

UC Davis

UC Davis Electronic Theses and Dissertations

Title

Using Aqueous and Enzyme-Assisted Extraction Processing to Develop a Novel Almond Milk

Permalink

<https://escholarship.org/uc/item/6kf254xm>

Author

Ruiz Hallstrom, Jessica Lynn

Publication Date

2024

Peer reviewed|Thesis/dissertation

Using Aqueous and Enzyme-Assisted Extraction Processing to Develop a Novel Almond Milk

By

JESSICA LYNN RUIZ HALLSTROM
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Food Science and Technology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

Juliana Leite Nobrega de Moura Bell, Chair

Christopher Simmons

Nitin Nitin

Committee in Charge

2024

Abstract

Almonds are an important crop for California and are an intrinsically nutrient-dense food, containing approximately 20% protein and 50-55% fat. Despite this, almond milk, a popular non-dairy beverage, is typically quite low in nutrients, containing only ~0.5% protein and ~1.25% fat. Most commercial almond milk is made by suspending a finely ground roasted almond paste in water with hydrocolloids and emulsifiers, which help keep the solids in suspension and improve mouthfeel. Due to this production method, the quantity of almond paste that manufacturers can use without solids settling out of solution is limited. Therefore, there is significant potential for both nutritional and sensory improvements through the development of alternative processing methods for almond milk.

The overarching goal of this thesis was to develop and optimize environmentally friendly sustainable processes such as aqueous (AEP) and enzyme-assisted extraction processes (EAEP) to produce almond milk with enhanced nutritional and sensory properties. AEP and EAEP enable the simultaneous extraction of proteins and lipids from organic matrices through protein solubilization and washing out of lipids into the aqueous medium. Extractions are performed under constant agitation and controlled pH and temperature conditions. The extraction is followed by centrifugation to separate the protein- and lipid-rich supernatant from the fiber-rich “insoluble” fraction. In the EAEP, enzymes like proteases can be added during the extraction to improve the solubilization of the proteins. Additionally, the use of enzymes can significantly impact the properties of the extracted proteins including their functionality, flavor, and biological activity. This thesis proposes the use of AEP and EAEP to produce almond milk that is rich in proteins and fats, is well-accepted by consumers, and is feasible for production.

Chapter 1 details the state of the art in retail non-dairy milks, using almond milk as a case study. Recent studies on emerging processing technologies for almond milk are presented, including a detailed explanation of AEP and EAEP processes, their mechanisms, and their potential as processing methods for almond milk.

Chapter 2 explores how preprocessing parameters (degree of roast and degree of grind) and methods (AEP vs. EAEP) affect the overall process extractability and the nutritional composition of the milks. Some particle size reduction is necessary for AEP and EAEP to free trapped materials from within cellular structures; therefore, the impact of degree of grinding was investigated. Almonds are typically roasted before grinding into paste, as roasting facilitates grinding through oil release and cell wall shrinkage and imparts desirable flavors to almonds. However, roasting can also denature proteins, potentially affecting their extractability. Consequently, the impact of degree of roast was also investigated. Three degrees of roast (unroasted, light roast, dark roast) and three degrees of grind (flour, butter, paste) of almonds were applied to almonds, resulting in a total of nine raw materials, each subjected to AEP and EAEP. The effects of roasting and grinding levels on protein and lipid extractability, as well as protein properties such as proximate composition, digestibility, surface charge, secondary structure, and surface hydrophobicity, were evaluated. Finer particle size was associated with higher extraction yields while increased roasting was found to reduce protein extraction, likely due to protein denaturation and aggregation. Based on yields, milk composition, and energy requirements, light-roasted almond butter was selected as the optimum material for almond milk production.

Chapter 3 builds off this knowledge, utilizing a commercially purchased food-grade light roasted almond paste to further investigate the impact of extraction parameters on the physicochemical and sensory properties of the milk. An innovative experimental design was employed in this study, utilizing both sensory and physicochemical feedback to optimize process development. Central composite designs (CCDs) were used for both AEP (varying levels of pH and solids-to-liquids ratio) and EAEP (varying levels of protease and solids-to-liquids ratio) for measuring the impact of these variables on physicochemical parameters such as extraction yields, protein content, protein immunoreactivity and antioxidant activity, and sensory descriptors of the milks such as creaminess, mouthfeel, bitterness, and nutty flavor. After initial descriptive analyses, six formulas were selected for further hedonic analyses, which led to a subsequent refinement of extraction parameters and a final hedonic analysis. Almond milk formulas with increased lipid and protein content and positive consumer acceptance were produced using both AEP and EAEP methods.

In Chapter 4, the selected AEP and EAEP formulations (Chapter 3) were scaled up to identify potential pitfalls. Previously performed at bench scale, extractions were performed in larger (15 L) quantities and subjected to ultra-high temperature (UHT) sterilization and homogenized at pilot-scale. These were compared with a typical “commercial” formula using the suspended solids method. These milks were then evaluated for proximate composition and protein properties (immunoreactivity, antioxidant activity, digestibility), as well as for emulsion stability, lipid oxidation, and viscosity over their shelf-life. AEP and EAEP milks were both significantly higher in protein and fat than the commercial milk formula, with enzyme leading to milks with reduced immunoreactivity and increased antioxidant activity. AEP and EAEP milk proteins exhibited significantly higher *in vitro* protein digestibility than the commercial formula. Oil

oxidation was low over the shelf-life of all milks. This work demonstrates the feasibility of scaling up these methods for large-scale production of these products.

In this work, we present a method for manufacturing almond milk with improved nutritional qualities and tailored physicochemical properties. The optimization of upstream processing and extraction parameters, driven by sensory and analytical data, was crucial for selecting conditions for scaling-up of these methods. This work offers almond milk processors the opportunity to develop nutritious products that appeal to consumers.

