

OFFICIAL METHODS OF ANALYSIS

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All 70 figures refer to sample. Proximate analysis is needed to adjust diet so that all comparisons between samples and ref. material shall be made with diets having same content of N, fat, ash, moisture, and crude fiber. These suggested levels of fat, ash, moisture, and crude fiber are desirable whenever proximate analysis of sample permits.

B. Experimental Animals

Laboratory rats, males, shall be from same colony, and maintained during period before weaning upon diet and under environmental conditions that will provide for normal development in all respects; weaned; ≥ 21 days of age but ≤ 28 days of age; range of individual rat wts among animals used shall be ≤ 10 g. When animals are transported from breeding colony to test laboratory, acclimation period of ≥ 3 days but < 7 should precede test.

C. Assay Groups

Assemble groups of ≥ 10 rats. In assay of each material provide 1 group that will receive ANRC ref. casein. One ref. casein group may be used for concurrent assay of > 1 assay material. When assembling of all groups is complete, total number of rats in each group must be the same, and av. wt of rats in any 1 group on day beginning assay period must not exceed by > 5 g av. wt of rats in any other group.

D. Assay Period

Throughout assay period keep each rat in individual cage and provide with appropriate assay diet and H_2O *ad libitum*. During assay period maintain all conditions of environment as uniform as possible with respect to each of groups being compared to ANRC ref. casein. Record body wt of each rat on beginning day of assay period and body wt and food intake of each rat at regular intervals, not > 7 days, and on 28th day after beginning of assay period.

E. Calculation and Tabulation of Results

Calc. av. 28 day wt gain and protein ($N \times 6.25$) intake per rat for each group. Calc. Protein Efficiency Ratio (PER) (wt gain/protein intake) for each group. Det. ratio $\times 100$ of PER for each assay group to PER for ANRC casein ref. group. Tabulate 28 day wt gains, protein intake, PER, and ratio $\times 100$ of sample PER to ANRC Ref. Casein PER for each assay group. Report protein quality of sample as ratio $\times 100$ of sample PER to ANRC Ref. Casein PER.

Refs.: JAOAC 43, 38(1960); 48, 847(1965).

982.30

Protein Efficiency Ratio

Calculation Method

First Action

A. Principle

Protein efficiency ratio is calcd from the essential amino acid composition of sample protein (DC-PER) or from both essential amino acid composition and enzymatic digestibility of sample protein (C-PER). Used together, C-PER and DC-PER models are capable of providing reliable ests of protein quality for majority of foods and food ingredients currently in use. Rat bioassay, 936.14, remains official method for detg protein quality; C-PER and DC-PER assays are alternative methods for routine quality control screening of foods and food ingredients. Use of both assays is recommended when estg protein quality to provide internal check. Experience indicates that, in rare cases, the 2 models will report quite different ests of protein quality. When this occurs, it should be regarded as a warning that the sample under analysis is probably:

(1) single-cell protein or protein surrounded by heavy cell walls (e.g., yeast or wheat bran), where DC-PER will overest. protein quality, or

(2) partially or completely predigested proteins (e.g., liq. protein supplements), where C-PER will underest. protein quality or

(3) protein sources known to possess significant quantities of proteolytic inhibitors (e.g., improperly heat-treated soy protein), where DC-PER will overest. protein quality.

For major discrepancies in PER predictions of the 2 models, use rat assay as assay of choice to est. protein quality.

Computational procedures for obtaining C-PER and DC-PER ests are too lengthy for repetitive hand calcn. For routine use of assay, it is recommended that algorithm be placed on computer.

B. Apparatus

(a) *Amino acid analyzer*.—Able to accurately measure individual amino acids at concns as low as 20 nmolar. Must be stdzd using known amino acid stds at least once every 24 h.

(b) *Hydrolysis tubes*.—Any std Kimax/Pyrex test tube or ampule ≥ 15 mL capacity.

(c) *Water-jacketed chamber*.—To fit on stir plate and connected to 37° circulating H_2O bath.

(d) *Water bath*.—55°.

(e) *pH meter*.—Having combination pH electrode and capable of reading to 0.01 pH unit.

C. Reagents

(a) *ANRC reference casein*.—Available from New Zealand Milk Products, 1269 N McDowell, PO Box 80816, Petaluma, CA 94975-8016; or Teklad, a Harlan Sprague Dawley, Inc., Co., PO Box 4220, Madison, WI 53711.

