Original Article

Estimation of inter-individual variability of protein requirement by indicator amino acid oxidation method

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The indicator amino acid oxidation (IAAO) method is a recently developed method to determine the protein requirement and is particularly useful for analyzing human subjects because of its minimal invasiveness. IAAO study is performed using two-phase regression analysis, with the break-point between these phases being the estimated average requirement. However, this method requires that the break-point lie within a certain range in advance, which is in practice difficult. Recently, the change-point regression model (CPRM) has been proposed to be more effective for two-phase regression analysis. There is also a need to re-evaluate the value corresponding to the recommended dietary allowance. Calculation of the recommended dietary allowance requires data on the average requirement and the inter-individual variability of this requirement. However, no inter-individual variability values have been reported in the IAAO method. The aim of this study was thus to estimate the inter-individual variation in protein requirement using CPRM. From seven IAAO studies, the inter-individual variability was estimated as a coefficient of variation of about 20%. The coefficient of variation of the protein requirement determined by IAAO study was wider than the ordinary coefficient of variation obtained from the nitrogen balance test.

Key Words: **protein requirement, change-point regression model, inter-individual variability, indicator amino acid oxidation, break-point**

Proteins are made up of 20 different L-amino acids linked by peptide bonds. They are important components of organisms, and their types differ depending on the number and types of amino acids of which they are composed, and the sequence of the peptide bonds. In humans, proteins cannot be biosynthesized from other nutrients in the body and must be taken in from the environment. Thus, proteins are essential nutrients. When there is a deficiency of proteins, kwashiorkor can occur.[1] When attempting to estimate protein and amino acid requirements, there are two major issues. One is that individuals vary in their demand for, and utilization of, these nutrients provided in the daily diet. The other is that unambiguous indicators of the dietary inadequacy of protein and amino acids can rarely be identified until gross dysfunction has developed.[2] Therefore, the requirement for protein is calculated as the estimated average requirement (EAR) and inter-individual variability, and the estimated recommended dietary allowance (RDA) is set as the dietary reference intake (DRI).[3] At the EAR, 50% of individuals in a group are below their requirement and 50% are above it. However, from a nutritional perspective, it is better to use the RDA value, reflecting the amount that satisfies about 98% of a population. Conventionally, the nitrogen balance method has been used as an experimental method for estimating protein or amino acid requirements.[4] However, in recent years, research using the indicator amino acid oxidation (IAAO) method has attracted attention as an alternative method for determining the requirements for indispensable amino acids.[5] Recently, the IAAO method has been applied not only to estimate the indispensable amino acid requirements but also to estimate protein requirements.[6–14] According to the theory of the IAAO method, if one indispensable amino acid in the diet contains less than the protein metabolic requirements (i.e., limiting amino acid), all other indispensable amino acids cannot be used for protein synthesis. Then, the unused amino acids are oxidized and irreversibly released in exhaled breath as CO_2. For example, consider using [1-^{13}C]phenylalanine (^{13}C-Phe) as an indicator amino acid. If the amount of limiting amino acids is less than the protein requirement, the amount of protein synthesis decreases and the amount of oxidation of the surplus ^{13}C-Phe increases. The carbon skeleton of ^{13}C-Phe is excreted as $^{13}CO_2$. This $^{13}CO_2$ excretion decreases until the ingested protein mass increases and the limiting amino acids in the free amino acid pool are equal to the protein requirement. If the limiting amino acids are supplied at a level greater than the amount required for the protein to be synthesized, there is no need to synthesize the protein anymore, so the excreted amount of exhaled $^{13}CO_2$ from the indicator amino acid will be constant. This break-point can be considered as the dietary protein requirement (Fig. 1).[2,5,15] Therefore, in the IAAO method, it is particularly important to estimate the break-point. The break-point is usually estimated using the two-phase linear regression model by the Seber method, and the 95% confidence interval (CI) of the break-point is calculated by Fieller's theorem.[16,17] To estimate the threshold value of the IAAO, in the models applied in previous studies, any pair of slopes for the test amino acid or protein (explanatory variable) was considered in the two regions before and after the break-point of the test amino acid. The Seber method assumes that the researcher knows a priori that the break-point exists within a certain range. In other words, it is not known whether there actually is a break-point. Hayamizu *et al.*[18] and Kato *et al.*[19] proposed a change-point regression model (CPRM) to resolve this issue, and demonstrated its feasibility by estimating L-lysine requirements. The CPRM has now begun to be applied to the estimation of protein requirements by the IAAO method.[9,11–13] Additionally, in conventional IAAO analysis, the key features are the interpretation of the break-point and the upper limit of its 95% CI. These values obtained by the Seber method and Fieller's theorem are the average of the requirement of protein and the latter is the upper limit of the 95% CI of the average required amount. In other words, nutritionally, those values are only determining the

