

Add the following:

^Rice Protein

DEFINITION
Rice Protein is derived from the bran, germ, and endosperm of *Oryza sativa* L. (Fam. Poaceae). It contains NLT 80.0% of total proteins on the dried basis.

IDENTIFICATION

• **A. PEPTIDE MAPPING**

Tris buffer: 1 M tris(hydroxymethyl)aminomethane prepared as follows. Dissolve 60.57 g of tris base in [water](#) and then dilute with [water](#) to 500 mL.

Solution A: 0.1% [formic acid](#) in [water](#) (v/v)

Solution B: 0.1% [formic acid](#) in [acetonitrile](#) (v/v)

Mobile phase: See [Table 1](#).

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	85	15
0.8	85	15
5.1	50	50
6.4	2	98
7.3	2	98
7.5	85	15
10	85	15

Extraction solution: 5 M urea and 50 mM tris(hydroxymethyl)aminomethane prepared as follows. Dissolve 300.3 g of [urea](#) in [water](#), add 50 mL of *Tris buffer*, and dilute with [water](#) to 1000 mL.

Reducing solution: 1 M dithiothreitol prepared as follows. Dissolve 1.697 g of [D,L-dithiothreitol](#)¹ in 11 mL of water. Aliquot the solution in 1.1-mL portions into 1.5-mL centrifuge tubes. [NOTE—The solution is stable for 2 freeze–thaw cycles if kept at –20° during the frozen part of the cycle.]

Digestion buffer: 100 mM ammonium bicarbonate prepared as follows. Transfer 3.953 g of [ammonium bicarbonate](#) into a 500-mL volumetric flask and dilute with water to volume.

Trypsin stock solution: 50 mg/mL of trypsin from bovine pancreas² in *Digestion buffer*. Aliquot the solution in 1.2-mL portions into 1.5-mL centrifuge tubes. [NOTE—The solution is stable for 2 freeze–thaw cycles if kept at –20° during the frozen part of the cycle.]

Trypsin solution: 500 µg/mL of trypsin in *Digestion buffer*, prepared as a 100-fold dilution of the *Trypsin stock solution*

Alkylating/protection solution: 0.5 M iodoacetamide prepared as follows. [CAUTION—Toxic substance. Use appropriate protection.] For each run per sample, dissolve 185 mg of iodoacetamide in 2 mL of [water](#) and mix. Prepare immediately before use.

Internal standard stock solution: 100 µg/mL prepared as follows. Transfer 40 mg of β-casomorphin 1-4³ to a 500-mL volumetric flask. Dissolve and dilute with phosphate buffered saline (PBS)⁴ to volume. Aliquot approximately 1-mL portions into appropriate vials and store

in a freezer.

Internal standard solution: 2000 ng/mL, prepared as a 50-fold dilution of the *Internal standard stock solution*

Sample solution: Weigh 500 mg of Rice Protein and add 10 mL of *Extraction solution*. Vortex and sonicate for 60 min. Centrifuge for 10 min at 4000 g. Transfer 4 mL of the supernatant to a new conical centrifuge tube and add 150 µL of the *Reducing solution*. Mix and heat at 55° for 30 min in a water bath. Cool to room temperature. Alkylate each sample by adding 1.2 mL of freshly prepared *Alkylating/protection solution*. Mix and store the tube protected from light for 30 min. Add 4 mL of *Digestion buffer* and mix. Add 250 µL of the *Trypsin solution* and incubate overnight at 37°. Add 400 µL of formic acid⁵ to quench the sample digestion. Invert several times carefully to avoid foaming. Centrifuge the sample for 10 min at 4000 g. Pass the supernatant through a 0.22-µm or 0.45-µm nylon syringe filter. Prepare a final sample solution by combining 100 µL of the sample supernatant, 100 µL of the *Internal standard solution*, and 800 µL of *Solution A* in an autosampler vial.

System suitability solution 1: Prepare a protein mixture with approximately 20% each of pea protein concentrate, rice protein concentrate, soy protein isolate, and milk protein concentrate (whey and casein) on the protein basis calculated by [Nitrogen Determination \(461\), Method II](#). Prepare *System suitability solution 1* by weighing 500 mg of this protein mixture and proceed as directed in the *Sample solution* starting at "add 10 mL of *Extraction solution*".

