as having a ratio of energy intake:BMR of < 1.1
138 [23]. During the data collection period, a 50 ml first void urine sample was also collected
139 from participants. All urine samples were centrifuged at $1800 \times g$ for 10 min at 4°C and stored
140 at -80°C for analysis.

141

142 **2.3 Urine analysis and metabolite quantification**

143 Urine samples were prepared for ^1H NMR spectroscopy by the addition of 250 μL phosphate
144 buffer (0.2 mol $\text{KH}_2\text{PO}_4/\text{L}$, 0.8 mol $\text{K}_2\text{HPO}_4/\text{L}$) to 500 μL urine. After centrifugation at 5360
145 $\times g$ for 5 min at 4°C , 10 μL sodium trimethylsilyl [2,2,3,3- $^2\text{H}_4$] propionate (TSP) and 50 μL
146 deuterium oxide (D_2O) were added to 540 μL of the supernatant. Spectra were acquired on a
147 600-MHz Varian NMR spectrometer by using the first increment of a nuclear Overhauser
148 enhancement spectroscopy pulse sequence at 25°C . Spectra were acquired with 16,384 data
149 points and 128 scans. Water suppression was achieved during the relaxation delay (2.5 s) and
150 the mixing time (100 ms). All ^1H NMR urine spectra were referenced to TSP at 0.0 parts per

151 million and processed manually with the Chenomx NMR Suite (version 7.7, Inc.; Edmonton,
152 Canada) by using a line broadening of 0.2 Hz, followed by phase and baseline correction. A
153 ¹H NMR spectrum was acquired for a proline betaine standard. This spectrum was added to
154 the Chenomx Spectral Reference Library using the company's recommended spectral
155 acquisition and formatting protocols. Proline betaine was identified and quantified by using
156 the Chenomx Profiler (version 7.7). Osmolality was measured by using an Advanced
157 Osmometer model 3D3 (Advanced Instruments, Norwood, MA). Aliquots of urine were
158 tested for osmolality with the use of micro-osmometry by freezing point depression, with
159 values reported as the number of solute particles, in moles, dissolved in a kilogram of urine.
160 Metabolite concentrations were normalized to osmolality where appropriate, by dividing the
161 metabolite concentration by the osmolality reading for the sample.

162

163 **2.4 Statistical analyses**

164 Paired sample t-tests were performed using IBM SPSS Statistics 20.0 to compare proline
165 betaine concentrations between intervention week one and intervention week three in the 24 h
166 and fasting urine samples. Ten participants were randomly selected from the 50 NutriTech
167 participants and served as a test set, the remaining 40 participants served as a training set.
168 Concentration curves were determined based on data from the training set and orange juice
169 intakes were predicted in the test set based on the proline betaine concentrations in urine
170 using curve-fitting software (WinCurveFit). Concentration curves were also determined using
171 data from the NutriTech total population (n = 50) and citrus intakes were predicted in the
172 NANS cohort. Bland and Altman plots were made via GraphPad Prism 6.0 to assess
173 agreement between the predicted (based on proline betaine concentrations) citrus intake and
174 actual (NutriTech intake) or recorded (food diary) citrus intake in the test set and the NANS

175 cohort [24]. The association between the actual intake and the predicted orange juice intake
176 was also examined using Spearman's correlations.

177

178 **3 Results**

179 **3.1 The NutriTech study population**

180 Characteristics of the NutriTech participants (n = 50) are presented in Table 1. The training
181 set (n = 40) comprised of 21 males and 19 females, with a mean age of 60 ± 4 y and a mean
182 BMI of 28.5 ± 3.6 kg/m². The test set (n = 10) comprised of four males and six females, a
183 mean age of 59 ± 5 y and a mean BMI of 29.2 ± 3.4 kg/m².

