

## Effects of methodological variation on assessment of riboflavin status using the erythrocyte glutathione reductase activation coefficient assay

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Riboflavin status is usually measured as the *in vitro* stimulation with flavin adenine dinucleotide of the erythrocyte enzyme glutathione reductase, and expressed as an erythrocyte glutathione reductase activation coefficient (EGRAC). This method is used for the National Diet and Nutrition Surveys (NDNS) of the UK. In the period between the 1990 and 2003 surveys of UK adults, the estimated prevalence of riboflavin deficiency, expressed as an EGRAC value  $\geq 1.30$ , increased from 2 to 46 % in males and from 1 to 34 % in females. We hypothesised that subtle but important differences in the detail of the methodology between the two NDNS accounted for this difference. We carried out an evaluation of the performance of the methods used in the two NDNS and compared against an ‘in-house’ method, using blood samples collected from a riboflavin intervention study. Results indicated that the method used for the 1990 NDNS gave a significantly lower mean EGRAC value than both the 2003 NDNS method and the ‘in-house’ method ( $P < 0.0001$ ). The key differences between the methods relate to the concentration of FAD used in the assay and the duration of the period of incubation of FAD with enzyme. The details of the EGRAC method should be standardised for use in different laboratories and over time. Additionally, it is proposed that consideration be given to re-evaluating the basis of the EGRAC threshold for riboflavin deficiency.

**Riboflavin status: Erythrocyte glutathione reductase activation coefficient: Methodology: Nutrition surveys**

The last two National Diet and Nutrition Surveys (NDNS) of the adult UK population have indicated an apparent shift in the prevalence of biochemical ariboflavinosis as measured by the erythrocyte glutathione reductase activation coefficient (EGRAC) assay. In the 1990 survey report of adults aged 19–65 years<sup>(1)</sup> data collected between 1986 and 1987 suggested that riboflavin deficiency was not a public health issue, with only 1 % of men and 2 % of women being classified as deficient (EGRAC  $\geq 1.30$ ). The mean EGRAC values for men and women were, respectively, 1.09 (SD 0.058) and 1.10 (SD 0.06). However, 14 years later, data collected between 2000 and 2001 for the 2003 survey of adults aged 18–65 years<sup>(2)</sup> reported that 46 % of men and 34 % of women were riboflavin deficient according to the same criterion (EGRAC  $\geq 1.30$ ). Mean EGRAC values had increased in men to 1.38 (SD 0.169) and in women to 1.40 (SD 0.194).

This was a surprising finding and could be explained by one or more of several possibilities including a fall in the dietary intake of riboflavin (or in the methodology used to estimate this), a decline in bioavailability of food riboflavin or an increase in requirements. Alternatively, the finding could be attributable to changes in the protocol of the biochemical method used to determine riboflavin status.

Both NDNS report using records of weighed food intakes to estimate dietary intake. Intake data showed a small and statistically insignificant increase in average intakes of

riboflavin from food between the two surveys. Dietary riboflavin intake increased from an average of 2.08 mg/d in men to 2.11 mg/d and in women from 1.57 to 1.60 mg/d between the two surveys. Total intake of riboflavin (including supplements) showed a similar increase. Total riboflavin intake increased in men from between 2.29 and 2.33 mg/d and in women from 1.84 to 2.02 mg/d between the two surveys.

The small increase in riboflavin intake from food between the 1990 and the 2003 report was mitigated to some extent when riboflavin intake was expressed relative to energy intake; in men the intake of riboflavin from food relative to energy increased from 0.20 to 0.22 mg/MJ and in women from 0.22 to 0.23 mg/MJ.

During the 1970s and 1980s considerable attention was paid to the performance of the EGRAC assay under different experimental conditions and it was shown that the concentration of substrates and the temperature and times of incubation of reagents with enzyme were all likely to affect results obtained<sup>(3–8)</sup>. Current reports of riboflavin status in populations around the world often lack methodological detail, and this makes it difficult to interpret some published results.

We hypothesise that the details of the protocol used for the estimation of riboflavin status using the EGRAC assay differed between the 1990 and 2003 NDNS and that this contributed to the apparent increase in the prevalence of riboflavin deficiency over the intervening period.