Acknowledgements

This work has been a labor of love (and almonds, so many almonds), and it truly would not have been possible without the unwavering support of my family, friends, peers, collaborators, and mentors. I'm where I am today because of them.

Prof. Juliana M.L.N. de Moura Bell: Thank you for your mentorship, guidance, and persistence. You pushed me to be a better scientist and taught me how to come up with creative solutions to projects. I am so grateful that I ended up in your lab. Thank you for guiding me through the first years to the very end of this exciting and fun project. It's been a pleasure working with and getting to know you.

Prof. Julien Delarue and Dr. Jane Ong: Thank you for collaborating with us on this project! Maybe one day 4J milk will be in stores.

Prof. Chris Simmons: Thank you for being an advocate for me and every student in the department, serving on both my QE and dissertation committees, as well as providing so much support in my graduate career.

Prof. Nitin Nitin: Thank you for serving on my dissertation committee and being so open to collaboration, and for your guidance over the years.

To my postdoc, graduate student, and undergrad intern lab mates, past and present: Thank you for teaching and supporting me, helping me with endless extractions, washing dishes, holding my hand (sometimes literally) through assays, commiserating when the nitrogen analyzer broke down again, and sharing laughs. It wouldn't have been possible without every single one of you.

To my cohort-mates: 2018 feels like decades ago, but I'm so glad we all started through it together. The Diffusive Dozen remains the greatest company.

To my friends near and far: Thank you for your love and support from back home in LA to all around the world.

To my family: Thank you for being my cheerleaders and my home. It was a little crazy to pack up and leave home to start a Ph.D. with an 8-month-old baby, and having your support has been lifesaving.

To my mother: I can never thank you enough. From moving us to Davis to driving all the way up here to babysit, this would never have worked without you. I know you're probably crying already reading this, and I'm glad I got that from you too. I love you mama.

To my husband: You are an actual saint. Thank you for bugging me to stop talking about it and start applying already. I couldn't, and wouldn't, have done this without you. Thank you, thank you, thank you.

To Sam: I dedicate this to you, my best little guy. Mommy loves you! Please remember to wash your hands.

Table of Contents

Section	Title	Page
1	Chapter 1: A review of current almond milk technology and proposed method for its improvement	1
1.1	Exploring almond milks in the shift to plant-based milks	1
1.2	Understanding almond composition and its transformation into milk alternatives	2
1.2.1	Almond proteins: composition, amino acid profile, and digestibility	3
1.2.2	Lipid composition and profile	5
1.3	Non-dairy milks	6
1.3.1	Current state-of-art on almond milk production and its impact on nutritional properties	7
1.4	Recent investigations into almond milk processing techniques	9
1.4.1	Non-thermal processing techniques: impact on almond milk quality and nutritional value	10
1.4.2	Fermentation as a tool for nutritional and functional improvement	12
1.4.3	Impact of processing strategies and conditions on almond milk protein digestibility	13
1.5	Optimizing almond milk's nutritional profile: future directions in protein enrichment and quality	14
1.6	Bridging the knowledge gaps: enhancing almond milk through focused research	16
1.6.1	Impact of roasting conditions on protein extractability and digestibility	16
1.6.2	Impact of particle size on extraction of protein and lipids	18
1.6.3	Impact of solids-to-liquids ratio on extractability and sensory properties	18
1.6.4	Impact of pH on extractability and protein physicochemical properties	19
1.6.5	The role of enzymes in optimizing total protein and oil extractability	19
1.6.6	Harnessing proteolysis for enhanced control of protein zeta-potential	20
1.6.7	Tailoring enzyme use for enhanced emulsion stability	21
1.6.8	Reducing almond protein immunoreactivity with proteolysis	21
1.6.9	Impact of enzymatic extraction on protein digestibility	22
1.6.10	Exploring the impact of proteolysis on the release of bitter peptides	23
1.6.11	Other extraction conditions	23
1.6.12	Potential challenges	24
1.6.13	Potential impact	24
1.7	Conclusions	24
1.8	Objectives	
1.9	References	26

2.	From solid dispersion to the simultaneous extraction of lipids and proteins: a bio-guided strategy to improve the nutritional and biological properties of almond milk	32
	Abstract	32
	Keywords	33
2.1	Introduction	34
2.2	Materials and methods	37
2.2.1	Almond processing	37
2.2.2	Characterization of almond starting materials	38
2.2.2.1	Particle size analysis	38
2.2.2.2	Starting material proximate analyses	39
2.2.3	Almond milk extraction using the AEP and EAEP	39
2.2.4	Quantifying oil and protein extractability	40
2.2.5	Confocal laser scanning microscopy	40
2.2.6	Starting material selection and almond milk compositional, nutritional, and physicochemical characterization	41
2.2.6.1	Impact of almond butter roasting on color of AEP and EAEP milks	42
2.2.6.2	Influence of almond butter roasting and extraction methods on in vitro protein digestibility of almond milk	42
2.2.6.3	Almond milk protein characterization via SDS-PAGE	43
2.2.6.4	Defatting of almond milk	43
2.2.6.5	Circular dichroism (CD) spectra	44
2.2.6.7	Surface hydrophobicity (H ₀) of defatted almond milk proteins	44
2.2.6.8	Zeta potential of defatted almond milk proteins	45
2.2.7	Statistical analyses	45
2.3	Results	45
2.3.1	Impact of roasting and grinding on particle size	45
2.3.2	Impact of roasting, grinding, and extraction methods on oil and protein extractability	46
2.3.3	Confocal laser scanning microscopy (CLSM)	50
2.3.4	Starting material selection and almond milk compositional, nutritional, and physicochemical characterization	51
2.3.5	Impact of almond butter roasting on color of AEP and EAEP milks	51
2.3.6	Influence of almond butter roasting and extraction methods on in vitro protein digestibility of almond milk	56
2.3.7	Almond milk protein characterization via SDS-PAGE	57
2.3.8	Circular dichroism (CD) spectra	58
2.3.9	Surface hydrophobicity and zeta-potential of almond butter milk proteins	60