184

185 **3.2 Proline betaine quantification**

186 Proline betaine was quantified using Chenomx Profiler and concentrations were compared
187 between intervention week one and intervention week three in the training set. Two samples
188 were missing for the 24 h analysis and one sample was missing for the fasting sample,
189 therefore data presented is based on 38 participants and 39 participants respectively. In both
190 the 24 h urine samples and the fasting urine samples proline betaine concentrations
191 significantly decreased from intervention week one to intervention week three ($p < 0.001$) in
192 response to decreasing orange juice consumption (Table 2). Proline betaine also decreased
193 significantly ($p < 0.001$) in both the 24 h urine and the fasting urine samples when normalized
194 for osmolality (Supporting Information Table S2).

195

196 **3.3 Development of calibration curves for prediction of orange juice intakes**

197 Calibration curves were determined using proline betaine concentrations and actual orange
198 juice intakes from the training set. This was completed for the 24 h and fasting urine, both

199 normalized and not normalized to osmolality. The calibration curve based on proline betaine
200 concentrations from the training set 24 h urine sample not normalized to osmolality is
201 presented in Figure 1 ($Y=1.63E-03*X+1.31E-01$). From this orange juice intake was
202 predicted for the test set ($n = 10$) (Supporting Information Table S3).

203

204 Bland and Altman plots, used to assess the agreement between actual orange juice intakes and
205 predicted orange juice intakes in the test set are presented in Figure 2. The 24 h urine sample
206 had less than 10 % of the observations fall outside the 95 % limits of agreement (the dotted
207 lines) (Figure 2A). Similar results are found with the fasting samples, as less than 10 % of the
208 observations also fall outside the 95 % limits of agreement (Figure 2C). The mean difference
209 (bias) between predicted and actual orange juice intake was small (43.1 and -18.1 g for the 24
210 h and fasting samples respectively). Overall these plots indicate good agreement between the
211 predicted and actual orange juice intakes. 24 h urine samples and fasting urine samples
212 normalized to osmolality also had less than 10 % of the observations fall outside the 95 %
213 limits of agreement. The mean difference (bias) between predicted and actual orange juice
214 intake was smaller for 24 h and fasting urine samples normalized to osmolality (9.8 and -4.1 g
215 respectively) (Figure 2B, Figure 2D).

216

217 The association between actual orange juice intakes and predicted orange juice intakes was
218 assessed using Spearman's correlations coefficient. Actual orange juice intake showed a
219 significant association with predicted orange juice (Supporting Information Table S4). The
220 spearman correlation was 0.712 ($p < 0.001$) and 0.710 ($p < 0.001$) for 24 h and fasting urine
221 respectively, while proline betaine concentrations normalized to osmolality in the 24 h urine
222 and the fasting urine samples had correlations of 0.859 and 0.919 ($p < 0.001$) respectively
223 (Supporting Information Table S4).

224 **3.4 Prediction of citrus intakes in an independent cohort**

225 The calibration curve determined using NutriTech participant's (n = 50) fasting urine proline
226 betaine concentrations was used to predict citrus intake for the NANS participants (n = 565).
227 Bland and Altman plots were used to assess the agreement between participant's self-reported
228 mean daily citrus intake and predicted citrus intakes from the participant's proline betaine
229 concentrations in the fasting urine sample (normalized and not normalized to osmolality)
230 (Figure 3A, Figure 3B). Mean daily citrus intake both normalized and not normalized for
231 osmolality had <5 % of the observations fall outside the 95 % limits of agreement. The mean
232 difference (bias) between recorded citrus intake and predicted citrus intake using proline
233 betaine concentrations not normalized to osmolality was 21.6 g (Figure 3A). The mean
234 difference (bias) between recorded citrus intake and predicted citrus intake using proline
235 betaine concentrations normalized to osmolality was smaller (4.3 g) (Figure 3B).
236 Disagreement between measurements was greatest for high predicted intakes. Twenty-two
237 participants were predicted to have higher citrus intake compared to the self-reported data.
238 Upon further investigation, seven participants were identified as under-reporters and three
239 participants were supplement users. When data was normalized to osmolality the number of
240 participants having predicted citrus intakes higher than recorded intakes was reduced (15
241 participants).

