

Absolute Quantification of Major Royal Jelly Protein Variants and Simultaneous Adulterant Identification to Assess Royal Jelly Product Quality and Authenticity

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Polymorphism-derived proteotypes named MRJPs are predominant in Royal Jelly (RJ). Each MRJP variant has distinguished health benefits. They may undergo a rapid structural change depending on storage conditions, and efficacies may be modified. Therefore, measuring the individual levels of each MRJP separately instead of the total protein concentration is crucial. In this study, it was aimed to develop an analytical method for the absolute quantification of MRJP variants. A novel MRJP purification workflow was also presented to obtain authentic standards for Native-LC-SEC-UV method development. Firstly, RJ proteome extract was subjected to tandem purification at FPLC. Purities were confirmed by SDS-PAGE. Protein annotation of each purified MRJP was accomplished by in-gel digestion followed by Orbitrap HRMS bottom-up proteomic strategy. Data was processed at Thermo PD software, sequences were retrieved from UniProtKB. Sample analysis was performed using the developed Native-SEC-UV method. In order to observe the effect of storage conditions samples stored at different temperatures were also analysed. Egg white powder, skimmed milk powder, and melamine as potential adulterants were also simultaneously screened. In real samples, MRJP-2 and MRJP-3 results were between 1.1-29.5 mg, and 0.6-22.8 mg respectively. Royalactin was quantified between 0.4-10.4 mg while Apisin was found between 1.2-78.2 mg. It was determined that storage conditions dramatically affect the protein composition and Apisin was the most fragile variant. Elevated temperatures or improper packaging promote protein loss induced by Maillard reaction. Furthermore, it was observed that the lyophilization process can induce MRJP3 to be fragmented and Royalactin to be oligomerized. The total protein amounts of these samples were also analyzed by Bradford, BCA, and Khejdahl assays. It has been observed that these methods have significant disadvantages. The non-protein components could potentially interfere with the accuracy of the measurements resulting in false positives with the BCA and Khejdahl assays. All these total protein assays cannot reflect the protein degradation and not indicate whether proteins are fragmented, denatured, or native form. So, they could not pinpoint improper storage induced protein loss. These results proved that adapting the novel LC-SEC-UV method into the QC workflows can reliably indicate the quality of the RJ products.

Absolute Quantification of Major Royal Jelly Protein Variants and Simultaneous Adulterant Identification to Assess Royal Jelly Product Quality and Authenticity

İsmail Emir Akyıldız (Ph.D.)

Balparmak R&D Center - Analytical R&D Manager
WG Leader of Authenticity & Characterization of Bee Products @ IHC

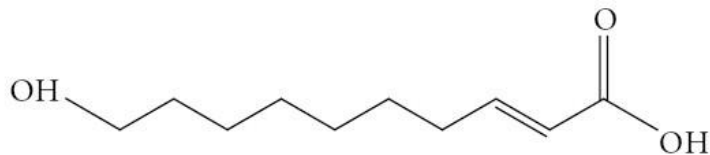
Scientific Contribution : Sinem RADAY, Sezer ACAR, Dilek UZUNÖNER, Emel DAMARLI
emir.akyildiz@balparmak.com.tr

Balparmak R&D Center, Istanbul / Türkiye

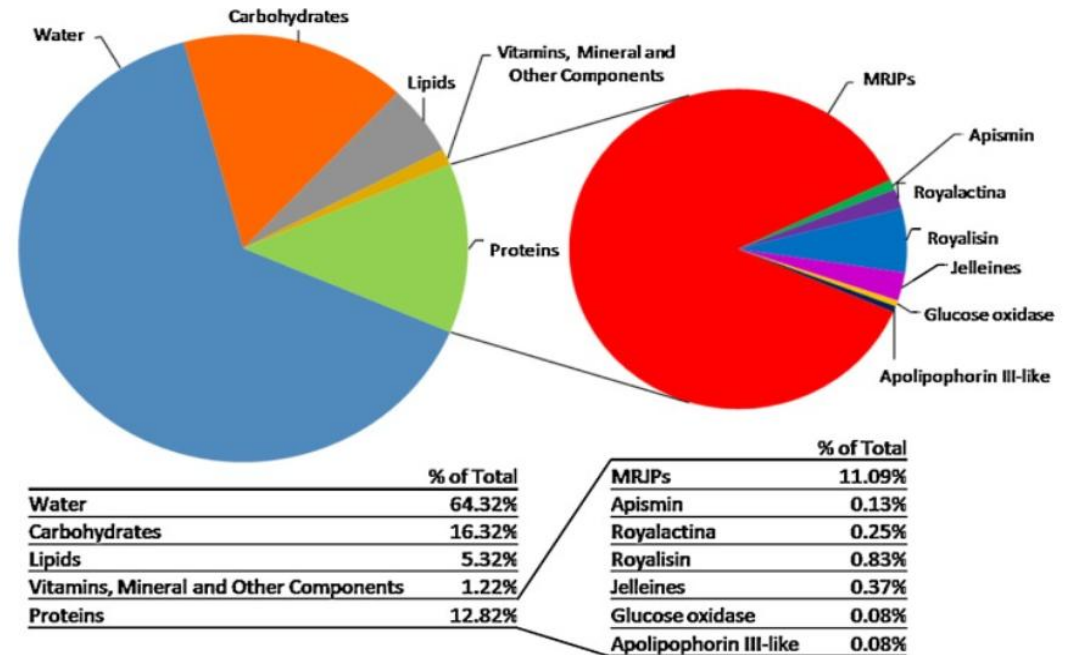


10-HDA% is stable and can be manipulated. Not a reliable quality, authenticity, and freshness indicator...

Total protein content is indispensable quality criteria for RJ !!!



Major Royal Jelly Proteins (MRJPs)

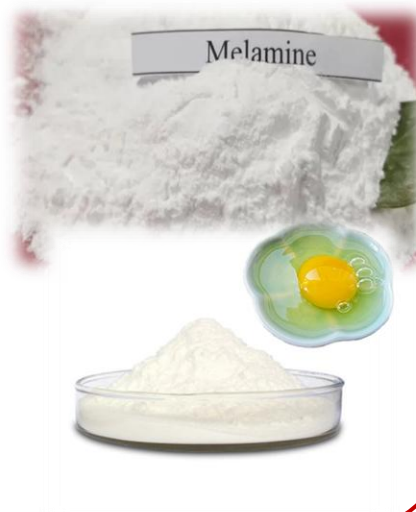


- The Yellow/MRJP protein family;
- 9 proteins encoded by different genes (MRJP-1 to 9).
- Conc. of > MRJP-3 are negligible in RJ...
- MRJPs play an important role in queen bee development and have many beneficial effects on human health !!!

RJ Protein Adulteration (Artificially Increased Protein Content)

Potential MRJP Mimickers

- Melamine
- Milk Powder
- Egg White Powder



PROTEINS

ADULTERATION



New analytical
techniques



Authenticity and
quality control

Commonly Used Assays for RJ Total Protein Quantification



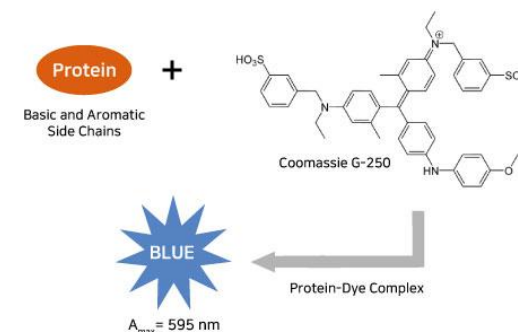
Kjeldahl – Total Nitrogen %

1. Quantifies amino acids, degraded proteins/peptides as well.
2. Does not give any information about the native/bioactive forms of MRJPs.
3. Does not pinpoint improper storage or extended shelflife induced protein loss.
4. Reports false positives.

Colorimetric Assays

Bradford Assay – Dye binding method

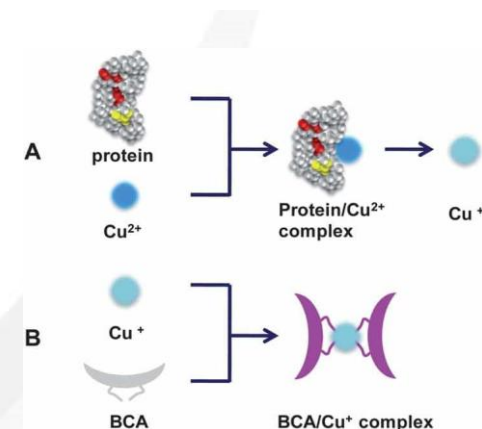
1. Coomassie dye binds to basic and aromatic amino acids, thus similar AA sequence of calibrant is needed for accurate quantification.
2. Reports false negatives.

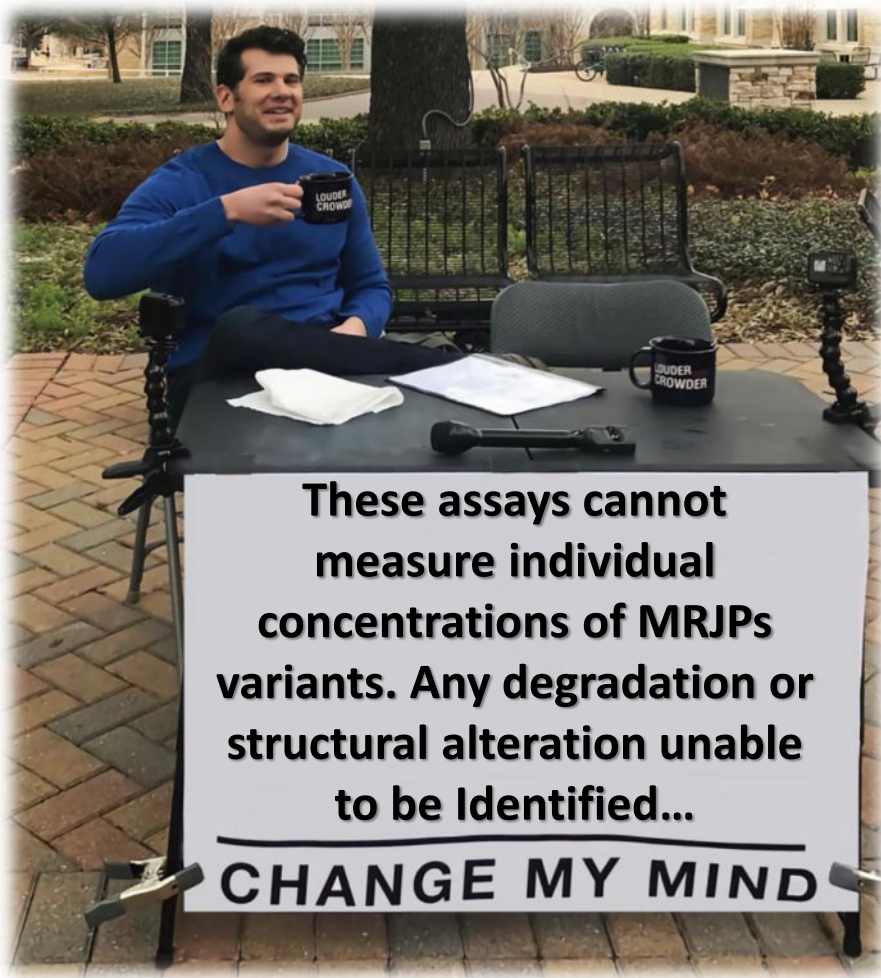


- Lowry Assay
- Biuret Assay
- BCA assay

Peptide bond Cu²⁺ reducing activity

1. Quantifies the degraded proteins/peptides as well.
2. Any reducing condition in final recipe cause false positives.
3. Does not pinpoint improper storage or extended shelflife.