(b) *Amino acid stds*.—ASP, THR, SER, GLU, PRO, GLY, ALA, VAL, MET, ILE, LEU, TRY, PHE, LYS, HIS, AMM, ARG, CYS, and TRP. Available from any amino acid analyzer supply house (e.g., Beckman Instruments, Inc., No. 338088; Pierce Chemical Co., Amino Acid Standard Kit 22, No. 20065).

(c) *Formic acid*.—Add 1 mL 30% H_2O_2 to 9 mL formic acid (88%). Let stand 1 h and cool to 0°.

(d) *Buffer soln*.—Use buffer recommended for sample diln for amino acid analyzer.

(e) *Enzyme solns*.—Use the following enzymes (Sigma Chemical Co.) or their equiv.: porcine pancreatic trypsin (Type IX), porcine intestinal peptidase (Grade I), bovine pancreatic α -chymotrypsin (Type II), bacterial protease (Pronase P or E). *Soln A*.—Dissolve 227 040 BAEE units of trypsin + 1860 BAEE units of α -chymo-trypsin + 0.520 L-leucine β -naphthylamide units of peptidase in 10 mL H_2O . *Soln B*.—Dissolve 65 casein units of bacterial protease in 10 mL H_2O . Store both solns on ice.

(f) *Control protein*.—Suspend 10 g ANRC Na caseinate (a) in 200 mL H_2O and adjust to pH 8 with NaOH. Maintain at pH 8 ≥ 1 h. Freeze-dry and det. N content by Kjeldahl method.

D. Nitrogen Determination

Det. N by 955.04C, 920.39A, 976.05A, or other appropriate Kjeldahl method.

E. Sample Hydrolysis

(a) *Acid hydrolysis*.—Place ca 0.1 g (weigh to 0.1 mg accuracy) sample in hydrolysis tube, add 10 mL 6N HCl, and mix. Freeze in dry ice-alcohol bath. Draw and hold vac. of ≤ 50 μ for 1 min; seal tube under vac. Hydrolyze 24 h at $110 \pm 1^\circ$. Cool, open tube, and filter hydrolysate thru Whatman No. 1 paper; rinse tube 3 times with H_2O and filter each rinse. Dry filtrate at 65° under vac. Dissolve dry hydrolysate in vol.

of buffer appropriate for amino acid analyzer. Store hydrolysate not >1 week before analysis. Use this hydrolysate to det. all amino acids except methionine, cystine and/or cysteine, and tryptophan.

(b) *Performic acid oxidation followed by acid hydrolysis.*—Place ca 0.1 g (0.1 mg accuracy) sample in hydrolysis tube, add 2 mL cold performic acid, and let sit overnight at 0–5°. Add 3 mL cold HBr + 0.04 mL 1-octanol (antifoam); immediately mix contents 30 s in ice-H₂O bath and evap. to dryness at 40° under vac. Add 10 mL 6N HCl to tube and perform acid hydrolysis as described above. This treatment will quant. convert methionine to methionine sulfone and cystine and/or cysteine to cysteic acid. Use this hydrolysate to det. methionine (MET) and cystine/cysteine (CYS).

(c) *Alkaline hydrolysis.*—Place ca 0.1 g (0.1 mg accuracy) sample into glass hydrolysis tube having Nalgene polypropylene centrifuge tube as internal liner. Add 25 mg hydrolyzed potato starch (omit if sample is high in starch). Add 0.6 mL fresh 4.2N NaOH + 0.04 mL 1-octanol. Mix contents 2 min under partial vac. Freeze tube contents in dry ice-alcohol bath. Draw and hold vac. $\approx 50 \mu$ 1 min; seal tube while under vac. Hydrolyze 22 h at $110 \pm 1^\circ$. Cool, open tube, and transfer contents to 5 mL vol. flask contg sufficient cold 6N HCl to neut. hydrolysate; dil. to vol. using buffer appropriate for amino acid analyzer. Centrf. or filter hydrolysate and store frozen. Use this hydrolysate to det. tryptophan (TRP).

