

Dyad Labs Protein Testing for USP Dietary Proteins Expert Panel

May 23, 2019



Outline

- Method Details
- Validation Data
- Testing on USP samples
- Opinion
- Considerations
- Next steps

Sample Preparation

- Weigh sample
- Add 5.0 M urea in 50 mM Tris buffer
- Reduce with 1 M dithiothreitol (DTT) for 30 min
- Alkylate in 0.5 M iodoacetamide for 30 min
- Digest with trypsin solution at 37°C overnight
- Quench with formic acid
- Filter with a disposable syringe and 0.22 μm nylon syringe filter
- Dilute (1:10) with 0.1% formic acid and 200 ng/mL internal standard, β -casomorphin 1-4 peptide

Chromatography

- Shimadzu Nexera
- Mobile phase A: 0.1% formic acid in water
- Mobile phase B: 0.1% formic acid in acetonitrile
- Flow Rate: 0.3 mL/min
- Column: C18, 150 × 2 mm, Synergi 4 μ Hydro 80 Å (P/N 00F-4375-B0; Phenomenex)
- Gradient:

Time (min)	A (%)	B (%)
Start	85	15
0.8	85	15
5.1	50	50
6.4	2	98
7.3	2	98
7.5	85	15
10.0	85	15

Peptide Sources

Common Name	Protein	Species	Peptide
Casein	α -S1-Casein	Bos taurus	Cas-1, Cas-2, Cas-3
Whey	β -Lactoglobulin	Bos taurus	Lac-1, Lac-2, Lac-3
Rice	Glutelin	Oryza sativa	Glu-1, Glu-2, Glu-3
Pea	Vicilin	Pisum sativum	Vic-1, Vic-2, Vic-3
Soy	Glycinin G1	Glycine max	Gly-1, Gly-2, Gly-3

Typical Sciex API 5500 Conditions

Ion Transfer Voltage	5000
Probe Temperature	600
Collision Gas	9
Curtain Gas	45
Ion Source Gas 1	70
Ion Source Gas 2	70
Entrance Potential	5

MS/MS Transitions

Peptide	ID	Peptide Sequence	Recommended Parameters			
			Q1/Q3	DP	CE	CXP
IS	β-Casomorphin	YFPF	523.3 / 261.1	80	30.0	20.0
Cas-1	Milk_1_Pep1	FFVAPFPEVFGK	692.9 / 920.5	110	26.9	45.0
Cas-2	Milk_1_Pep2	YLGYLEQLLR	634.4 / 991.6	120	26.4	25.0
Cas-3	Milk_1_Pep3	HQGLPQEVLNENLLR	880.5 / 970.6	65	41.8	25.0
Lac-1	Milk_2_Pep1	VYVEELKPTPEGDLEILLQK	1157.1 / 501.3	30	58.8	35.0
Lac-2	Milk_2_Pep2	VLVLDTDYK	533.3 / 853.5	80	21.0	47.0
Lac-3	Milk_2_Pep3	LIVTQTMK	467.3 / 707.4	80	20.0	35.0
Glu-1	Pea_2_Pep1	EGSLLLPHYNSR	693.4 / 773.6	120	34.0	41.0
Glu-2	Pea_2_Pep2	GDFELVGQR	510.8 / 572.5	75	25.0	30.0
Glu-3	Pea_2_Pep3	GPIYSNEFGK	556.3 / 844.5	85	24.4	40.0
Vic-1	Rice_2_Pep1	ALPNDVLANAYR	658.9 / 566.8	110	27.6	25.0
Vic-2	Rice_2_Pep2	LQAFEPPIR	487.3 / 732.5	85	23.2	40.0
Vic-3	Rice_2_Pep3	GDEFGAFTPIQYK	736.9 / 1024.8	120	35.0	20.0
Gly-1	Soy_2_Pep1	VLIVPQNFVVAAR	713.4 / 1001.6	120	29.0	45.0
Gly-2	Soy_2_Pep2	VFDGELQEGR	575.3 / 903.4	100	27.2	50.0
Gly-3	Soy_2_Pep3	LNALKPDNR	520.8 / 629.3	95	29.1	40.0

Data Processing

- Raw materials identified using PCR are used to set the Method Reporting Limit (MRL)
- If all three peptides from same protein have larger peak area than the MRL (1000 ppm), a positive identification is made