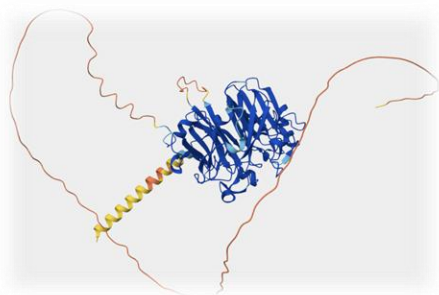




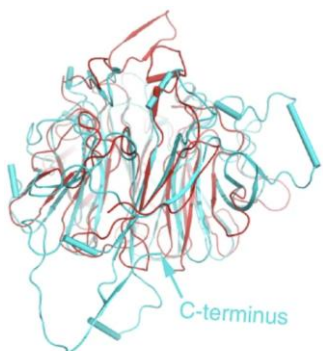
AIM OF THE STUDY

Aimed to develop a single shot, practical analytical method for the quantification of both total protein content and each MRJP variants in their native forms by harnessing novel LC-SEC-UV technique.

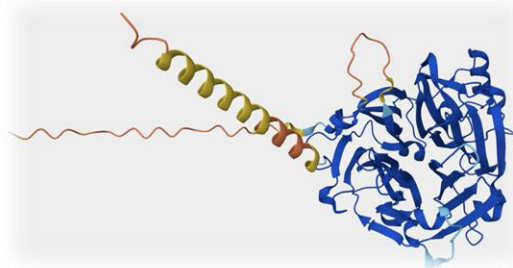
Potential adulterants such as egg white, melamine, and milk powder were also **tried to be monitored simultaneously !!!**



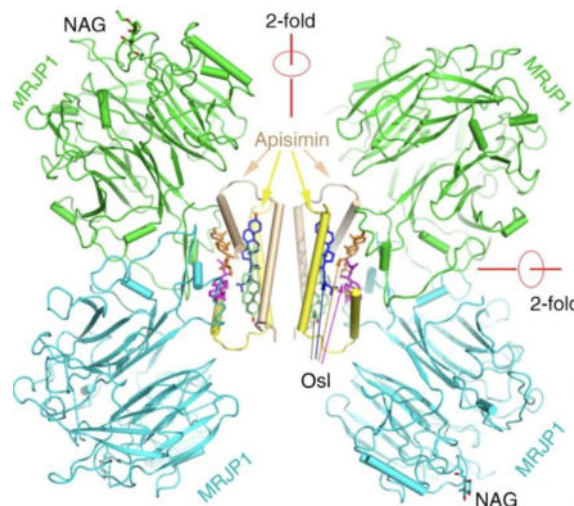
MRJP-3



**MRJP-1 monomer
(Royalactin)**



MRJP-2



**Apisin (A Heteroligomer
complex; MRJP-1 & Apisimin
(conjugation peptide), and
methylenecholesterol)**

A Glimpse of Preliminaries



E><pasy
Compute pI/MW

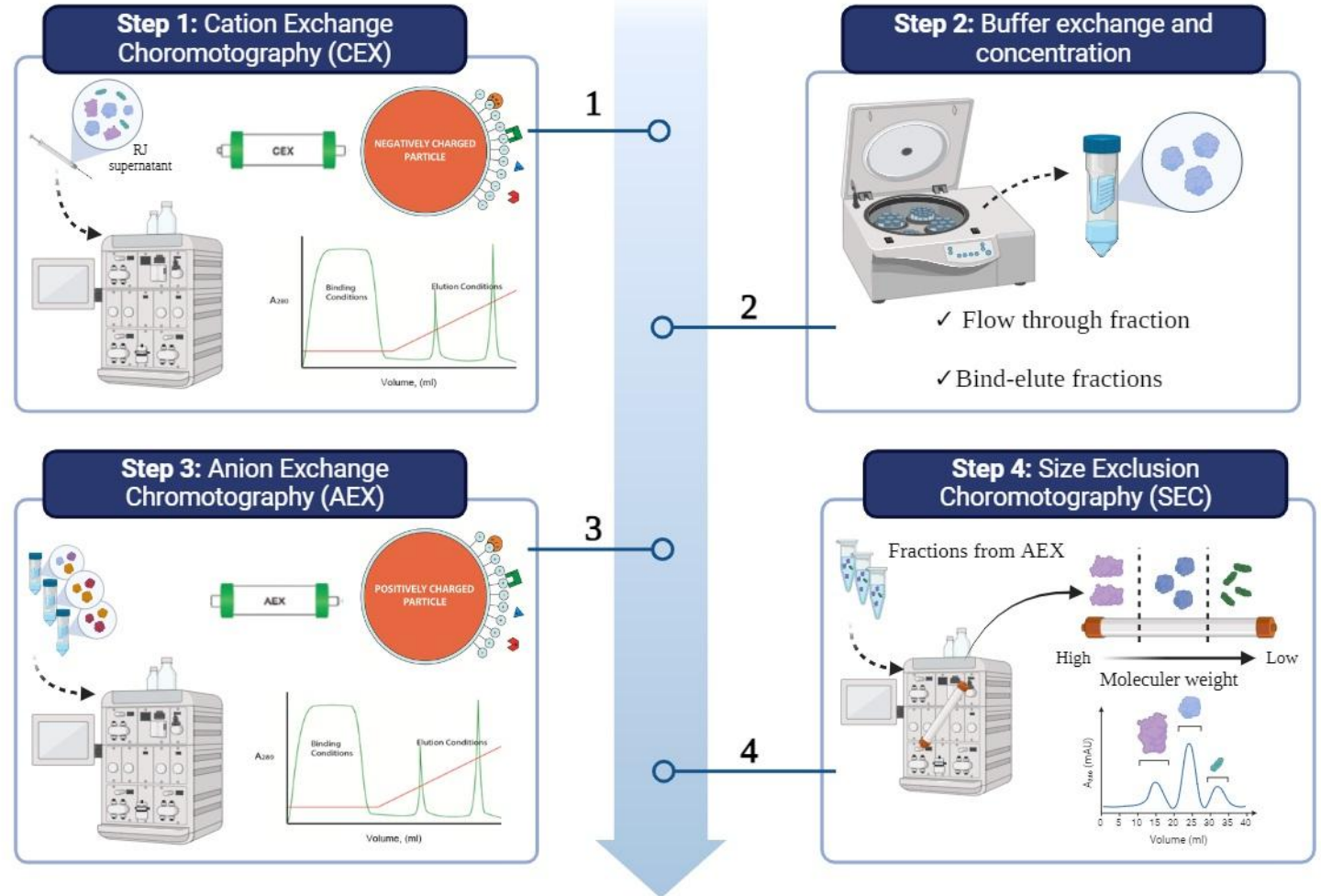
**pI and MW predictions of MRJPs for setting
up the spot on purification strategy...!!!**

Protein accession	Protein name	Theoretical	
		MW (kDa)	pI
O18330	MRJP-1	48.9	5.3
O77061	MRJP-2	51.0	7.2
Q17060	MRJP-3	61.6	6.9
Meta-analysis	Apisin (MRJP-1 Oligomer)	280 - 450	≈ 5.3

3 Steps - Tandem MRJP Purification Scheme

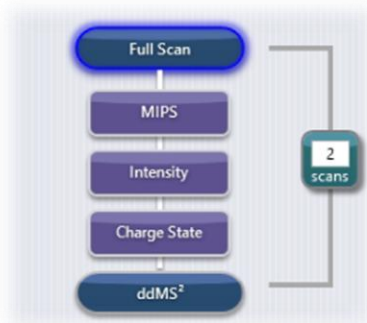
Obtaining the RJ Crude Protein Isolate by extraction
using an acidic buffer (pH 4.6 citrate buffer)

CIPP Purification Strategy

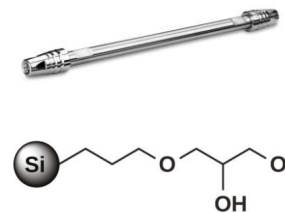
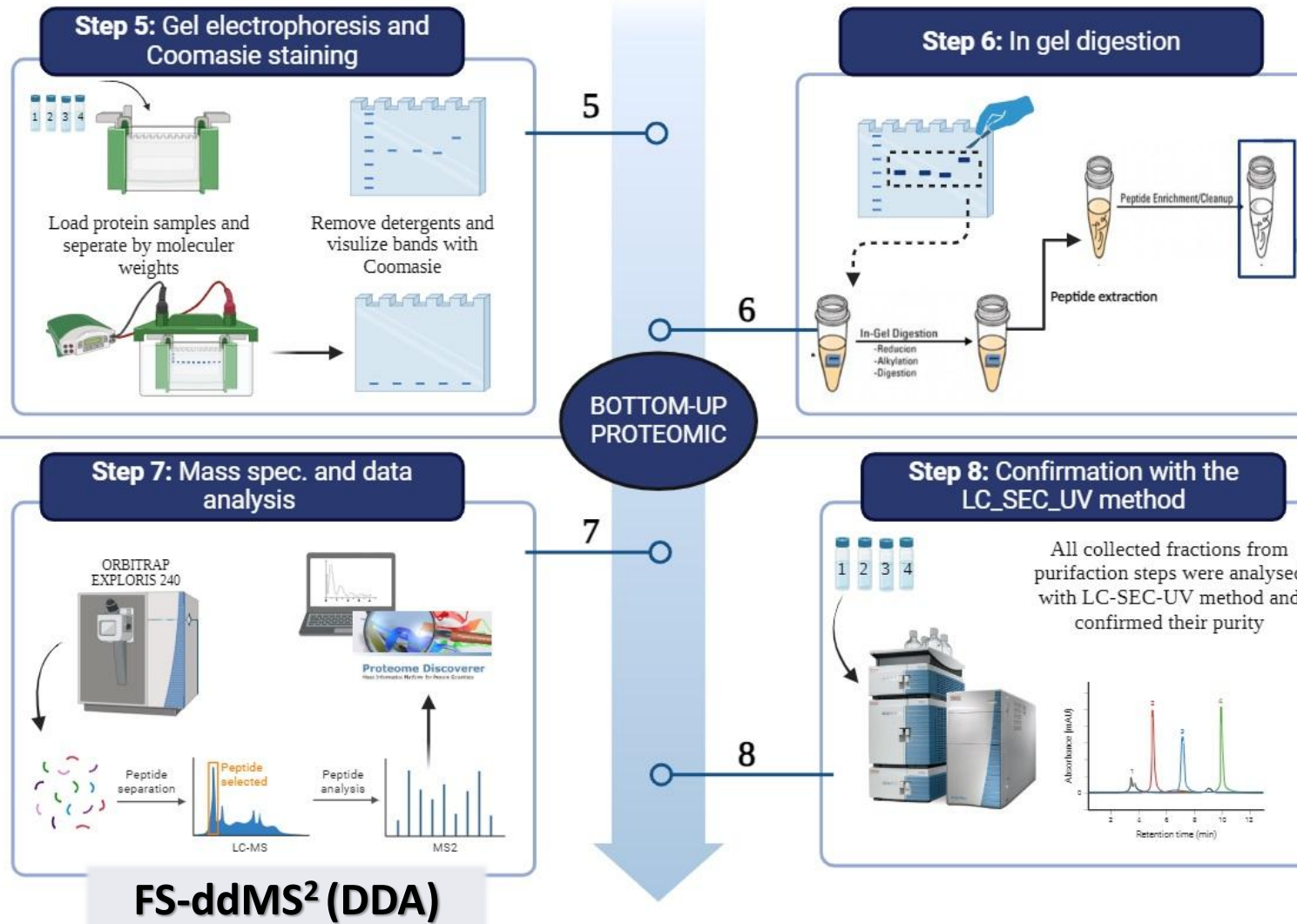