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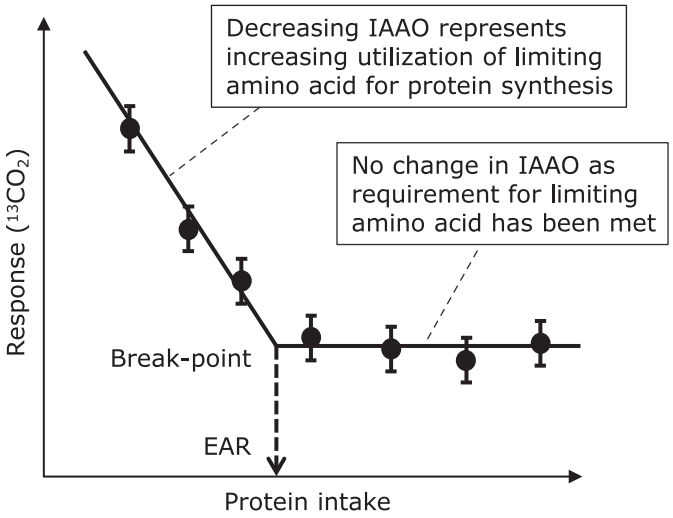

Fig. 1. The oxidation pattern of amino acids in studies using the indicator amino acid oxidation (IAAO) method. The lines represent kinetic responses to graded intakes of the test amino acid. The change-point in the oxidation response has been proposed as the physiological requirement of the test amino acid for the average individual in a population.

EAR and upper limit of the 95% CI of EAR. From a nutritional perspective, it is more important that to determine the RDA value than the EAR. The RDA can be determined using the EAR and information on the inter-individual error of the required amount.[2,3] Against this background, the aim of this study is to estimate the inter-individual variability of the break-point to estimate the RDA of protein based on the IAAO method.

Materials and Methods

Indicator amino acid oxidation studies of protein requirement. Individual data of the IAAO profile are required to estimate inter-individual variability of the break-point. We used IAAO profile data obtained in the following studies to estimate inter-individual variability. A summary of each study is provided below and in Table 1.

1) Elango *et al.*[7] This was a human study investigating the protein requirements of school-age children (6–10 years of age) as determined by the IAAO method, in which the oxidation of ^{13}C-Phe was measured as the indicator amino acid. The requirement value of protein was determined in seven subjects (five boys and two girls) by examining the effects of varying dietary protein intake on phenylalanine flux. Test protein was given as a crystalline L-amino acid mixture based on the composition of proteins in egg. The subjects consumed seven different levels of test protein (0.10, 0.52, 0.90, 1.30, 1.72, 2.10, and 2.50 g/kg/day). The estimated break-point was 1.30 g/kg/day with the upper limit of its 95% CI of 1.55 g/kg/day as determined by Fieller's method. The authors concluded that the RDA was 1.55 g protein/kg/day.

2) Kato *et al.*[8] This study aimed to determine the protein requirements in endurance athletes during an acute 3-day controlled training period using the IAAO method. Six male endurance-trained subjects performed a bout of endurance exercise prior to consuming variable amounts of test protein (0.2–2.8 g/kg/day). The estimated break-point was 1.65 mg/kg/day and the upper limit of its 95% CI was 1.83 g/kg/day. The authors concluded that the recommended protein intake was 1.83 g/kg/day for those undergoing endurance training.

3) Bandegan *et al.*[9] This study aimed to assess the viability of the current dietary protein requirement for young male body-builders (mean age: 22.6 years) on a non-training day. Eight male subjects consumed variable amounts of test protein (0.1–3.5 g/kg/day). The estimated break-point and the upper limit of its 95% CI were estimated as 1.7 and 2.2 g/kg/day, respectively. Therefore, the authors concluded that the RDA for young male bodybuilders was 2.2 g/kg/day.