242

243 **4 Discussion**

244 The present study has made significant advancements in the dietary biomarker field.
245 Primarily, the development of calibration curves successfully enabled proline betaine to be
246 used to estimate citrus intakes in a large cross-sectional study. Furthermore, this was
247 supported by demonstrating a dose-response relationship between proline betaine and orange
248 juice intake. This approach, using dietary biomarkers to quantify food or beverage intake can

249 be developed and utilized in future studies, therefore aiding the translation of these
250 biomarkers into practice.
251
252 Our study is an important demonstration of the successful use of dietary biomarkers. The
253 study design enabled the examination of the dose-response relationship between the
254 biomarker and actual food intake. Importantly, the orange juice was consumed as part of a
255 mixed meal, which is more reflective of habitual dietary intake and demonstrates the
256 sensitivity of proline betaine as it can still classify participant's intakes irrespective of other
257 components of the diet. Demonstration of the use of the developed calibration curves to
258 predict intake in the cross-sectional study was a valuable aspect of this study. Proline betaine
259 concentrations successfully predicted citrus intakes. The ability to predict mean daily citrus
260 intake is important for future use of proline betaine as a marker of habitual intake of citrus
261 fruit. Although the calibration curves were built using orange juice the use of the NANS
262 cohort demonstrated their ability to predict citrus intake which included both juices and fruit.
263
264 Both 24 h urine and fasting urine samples were examined in this study. Interestingly, the
265 results indicate that the fasting samples performed well and once corrected for osmolality
266 outperformed the 24 h samples. This is particularly important for nutritional epidemiology
267 where many cohort studies have fasting samples collected and not 24 h urine samples. For
268 future studies it also demonstrates the potential use of fasting samples once corrected for
269 osmolality. Collection of a fasting sample is less burdensome on the volunteer and should
270 enable easier compliance within studies. In the current study fasting samples were used to
271 predict citrus intake in the free-living NANS population. Bland and Altman plots
272 demonstrated good agreement between predicted and recorded intakes. The disagreement
273 observed between methods in the NANS cohort was predominantly as a result of higher

274 predicted intakes compared to the self-reported intakes. Interestingly, when participants with
275 self-reporting issues (under-reporters, overweight or obese participants) were taken into
276 account, this disagreement accounted for less than 2 % of the total population. Agreement was
277 further improved between predicted and recorded intakes when samples were normalized to
278 osmolality. In both datasets in the current study normalization with osmolality improved the
279 agreement between predicted and actual/reported intakes. A previous study also reported the
280 importance of normalizing urine to osmolality for the detection of changes in metabolite
281 profiles [25].

282

283 While there has been significant interest in using metabolomics to identify dietary biomarkers
284 there has been a lack of studies demonstrating the use of such biomarkers in predicting intake.
285 In a recent study a dose-response relationship between tartaric acid and grape intake was
286 demonstrated [26]. Tartaric acid was subsequently quantified in participants (n=19) following
287 four four-day dietary interventions which included 0 g/d, 50 g/d, 100 g/d, and 150 g/d of
288 grapes in standardized diets in a randomized controlled trial. Predicted grape intake was found
289 to be most accurate for 24 h urine samples compared to spot urine samples ($r^2 = 0.90$) [26]. In
290 relation to citrus fruit biomarkers, Lloyd and colleagues demonstrated the potential
291 quantitative relationship between proline betaine and citrus fruit consumption as urinary
292 proline betaine levels differed among low, medium and high citrus consumers after an
293 overnight fast [17]. However, estimations of consumption were based on self-reporting data
294 from an FFQ and the dose-response of proline betaine with citrus intake was not investigated.
295 Proline betaine has also been identified as a biomarker of citrus intake using three study
296 designs; a short term intervention where an acute dose of orange/grapefruit juice was
297 consumed, a medium term intervention where orange juice was consumed regularly for one
298 month, and a cohort study where high or low consumers of citrus products were identified