**Abbreviations:** EGRAC, erythrocyte glutathione reductase activation coefficient; GSSG, oxidised glutathione; NDNS, National Diet and Nutrition Survey.

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The present study describes a systematic investigation of the effects on EGRAC values in stored blood samples, of using the analytical methods from the 1990 and 2003 NDNS. Results are compared with the method currently used in our laboratory<sup>(9)</sup>.

## Methods

### *Study population and blood handling*

In order to compare the different protocols it was necessary to use a source of samples that had a wide range of EGRAC values, representative of those seen in the UK population. Samples were available from a previous riboflavin intervention study carried out in 2005 involving sixty-one healthy volunteers from the Sheffield area. The median age of recruits was 37 years (range 20–64 years), of whom thirteen were males and forty-eight females. Mean baseline EGRAC was 1.45 (SD 0.17), with a range of values between 1.14 and 2.01. Volunteers were excluded from the study if they smoked or took multivitamin supplements. Samples were available pre-supplementation and following an 8-week riboflavin supplementation with either 1.5 or 10 mg/d, and these provided 122 samples in total. That study was approved by the North Sheffield Local Research Ethics Committee (NS0361713). At the time of blood collection, blood was processed in the following manner: whole blood was collected into EDTA tubes and centrifuged at 1000g for 10 min, the plasma and buffy coat were removed and the erythrocytes washed twice with PBS (pH 7.4). The packed erythrocytes were re-suspended in an equal volume of distilled water and frozen at  $-80^{\circ}\text{C}$  for storage (the haemolysate). Samples were stored from mid-2005 until analysis for the present study in mid-2007.

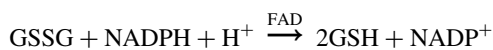
For the study described here it was possible to use spare haemolysates that had not been previously thawed and refrozen.

### *Dietary information*

It was considered useful to be able to examine the relationship between estimated riboflavin intake and EGRAC value, using the different protocols. Dietary intake data were available for fifty-five of the sixty-one volunteers who had completed a FFQ before the intervention. The FFQ was a modified version of that used and validated by the European Prospective Investigation of Cancer and Nutrition study<sup>(10)</sup>. Dietary data were analysed by SPSS (SPSS Inc., Chicago, IL, USA) using food composition information from the 5th edition of *The Composition of Foods*<sup>(11)</sup>.

### *Principle of the erythrocyte glutathione reductase activation coefficient assay*

The EGRAC assay utilises the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH) by the flavin-dependent enzyme glutathione reductase (EC 1.6.4.2), with the concomitant oxidation of NADPH.



FAD is an essential cofactor that is bound tightly to the enzyme, and is required for maximum activity. NADPH is

used as the electron donor and the disappearance of NADPH during the reaction is monitored spectrophotometrically at 340 nm. The rate of disappearance of NADPH is equivalent to the rate of enzyme activity. This activity can be stimulated *in vitro* by incubating with FAD. The ratio of stimulated activity to unstimulated activity represents the activation coefficient and reflects the intracellular saturation of the enzyme with FAD, which is determined primarily by riboflavin intake. An EGRAC value of 1.0 indicates complete saturation of the enzyme with FAD *in vivo* and increasing values of EGRAC reflect a progressive fall in intracellular FAD. An EGRAC value of 1.30 or greater is conventionally interpreted as riboflavin deficiency<sup>(5,12,13)</sup>. Thus, the EGRAC assay consists of a period of pre-incubation of an erythrocyte haemolysate (enzyme) with or without added FAD and a subsequent incubation with GSSG and NADPH, during which time the latter becomes oxidised and the former becomes reduced.

During the early development of the EGRAC assay there were some inconsistencies in results produced from different laboratories and several factors have been reported to influence the performance of the assay. Glatzle *et al.*<sup>(5)</sup> described how choosing the final concentration of FAD for the pre-incubation was a fine balance between having sufficient concentration of FAD for stimulation of the enzyme and the problem of inhibition by high concentrations of FAD. This inhibitory effect of high concentrations of FAD was also reported by other workers<sup>(8,13)</sup>. The effect was thought to be the result of a contaminant in the FAD and although never confirmed, is a plausible explanation because various compounds, including flavin mononucleotide, have been shown to be potent inhibitors of glutathione reductase<sup>(14)</sup>. Hartman *et al.*<sup>(15)</sup> identified a contaminant in commercial FAD with similar chromatographic characteristics to flavin mononucleotide.