4) Wooding *et al.*[10] This study aimed to determine the dietary protein requirement in active women performing variable-intensity intermittent exercise. Six women (mean age: 22.1 years) consumed variable amounts of test protein (0.2–2.66 g/kg/day) 8 h after performing the exercise. The estimated break-point and the upper limit of its 95% CI were 1.41 and 1.71 g/kg/day, respectively. Therefore, the authors concluded that the RDA for women performing variable-intensity intermittent exercise was 1.71 g/kg/day.

5) Bandegan *et al.*[11] This study aimed to determine the dietary protein requirement of healthy young endurance-trained men 24 h after exercise by the IAAO method. Eight men were each repeatedly studied 24 h after the exercise with protein intakes ranging from 0.3 to 3.5 g/kg/day. Test protein was fed as an amino acid mixture based on the composition of proteins in egg. The estimated break-point was 2.1 g/kg/day and the upper limit of its 95% CI was calculated as 2.6 g/kg/day.

6) Rafii *et al.*[12] This study was conducted to determine the protein requirement of independently living women aged >65 years by the IAAO method. Twelve subjects participated in this study, with test protein intake ranging from 0.2 to 2.0 g/kg/day. Test protein was given as a crystalline L-amino acid mixture based on the composition of proteins in egg. The estimated break-point and the upper limit of its 95% CI were 0.96 and 1.29 g/kg/day, respectively.

7) Rafii *et al.*[13] This study was conducted to determine the protein requirements of men aged >65 years. Six subjects were studied and each individual was tested seven times with test protein intake ranging from 0.2 to 2.0 g/kg/day. Test protein was given as a crystalline L-amino acid mixture based on the composition of proteins in egg. The estimated break-point and the upper limit of its 95% CI were 0.94 and 1.24 g/kg/day, respectively.

Table 1. Characteristics of IAAO studies for protein requirement

Study	Dose (g/kg/day)	Subject	*n*	Age (years)	Height (cm)	Body weight (kg)	REE (kcal/day)	Break point (g/kg/day) [upper 95% CI]
Elango 2011[7]	0.1–2.56	Healthy children	7	8.4 (1.4)	132.6 (19.8)	31.9 (11.4)	1,147 (289)	1.30 [1.55]
Kato 2016[8]	0.2–2.8	Healthy adult male (athlete)	6	28 (4)	173.3 (4.0)	64.5 (10)	1,624 (274)	1.53 [1.70]
Bandegan 2017[9]	0.1–3.5	Healthy adult male (athlete)	8	22.5 (1.7)	170 (10)	83.9 (11.6)	1,871 (26)	1.70 [2.20]
Wooding 2017[10]	0.2–2.66	Healthy adult female (athlete)	6	21.2 (1.96)	171.9 (3.4)	68.8 (4.1)	1,684 (59)	1.41 [1.71]
Bandegan 2019[11]	0.3–3.5	Healthy adult male	8	26.6 (5.8)	—	68.2 (2.0)	1,698 (—)	2.10 [2.60]
Rafii 2015[12]	0.2–2.0	Healthy elderly female	12	74.3 (7.4)	—	63.2 (9.8)	1,210 (105)	0.96 [1.29]
Rafii 2016[13]	0.2–2.0	Healthy elderly male	6	71.3 (4.5)	177 (10.3)	87.2 (12.7)	1,560 (220)	0.94 [1.24]

REE, resting energy expenditure. Mean (SD).

Break-point estimation of individuals by CPRM. The test protein dose and ^{13}C-Phe oxidation level of each individual in each study were obtained from the plots of IAAO profiles using the software PlotDigitizer X.[20] Prior to the analysis, we compared the reproducibility of the actual data with the data obtained from PlotDigitizer X using the data from the paper by Zello *et al.*,[21] which reported the raw data of IAAO; and we confirmed that $r = 1$ ($y = 0.9996x + 0.0045$). Therefore, we determined that, using the data obtained from PlotDigitizer X, break-point estimation was feasible. The digitalization of plot data was performed in triplicate, and the average value was used for break-point estimation.