299 from a 24 h recall [15]. The focus of this study however was on the discovery of biomarkers
300 and therefore did not examine the dose-response. Furthermore, previous studies have shown
301 that proline betaine has a relatively short half-life; however, this did not seem to impact on its
302 ability to predict habitual dietary intake. An earlier study targeted four metabolites in 24 h
303 urine samples following the consumption of controlled diets containing low red meat (60 g/d),
304 medium red meat (120 g/d) and high red meat (420 g/d) [6]. Two metabolites demonstrated a
305 dose-response relationship with meat intake, increasing as the amount of meat in the diet
306 increased, however no further practical use of these biomarkers in quantifying red meat intake
307 were demonstrated [6]. The current study used a well-controlled intervention study to develop
308 calibration curves which enabled prediction of intake in a free-living cross-sectional cohort
309 marks a very significant step forward in the field of dietary biomarkers.

310

311 There are a number of strengths associated with the present study. Primarily this study reveals
312 how a dietary biomarker, discovered through a metabolomics based approach can be used to
313 successfully predict food intakes in a large cross-sectional study. Thus clearly demonstrating
314 the potential application of dietary biomarkers in dietary assessment. Furthermore, the
315 NutriTech food intake study provides a successful strategy for dietary biomarker
316 identification, enabling the assessment of the dose-response relationship between the
317 biomarker and food source. However, it must be noted that although there was excellent
318 agreement between predicted citrus intake and self-reported citrus intakes, further
319 interventions with repeated measurements over time may be needed to assess the dose-
320 response relationship for long term intakes. It is also important to acknowledge that this work
321 reflects a food intake biomarker and addresses the significant issue of improving estimations
322 of food intake. However, we did not assess if this results in improvements in nutrient intake
323 data.

324

325 The present study represents an important advancement in biomarker research by
326 demonstrating the utility of calibration curves to successfully quantify intakes of citrus food.
327 This study illustrates a clear dose-response relationship between actual food intake and a
328 dietary biomarker in a mixed meal setting. The results presented here are very promising for
329 the field of dietary biomarkers; however more studies on dose-response relationships are
330 essential for further progression in this area. This work will pave the way for further
331 development of dietary biomarkers that can be used to predict unbiased intakes and that can
332 be used to obtain more reliable risk estimates in diet-disease analyses.

333 **Author contributions**

334 H. G. conducted research, analyzed data and prepared the manuscript. C. J. R. M. assisted in
335 the statistical analyses, M. R., G. F., B. A. M., A. P. N., J. W., A. F. and M. J. G. provided
336 essential materials, L. B. designed research, conducted research, analyzed data and prepared
337 the manuscript. All authors read and approved the final manuscript.

338

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347

348 **Conflict of interest statement**

349 The authors have declared no conflict of interest.

350

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Table 1 NutriTech food intake study characteristics ^{a)}

Characteristics	Training set (n = 40)	Test set (n = 10)
Gender	21 (M) 19 (F)	4 (M) 6 (F)
Age (y)	60 ± 4	59 ± 5
BMI (kg/m ²)	28.5 ± 3.6	29.2 ± 3.4
Systolic blood pressure (mm Hg)	133.5 ± 15.9	127.8 ± 14.4
Diastolic blood pressure (mm Hg)	78.8 ± 10.6	74.2 ± 11.4

^{a)}Data are Mean ± SD (all such vales). 10 participants were randomly selected from the 50 NutriTech food intake participants and served as the test set, the remaining 40 participants served as a training set.