Purity verification

Bottom-up proteomic for annotation and MRJP reference validation



Purity Check and Annotation Experiments for Each MRJP





Proteome Discoverer 2.2

Mass Informatics Platform for Protein Scientists

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This program is protected by copyright law and international treaties as described in Help About.

thermo
scientific

Proteome Discoverer (PD) Software Workflows

Study Definition | Input Files | Samples | Analysis Results | Workflow

Parameters of 'Sequest HT'

Hide Advanced Parameters

1. Input Data	
Protein Database	contaminants.fasta; PD_Contaminants.fasta
Enzyme Name	Trypsin (Full)
Max. Missed Cleavage Sites	2
Min. Peptide Length	6
Max. Peptide Length	144
Max. Number of Peptides Reported	10

2. Tolerances	
Precursor Mass Tolerance	10 ppm
Fragment Mass Tolerance	0.05 Da
Use Average Precursor Mass	False
Use Average Fragment Mass	False

3. Spectrum Matching	
Use Neutral Loss a Ions	True
Use Neutral Loss b Ions	True
Use Neutral Loss y Ions	True
Use Flanking Ions	True
Weight of a Ions	0
Weight of b Ions	1
Weight of c Ions	0
Weight of x Ions	0
Weight of y Ions	1
Weight of z Ions	0

4. Dynamic Modifications	
Max. Equal Modifications Per Peptide	3
Max. Dynamic Modifications Per Peptide	4
1. Dynamic Modification	Oxidation / +15.995 Da (M)
2. Dynamic Modification	None
3. Dynamic Modification	None
4. Dynamic Modification	None
5. Dynamic Modification	None
6. Dynamic Modification	None

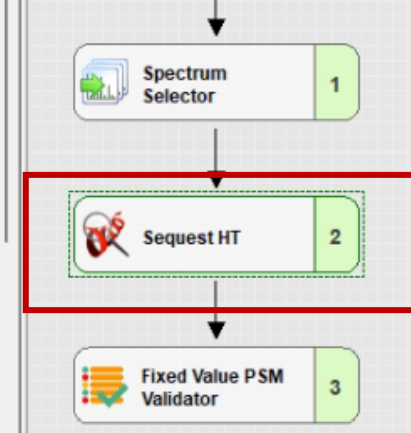
5. Dynamic Modifications (peptide terminus)	
1. N-Terminal Modification	None
2. N-Terminal Modification	None

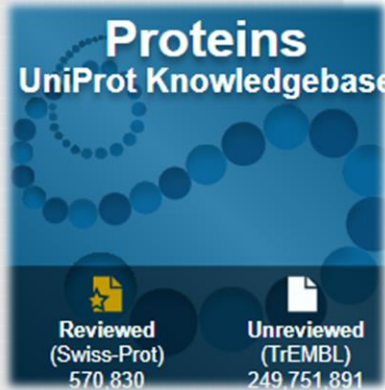
Protein Database
The sequence database(s) to be searched.

Hide Advanced Parameters

1. Input Data	
Protein Database	otkb_apis_mellifera_AND_reviewed_t_2023_09_22.fasta
Enzyme Name	contaminants.fasta
Max. Missed Cleavage Sites	☐ Homo sapiens (sp_canonical TaxID=9606) (v2023-09-22).fasta
Min. Peptide Length	☒ PD_Contaminants_2015_5.fasta
Max. Peptide Length	☒ uniprotkb_apis_mellifera_AND_reviewed_t_2023_09_22.fasta
Max. Number of Peptides Reported	☐ uniprotkb_bovine_AND_reviewed_true_2023_08_10.fasta
	☐ uniprotkb_taxonomy_id_425011_2023_08_10.fasta

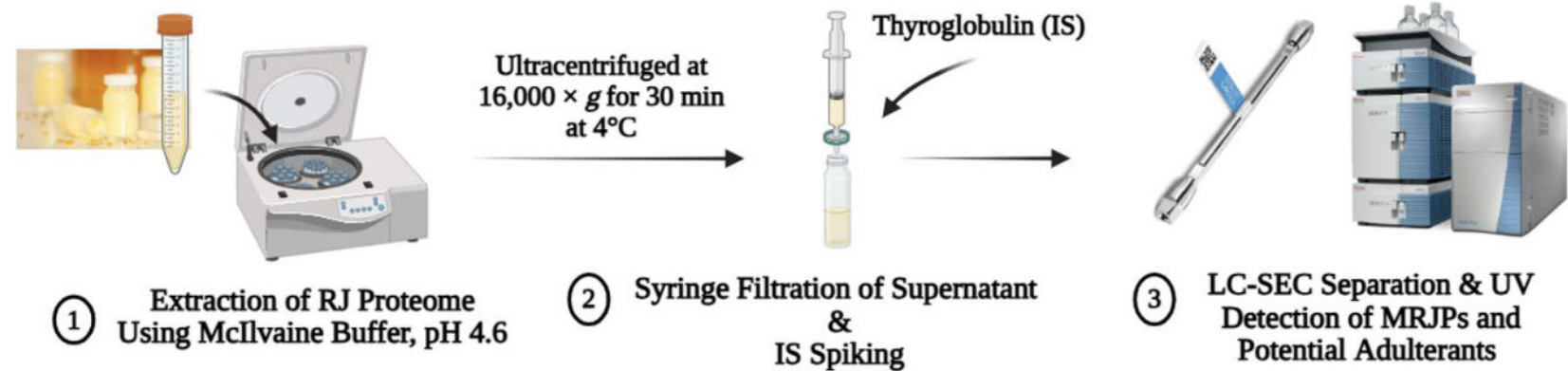
2. Tolerances	
Precursor Mass Tolerance	





Reviewed (Swiss-Prot)	Unreviewed (TrEMBL)
570 830	249 751 891

LC-SEC-UV Method Development and Comparison Assays



Total Protein Quantification of Market Samples Using Traditional Bradford, BCA and Kjeldahl Assays

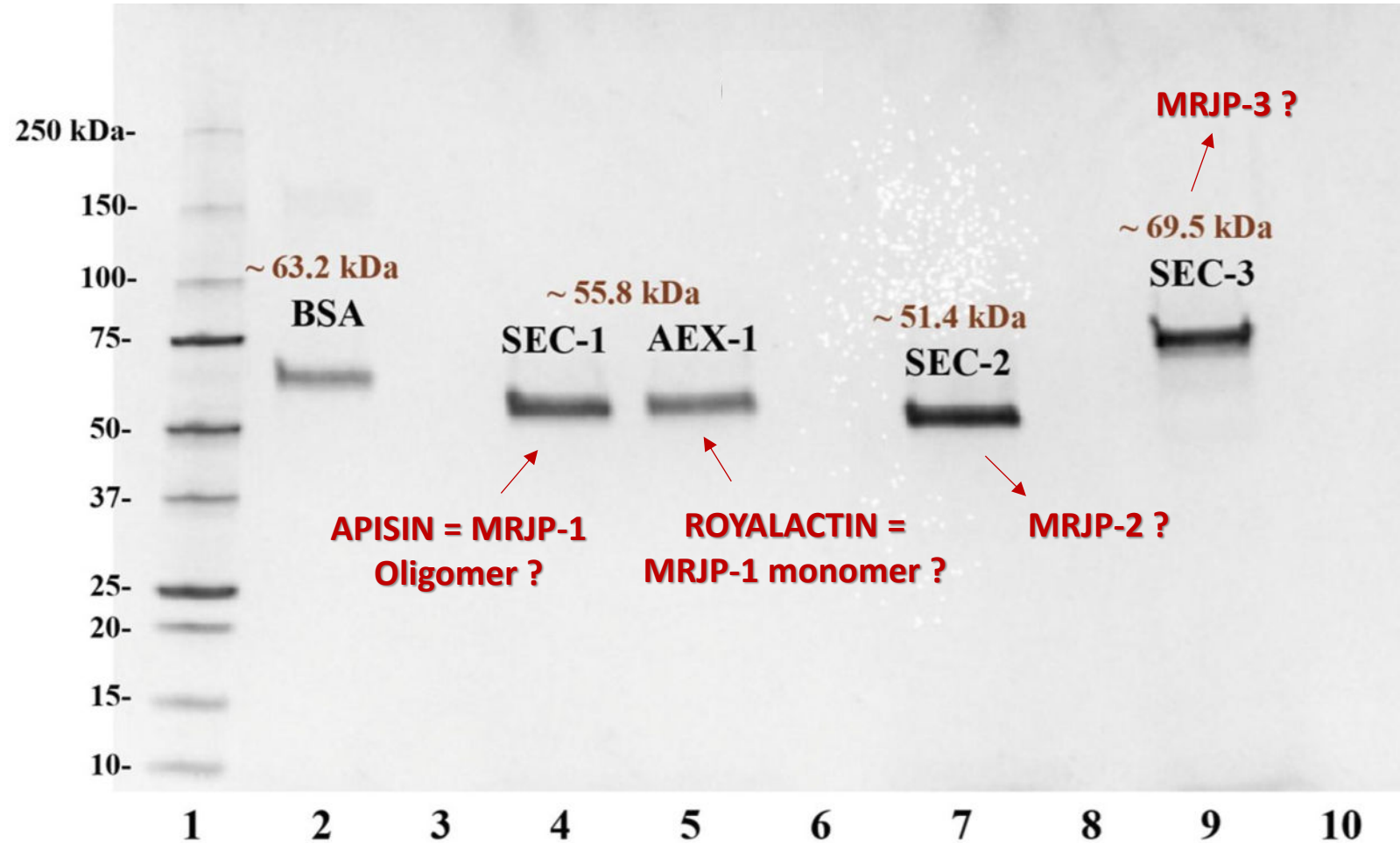
VS

- The Xbridge® Premier Protein SEC column (250 Å, 7.8 × 300 mm, 2.5 µm and MaxPeak Premier Protein SEC Guard column.
- Run time; 70 min
- Flow Rate; 0.25 mL/min
- MP; 2X PBS
- UV Detection; 215 nm
- Inj vol; 20 µL

SDS-PAGE Analysis – Purity Check for MRJPs

Illustration of SDS-PAGE analysis results of each purified protein fraction !!