We used CPRM for estimation of the break-point of individuals because it is an analytical method that compensates for the problems of the Seber method.[18] The following is statistical model for IAAO data analysis:

$$y_{id} = \alpha + \beta_1 I\,(x_{id} < x_{bp})\,(x_{id} - x_{bp}) + \varepsilon_{id} \quad \text{(Eq. 1)}$$

$(i = 1, 2, \ldots, n,\ d = 1, 2, ..., \mathrm{D})$

where n is the number of subjects enrolled in the study, D is the dose level of the test protein, y_{id} is the observation at the dose of the test protein of i, x_{id} is the dose level of the test protein of the i-th subject, ε_{id} are random errors that are independently normally distributed with mean 0 and variance σ^2, and $I\,(x_{id} < x_{bp}) = 1$ if x_{id} is equal to or more than x_{bp} and 0 otherwise. x_{bp} is the individual break-point of the i-th subject. This model has one regression slope for x_{id}; the slope is β_1 for x_{bpi} less than x_{cp} and becomes constant at $y = \alpha$ for x_{id} equal to or more than x_{bp}. The model based on Eq. 1 is a special case of widely used linear models, and thus the maximum restricted likelihood method or the maximum likelihood method can provide estimates of unknown parameters other than the break-point.[22] In CPRM, the break-point is determined as a value maximizing the likelihood or the restricted likelihood function over the break-point (profile likelihood).[18,23] The model minimizing the Akaike information criterion (AIC) was regarded as the model with the best fit break-point.[18,24] To check whether the individual break-point was successfully detected in the IAAO study, we compared the model with the following model without a break-point for each individual's data.

$$y_{id} = \alpha + \beta_1 x_{id} + \varepsilon_{id} \quad \text{(Eq. 2)}$$

Comparing the AICs of Eq. 1 and Eq. 2, if AIC of Eq. 1 showed a lower value by 2 or more than that of Eq. 2, Eq. 1 was judged to be a more suitable model than Eq. 2, and it was judged that there was a break-point.

Individual data were excluded according to the following criteria: 1) the break-point was not detected in the test protein intake range by CPRM analysis or 2) the number of individual data points was small (e.g., only two points).

The break-points calculated with CPRM were tested using the Kolmogorov–Smirnov test for analysis of the normality of the data distribution. A p value ≤0.20 was considered statistically significant. All statistical analyses were conducted using R3.6.1.[25]

Results

Initially, we used the CPRM method to estimate the break-point of the entire study group, the results of which are shown in Table 2. In comparison with the break-point values reported in the previous papers, some showed similar values, but one was markedly different.[9] Next, the break-points in individuals were estimated and the inter-individual variability was calculated. The Kolmogorov–Smirnov test indicated tentatively that the individual break-point data were normally distributed ($p = 0.92$). An example of an individual IAAO profile and analytical results are shown in Fig. 2 and Table 3, respectively. In the study by Elango *et al.*,[7] the break-point of subject No. 5 could not be estimated by CPRM in the protein intake range; in other words, the existence of a break-point could not be confirmed, so the data were excluded. The mean, SD, and coefficient of variation (CV) of break-point were 1.46 g/kg/day, 0.14 g/kg/day, and 9.5%, respectively. In the study by Kato *et al.*,[8] subject No. 2 had IAAO data measured at only two points and thus his individual break-point could not be estimated, so his data were excluded. The mean, SD, and CV of break-point were 1.55 g/kg/day, 0.53 g/kg/day, and 34.3%, respectively. In the study by Bandegan *et al.*,[9] the break-points of subjects No. 3 and No. 8 could not be estimated by CPRM in the protein intake range, so their data were excluded. The mean, SD, and CV of break-point were 2.22 g/kg/day, 0.40 g/kg/day, and 18.0%, respectively. In the study by Wooding *et al.*,[10] the break-points of subjects No. 2 and No. 4 were not estimated by CPRM in the protein intake range, so their data were excluded. The mean, SD, and CV of break-point were 1.32 g/kg/day, 0.25 g/kg/day, and 18.7%, respectively. In the study by Bandegan *et al.*,[11] the break-points of subjects No. 5, No. 7, and No. 8 could not be estimated by CPRM in the protein intake range, so their data were excluded. Subject No. 2 had IAAO data measured at only three points, so his individual break-point could not be estimated and his data were excluded. The mean, SD, and CV of break-point were 1.82 g/kg/day, 18.5 g/kg/day, and 10.1%, respectively. In the study by Rafii *et al.*,[12] the break-points of subjects No. 1, No. 3, No. 4, and No. 10 could not be estimated by CPRM in the protein intake range, so their data were excluded. Subjects No. 8 and No. 9 had IAAO data measured at only two or three points, so their individual break-points could also not be estimated and their data were excluded. The mean, SD, and CV of break-point were 1.17 g/kg/day, 0.18 g/kg/day, and 15.5%, respectively. Finally, in the study by Rafii *et al.*,[13] the break-point of subject No. 4 could not be estimated by CPRM in the protein intake range, so his data were excluded. The mean, SD, and CV of break-point were 1.06 g/kg/day, 0.18 g/kg/day, and 23.1%, respectively. Among all of the studies, the minimum CV was 9.5% and the maximum CV was 34.3.[8,9] The weighted mean CV was calculated as 18.5%, which was larger than the CV (12.5%) obtained from nitrogen balance studies.[3]