2.4	Conclusion	62
2.5	Acknowledgements	64
2.6	References	64
3.0	Optimizing the nutritional profile and sensory attributes of almond milks using aqueous and enzymatic extraction methods	69
	Abstract	69
	Keywords	69
3.1	Introduction	70
3.2	Materials and methods	73
3.2.1	Materials	73
3.2.2	Characterization of almond paste	73
3.2.3	Leveraging experimental design tools to elucidate the impact of extraction methods on almond milk properties	74
3.2.4	Extraction of almond milk using the AEP and EAEP	74
3.2.5	Proximate analyses	76
3.2.6	Oil Droplet Diameter of AEP and EAEP milks	77
3.2.7	Viscosity of AEP and EAEP milks	77
3.2.8	Molecular weight of AEP and EAEP milk proteins	77
3.2.9	Defatted AEP and EAEP Milk Protein Analyses	77
3.2.9.1	Degree of hydrolysis and zeta potential of defatted AEP and EAEP milk proteins	78
3.2.9.2	Immunoreactivity of AEP and EAEP milk proteins	78
3.2.9.3	Antioxidant capacity of AEP and EAEP milks	79
3.2.10	Statistical analysis of physicochemical assays	79
3.2.11	Sensory analysis of AEP and EAEP milks	79
3.2.11.1	Descriptive analysis	80
3.2.11.2	Consumer tests	81
3.2.11.3	Statistical analysis of sensory data	83
3.3	Results	83
3.3.1	Impact of extraction conditions on the composition of AEP and EAEP milks and their effect on oil and protein extractability	83
3.3.1.1	Protein content of AEP and EAEP milks and total protein extractability	84
3.3.1.2	Oil content of AEP and EAEP milks and total oil extractability	87
3.3.1.3	Carbohydrate and ash content of AEP and EAEP milks	88
3.3.2	Impact of extraction conditions on oil droplet diameter and milk viscosity	88
3.3.3	Impact of extraction conditions on degree of hydrolysis	92
3.3.4	SDS-PAGE	93
3.3.5	Zeta-potential	94

3.3.6	Immunoreactivity by ELISA sandwich assay	95
3.3.7	Total antioxidant activity	96
3.3.8	Descriptive analyses	97
3.3.9	Leveraging hedonic analyses to guide AEP and EAEP milk formulations	98
3.3.10	Composition and physicochemical characteristics of optimized AEP and EAEP milks	102
3.4	Conclusions	106
3.5	Acknowledgements	107
3.6	References	107
4.0	Scaling aqueous and enzyme-assisted extraction for enhanced almond milk production	111
	Abstract	111
	Keywords	112
4.1	Introduction	113
4.2	Materials and methods	115
4.2.1	Materials	115
4.2.2	Almond milk extractions and commercial milk formulation	116
4.2.3	Pasteurization and homogenization	117
4.2.4	Proximate analyses	117
4.2.5	Total protein and oil extraction	118
4.2.6	Antioxidant capacity of AEP, EAEP, and commercial milks via ABTS and ORAC assays	118
4.2.7	Allergenicity potential of AEP and EAEP milks by sandwich ELISA	119
4.2.8	In vitro protein digestibility of AEP and EAEP milks	120
4.2.9	Impact of production method, pasteurization, and homogenization on milk shelf-life	121
4.2.9.1	Peroxide value	121
4.2.9.2	Secondary oxidation markers via TBARS	121
4.2.9.3	Oxylipin analysis	122
4.2.9.3.1	Sample preparation	123
4.2.9.3.2	Ultra-high-pressure-liquid chromatography mass-spectrometry analysis	124
4.2.9.4	Emulsion stability over shelf-life: creaming index, oil droplet diameter, and viscosity	124
4.2.10	Statistical analyses	126
4.3	Results	126
4.3.1	Proximate compositions of AEP, EAEP, and commercial almond milks	126
4.3.2	Total protein and oil extraction yields	127
4.3.3	Antioxidant activity of the milks by ABTS and ORAC assays	128
4.3.4	Allergenicity of the milks via S-ELISA	130

4.3.5	IVPD of AEP and EAEP milks	132
4.3.6	Understanding the impact of extraction and processing methods on milk shelf-life and quality	134
4.3.6.1	Primary and secondary oxidation markers	134
4.3.6.2	Oxylipin content of milk through shelf-life	135
4.3.6.3	Emulsion stability of the milks via oil droplet size and creaming index	139
4.3.6.4	Viscosity of milks	142
4.4	Conclusions	143
4.5	Acknowledgements	145
4.6	References	145