AOAC Method

- Protein identification method received AOAC First Action status in December 2017
 - AOAC Official Method 2017.11: Identification of Pea, Rice, and Soy Proteins in Raw Materials and Finished Goods
 - AOAC Official Method 2017.12: Identification of Milk Proteins in Raw Materials and Finished Goods

Validation – Selectivity, POI

- Negative Selectivity: Two different matrices, six replicates each, no analyte peaks detected
- Positive Selectivity: Matrix with analyte only for each protein, no other analyte peaks detected, other than the analyte of interest
- Probability of Identification at low concentration: 18 different matrices with MRL at 100 ppm for each (except rice at 500 ppm); all confirmed presence, except rice was 17/18 confirmed.
- Probability of Identification at high concentration: Two different matrices, six replicates each all confirmed presence of analyte at 10,000 ppm.

Validation - Precision

- Low and High concentrated sample was prepared six times. %RSDs for each peptide shown below:

%RSDs	Casein			Whey			Rice			Pea			Soy		
	Pep-1	Pep-2	Pep-3	Pep-1	Pep-2	Pep-3	Pep-1	Pep-2	Pep-3	Pep-1	Pep-2	Pep-3	Pep-1	Pep-2	Pep-3
Low (1% protein)	9	15	15	29	12	16	28	19	179	15	9	3	22	19	66
High (20% protein)	4	5	5	6	8	10	4	5	6	5	7	7	5	5	6

Validation – Reinjection Reproducibility

- Samples were stored at 1-8C for 5 days and then reinjected. The following are % peak areas compared to original:

%Original Peak Area	Casein			Whey			Rice			Pea			Soy		
	Pep-1	Pep-2	Pep-3	Pep-1	Pep-2	Pep-3	Pep-1	Pep-2	Pep-3	Pep-1	Pep-2	Pep-3	Pep-1	Pep-2	Pep-3
#1	108	109	116	127	119	72	115	117	114	117	120	114	115	108	96
#2	107	110	117	122	124	72	116	117	115	105	117	111	111	106	97
#3	104	106	113	120	112	78	119	114	107	103	108	97	116	109	100
Average	106	108	115	123	118	74	117	116	112	108	115	107	114	108	98
%RSD	2%	2%	2%	3%	5%	5%	2%	1%	4%	7%	5%	8%	2%	1%	2%

Validation - Interference

- The following compounds were analyzed in blank matrix and no analyte peaks were found:

- Creatine
- Beta alanine
- Histidine
- Taurine
- Serine
- Citrulline
- Caffeine
- Phenylalanine
- Tryptophan
- Metanine
- Urea
- Methionine
- Isoleucine
- Arginine
- Glycine
- Lysine
- Leucine
- Valine
- Threanine
- Tyrosine

Testing USP Samples – Round 1

- Dyad tested the following samples on 10-23-18:

Dyad SID	Description	Result	Protein
506962	BiPRO	Conform	Whey
506963	Glycomacropeptide (GMP)	Conform	Whey
506964	Alpha-Lactalbumin	Conform	Whey/Casein
506965	Whey Protein Concentrate 80%	Conform	Whey
506966	IsoChill 9000	Conform	Whey/Casein
506967	Hilmar Whey Protein Iso 9000	Conform	Whey
506968	Hilmar Whey Protein Iso 9400	Conform	Whey
506969	Hilmar Whey Protein Conc 8000	Conform	Whey

Testing USP Samples – Round 2

- Dyad tested fish gelatin samples on 03-07-19:

Dyad SID	Description	Result
516694	Fish Gelatin	Does not Conform
516694	Fish Gelatin	Does not Conform

- Some peptides had peaks:
 - Gly-3 (~800% of MRL)
 - Cas-1 (~0.5% of MRL)
 - Cas-2 (~1% of MRL)

Opinion

- Dyad believes that the method is suitable to put into USP whey monograph

Considerations

- LC/MS/MS is becoming a more common instrument in labs that test dietary supplements and foods
- Estimated cost for analysis, including labor, instrumentation, reagents, consumables:
 - \$197/first sample
 - \$31/each additional sample

Next Steps

- Are the peaks in the fish gelatin samples peptides from fish gelatin protein or something else? Does this matter?
- Different protein sources can be added to the method as needed.