F. Amino Acid Analysis

Analyze each of the 3 hydrolysates using parameters optimal for amino acid analyzer being used. Use std amino acid solns to calibrate analyzer at least every 24 h. Each amino acid peak should have $\geq 85\%$ resolution. When alkaline hydrolysate is analyzed, tryptophan must be sepd from lysinoalanine. Compute for each of the following amino acids, the uncorrected g/16 g N: ASP, THR, SER, GLU, PRO, GLY, ALA, VAL, MET, ILE, LEU, TRY, PHE, LYS, HIS, AMM, ARG, CYS, and TRP according to:

$$\begin{aligned} &\text{g amino acid (uncorrected)/16 g spl. N} \\ &= (\eta \text{ moles aa} \times \text{initial spl. vol. (mL)} \times \text{MW aa}) / \\ &\quad (\text{vol. spl. injected (mL)} \times \text{spl. wt (g)} \\ &\quad \times \% \text{N for spl.} \times 6.25 \times 10^3) \end{aligned}$$

Compute percentage recovery by detg N content for each amino acid:

$$\begin{aligned} &\text{g N contributed by each aa/16 g spl. N} \\ &= (14 \times \text{No. of N atoms in aa/MW aa}) \\ &\quad \times (\text{uncorrected g aa/16 g spl. N}) \end{aligned}$$

$$\% \text{ Recovery} = \Sigma (\text{g aa N for each aa/16 g spl. N}) \times 100$$

Note: If percent recovery is <86 or >105, error was made in hydrolysis procedure (weighing errors, diln, instrument calibration) or in computational process of percent recovery. Hydrolysis, analysis, and/or computation of percent recovery must be repeated until percent recovery falls within 86–105 tolerance before proceeding further. Adjust amino acid profile to normalize to 95% hydrolysis:

$$\text{Correction factor} = 95\% / \% \text{ recovery}$$

Note: For each amino acid, compute the corrected g/100 g protein by:

$$\begin{aligned} &\text{g amino acid/16 g N (corrected)} \\ &= \text{correction factor} \times \text{g aa/16 g N} \end{aligned}$$

In Vitro Protein Digestion—For C-PER

Use sample or control wt contg 10 mg N.

Place appropriate quantity of control protein, ANRC Na caseinate (f) or sample, in labeled vial contg mag. stirring bar. Add 10 mL H₂O and let soak 1 h. Using pH meter, 37° bath,

and stirrer, equilibrate sample and control to pH 8 ± 0.03 at 37° by addns of dil. HCl and NaOH. At this time also equilibrate enzyme solns to pH 8 ± 0.03 at 37°. Replace enzymes on ice; hold sample and control at 37°.

To equilibrated control vial, add 1 mL enzyme soln A while stirring. Exactly 10 min after addn of soln A, add 1 mL enzyme soln B, and then transfer vial to 55° H₂O bath. Exactly 19 min after adding soln A, transfer vial back to 37° bath, insert pH electrode, and read pH at 20 min. pH of casein control should read 6.42 ± 0.05 at 20 min. After proper pH reading is obtained for control, carry each sample thru identical procedure and read 20 min pH (X) for each. Calc. % protein digestibility as

$$\% \text{ Digestibility} = 234.84 - 22.56(X)$$

H. Computing the C-PER

Compute C-PER using % digestibility and g amino acid/16 g N of: LYS, MET + CYS, THR, ILE, LEU, VAL, PHE + TRY, and TRP. When combining ME + CYS and PHE + TYR, the CYS and TYR can be no >50% of MET + CYS and PHE + TYR totals, resp. For example, if g amino acid/16 g N were: MET = 2 and CYS = 3, use 4 for MET + CYS total, because max. CYS can only be 50% of MET + CYS total.

Step 1: Express each essential amino acid as percentage of FAO/WHO std:

$$\begin{aligned} \% \text{ FAO} &= [(g \text{ aa/16 g N}) / \text{FAO/WHO std}] \\ &\quad \times \% \text{ digestibility} \end{aligned}$$

where FAO/WHO std is assumed to be: LYS = 5.44, MET + CYS = 3.52, THR = 4.00, ILE = 4.00, LEU = 7.04, VAL = 4.96, PHE + TYR = 6.08, TRP = 0.96.

Step 2: Examine each percentage of FAO/WHO std and adjust as follows: (a) If all percentages are >90% (before rounding to nearest integer) of FAO/WHO std, and LEU is <135% (before rounding to nearest integer) proceed to Step 3; otherwise, (b) if any percentage is >100, reduce to 100 and proceed to Step 3.

Step 3: Compute the following for sample protein and reference casein:

$$X = \Sigma [(1/\% \text{ FAO/WHO for each aa})(\text{wt})]$$

$$Y = \Sigma \text{ wts used}$$

Wts to be used in Step 3 computations:

% FAO/WHO*	Wt
≥ 100	1
91–99	2
81–90	2.83
71–80	4
61–70	5.66
51–60	8
41–50	11.31
31–40	16
21–30	22.83
11–20	32
0–10	45.25

* Round to nearest integer.

Step 4: Divide the sum of wts (Y) by sum of reciprocals (X) for both sample protein and ref. casein. Results will be termed essential amino acid scores for sample and casein.