Table 2. Comparison of break-point and upper 95% CI on reported data in article and re-estimated data by CPRM

Study	Reported data (g/kg/day)		CPRM (g/kg/day)	
	Break-point	95% CI (UL)	Break-point	95% CI (UL)
Elango 2011[7]	1.25	1.55	1.42	1.59
Kato 2016[8]	1.65	1.83	1.65	1.87
Bandegan 2017[9]	1.70	2.20	2.20	2.31
Wooding 2017[10]	1.41	1.71	1.39	1.59
Bandegan 2019[11]	2.10	2.60	1.92	2.15
Rafii 2015[12]	0.96	1.29	1.06	1.23
Rafii 2016[13]	0.94	1.24	1.10	1.26

UL; upper limit.

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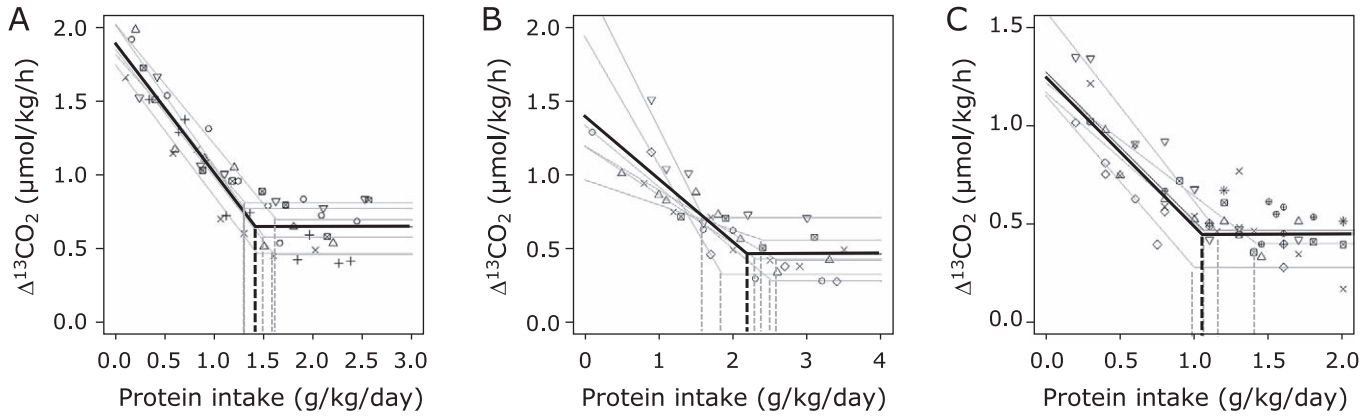

Fig. 2. An example IAAO profile by CPRM. Bold black line indicates the IAAO profile of overall subjects. Gray lines indicate individual IAAO profiles. (A) Elango,[7] (B) Bandegan,[9] (C) Rafii.[12]

Table 3. Inter-individual variability of break-point

Study	Inter-individual variability		
	mean	SD	CV (%)
Elango 2011[7]	1.46	0.14	9.5
Kato 2016[8]	1.55	0.53	34.3
Bandegan 2017[9]	2.22	0.40	18.0
Wooding 2017[10]	1.32	0.25	18.7
Bandegan 2019[11]	1.82	0.19	10.1
Rafii 2015[12]	1.17	18.1	15.5
Rafii 2016[13]	1.06	0.25	23.1

Discussion

In this study, we performed CPRM to determine the inter-individual variability of protein requirements based on the IAAO. We believe that the model based on Eq. 1 is appealing since the amount of indicator amino acid (^{13}C-Phe) oxidation is anticipated to be constant once the metabolite used as the test amino acid is saturated. AIC enables us to perform a data-oriented analysis for an unknown break-point.[18] The data used for estimating inter-individual variability are reproduced data obtained from the seven articles described in Table 1. Only studies reporting IAAO plots that allow individual identification were included.