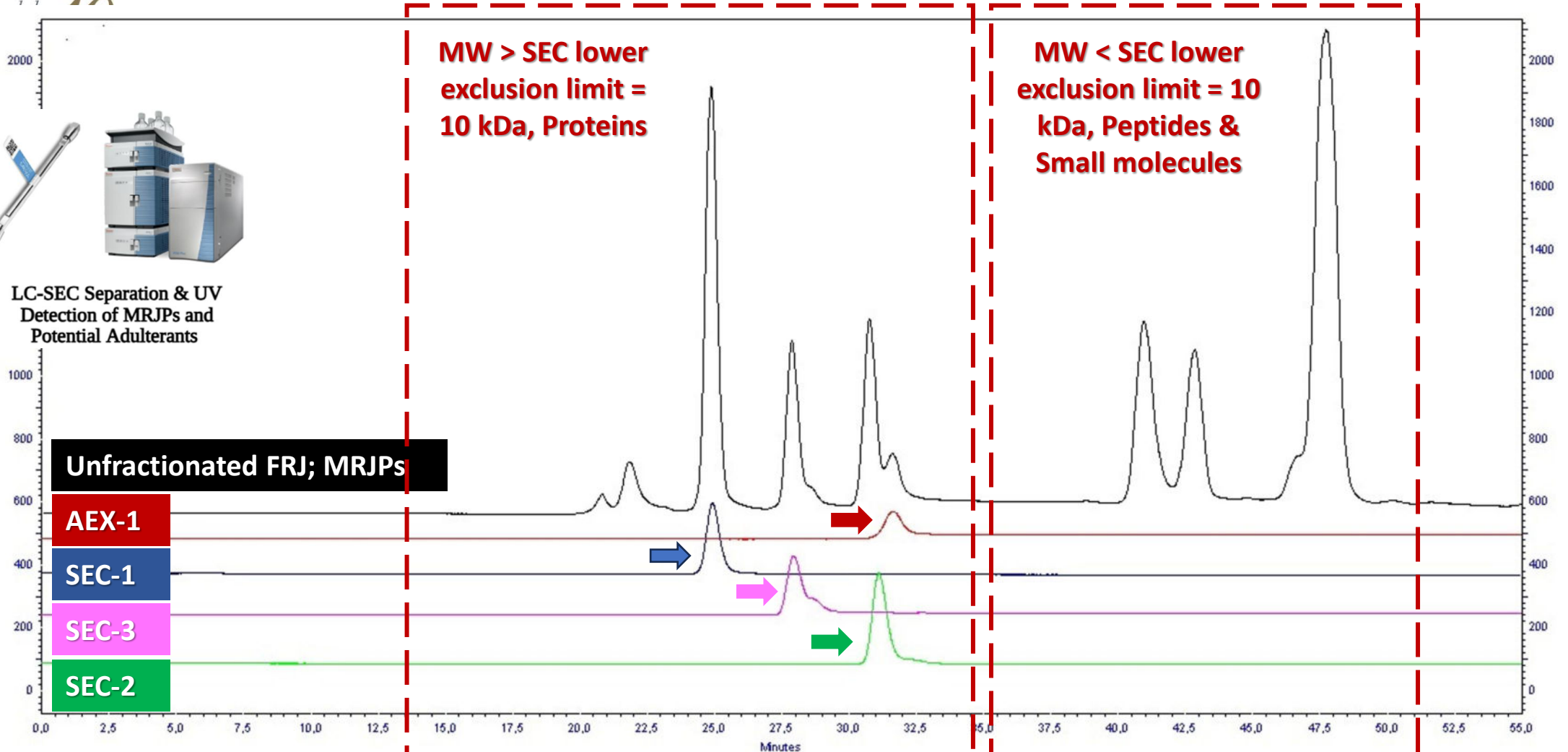
SDS-PAGE
Image – pure
protein bands
indicate
successful
purification
scheme !!!



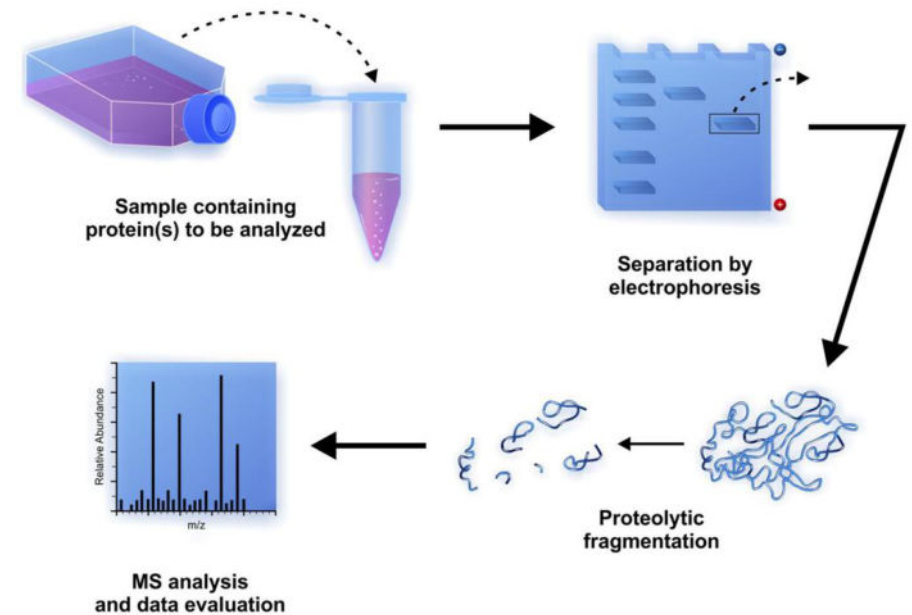
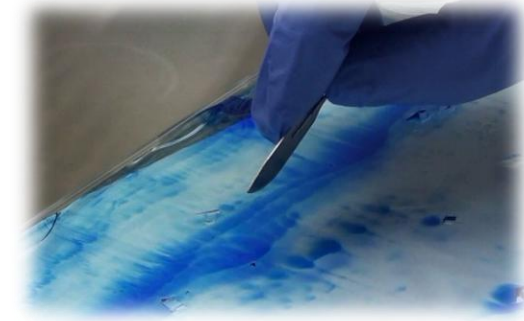
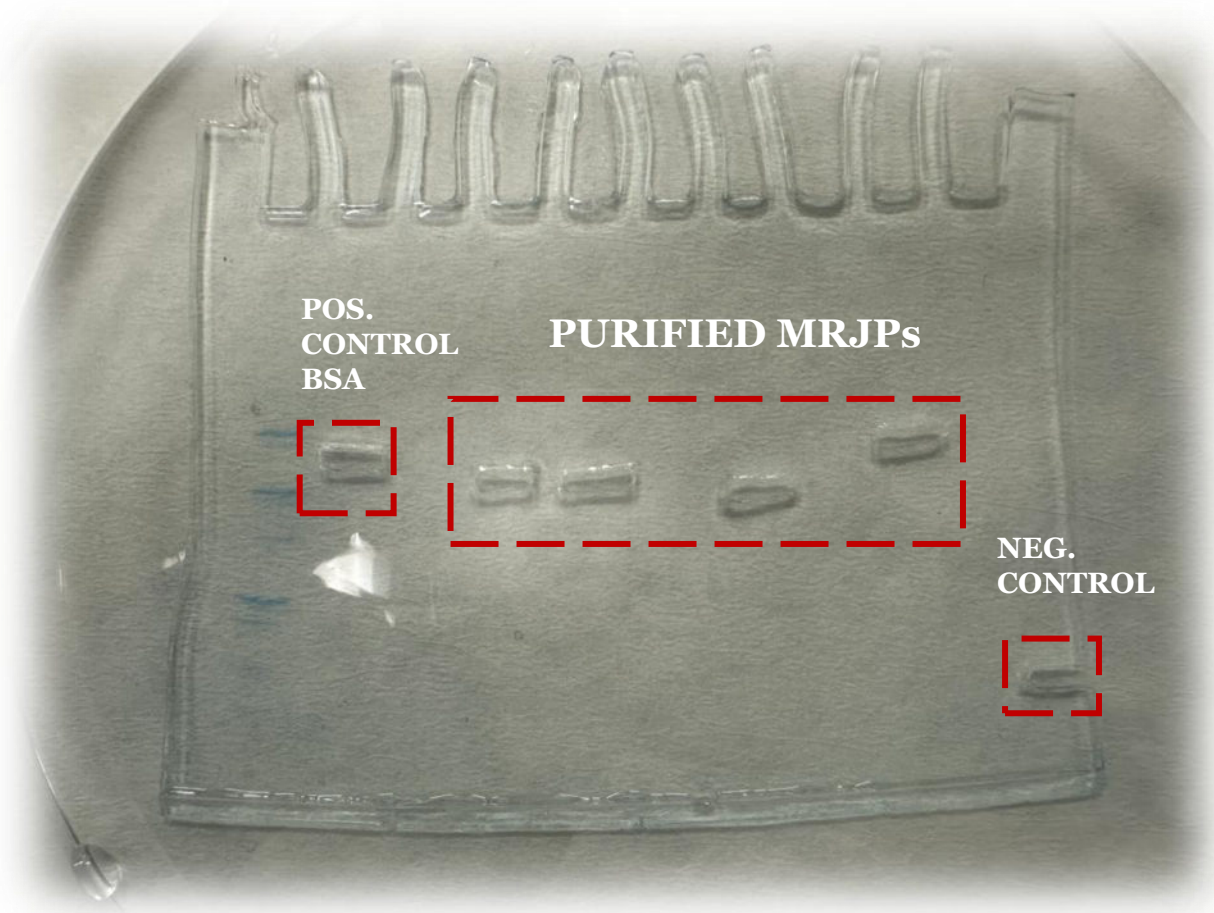
Purity Check-2; Native LC-SEC-UV Method Chromatograms Belongs to FRJ and Pure MRJPs