In 1982, Thurnham & Rathakette<sup>(7)</sup> conducted a study of glutathione reductase activity that suggested that the duration and temperature of the pre-incubation of the haemolysate with FAD could also affect the EGRAC values obtained. They stressed the importance of leaving enough time to allow for maximum binding between enzyme and FAD, recommending 15 min as the optimal time but stated that pre-incubation at 37 or 35°C did not affect the EGRAC value obtained. Bayoumi & Rosalki<sup>(3)</sup> and Powers *et al.*<sup>(9)</sup> were the only workers at this time to use an incubation period of 30 min although the concentrations of FAD were very different, 10 and 2  $\mu\text{M}$ , respectively. Glatzle *et al.*<sup>(5)</sup> used only a 5 min pre-incubation but with a relatively high FAD concentration of 8.3  $\mu\text{M}$ . By lowering the FAD concentration to 0.83  $\mu\text{M}$ , Glatzle *et al.*<sup>(5)</sup> were able to abolish EGRAC values below 1.00 but with a concomitant lowering of EGRAC values in the upper range suggesting that there was insufficient time for binding of FAD to the enzyme, and probably also insufficient FAD for maximum activation.

Garry & Owen<sup>(13)</sup> in 1976 used a very high concentration of FAD (46  $\mu\text{M}$ ) during the pre-incubation with haemolysate to achieve maximum binding of FAD to the enzyme and then diluted the samples to a final FAD concentration of 1  $\mu\text{M}$ . They reported no EGRAC values less than 1.00.

It is clear, therefore, that the exact conditions of the assay are vitally important when comparing results from different surveys and for this reason we have tried to replicate as closely as possible the assay conditions that were used for

the last two NDNS reports, taking into account the method that they were based on and the reported modifications that were made.

### The assays

The three methods used for comparison were those reportedly used in the 1990 and 2003 NDNS and the in-house method<sup>(9)</sup> currently used in our laboratory. The basic protocol will be described first, followed by the modifications made for the individual methods.

**Reagents.** All reagents were prepared and diluted in phosphate buffer (0.1 M-potassium phosphate containing 2.5 mM-EDTA, pH 7.4). FAD, NADPH and GSSG (Sigma) were made up as concentrated stock solutions as follows: FAD, 1.83 mM; NADPH, 60 mM; GSSG, 82 mM. These were stored at  $-20^{\circ}\text{C}$  until the day of use. Working solutions were prepared fresh each day by diluting the stock solutions to achieve final concentrations in the assays as shown in Table 1.

Sample haemolysates were thawed at the beginning of each day, centrifuged at 13 000 g for 2 min to remove cell debris and diluted 1:61 in buffer to produce the working haemolysate. This dilution was repeated if necessary to give enzyme activity in the linear range. The dilution step was performed immediately before each analysis to prevent deterioration on standing for long periods of time. Thawed haemolysates were kept at  $4^{\circ}\text{C}$  and protected from light. To ensure there was no bias, the order of analysis of each method was rotated on a daily basis.

**Protocol.** The individual conditions for each of the assays will be described in more detail in the next section.

Two aliquots (120  $\mu\text{l}$ ) of diluted haemolysate from each sample were placed in the sample cups of a COBAS rotor and 20  $\mu\text{l}$  FAD (stimulated) were added to one and 20  $\mu\text{l}$  phosphate buffer (unstimulated) were added to the other. Each sample was analysed in duplicate. The rotor was then placed in an incubator at  $37^{\circ}\text{C}$  for the required time, for the pre-incubation. Care was taken to maintain a constant temperature, which was monitored continuously. After incubation with FAD, the activity of glutathione reductase was measured using a COBAS BIO centrifugal analyser (Roche, Switzerland). During the programmed run 90  $\mu\text{l}$  GSSG were transferred to 80  $\mu\text{l}$  haemolysate and the reaction was initiated by the addition of 70  $\mu\text{l}$  NADPH. The reaction was monitored at 340 nm with readings taken every 10 s for 5 min. The enzyme activity was expressed as  $\mu\text{mol}$  NADPH oxidised/min and the EGRAC value was calculated as: Activity of stimulated sample/Activity of unstimulated sample.