Table 2 Mean proline betaine concentrations in the 24 h urine (n = 38) and fasting urine samples (n = 39) ^{a)}

mmol/L	Week 1	Week 3	P ^{b)}
24 h proline betaine	0.74 ± 0.32	0.20 ± 0.09	<0.001
Fasting proline betaine	0.71 ± 0.34	0.20 ± 0.10	<0.001

^{a)}Data are Mean ± SD.

^{b)}Paired sample t-test was used to examine the differences between intervention week 1 and week 3.

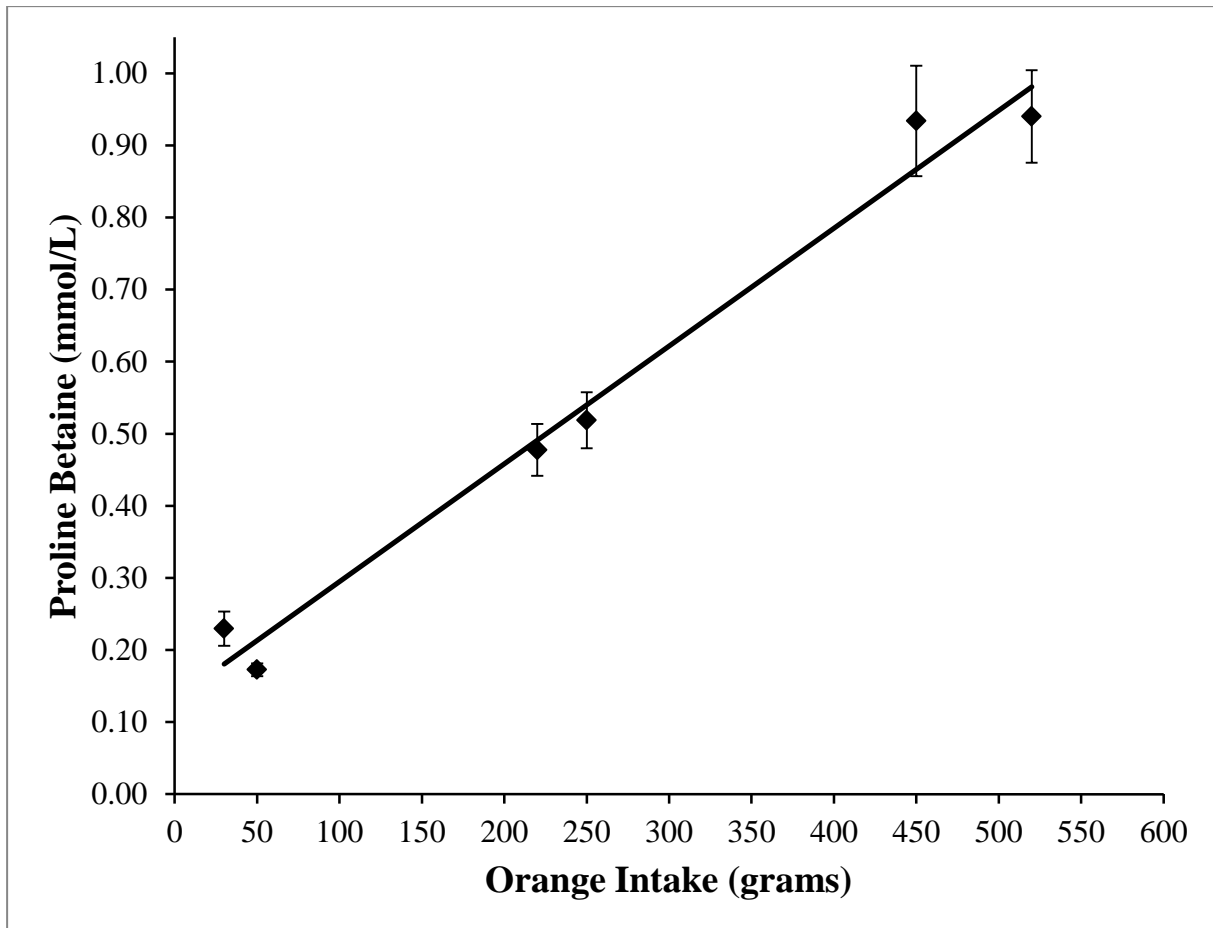


Figure 1. Calibration curve using the 24 h urine samples (mean and error bars (SEM) are presented). The x-axis; actual orange juice intake (grams) during the NutriTech food intake study, the y-axis; proline betaine concentrations measured in urine (mmol/L). Each point represents average proline betaine concentration for a particular orange juice intake.