Step 5: Divide score of sample by score of ref. casein. Result expresses sample as the ratio of ref. casein, and is termed RATIO. If RATIO is >0.99 and <1.01, then PER of sample is 2.5 and program should terminate at this point, i.e., the sample is casein or its equiv.

Step 6: Compute the following: $Z = \text{RATIO} \times 2.5$.

Step 7: Compute 4 discriminant values to det. group into which sample is to be classified. Discriminant equations are:

$$\begin{aligned}\text{Group 1} &= -671.8418 - 6.57689(\text{LYS}) + 3.56696(\text{MET} + \text{CYS}) + 13.10145(\text{THR}) + 2.54503(\text{ILE}) + 16.9981(\text{LEU}) - 0.43395(\text{VAL}) - 11.5244(\text{PHE} + \text{TYR}) + 31.55321(\text{TRP}) + 14.59278(\text{Digestibility}) \\ \text{Group 2} &= -666.4492 - 2.78584(\text{LYS}) + 5.17441(\text{MET} + \text{CYS}) + 13.08564(\text{THR}) + 4.61808(\text{ILE}) + 16.22603(\text{LEU}) - 1.63223(\text{VAL}) - 10.13673(\text{PHE} + \text{TYR}) + 32.60196(\text{TRP}) + 14.11668(\text{Digestibility}) \\ \text{Group 3} &= -619.0813 - 3.13909(\text{LYS}) + 4.26918(\text{MET} + \text{CYS}) + 10.00988(\text{THR}) - 1.42144(\text{ILE}) + 15.7547(\text{LEU}) + 5.6604(\text{VAL}) - 11.28705(\text{PHE} + \text{TYR}) + 30.49168(\text{TRP}) + 13.79953(\text{Digestibility}) \\ \text{Group 4} &= -744.7122 - 0.37674(\text{LYS}) + 6.03697(\text{MET} + \text{CYS}) + 11.51527(\text{THR}) + 1.63251(\text{ILE}) + 17.29687(\text{LEU}) + 3.0294(\text{VAL}) - 11.5033(\text{PHE} + \text{TYR}) + 37.88725(\text{TRP}) + 14.68169(\text{Digestibility})\end{aligned}$$

Step 8: Compute C-PER by examining the 4 group values computed in Step 7. Choose group number that has largest value and use that number to pick correct C-PER equation. For PER predictions, use following group equations when digestibility was estd by the 4 enzyme procedure:

$$\begin{aligned}\text{Group 1: C-PER} &= 1.12683 - 1.61426(\text{Z}) + 0.99306(\text{Z}^2) \\ \text{Group 2: C-PER} &= -7.25391 + 8.14063(\text{Z}) - 1.79517(\text{Z}^2) \\ \text{Group 3: C-PER} &= 4.30469 - 1.99609(\text{Z}) + 0.45996(\text{Z}^2) \\ \text{Group 4: C-PER} &= 12.75 - 8.21484(\text{Z}) + 1.66016(\text{Z}^2)\end{aligned}$$

1. Computing the DC-PER

DC-PER is computed using steps just described in computing C-PER, with one additional step—percent protein digestibility is computed from amino acid profile instead of being detd via in vitro procedure. Coefficients for discriminant equations (Step 7) and PER predictive equations (Step 8) are also changed.

Compute digestibility from amino acid profile as follows:

Step 1: Compute the 3 group discriminant values for sample and ref. casein.

$$\begin{aligned}\text{Group 1} &= -203.7537 - 2.59402(\text{LYS}) + 9.27153(\text{LEU}) + 19.36964(\text{ASP}) + 4.19676(\text{PRO}) + 12.46035(\text{CYS}) + 34.3075(\text{AMM}) \\ \text{Group 2} &= -150.3707 - 0.78115(\text{LYS}) + 7.6239(\text{LEU}) + 15.46558(\text{ASP}) + 3.8947(\text{PRO}) + 12.79949(\text{CYS}) + 29.74493(\text{AMM}) \\ \text{Group 3} &= -155.9532 + 4.61135(\text{LYS}) + 7.85429(\text{LEU}) + 13.25949(\text{ASP}) + 4.68431(\text{PRO}) - 13.2907(\text{CYS}) + 19.89403(\text{AMM})\end{aligned}$$

Examine resulting discriminant values for sample protein and ref. casein, and choose group number associated with highest discriminant value. Use group number to det. which digestibility equation to use. If for sample, group equation No. 3 has highest value, then use digestibility equation No. 3 below to compute sample digestibility. If ref. casein had highest value from group No. 2 equation, then use digestibility equation No. 2 below to compute digestibility for casein.