System suitability solution 2: Spike 0.1% each of pea protein concentrate, rice protein concentrate, soy protein isolate, and milk protein concentrate (whey and casein) on the protein basis calculated by [Nitrogen Determination \(461\), Method II](#) into a negative control matrix (branched-chain amino acids containing L-leucine, L-valine, and L-isoleucine in 2:1:1 ratios). Prepare *System suitability solution 2* by weighing 500 mg of protein and amino acid mixture and proceed as directed in the *Sample solution* starting at "add 10 mL of *Extraction solution*".

Chromatographic system

(See [Chromatography \(621\), System Suitability](#).)

Mode: UHPLC

Detector: Triple quadrupole mass spectrometer with electrospray ionization⁶

Scan type: Multiple reaction monitoring (MRM)

Ionization mode: Positive

Transition parameters: See [Table 2](#).

Table 2

Protein Analyte	Peptide	Q1 Mass-to-Charge Ratio (m/z)	Q3 Mass-to-Charge Ratio (m/z)	Collision Energy
Rice (glutelin)	ALPNDVLANAYR	658.9	566.8	26
	LQAFEPIR	487.7	732.5	23
	GDEFGAFTPIQYK	736.9	1024.8	33
Pea (vicilin)	EGSLLLPHYNSR	693.4	773.6	40
	GDFELVGQR	510.8	572.5	26
Soy (glycinin G1)	VLIVPQNFVVAAR	713.4	1001.6	33
	VFDGELQEGR	575.3	903.4	29
	LNALKPDNR	520.8	629.3	32
Casein (α-S1-casein)	FFVAPFPEVFGK	692.9	920.5	29
	YLGYLEQLLR	634.4	991.6	33
	HQGLPQEVLENLLR	880.5	1324.7	51
Whey (β-lactoglobulin)	VYVEELKPTPEGDLEILL QK	1157.1	1453	60



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Protein Analyte	Peptide	Q1 Mass-to-Charge Ratio (m/z)	Q3 Mass-to-Charge Ratio (m/z)	Collision Energy
	VLVLDTDYK	533.3	853.5	23
	LIVTQTMK	467.3	707.4	21
Internal standard (β-casomorphin 1-4)	YPFP	523.3	261.1	30

Ion transfer voltage: 5500 V

Probe temperature: 500°

Interface heater: On

Curtain gas: 20

Ion source gas 1: 40

Ion source gas 2: 50

Collision gas: High

[NOTE—Operating conditions may vary for collision energy, ion transfer voltage, probe temperature, interface heater, curtain gas, ion source gas 1 and 2, and collision gas.]

Column: 2-mm × 15-cm; 4-μm packing [L1](#)

Column temperature: 30°

Flow rate: 0.3 mL/min

Injection volume: 5 μL

Valve schedule: See [Table 3](#).

Table 3

Time (min)	Position	Destination
0	A	Waste
1	B	MS analysis
9	A	Waste

System suitability

Samples: *System suitability solution 1* and *System suitability solution 2*

Suitability requirement: Presence of all peptide peaks in [Table 2](#) in both suitability solutions.

Analysis: Separately inject the *Sample solution* and *System suitability solution 2* into the UHPLC-MS system and record the spectrograms.

Calculate the peak area ratio of the sample:²

$$\text{Result} = R_U/R_{IS}$$

R_U = peak area of analyte

R_{IS} = peak area of the internal standard

Acceptance criteria: Rice Protein is confirmed as present in the sample if its peak area ratio is higher than that of *System suitability solution 2* for all peptide transitions described in [Table 2](#). The presence of other peptides in [Table 2](#) or with an absence of characteristic rice protein peptides needs to be investigated further.

• B. AMINO ACID PROFILE

0.2 M hydrochloric acid: 0.2 M [hydrochloric acid](#) prepared as follows. Transfer 400 mL of water into a 0.5-L volumetric flask, slowly add 8.35 mL of 12.1 M [hydrochloric acid](#), dilute with water to volume, and mix well.

0.1 M hydrochloric acid: 0.1 M [hydrochloric acid](#) prepared as follows. Transfer 750 mL of water into a 1-L volumetric flask, slowly add 8.3 mL of 12.1 M [hydrochloric acid](#), dilute with water to volume, and mix well.