**Method comparison.** The specific conditions used in each of the three methods under comparison are summarised in Table 1. The concentrations of reagents represent the final concentrations in the assay.

The assay used for the 1990 NDNS was a modification of a method described by Thurnham & Rathakette<sup>(7)</sup>. In the 1990 survey the Kone Discrete Analyser was used to automate the process as described in Appendix J of the survey report<sup>(1)</sup>. The pre-incubation time with FAD was 15 min, which was the shortest of all three methods, and the concentration of FAD was the highest (3  $\mu\text{M}$ ). We replicated the method using a COBAS BIO centrifugal analyser. The volumes that had been used for the pre-incubation or the assay were not stated in the 1990 report and this was considered a potentially important factor in influencing the time it might take for the reagents to reach the correct temperature. We therefore examined the effect of using two different volumes, the in-house method volume of 140  $\mu\text{l}$  (representative of the volumes used in the COBAS BIO autoanalyser) and a larger volume (2.8 ml) more representative of volumes used in manual assays. The latter pre-incubation was performed in 5 ml tubes and 140  $\mu\text{l}$  were transferred to the COBAS sample cups prior to the enzyme analysis on the COBAS BIO centrifugal analyser.

The assay used for the 2003 NDNS was based on a method described by Glatzle *et al.*<sup>(5)</sup>. For the 2003 NDNS, the method was modified for use on a COBAS FARA centrifugal analyser as described in Appendix O of the survey report<sup>(2)</sup>. The time of pre-incubation had been increased to 30 min and the concentration of FAD reduced to 1.5  $\mu\text{M}$ . For the purpose of the present study, the conditions were replicated exactly and samples run on the COBAS BIO centrifugal analyser.

The in-house Powers method is based on that described by Glatzle *et al.*<sup>(5)</sup>, modified for use on the COBAS BIO centrifugal analyser<sup>(9)</sup>. This is the method currently used in our laboratory and has been optimised with a pre-incubation time of 30 min and a final FAD concentration of 2  $\mu\text{M}$ .

**Precision.** An in-house quality control sample was prepared and run at the beginning and end of each batch of twelve samples. Quality controls consisted of 500  $\mu\text{l}$  aliquots of haemolysate prepared from a 50 ml blood sample processed and stored in the same way as the study samples had been. The within-batch CV was calculated by analysing ten quality control aliquots at the beginning of the study. The between-batch CV was calculated from the mean of the two quality control values from each of twelve runs over the duration of the study.

**Table 1.** Details of assay conditions

Method	NADPH ( $\mu\text{mol/l}$ )*	GSSG (mmol/l)*	FAD ( $\mu\text{mol/l}$ )*	Pre-incubation time (min)	Pre-incubation temperature ( $^{\circ}\text{C}$ )†	Pre-incubation volume
1990 NDNS	80	0.889	3.0	15	37	140 $\mu\text{l}$ /2.8 ml
2003 NDNS	80	0.889	1.5	30	37	140 $\mu\text{l}$
Powers (in-house assay)	137	0.590	2.0	30	37	140 $\mu\text{l}$

GSSG, oxidised glutathione; NDNS, National Diet and Nutrition Survey.

\* Final concentration in the complete assay medium.

† The same temperature was used for the assay incubation.

### Data handling

The EGRAC values from 122 samples were shown to be normally distributed (Kolmogorov–Smirnov test,  $P > 0.05$ ) for the 2003 NDNS method and the Powers method but not normally distributed for the 1990 NDNS method, and therefore a Wilcoxon matched-pair test was used to compare the median values between the three methods.

Correlations between EGRAC values for each of the methods and between the methods and dietary intake data were carried out using Pearson's correlations. All analyses were performed using SPSS software (SPSS 11 for Mac OS X).

The bias between the two survey methods was assessed using a Bland–Altman plot (GraphPad Prism 9 for Mac; GraphPad, San Diego, CA, USA). Bland–Altman is routinely used for method comparison and looks at the differences in the results of two methods and plots them against the mean value for both methods. This approach allows any systematic shift between methods to be seen more clearly and the bias calculated provides additional information to correlations and scatterplots alone<sup>(16,17)</sup>.