Index of Figures

Figure	Title	Page
1.1	Manufacturing process for almond paste for almond milk production.	7
1.2	Schematic representation of typical almond milk manufacturing process	8
2.1	Conventional and aqueous and enzyme-assisted aqueous extraction processing.	37
2.2	Almond roasting and grinding conditions to produce AEP and EAEP milks.	38
2.3	Impact of roasting, particle size, and extraction methods (AEP vs. EAEP) on almond oil a) and protein extractability.	49
2.4	Confocal laser scanning microscopy images of almond flour materials, AEP insolubles, and EAEP insolubles.	53
2.5	Confocal laser scanning microscopy images of almond butter starting materials, AEP insolubles, and EAEP insolubles.	54
2.6	Confocal laser scanning microscopy images of almond paste starting materials, AEP insolubles, and EAEP insolubles	55
2.7	In vitro protein digestibility of almond milks produced by different roasting and extraction methods (AEP/EAEP) using the INFOGEST method.	57
2.8	Almond milk protein molecular weight by SDS-PAGE analysis on AEP and EAEP almond butter milks.	58
2.9	Impact of roasting and extraction methods on surface hydrophobicity and zeta potential of butter milk defatted surface proteins at pH 7.	62
3.1	Impact of extraction conditions on total protein a) and oil b) extraction yields of AEP and EAEP milks.	88
3.2	Characterization of AEP and EAEP milks at different experimental conditions.	91
3.3	SDS-PAGE protein gels for AEP (a) and EAEP (b) milk proteins.	94
3.4	CVA score plot of formulated almond milks with top 5 contributing attributes.	98
3.5	Results of first consumer tests. a) Means of liking scores of selected AEP and EAEP milks. Samples with different letters were significantly different from each other as determined by Tukey's HSD. b) Correspondence analysis of CATA attributes for selected AEP and EAEP milks. c.) External preference mapping of liking scores of selected AEP and EAEP milks.	101
3.6	a) Means of liking scores of samples from consumer test 2. Samples with different letters were significantly different from each other as determined by Tukey's HSD. b) Correspondence analysis of CATA attributes from consumer test 2.	105

4.1	Pasteurized and homogenized milks.	117
4.2	a) Total protein extraction (TPE, %) of AEP and EAEP almond milks. B) Total oil extraction (TOE, %) of AEP and EAEP almond milks.	128
4.3	Total antioxidant activity of milks as determined by a) ABTS and b) ORAC assays; immunoreactivity by S-ELISA as measured by c) ppm amandin and d) mg amandin detected/kg protein in milk.	131
4.4	<i>In vitro</i> protein digestibility of commercial (C), AEP, and EAEP almond milk formulas.	132
4.5	Secondary lipid oxidation products via TBARS assay over shelf-life of commercial (C), AEP, and EAEP milks.	137
4.6	Creaming index (a) and dynamic viscosity (mPa s) at 50 s ⁻¹ (b) of milks over shelf-life of commercial (C), AEP and EAEP milks.	141

Index of Tables

Table	Title	Page
1.1	Typical macronutrient composition of <i>Prunus dulcis</i> almond on wet basis.	3
1.2	Amino acid composition of almonds.	4
1.3	Comparative macronutrient composition of commercial almond milks.	9
2.1	Influence of roasting and grinding on almond particle size distribution as measured by dynamic laser scattering.	46
2.2	Impact of roasting and extraction methods on the macronutrient composition of almond milks produced via AEP and EAEP compared to an average commercial almond milk.	51
2.3	Impact of almond butter roasting on color (CIE L*a*b* values) of almond starting materials.	52
2.4	Secondary structure composition of almond “butter” milk proteins as determined by circular dichroism in the far UV region (190-250 nm).	59
3.1	Solids-to-liquid ratio and extraction pH levels evaluated in the production of AEP milks.	75
3.2	Table 3.2. Solids-to-liquid ratio and extraction pH levels evaluated in the production of EAEP milks.	75
3.3	Sensory attributes, techniques, and definitions of almond milks, as evaluated by trained panelists.	81
3.4	Impact of extraction conditions on the macronutrient composition of AEP and EAEP almond milks.	85
3.5	Two-way analysis of variance (ANOVA) of the estimated regression models AEP and EAEP milk physicochemical properties.	87
3.6	Extraction conditions and proximate composition of final AEP and EAEP milks.	104
4.1	Proximate composition of AEP, EAEP, and Commercial Milks.	127
4.2	Oxylipin concentration (pmol/mL) in commercial (C), AEP (A), and EAEP (E) milks at beginning (T0) and end (Tend) of shelf-life.	137
4.3	D (4,3) oil droplet mean diameter (mm) of almond milks over shelf-life.	140

List of Acronyms

ABTS: 2,20-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay
AEP: Aqueous extraction processing
ANOVA: Analysis of variance
CATA: Check-all-that-apply
CCD: Central composite design
CD: Circular dichroism
CHO: Carbohydrate
CVA: Canonical variate analysis
CSLM: Confocal laser scanning microscopy
DH: Degree of hydrolysis
EAEP: Enzyme-assisted extraction processing
ELISA: Enzyme-linked immunosorbent assay
ESL: Extended shelf-life
H₀: Surface hydrophobicity
HPP: High pressure processing
IVPD: *In vitro* protein digestibility
kDa: Kilodaltons
MANOVA: Multiple analysis of variance
ORAC: Oxygen radical antioxidant capacity assay
PCA: Principal component analysis
PDCAAS: Protein digestibility corrected amino acid score
PEF: Pulsed electric field
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLR: Solids-to-liquid ratio
TBARS: Thiobarbituric acid reactive substances assay
TOE: Total oil extraction
TPE: Total protein extraction
UHT: Ultra high temperature
UPLC: Ultra-high pressure liquid chromatography tandem mass spectrometry

Chapter 1: A review of current almond milk technology and proposed method for its improvement

1.1 Exploring almond milks in the shift to plant-based milks

Feeding a growing global human population that is expected to reach 9 billion by 2030 is compounded by the impact that our global food production processes have on the climate. One approach to reducing the environmental impact of the food system is the widespread adoption of a more plant-based diet to reduce the impact of animal agriculture^{1,2}. Plant-based milks are included in this strategy, producing far fewer greenhouse gas emissions than dairy milk³. These beverages are water-based mixtures made from nuts, seeds, legumes, and grains, crafted to serve as analogues to cows' milk. They serve as an important dietary staple for those who cannot or choose to not consume dairy, such as those with allergies or lactose intolerance, vegans, and those with religious prohibitions against it. Increasingly, consumers who purchase dairy milk are also incorporating plant-based milks into their diets, whether for variety or to reduce their dairy consumption⁴.