③ LC-SEC Separation & UV Detection of MRJPs and Potential Adulterants

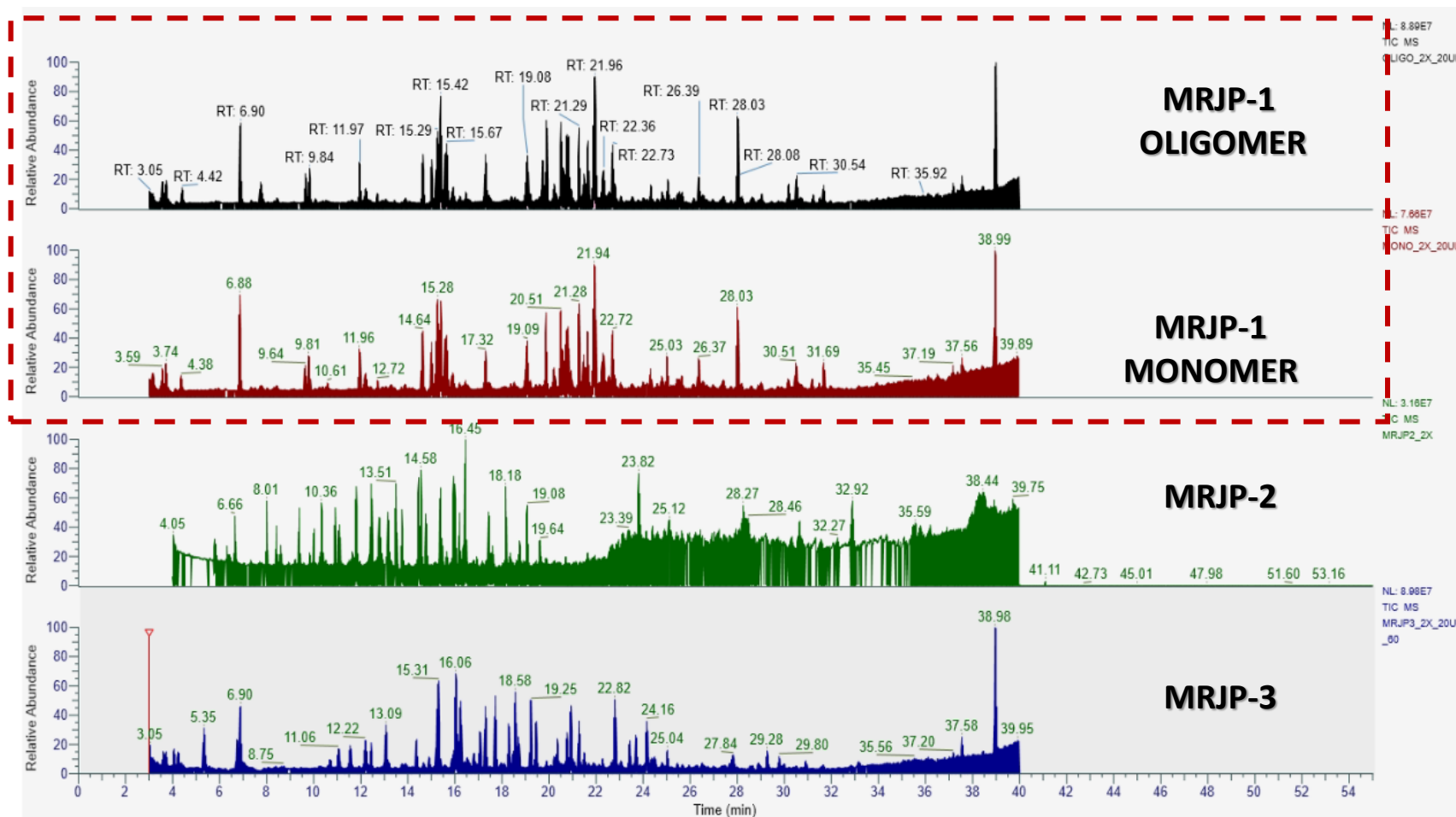


Band Excision Prior to In-gel Digestion Procedure



Bottom-up - Untargeted Proteomics Raw Data

TICs – Full Scan MS1 level



Peptide Mass Fingerprints (PMFs) highlight identical sequences of oligo and mono MRJP-1, while distinct MRJP-2 and MRJP-3 peptide profiles were observed !!!

Proteome Discoverer – Protein Annotation

MRJP-2

Proteins														Protein Groups		Peptide Groups		PSMs		MS/MS Spectrum Info		Input Files		Specialized Traces		Study Information	
	Checked	Master	Accession	Description	Coverage [%]	# Peptides	# PSMs	# Unique Peptides	# AAs	Mw [kDa]	calc. pI	Score S	# Peptides	# Protein Groups													
1			O77061	Major royal jelly protein 2 OS=Apis mellifera OX=7460 GN=	56%	20	163	20	452	51.0	7.27	512.65	20	1													
2			P00761	Trypsin OS=Sus scrofa PE=1 SV=1	25%	4	29	4	231	24.4	7.18	91.43	4	1													
3			P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1	2%	1	1	1	644	66.0	8.12	2.41	1	1													

MRJP-2 Identification

Hide Associated Tables

Protein Groups

Peptide Groups

PSMs

MS/MS Spectrum Info

	Checked	Confidence	Sequence	Modifications	# Protein Groups	# Proteins	# PSMs	Master
1	☒		NTHCVNNNGNDNIQNTNNGDNNQK	1xCarbamidomethyl [C4]	1	1	6	077061
2	☒		SQFGENNVYQGSSEILNTQSLAK		1	1	10	077061
3	☒		IKEELPHFVGSNKPVK		1	1	14	077061
4	☒		IKEELPHFVGSNKPVKDEYMLVLSNR		1	1	4	077061
5	☒		GDALIVYQNADDSFHR		1	1	10	077061
6	☒		GGLVSLAVQAIDLANTLVYMAHDK		1	1	5	077061
7	☒		IVNDDFNFDVNFNR		1	1	12	077061
8	☒		LLKPYPDWFAEFK		1	1	12	077061
9	☒		QAAIQSGEYDHTK		1	1	7	077061
10	☒		QNLEMAQNDR	1xOxidation [M5]	1	1	10	077061
11	☒		DEYMLVLSNR		1	1	2	077061
12	☒		YFDYDFGSEER		1	1	8	077061
13	☒		YDGVPSLTNIVSGK		1	1	7	077061
14	☒		IAIDKFDR		1	2	4	077061
15	☒		QNLEMAQNDR		1	1	2	077061
16	☒		SLNVIHEWK		1	1	9	077061
17	☒		LHVFDLK		1	1	8	077061
18	☒		MTIDGESFTLK		1	1	5	077061
19	☒		TLQMIAGMK		1	1	8	077061
20	☒		MTIDGESFTLK	1xOxidation [M1]	1	1	1	077061
21	☒		NYPFDVDQWR		1	2	8	077061
22	☒		TFVTILR		1	1	6	077061
23	☒		TLQMIAGMK	1xOxidation [M8]	1	1	3	077061
24	☒		TLQMIAGMK	2xOxidation [M4, M8]	1	1	2	077061

☐ Include PSMs that are Filtered Out

Coverage: 55.97%

Found Modifications:

C

O

Carbamidomethyl (C)

Oxidation (M)

Sequence

Modification List

1

MTFWLEMMV

81

ILRYDGVP

161

KTSNHLKQ

241

DGSEFTLR

321

QNSAVGCK

401

LGANVKEL

|--|

Protein Identification Details

Coverage ProteinCard

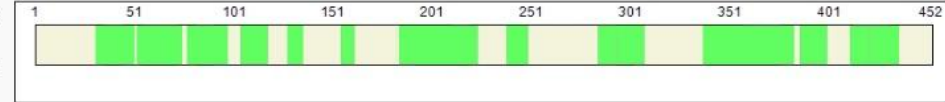
Major royal jelly protein 2 OS=Apis mellifera OX=7460 GN=MRJP2 PE=1 SV=1

- ☐ Annotate PTMs reported in Uniprot
- ☐ Show only PTMs
- ☐ Include PSMs that are Filtered Out

Coverage: 55.97%

Found Modifications:

- C Carbamidomethyl (C)
- O Oxidation (M)



Sequence	Modification List
1	1 11 21 31 41 51 61 71
Modifications 077061	1 MTRWLMVAC LGIACQGAIV RENSPPNLEK SLNVIHEWKY FQYDFGSEER RQAATQSGEY DHTKNYPFDV DQWRDRTFTV
Modifications 077061	81 ILRYDGVPSLT LNVIISGKTGK GGRLLKPYPD WSFAEFKDCS KIVSAFKIAI DRFDRLWVLD SGLVNRTPVP CAPKLEHFDL
Modifications 077061	161 KTSNHLNQIE IPHDIADVAT TKGGLVSLA VQAIDLANTLVYMAHKGDA LIVYQADDS FHLRTSNTFD YDPRYAKMTI
Modifications 077061	241 DGESEFTLNG ICGMALSPVT NNLYSPLAS HGLYYVNTAP FMKSGFGENN VQYQGSSEIL NTQSLAKAVS KNGVLEFGLV
Modifications 077061	321 CNSAVGCWNE HQSLQRQNL E MVAQNDRTIQ MIAQMKIKEE LPHFVGSNKP VKDEYMLVLS NRMQKIVNDD FNFDDVNFRI
Modifications 077061	401 LGANVKELIR NTHCVNNNGN DNIQNTNNGN DNNQKNNKGN ANNQKNNNGN DN