## Results

### Precision

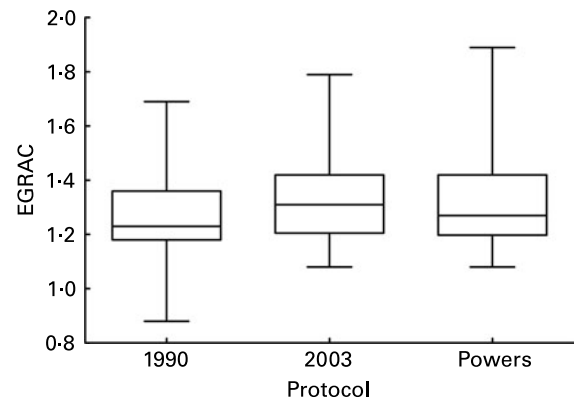
The within-batch CV for all three methods was  $< 5\%$ . The between-batch CV in EGRAC values using the 1990 NDNS method was higher than for the other methods; 6.4% for the large volume assay and 3.53% for the small volume assay compared with 1.8 and 1.7% for the 2003 NDNS and Powers methods, respectively.

### Method comparison

**Differences in mean values.** There was no statistically significant difference in the EGRAC values obtained using the 1990 NDNS method at small or large volumes ( $P = 0.99$ ). Comparison with the 2003 NDNS method and the Powers method were therefore made using only the data from the small volume assay.

The EGRAC values obtained for the three methods can be seen in Fig. 1. The median values for each method ( $n = 121$ ) were as follows: 1990 NDNS method, 1.23 (range 0.88–1.61); 2003 NDNS method, 1.31 (range 1.08–1.79); Powers method, 1.27 (range 1.08–1.89). The results for the 2003 NDNS method and Powers methods were both significantly higher than the 1990 NDNS method ( $P < 0.0001$ ). The results for the 2003 NDNS method did not differ significantly from the Powers method ( $P > 0.05$ ). Additionally, 3% of samples measured using the 1990 NDNS method gave EGRAC values  $< 1.0$ .

**Associations between values.** Associations between values obtained using different methods were compared using Pearson's correlation. Fig. 2 shows the strong correlation ( $r = 0.769$ ,  $P < 0.001$ ) between values obtained using the methods from the 1990 and 2003 NDNS. This was further examined by a Bland–Altman plot (Fig. 3(a)), which indicated an average positive bias of 0.057 in the 2003 NDNS method; the figure also suggests a trend towards a larger positive bias at the lower end of EGRAC values. This was clearer when



**Fig. 1.** A comparison of erythrocyte glutathione reductase activation coefficient (EGRAC) values using the three methods (1990, 1990 National Diet and Nutrition Survey (NDNS); 2003, 2003 NDNS; Powers, in-house assay). The box and whisker plot shows medians, 75% limits and ranges for measurements made on 121 blood samples.

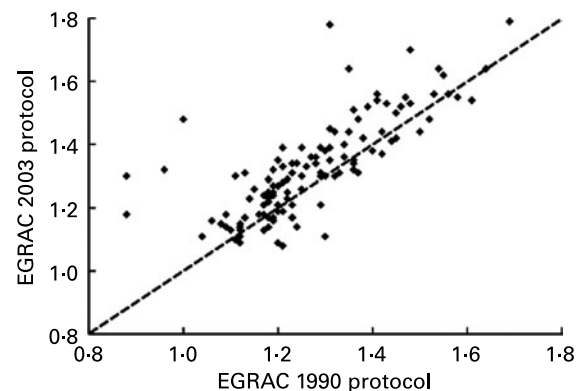
only data from post-supplementation samples were examined; the average positive bias was 0.068 (Fig. 3(b)).

To investigate further the particularly higher values for the 2003 NDNS method in the lower range of EGRAC values, values were compared according to quartiles. The 1990 NDNS values were divided into quartiles and the means for each quartile compared with values obtained for the equivalent samples, using the 2003 NDNS method (Fig. 4). The difference in the values obtained using the two methods was more evident in the lowest two quartiles.