Many plant-based milks, such as soymilk or coconut milk, have existed for centuries. However, in recent years, others such as almond milk have significantly increased in popularity⁵. Soymilk was the leading plant-based milk in the United States until 2013, when it was surpassed by almond milk. Almond milk remains the most popular plant-based milk to the present day, with an expected global market value of \$9.6B by 2029⁶. However, in recent years, almond milk has faced criticism for its high environmental impact, particularly in terms of water usage, compared to other plant-based milks⁷. Additionally, almond milk has been noted for its low protein content compared to bovine milk^{8,9}.

A variety of comprehensive reviews on plant-based milks has been conducted in recent years^{10–16}. The purpose of this review is to highlight some of the challenges associated with currently

available plant-based milks, using almond milk as a case study, and to summarize recent research on the subject. Due to the transformational potential of aqueous and enzyme-assisted aqueous extraction processes (AEP/EAEP) in addressing challenges related to the production of plant-based milks, including improvements in nutritional value and quality, this review will highlight and discuss key knowledge gaps in the field. It will focus on advancing our understanding of how upstream unit operations, extraction methods and conditions impact the physiochemical and nutritional properties of the final product. Importantly, this review will explore the potential for manipulating these processes and conditions to tailor the functional, organoleptic, and bioactive properties of the finished milk.

1.2. Understanding almond composition and its transformation into milk alternatives

Almonds, the seeds of sweet almond (*Prunus dulcis*) trees, are a widely consumed snack and ingredient in foods of many different cultures. They are intrinsically nutrient-dense, with high levels of protein (~20% by weight) and healthy fats (~50% by weight), facilitating their use in a wide range of food applications like almond milk (Table 1.1)^{17,18}. Almond carbohydrate content varies between varieties, comprising a mix of starches, sugars, and polysaccharides. Sucrose has been identified as the major sugar in almonds, along with raffinose, and minor quantities of glucose, fructose, and the sugar alcohols sorbitol and inositol¹⁸. Almonds are also high in dietary fiber (14.0-26.6 g/100 g). Almonds also contain a variety of micronutrients, such as potassium (465-1,500 mg/100 g), calcium (160-633 mg/100 g), and phosphorus (310-938 mg/100g)¹⁹. This high nutritional density contrasts with the relatively low nutrient density of almond milk, which, as discussed later in this review, results from current production methods. Despite being rich in nutrients, almonds remain an underutilized base for plant-based milk.

Table 1.1. Typical macronutrient composition of *Prunus dulcis* almond on wet basis.

Nutrient	Range of means g/100 g almonds
Moisture	3.3-6 ^{18,20}
Carbohydrate	14-26.3 ¹⁹
Fiber	10.4-13.0 ²¹
Sugars	3.5-7.4 ¹⁸
Protein	16-24 ^{18,20}
Lipids	32-66 ^{18,19,22}
Oleic Acid	59.2-73.80% ^{22*}
Linoleic Acid	19.49-33.29% ^{22*}
Palmitic Acid	5.15-6.65% ^{22*}
Palmitoleic	0.31-0.57% ^{22*}
Stearic Acid	0.24-1.66% ^{22*}

*Fatty acids presented as % of total lipid composition.

1.2.1. Almond proteins: composition, amino acid profile, and digestibility

Nonpareil almonds, the most widely grown variety in the United States, contain a protein digestibility corrected amino acid score (PDCAAS) of ~47%²⁰, compared to 100% for whey protein²³, indicating the protein is limiting in amino acids; specifically lysine, methionine, and tyrosine. The amino acid profile of almond protein is presented in Table 1.2. Though incomplete, this protein is highly digestible, with a total *in vivo* protein digestibility of ~80%^{20,24}. In almonds, proteins are concentrated in protein bodies ranging from 2-12 μm in diameter within the parenchymal cells²⁵. Amandin, an 11S globulin is the primary storage protein in almonds²⁶, accounting for 65-70% of all water-extractable proteins. Amandin is a hexameric protein with an

Table 1.2. Amino acid composition of almonds.

Amino Acid	%
Alanine	1.10-4.86 ^{20,27,28}
Arginine	2.36-13.44 ^{20,27,28}
Aspartate	2.50-26.78 ^{20,27,28}
Cysteine	0.26-0.79 ^{20,27,28}
Glutamate	5.60-35.12 ^{20,27,28}
Glycine	1.48-6.88 ^{20,27,28}
Histidine	0.50-2.97 ^{20,27,28}
Isoleucine	0.82-4.63 ^{20,27,28}
Leucine	1.52-7.46 ^{20,27,28}
Lysine	0.59-3.06 ^{20,27,28}
Methionine	0.18-0.81 ^{20,27,28}
Phenylalanine	1.16-6.45 ^{20,27,28}
Proline	0.88-5.09 ^{20,27,28}
Serine	0.88-4.56 ^{20,27,28}
Tryptophan	0.18-0.70 ^{20,27,28}
Tyrosine	0.65-3.44 ^{20,27,28}
Valine	0.93-5.80 ^{20,27,28}
Essential amino acids	28.55-30.31 ^{20,27,28}
Sulfur-containing amino acids	1.16-1.32 ^{20,27,28}
Aromatic amino acids	9.10-9.82 ^{20,27,28}
Hydrophobic amino acids	38.05-39.44 ^{20,27,28}

Amino acid percentages of protein as determined from ground almonds^{20,28} or AEP/EAEP skins from blanched almond flour²⁷.

estimate of 56.4% β -turns and 14% α -helices²⁹. The major polypeptides comprising amandin are the 42-45 kDa acidic and 20-22 kDa basic subunits, linked by disulfide bonds^{30,31}.