$$(Y=1.63E-03*X+1.31E-01)$$

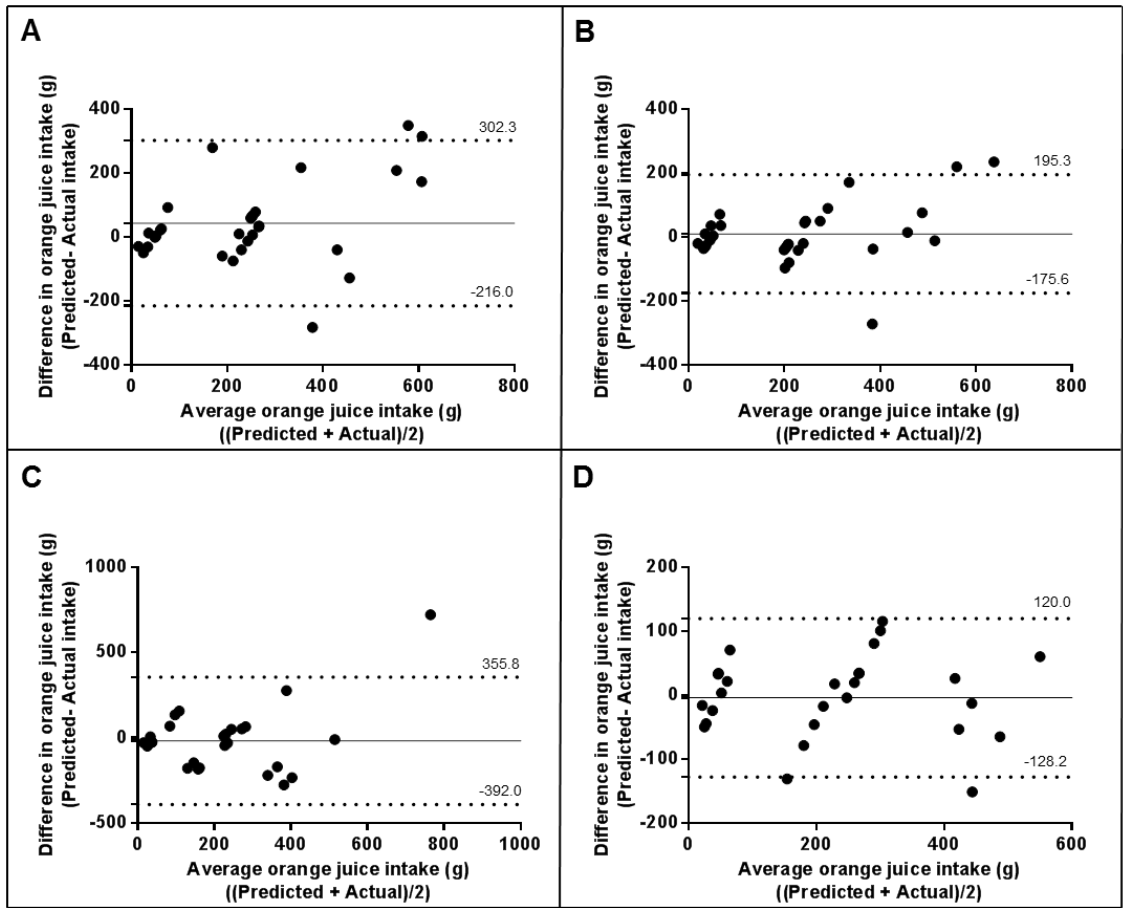


FIGURE 2

Figure 2. Bland and Altman plots for the test set **A:** Orange juice intakes were predicted from proline betaine concentrations measured in 24 h urine samples **B:** Orange juice intakes were predicted from proline betaine concentrations measured in 24 h urine samples normalized to osmolality **C:** Orange juice intakes were predicted from proline betaine concentrations measured in fasting urine samples **D:** Orange juice intakes were predicted from proline betaine concentrations measured in fasting urine samples normalized to osmolality, with mean difference and limits of agreement.

The solid line represents the mean difference and the dotted line represents the limits of agreement. ‘Predicted’ indicates the predicted orange juice intake based on urinary proline betaine concentrations. ‘Actual’ indicates the actual orange juice intakes according to the NutriTech study taking into account leftovers.