### Diet and riboflavin status

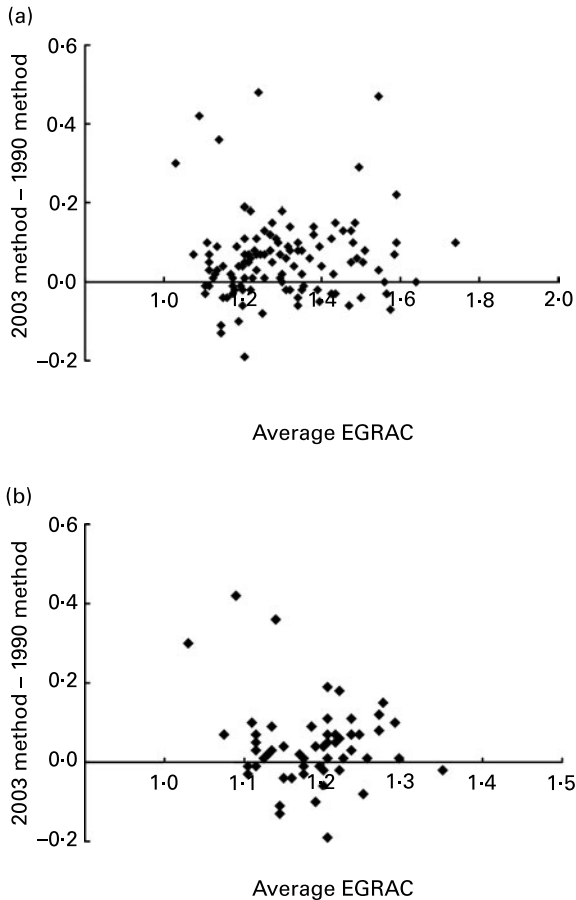
The mean riboflavin intake for the study sample population was 2.27 (SD 0.76) mg/d.

The correlation between EGRAC values and riboflavin intake data was examined for each of the three methods. Neither the 1990 nor 2003 NDNS method EGRAC values were significantly correlated with estimated dietary intakes ( $r = -0.151$ ,  $P = 0.277$  and  $r = -0.242$ ,  $P = 0.075$ , respectively).

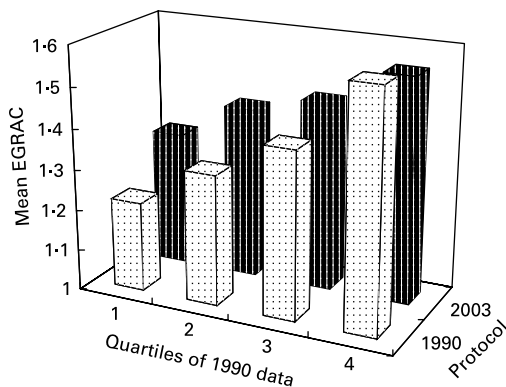


**Fig. 2.** Association between erythrocyte glutathione reductase activation coefficient (EGRAC) values obtained using the 1990 and 2003 National Diet and Nutrition Survey (NDNS) methods. Pearson's correlation analysis was conducted on EGRAC measurements made using the methods used for the 1990 and 2003 NDNS. The association was highly significant ( $r = 0.769$ ,  $P < 0.001$ ). ---, Line of equality.





**Fig. 3.** (a), Bland–Altman plot of erythrocyte glutathione reductase activation coefficient (EGRAC) data from the 1990 and 2003 National Diet and Nutrition Survey (NDNS) methods. Values are differences in EGRAC values for each of 121 measurements made using the 2003 and 1990 NDNS methods plotted against the average value for each pair. (b), Bland–Altman plot of post-intervention EGRAC data from the 1990 and 2003 NDNS methods. Results are plotted from only those blood samples collected after supplementation with riboflavin.



**Fig. 4.** Comparison of erythrocyte glutathione reductase activation coefficient (EGRAC) values for the 1990 and 2003 National Diet and Nutrition Survey (NDNS) methods according to quartiles. The EGRAC values generated using the method for the 1990 NDNS were split into quartiles and the means for each quartile compared with values obtained for the equivalent samples, using the 2003 NDNS method. The difference in the values obtained using the two methods was greatest in the lowest two quartiles.

EGRAC values generated using the Powers method were significantly inversely correlated with riboflavin intake ( $r = -0.290$ ,  $P = 0.032$ ).