A variety of other proteins such as oleosins (proteins embedded on the surface of almond oil bodies) are present throughout the seed. Almond oleosins have been extracted and purified and found to exhibit emulsifying properties³². Studies examining the functional properties of almonds such as emulsification and foaming capacity, oil and water absorption capacity, and hydrophobicity have yielded varying results, likely due to differences in the unit operations used to process almonds and protein extraction methods employed in these studies. Extraction and recovery methods impact the functional and nutritional properties of proteins; however, this variability raises important research questions, as it could significantly impact the performance of almond proteins in milk production. Sharma et al.³³, Amirshaghghi et al.³⁴, and Dias & de Moura Bell²⁷ have found that native almond proteins demonstrate 2.4-2.6 g water/g protein water absorption capacity as well as 3.1-3.6 g oil/g protein oil holding capacity^{27,33-35}, indicating a potential for water- and fat-binding in a milk formula. Almond protein demonstrates a very low least gelling concentration at pH 4 (8% w/v) and pH 7-8 (4% w/v)^{30,33,36}, indicating that it is highly effective at gelation, which could be beneficial for creating an almond milk with a desirable viscosity and mouthfeel.

1.2.2. Lipid composition and profile

Almonds are naturally high in lipids, containing 49.1-66.4% fat²². The lipids within oil-bearing seeds and nuts are concentrated in oil bodies (oleosomes) within the cotyledon. In almonds, oleosomes can be up to 3 μm in diameter²⁵. Almond oil is primarily composed of oleic acid (~65%), followed by linoleic (~27%) and palmitoleic acids (~0.4%)²². When recovered from blanched almond flour extracted via a variety of extraction methods (AEP, EAEP, hexane), the

majority of almond oil fatty acids (>99%) were in the esterified form, with 99% as triacylglycerols, and small amounts of phytosterol esters, phospholipids, and free fatty acids (0.1%, 0.3% and 0.5%, respectively)³⁷. As the majority of oil in almonds is monounsaturated, it is relatively resistant to oxidation¹⁹. In the same study, almond oil was found to have very low levels of traditional oxidation markers (peroxide value and p-anisidine). This oil is widely used in food and cosmetic products and has been studied and used traditionally for its potential health benefits³⁸. Almond oil is typically neutral-tasting, and its mono- and poly-unsaturated fats contain the potential for added nutritional benefit in almond milk.

1.3. Non-dairy milks

Non-dairy milks are formulated from a wide variety of crop sources. Soybeans, almonds, rice, oats, flax seeds, coconut, peas, cashew, and hemp seeds are some of the more popular milk bases on the current retail market⁵. Only soymilk, however, typically contains protein levels similar to those of bovine milk, with a protein digestibility-corrected amino acid score (PDCAAS) that is nearly as high as bovine milk^{12,39}. Many of these non-dairy milks are also sweetened with high quantities of sugar, often making them higher in calories than dairy milk despite their low protein content⁹. With the exception of coconut milk, many of these milks are also relatively low in fat compared to bovine milk, even though many of their original base ingredients are excellent sources of polyunsaturated fats^{9,18}. This is largely due the traditional non-dairy milk manufacturing process, which requires suspending insoluble solids within the milks, limiting the amount of plant material that can be used without sedimentation of the solids.

1.3.1. Current state-of-art on almond milk production and its impact on nutritional properties

The majority of almond milks on the market in the United States are manufactured via suspension of a fine almond paste in an aqueous solution⁷. In this process, dehulled deskinmed roasted almonds are ground into a very fine paste (Fig. 1.1), then mixed with water along with an emulsifier (typically lecithin); and thickening agents such as carrageenan, locust bean gum, and gellan gum, among others. Manufacturers can also add optional sweeteners and flavoring ingredients⁷.

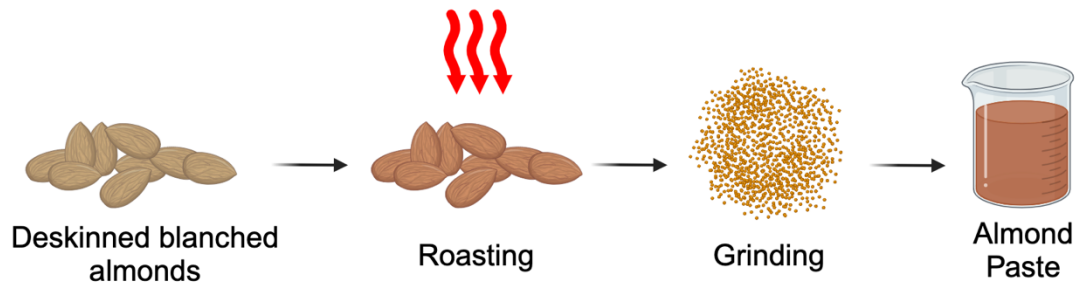


Figure 1.1. Manufacturing process for almond paste for almond milk production. Created with BioRender.com.

The milk is then pasteurized using ultrahigh temperature (UHT) processing (125-148 °C for 2-10 s)¹⁴ (Fig 1.2). Homogenization is also performed on the milk to reduce the diameter of suspended oil droplets and create a finer emulsion. The milk is then packaged aseptically in cartons for storage and sale at ambient temperature (90-170 d shelf-life), or packaged in a non-aseptic container for an extended shelf life (ESL) (90-170 d) under refrigerated storage⁴⁰.