OK Help

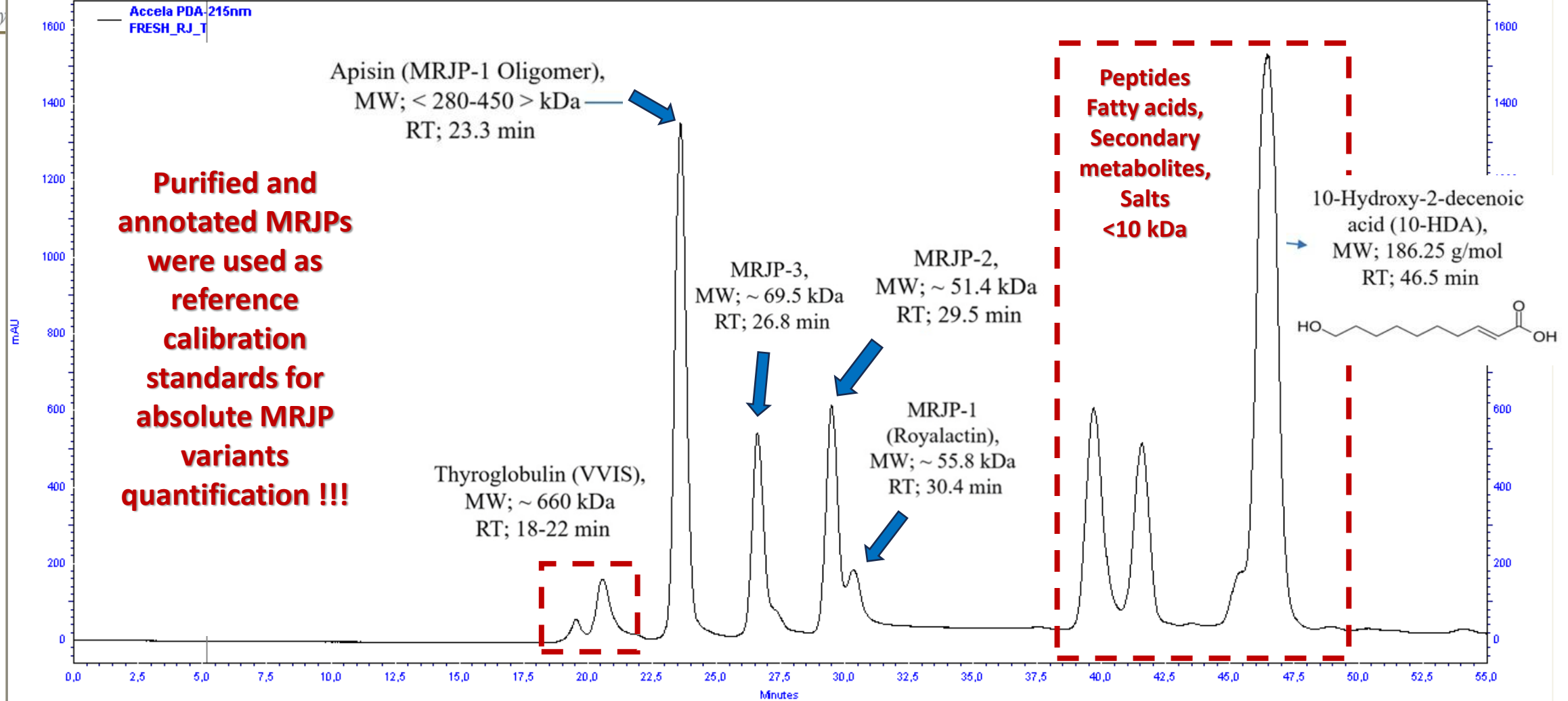
Protein Annotation Overall Success Scores

LC-HRMS identification results of the MRJPs and positive control (BSA)

Protein accession no ¹ .	Protein name ²	Score ³	Theoretical ⁴		No. of unique peptides	PSMs ⁵	Sequence coverage ⁶ (%)	Gel fractions ⁷
			MW (kDa)	pI				
O18330	Major royal jelly protein-1	494.34	48.9	5.34	20	143	57	Lane 4; SEC-1
O18330	Major royal jelly protein-1	428.66	48.9	5.34	20	124	57	Lane 5; AEX-1
O77061	Major royal jelly protein-2	512.65	51.0	7.27	20	163	56	Lane 7; SEC-2
Q17060	Major royal jelly protein-3	729.17	61.6	6.96	19	255	41	Lane 9; SEC-3
P02769	Bovine serum albumin	270.48	69.2	6.18	17	101	36	Lane 2; BSA

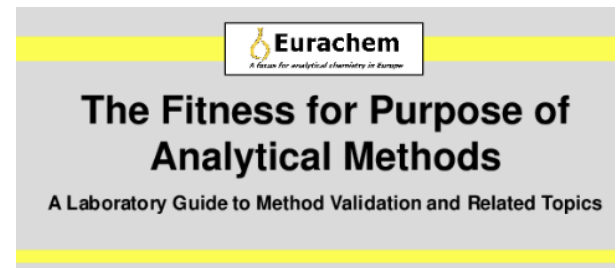
Native LC-SEC-UV Method Final View After Annotations

Dünyay



Validation For Quantitative Native LC-SEC-UV Method

Summary of the method performance results..!!!



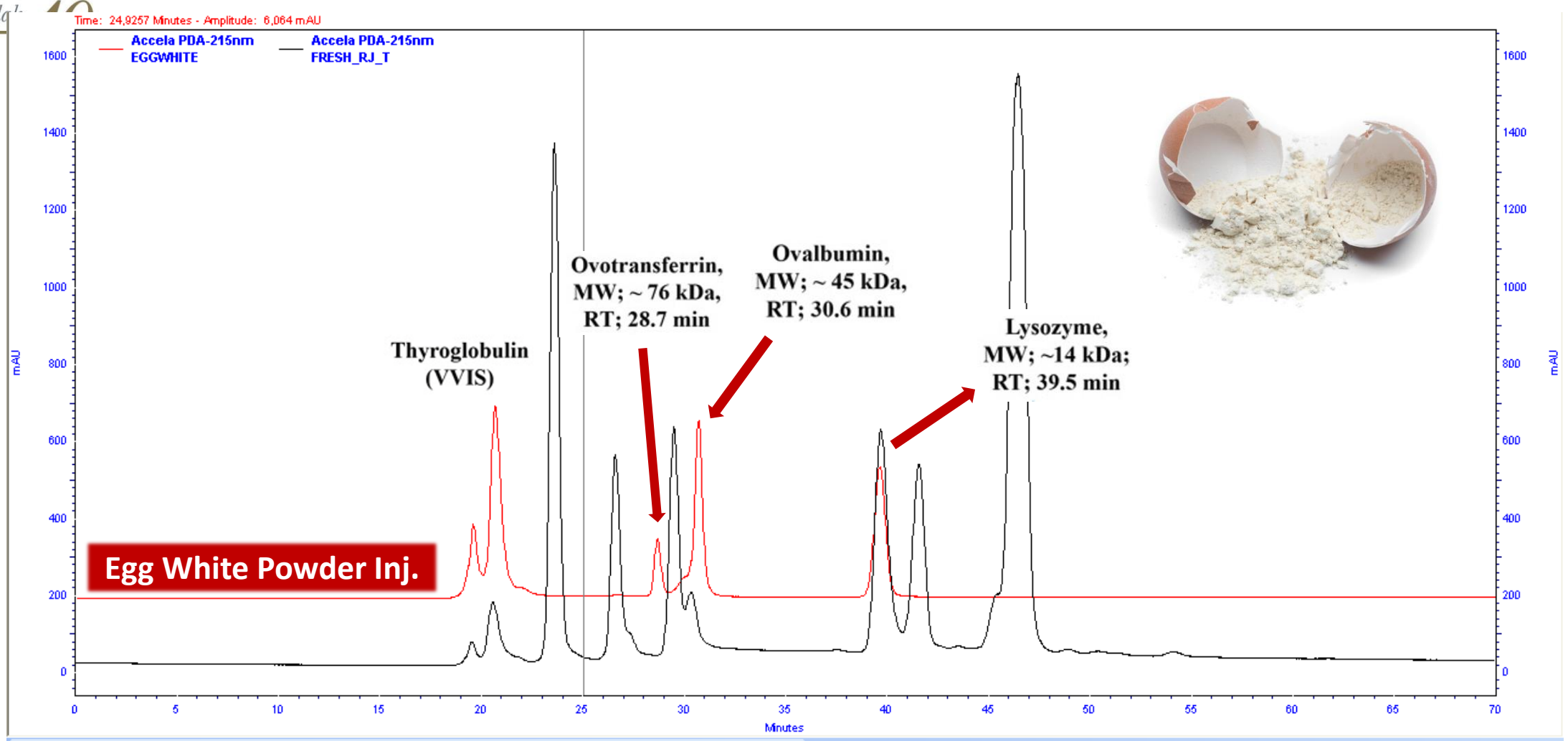
(3rd ed. 2025)

Analytes	Sensitivity		Recovery/Accuracy	Precision		Linearity
	CC α (mg/g)	CC β (mg/g)	Mean, % (n=7), (5.0 mg/g)	mean RSD _I %, (n=7), (5.0 mg/g)	mean RSD _R %, (n=7), (5.0 mg/g)	R ²
MRJP-1 Oligomer	0.7	0.8	94.7	6.5	9.8	0.998
MRJP-1 monomer	0.8	0.9	109.5	7.9	9.2	0.999
MRJP-2	0.7	0.7	98.6	6.6	8.5	0.997
MRJP-3	0.7	0.8	102.2	9.7	10.2	0.998

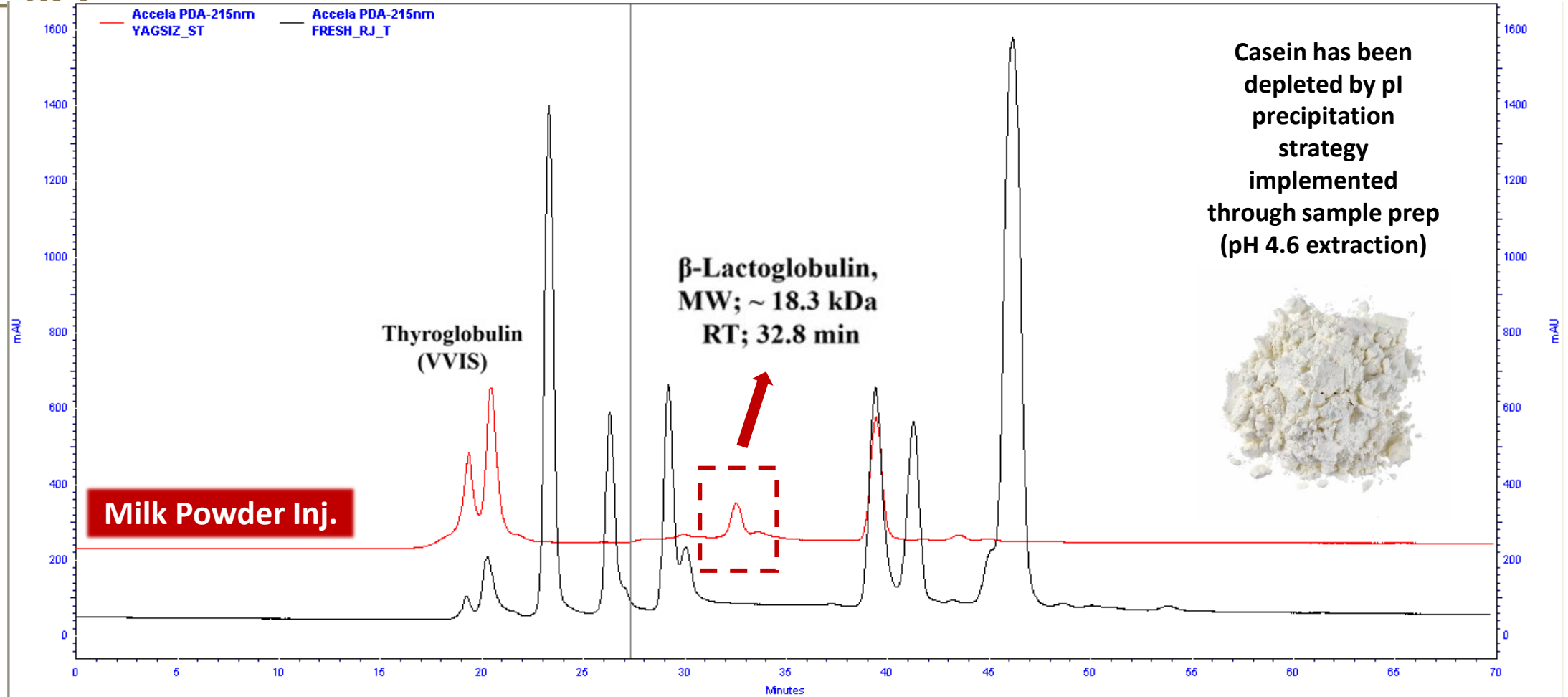
^aCC α ; Decision limit, ^bCC β ; Detection capability, ^cRSD_I; Relative Standard Deviation of Intra-day Precision,

^dRSD_R; Relative Standard Deviation of Inter-day precision.