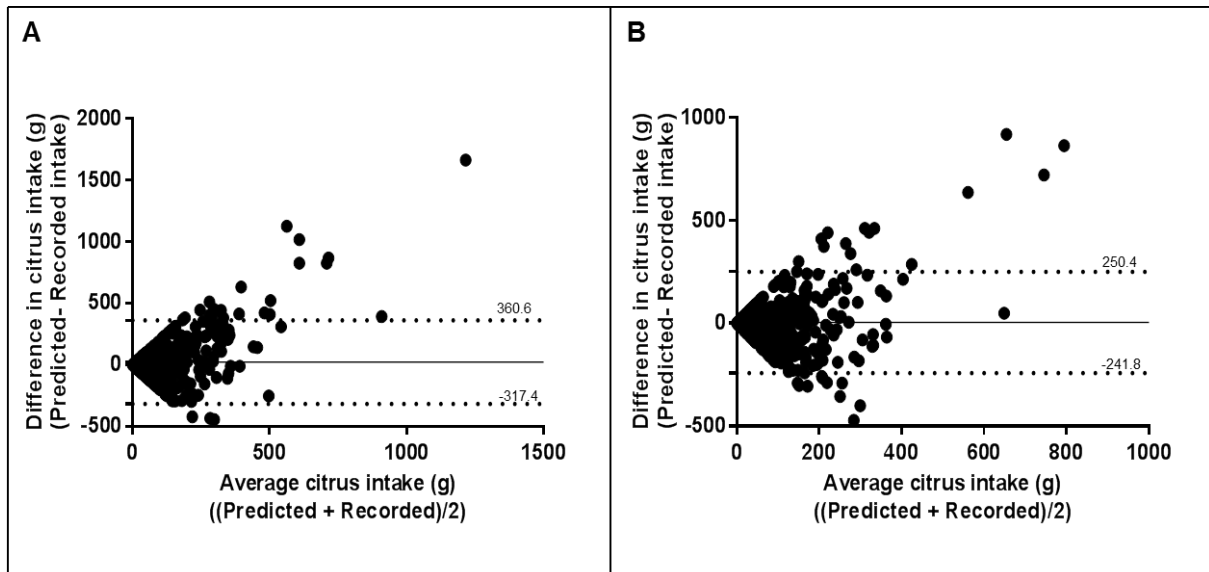


FIGURE 3

Figure 3. Bland and Altman plots for NANS **A:** Agreement between recorded mean daily citrus intake and citrus intakes predicted from proline betaine concentrations in a fasting sample **B:** Agreement between recorded mean daily citrus intake and citrus intakes predicted from proline betaine concentrations in a fasting sample normalized to osmolality, with mean difference and limits of agreement.

The solid line represents the mean difference and the dotted line represents the limits of agreement. ‘Predicted’ indicates the predicted citrus intake based on urinary proline betaine concentrations in a fasting sample. ‘Recorded’ indicates citrus intake recorded using the four-day food diary.