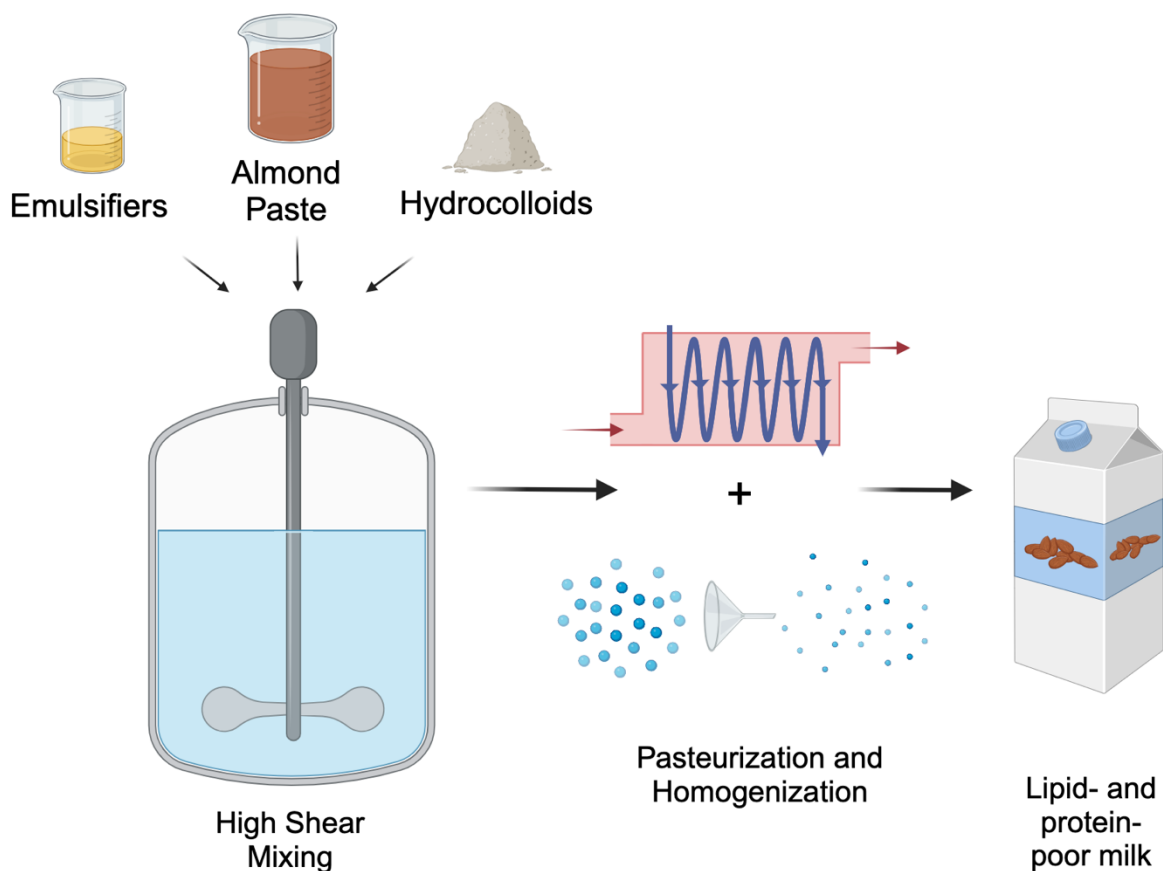


Figure 1.2 Schematic representation of typical almond milk manufacturing process. Created with BioRender.com.

To achieve a consistency and smooth mouthfeel similar to that of dairy milk, the almond paste is typically limited to ~2% of the total formulation. Considering that California almonds contain approximately 20% protein^{18,21}, almond milks typically provide less than 0.5 g protein per 100 g. In contrast, bovine milk contains roughly 3.5 g protein per 100 g⁹. This disparity prevents most almond milks from serving as a true analog to bovine milk.

The nutritional composition of 11 retail almond milks is listed in Table 1.3. Some almond milks have higher protein content due to an increased ratio of almond paste to water in the product; however, these typically have significant sedimentation of almond solids and must be shaken

vigorously prior to consumption. Due to their gritty texture, these milks do not mimic the consistency or drinking experience of bovine milk.

Table 1.3. Comparative macronutrient composition of commercial almond milks.

Brand	Fat (%)	Carbohydrates (%)	Protein (%)	% Almond in milk
A	1.25	0.83	0.42	1.98
B	1.25	0.83	0.42	1.98
C	1.04	0.42	0.42	1.98
D	1.25	0.83	0.42	1.98
E	1.04	3.33	0.42	1.98
F	1.27	0.42	0.42	2.01
G	4.58	1.25	2.08	9.92
H	3.98	0.88	1.77	8.43
I	3.38	0.42	1.27	6.03
J	4.58	0.83	2.08	9.92
K	1.25	0.83	0.42	1.98

Calculated from data collected in June 2019. Macronutrients were calculated from Nutrition Facts labels of almond milk products. Almond percentage in the milks was calculated using an assumption of 21 g protein/100 g almonds. Brands G-J exhibited higher protein levels but contained visible sedimented almond insoluble particles within their containers.

The need to suspend almond paste to maintain a fluid consistency limits the potential for protein fortification in almond milk. Therefore, a new approach is necessary to develop an almond milk that is higher in protein and lipids to satisfy consumers' needs and make it a better bovine milk analog.

1.4. Recent investigations into almond milk processing techniques

Most current studies on almond milk do not utilize the conventional manufacturing method employed by the food industry, which involves suspending finely ground roasted almond paste in water with emulsifiers and hydrocolloids. Instead, many of them are made by blending almonds with water; soaking almonds, draining them, and then blending them with water; or a simple aqueous extraction (blending almonds with water and straining out the solids). This review

presents a summary of recent research on various processing methods for almond milk, along with studies examining the impact of processing on milk digestibility.

1.4.1. Non-thermal processing techniques: impact on almond milk quality and nutritional value

Thermal processing of milk is an effective preservation method; however, it can have deleterious effects on the sensory properties of the finished milk, such as the generation of undesirable flavors and browning⁴¹. Though UHT processing is theoretically a relatively gentle process due to its brief holding time at high temperature, typically just a few seconds long, this does not account for the product's come-up time and cooling time, during which quality changes can occur, especially in indirect heat exchangers. To that end, a variety of nonthermal processes have been investigated as an alternative to traditional heat processing of almond milk.