## Discussion

The erythrocyte glutathione reductase activation coefficient has been used extensively to assess the riboflavin status of various populations. It is clear from work in the 1970s and 1980s that the coefficient can be affected by several methodological factors, as discussed.

The assay methods used in the last two diet and nutrition surveys of the adult population of the UK differed in several important respects. We have investigated whether these differences in methodology could have contributed to the substantial increase in EGRAC values reported in the 2003 NDNS compared with the 1990 NDNS of UK adults.

The EGRAC values obtained using the conditions reported for the 1990 NDNS method were significantly lower than those obtained using either the 2003 NDNS method or our own in-house method, the Powers method<sup>(9)</sup>. The 1990 method also gave a proportion of values  $< 1.0$ . The 1990 method used a shorter pre-incubation time and a higher concentration of FAD than either of the other two methods. This confirms reports that a shorter pre-incubation period can underestimate EGRAC values because less time is allowed for the FAD to bind to the enzyme in the haemolysate<sup>(7)</sup>. It also supports early reports that the use of a higher concentration of FAD is inadvisable as it can introduce a level of inhibition. Garry & Owen<sup>(13)</sup> reported that enzyme activity dramatically declined at FAD concentrations above  $5.0 \mu\text{M}$ .

The EGRAC values obtained from the two survey methods show a strong correlation. The Bland–Altman plot indicates a positive bias towards values for the 2003 method. This is illustrated more clearly when data are analysed by quartiles (Fig. 4), where it is shown that differences between methods are greater at the lower EGRAC values. This can be explained by sub-optimal binding of FAD with enzyme at short incubation times and also suggests that the higher FAD concentration could be having a small inhibitory effect, with the associated generation of EGRAC values  $< 1.0$ , an observation also reported by Garry & Owen<sup>(13)</sup> when using a high concentration of FAD.

EGRAC values generated by the 2003 NDNS method reflected dietary intake more closely than the 1990 method. This is a further indication that the 2003 NDNS method might be better able to reflect riboflavin status. Additionally, although the reliability of all methods was good, between-batch precision data indicated that the 2003 NDNS method was better than the 1990 method. The increased incubation time could have resulted in more efficient and reproducible binding with the FAD.

In summary, we have shown that the two methods used in the last two NDNS reports differed not only in the detail of the protocol but also produced significantly different results in our hands. Although the differences reported here would probably not account entirely for the discrepancy between the EGRAC values in the two reports, they do support the hypothesis that the different methods will have contributed to some extent to the increase in EGRAC values for the UK adult population between the years 1990 to 2003.

How might other factors have contributed to the apparent increased prevalence of riboflavin deficiency between the two surveys? As stated in the introduction, there is no evidence to suggest that riboflavin intake fell in the period between the two surveys. The method used for dietary data collection and the software used for dietary analysis were the same for both surveys. It is difficult to see how a decline in the bioavailability of riboflavin might come about, other than through a shift in the food sources of riboflavin, with an associated difference in bioavailability. The two NDNS reported the food sources of riboflavin, and there was little evidence for any difference between the surveys, except possibly for riboflavin from meat. From the 1990 to the 2003 NDNS there was a fall in the contribution that meat made to riboflavin intake; from 22 to 16% for men, and from 21 to 13% for women (with the biggest change in liver, being from 6 to 2% in men, and from 7 to 1% in women). However, there is no published evidence to suggest that the bioavailability of riboflavin from different foods differs significantly. A recent study of riboflavin bioavailability from milk and spinach using isotopically labelled riboflavin and kinetic modelling of absorption was unable to demonstrate a significant difference in riboflavin absorption from these foods<sup>(18)</sup>. This does not rule out possible effects of a reduction in the percentage of riboflavin derived from meat and meat products on EGRAC over the two NDNS.

Today researchers continue to use a variety of methods and are still reporting EGRAC values of less than 1.00<sup>(19,20)</sup>. The results of the present study support the need for a standardised method for EGRAC measurements in nutrition surveys and clear recording of the specific details of the protocol used. Consideration needs also to be given to the appropriateness of the current threshold (EGRAC  $\geq$  1.30) used by many groups to indicate riboflavin deficiency.

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