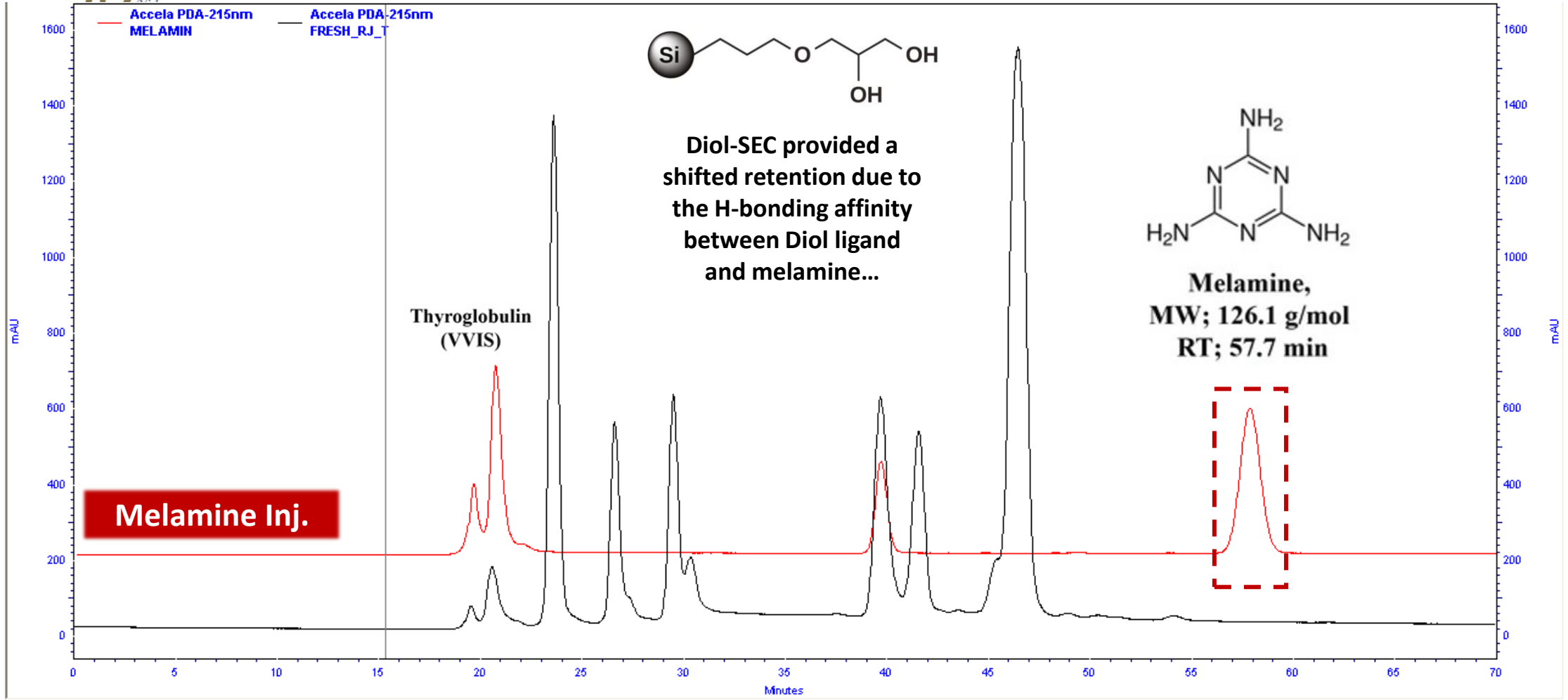
Native LC-SEC-UV Method; MRJP Mimetics Egg White Powder



Native LC-SEC-UV Method; MRJP Mimetics Milk Powder



Native LC-SEC-UV Method; MRJP Mimetics Melamine



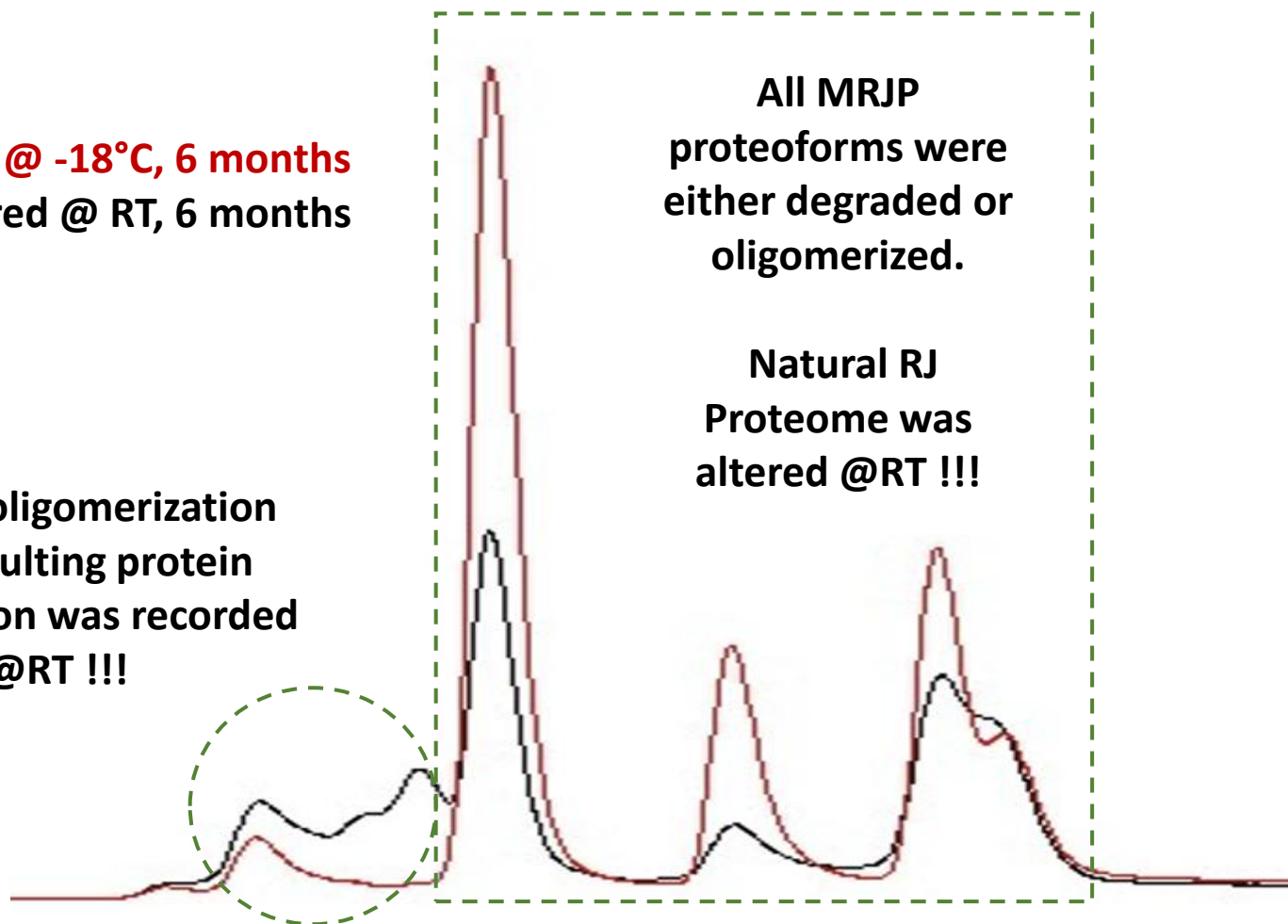
Degredation and Oligomerization Pattern of MRJPs Under Different Storage Conditions

Fresh RJ Stored @ -18°C, 6 months
Fresh RJ Stored @ RT, 6 months

Protein oligomerization
and resulting protein
aggregation was recorded
@RT !!!

All MRJP
proteoforms were
either degraded or
oligomerized.

Natural RJ
Proteome was
altered @RT !!!

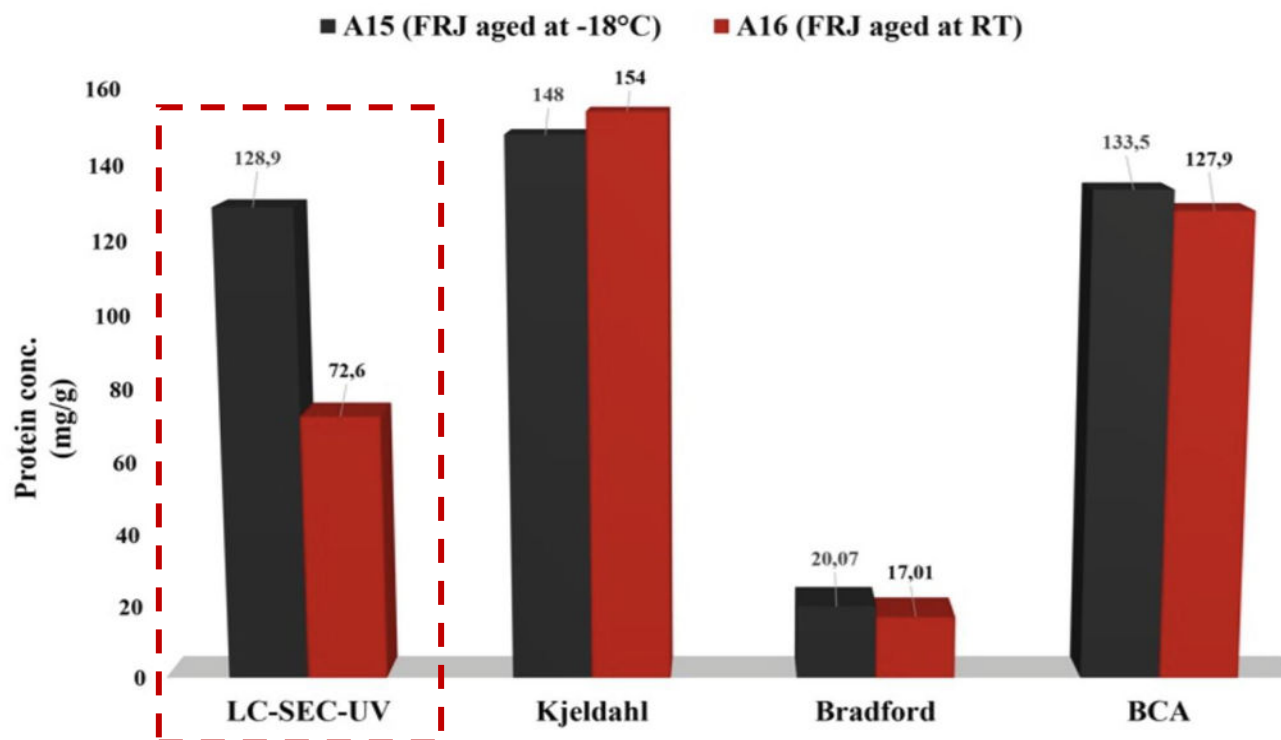


**Occuring Maillard
Reaction and Advance
Glycation of the
Proteins was the root
cause !!!**

Degredation and Oligomerization Patterns of MRJPs under Different Storage Conditions