Prior to estimating inter-individual variability, break-point estimation of the entire study population was performed by CPRM, and one study showed estimates that differed significantly from the original paper (Table 2).[9] It is expected that the reason for this difference is that the analysis was performed using reproduced data. However, the reproducibility was extremely high ($r = 1$) and is not considered to be the cause of the break-point differences reported in the present paper. The break-point estimation in these two papers was performed using mixed-effects CPRM, citing the study by Hayamizu *et al.*[18] The CPRM model used in these two papers was as follows.

$$y_{id} = \beta_0 + b_i + \beta_1 I(x_{id} > x_{bp})(x_{id} - x_{bp}) + \varepsilon_{id} \quad \text{(Eq. 3)}$$

b_i is the within-subject correlation obtained by incorporating a random intercept. Our point of interest about Eq. 3 is the direction of the inequality sign of the indicator function. From the theory of the IAAO profile pattern, the direction of the inequality sign of Eq. 3 is clearly opposite. This may be one of the causes of the difference in the estimated break-point values. The differences in break-points in other studies may be due to differences between the Seber method and CPRM.

Estimation of the inter-individual variation of break-points requires evaluation of the data distribution. Although the number of break-points obtained this time was small and the study conditions were different (especially for studies of athletes), their exhibition of a normal distribution was tentatively indicated; therefore, we performed inter-individual analysis assuming the presence of a normal distribution. It is known that the inter-individual variability obtained by the nitrogen balance test, for which substantial data have been obtained, follows a log-normal distribution.[2] It is necessary to increase the number of reports on the estimation of protein requirement by the IAAO method and to re-examine the data distribution at break-points. The break-point variability was expressed as CV and varied between studies (range: 9.5–34.3%) (Table 3). Kato *et al.*,[8] who reported the highest CV value, performed the IAAO test after applying the exercise training load. The IAAO test has been interpreted to represent the metabolic demand at the time the test is performed. The magnitude of the effects of exercise training differed for each individual, and was therefore speculated to be reflected in the CV value. The CV values of the IAAO studies for athletes performed at 24 h after exercise loading and at rest were as small as 10.1% and 18.0%, respectively.[9,11]

One of the most standard value is the estimation of protein requirement in adult men. Humayun *et al.*[9] reported that the protein requirement of adult men as determined by the IAAO method break-point and the upper limit of its 95% CI were 0.93 and 1.24 g/kg/day respectively. However, the paper did not provide IAAO plot information for individuals and could not be used to estimate inter-individual variability by CPRM. Therefore, we conducted an estimation of the RDA of the adult protein requirement using the CV of the IAAO studies that evaluated adults or the elderly. Three IAAO trials in adults or the elderly, performed in a steady state, were conducted without exercise loading.[9,12,13] The weighted mean of CVs of these studies was 19.0%. The estimated RDA was calculated using the reported EAR and CV as 1.28 g/kg/day, and the value was similar to that reported in the paper by Humayun *et al.*[9] (1.24 g/kg/day).

With regard to this study, there are two issues to be addressed in future work. First, appropriate criteria for the model selection for profiles should be established. From the observation of the individual IAAO profile, in some cases a sloping line, not a horizontal one, was indicated after the break-point (second-phase line) (data not shown). Where the slope of the second-phase line is included in the CPRM, it is known to affect the break-point estimation.[18] However, at present, there are not enough reports to

set the criteria for model selection, so we worked under the assumption that the slope of the second-phase line is 0, as interpreted under the standard theory of the IAAO method. Therefore, it may be necessary to consider increasing the number of application data in the future. Second, it is necessary to consider the case where the break-point of an individual cannot be detected by CPRM, or the case where the individual break-point is an outlier even though the plot of IAAO is sufficiently measured. This issue needs to be addressed in terms of nutritional physiology.

In conclusion, the present study showed that the estimated inter-individual variability of the protein requirement with the IAAO method in adults is about 20% as the CV. The requirement as reflected in the CV determined by IAAO study was wider than the ordinary CV obtained from the nitrogen balance test.

Author Contributions

KH was responsible for conceiving and designing the study and drafting the manuscript. YA performed running of the CPRM program and data reproduction. NI provided physiological advice regarding protein metabolism. MN provided advice on the study and revision of the manuscript.

Abbreviations

AIC	Akaike information criterion
CPRM	change-point regression model
CI	confidence interval
CV	coefficient of variation
EAR	estimated average requirement
IAAO	indicator amino acid oxidation
RDA	recommended dietary allowance

Conflict of Interest

No potential conflicts of interest were disclosed.

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