Group 1

$$\begin{aligned}\text{Digestibility} &= 67.8263 + 0.60144(\text{LYS}) - 1.73309(\text{LEU}) \\ &+ 2.48377(\text{ASP}) + 2.03523(\text{PRO}) - 0.97312(\text{CYS}) - 6.44299(\text{AMM})\end{aligned}$$

Group 2

$$\begin{aligned}\text{Digestibility} &= 160.5607 + 5.7998(\text{LYS}) - 2.20744(\text{LEU}) \\ &- 7.35627(\text{ASP}) - 0.85275(\text{PRO}) + 6.11058(\text{CYS}) - 14.54944(\text{AMM})\end{aligned}$$

Group 3

$$\begin{aligned}\text{Digestibility} &= 116.5451 + 0.99537(\text{LYS}) - 4.37473(\text{LEU}) \\ &- 0.10243(\text{ASP}) - 0.06304(\text{PRO}) - 0.14005(\text{CYS}) + 3.48679(\text{AMM})\end{aligned}$$

Previous Step 1 (C-PER procedure) now becomes 1-A. Steps 2-6 remain as before.

Step 7: Substitute following discriminant group equations:

$$\begin{aligned}\text{Group 1} &= -350.9675 + 2.34642(\text{LYS}) - 8.60862(\text{MET} + \text{CYS}) - 13.80721(\text{THR}) + 11.71013(\text{ILE}) + 11.7984(\text{LEU}) - 12.10787(\text{VAL}) + 9.68089(\text{PHE} + \text{TYR}) + 46.88927(\text{TRP}) + 7.291(\text{Digestibility}) \\ \text{Group 2} &= -454.6516 + 7.83575(\text{LYS}) - 14.3054(\text{MET} + \text{CYS}) - 15.64592(\text{THR}) + 13.32306(\text{ILE}) + 14.1817(\text{LEU}) - 17.40405(\text{VAL}) + 12.36894(\text{PHE} + \text{TYR}) + 64.39914(\text{TRP} + 8.00712(\text{Digestibility})) \\ \text{Group 3} &= -405.9275 + 5.01252(\text{LYS}) - 8.46439(\text{MET} + \text{CYS}) - 15.014(\text{THR}) + 10.1986(\text{ILE}) + 11.91023(\text{LEU}) - 9.50181(\text{VAL}) + 9.46879(\text{PHE} + \text{TYR}) + 49.43095(\text{TRP}) + 7.78124(\text{Digestibility}) \\ \text{Group 4} &= -488.5569 + 9.3207(\text{LYS}) - 11.36379(\text{MET} + \text{CYS}) - 15.24675(\text{THR}) + 10.60119(\text{ILE}) + 13.93578(\text{LEU}) - 12.14625(\text{VAL}) + 10.15707(\text{PHE} + \text{TYR}) + 63.1489(\text{TRP}) + 8.22588(\text{Digestibility})\end{aligned}$$

Step 8: Substitute the following predictive equations:

$$\begin{aligned}\text{Group 1: DC-PER} &= 1.254 - 2.04932(\text{Z}) + 1.30629(\text{Z}^2) \\ \text{Group 2: DC-PER} &= -4.08594 + 5.125(\text{Z}) - 1.08398(\text{Z}^2) \\ \text{Group 3: DC-PER} &= 4.66406 - 2.29297(\text{Z}) + 0.50586(\text{Z}^2) \\ \text{Group 4: DC-PER} &= 10.44141 - 5.93359(\text{Z}) + 1.13281(\text{Z}^2)\end{aligned}$$

Ref.: JAOAC 65, 798(1982).

974.31

Bioavailability of Iron Rat Hemoglobin Repletion Bioassay First Action 1974

A. Apparatus and Reagents

(a) *Colorimeter*.—Bausch & Lomb Spectronic 20 (Milton Roy Co., Analytical Products Div., 820 Linden Ave., Rochester, NY 14625), or equiv.

(b) *Basal diet*.—(Antibiotic may be added if desired.)

Ingredient	%
Glucose, H ₂ O	49.38
Vitamin-free casein	20.00
Degraded yellow corn meal	15.00
Gelatin	5.00
Corn oil	5.00
Monosodium phosphate	2.00
Calcium carbonate	2.00
KCl	0.50
Iodized salt	0.50
Trace mineral premix, (c)	0.27
Choline chloride	0.15
Vitamin premix, (d)	0.10
<i>DL</i> -Methionine	0.10