	Serving Size (mg)	Individual MRJP conc. by novel LC-SEC-UV method (per service, mg)				Total Protein conc. (per service, mg)				Total Protein conc. (per service, %)			
		MRJP 1 Oligomer	MRJP 3	MRJP 2	MRJP 1 monomer	LC-SEC-UV (Novel method)	Kjeldahl	Bradford	BCA	LC-SEC-UV (Novel method)	Kjeldahl	Bradford	BCA
Fresh Royal Jelly (-18°C)	1000	66	22	29	13	128,9	148,0	20,7	133,5	12,9	14,8	2,1	13,4
Fresh Royal Jelly (RT)	1000	30	8	20	15	72,6	154,0	17,1	127,9	7,3	15,4	1,7	12,8

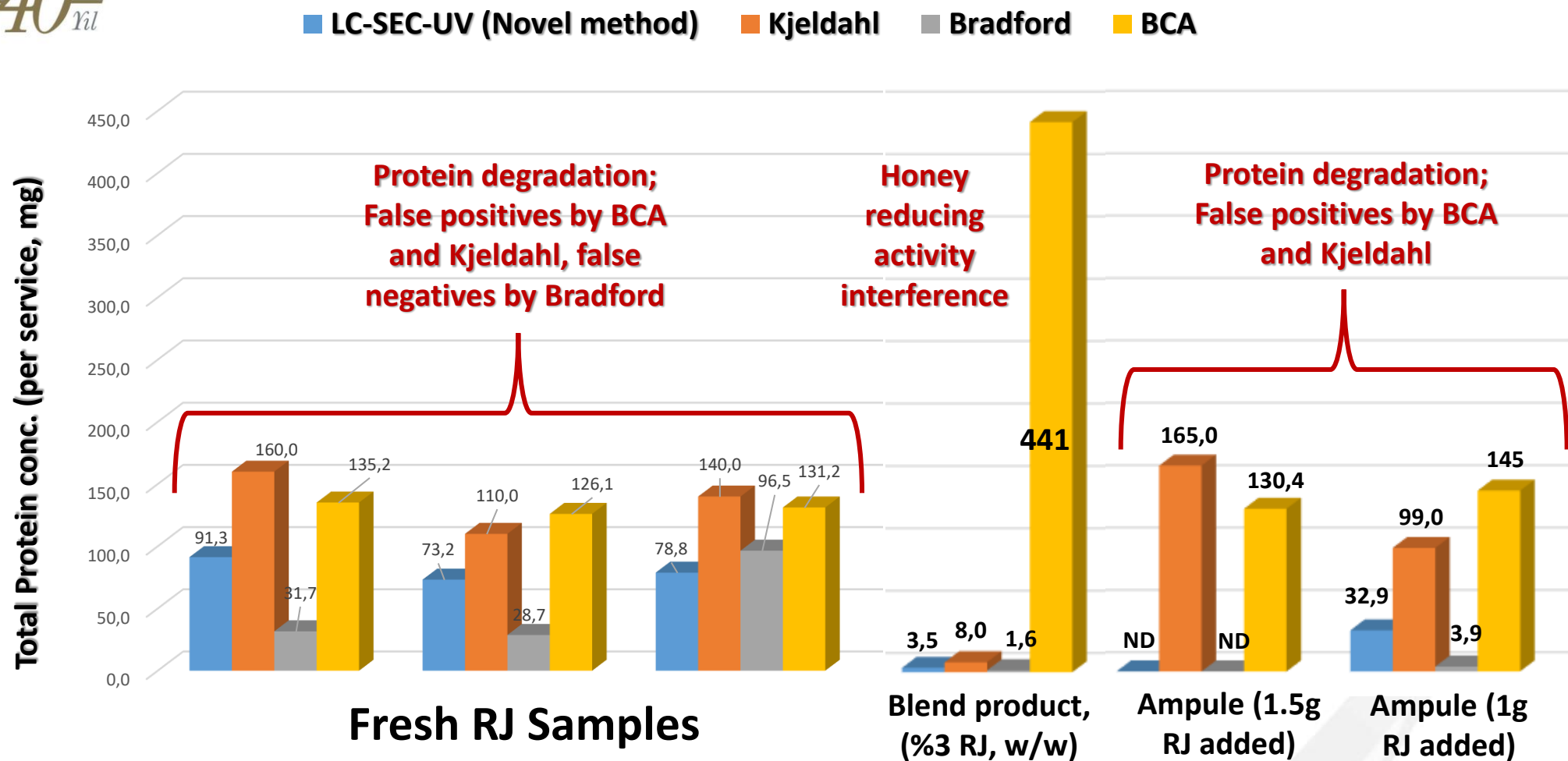
BCA, Bradford, and Kjeldahl assays are not efficient to uncover the 43.5% native protein loss !!!

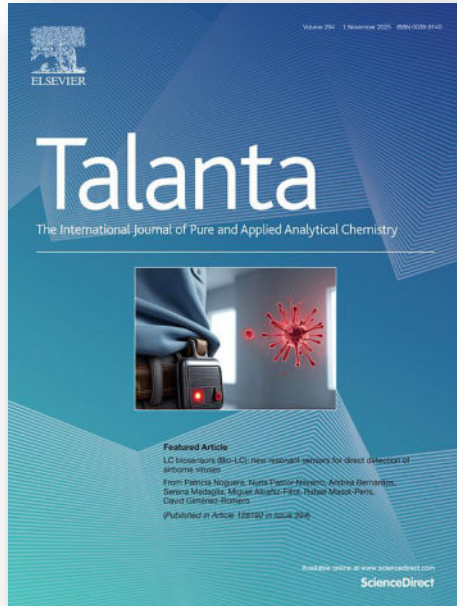


Real Sample Analysis (n=15)

Market Samples	Serving Size (mg)	MRJP conc. by LC-SEC-UV method (per service, mg)				Total Protein conc. (per service, mg)				Total Protein conc. (per service, %)				%HDA w/w
		MRJP 1 Oligomer	MRJP 3	MRJP 2	MRJP 1 monomer	LC-SEC-UV (Novel method)	Kjeldahl	Bradford	BCA	LC-SEC-UV (Novel method)	Kjeldahl	Bradford	BCA	
lyophilized RJ	300	49,2	16,2	19,1	10,3	94,2	126,0	42,3	125,2	31,4	42,0	14,1	41,7	4,0
Fresh Royal Jelly	1000	46,2	16,1	23,1	6,0	91,3	160,0	31,7	135,2	9,1	16,0	3,2	13,5	1,4
lyophilized RJ	1000	13,1	5,7	10,2	3,1	30,2	36,0	10,4	29,4	3,0	3,6	1,0	2,9	0,1
lyophilized RJ	1000	43,1	12,5	24,1	6,1	85,2	95,0	45,6	99,0	8,5	9,5	4,6	9,9	1,5
Fresh Royal Jelly	1000	36,3	12,2	19,3	6,2	73,2	110,0	28,7	126,1	7,3	11,0	2,9	12,6	1,4
Fresh Royal Jelly	1000	40,7	11,2	22,1	6,0	78,8	140,0	96,5	131,2	7,9	14,0	9,6	13,1	1,5
Syrup (%3 RJ)	1000	1,2	1,2	1,1	1,3	4,1	8,0	0,9	28,4	0,4	0,8	0,1	2,8	0,1
Fresh Royal Jelly	1000	40,2	15,1	25,3	9,1	88,3	68,0	37,8	115,4	8,8	6,8	3,8	11,5	2,2
Fresh Royal Jelly	1000	47,2	21,1	24,1	8,3	100,3	130,0	28,0	123,8	10,0	13,0	2,8	12,4	2,5
Blend, (%3 RJ, w/w)	1000	1,1	1,2	1,1	ND	3,5	8,0	1,6	441,0	0,4	0,8	0,2	44,1	0,2
lyophilized RJ	1000	78,2	23,1	29,2	10,5	140,9	100,0	26,2	7743,3	14,1	10,0	2,6	774,3	1,7
Ampule (1.5g RJ added)	15000 uL	ND	ND	ND	ND	ND	165,0	ND	130,4	ND	1,1	ND	0,9	0,1
Ampule (1g RJ added)	9000 uL	11,1	2,2	11,0	9,5	32,9	99,0	3,9	145,0	0,4	1,1	ND	1,6	0,1
Fresh Royal Jelly	1000	35,2	13,1	20,6	8,1	75,8	68,0	36,2	144,7	7,6	6,8	3,6	14,5	1,4
lyophilized RJ	750	29,2	10	13	4	55,6	65,0	8,9	80,9	7,4	8,7	1,2	10,8	0,9

Real Sample Analysis (n=15)





Publication – Under Review

Peer review status

Quantification of Major Royal Jelly Protein Variants and Concurrent Fraud Identification to Confirm Royal Jelly Quality Employing the Native LC-SEC-UV Methodology

- Reviews completed: 1
- Review invitations accepted: 2
- Review invitations sent: 2+

Under Review

Last review activity: 3rd
September 2025 ⓘ

Watch to learn what we're doing behind
the scenes ➤

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Need more help?

Conclusion



- **MRJPs are better quality indicators. Traditional protein assays are not convenient for MRJP quantification and freshness assessment.**
- **We developed a non-sophisticated native mode LC-SEC-UV protocol that enables the quantitative analysis of individual and total MRJPs and concurrently identifies any protein mimickers.**
- **A novel 3-step tandem purification workflow was demonstrated to get MRJP references.**
- **Adapting the new LC-SEC-UV method to QC activities will pave the way for preserving the integrity of RJ products.**

Behind Every Complex Matrix is a Team Who Knows How to Decode It ...!!!