Several studies have been performed on the use of ultrasonication of almond milk. Ultrasonication has been used in various plant-based milks for a variety of aims, such as reducing sedimentation and oil droplet size, increasing emulsion stability, and killing pathogens⁴². This process works through acoustic cavitation, or the formation, expansion, and collapsing of bubbles in a liquid. Ultrasonication has been demonstrated to decrease particle sedimentation in almond milk⁴³ as well as slightly increase *in vitro* protein digestibility⁴⁴.

Manzoor et al.⁴⁵ investigated the effect of thermosonication, or temperature-controlled ultrasound, on soaked and extracted almond milk at 40 kHz/600 W, and temperatures of 30, 45, and 60 °C. The authors found that thermosonication increased the bioavailability of many compounds such as total phenolics, flavanols and total flavonoids, as well as the antioxidant activity of the milks. Thermosonication also resulted in a significant reduction in unwanted residual enzymes peroxidase and lipoxygenase.

Though not through ultrasonication, Faraloni et al.⁴⁶ investigated the effect of hydrodynamic cavitation on almond milk and found that it was an effective antimicrobial processing step, along with low-temperature pasteurization, that preserved nutritional properties of the milk through processing. This processing method is promising for increasing the quality of almond milk, though its scalability has not been studied.

Commercial almond milk is typically homogenized to create a more stable emulsion. To accomplish this, the milk is homogenized, typically at pressures between 20 and 50 MPa (200-500 bar), via flowing through a small gap at high pressure⁴⁷. This deforms the surfaces of fat droplets within the milk, reducing their size. Higher homogenization pressures such as 62-200 MPa have been investigated for various benefits, such as reduction of immunoreactivity and improvement of emulsion strength, especially in combination with low heat treatment, through altering the physicochemical structure of the proteins and oil bodies⁴⁸⁻⁵¹. However, it may also negatively impact lipid oxidation. More research is needed to further assess its benefits and feasibility for use in industry.

Pulsed electric field (PEF) is a novel processing method in which a material is treated with short pulses of high-voltage electricity in lieu of traditional thermal processing⁵². It is most effective in liquid foods, thus it has high potential for use in almond milk⁵³. Similar to ultra-high pressure homogenization, PEF of almond milk at electric field strength of 7, 14, 21, and 28 kVcm⁻¹ for 200 μ s has been found to reduce fat droplet size in almond milk, yielding a more stable emulsion than untreated almond milk⁵⁴. PEF of almond milk has also been shown to induce an increase of antioxidant capacity, and reduction in lipoxygenase and peroxidase activity⁵⁵. This method has yet to be investigated for its impact on sensory properties of almond milk, as well as feasibility at scale.

High pressure processing (HPP) is another alternative processing strategy to thermal processing for use in almond milk. HPP utilizes high pressures up to 600 MPa to inactivate microbes while preserving qualities typically negatively impacted by thermal treatment⁵⁶. HPP has been found to significantly decrease the immunoreactivity of amandin in almond milk, though it did lead to significant aggregation, reducing protein solubility⁵⁶. This process has also not been studied for scalability, nor impact on sensory qualities of the resultant almond milk.

1.4.2. Fermentation as a tool for nutritional and functional improvement

Fermentation of almond milk has been investigated as another processing method of interest. Bernat et al.⁵⁷ utilized high pressure homogenization (see Section 5.2) followed by fermentation with *Lactobacillus reuteri* and *Streptococcus thermophilus* to produce a probiotic almond milk drink. The fermentation process was found to have a weak gelling effect on the milk, improving its serum retention capacity (43-48%) compared to unfermented milk (36%). Al-Zahrani and Shori⁵⁸ inoculated almond milk with four different *Lactobacillus* strains (*L. rhamnosus*, *L. plantarum*, *L. casei*, and *L. acidophilus*) and found that all four strains improved total phenolic content, total flavonoid content, and antioxidant activities of the milks over 7-21 days compared to control. These microbes exhibit metabolic activities such as the production of enzymes that facilitate the release of phenols, demonstrating the potential to produce almond milk with improved antioxidant activity. Finally, Lipan et al.⁵⁹ inoculated almond milk with *L. plantarum*, and following 18 h of incubation, spray-dried the milk with maltodextrin as a carrier. The spray-dried milks were found to have bacterial viability up to 8 months of storage at 4 °C and 6 months at 22 °C. Fermentation can have a significant impact on the physicochemical and sensory properties of almond milk; thus, this would require more investigation to assess its feasibility at scale.

1.4.3. Impact of processing strategies and conditions on almond milk protein digestibility

The digestibility of proteins is critical to their true nutritive value to humans. In lieu of performing human digestion studies, *in vivo* animal studies and *in vitro* (either static or dynamic) digestibility assays have been developed for analyzing this property. *In vitro* methods typically consist of an oral phase, where food is subjected to a simulated salivary solution, followed by gastric and intestinal phases with their associated simulated solutions⁶⁰. Almond milk digestibility has been studied thoroughly in recent years using *in vivo* and *in vitro* methods. Martinez-Padilla et al.⁶¹ utilized a static *in vitro* method to investigate the digestibility of proteins in eight commercial plant-based milks, including almond milk. The authors found that almond milk had a similar total digestibility (25.19%) to cow's milk (26.43%) when using a trinitrobenzene sulfonic acid (TNBS)-based assay to calculate total digestibility. This value is lower than other findings on milk digestibility due to its quantification methods; for example, the INFOGEST method, with which bovine milk has been found to have an IVPD of 86-91%⁶². However, the overall quality of almond proteins is lower than that of milk proteins due to its limiting amino acids (typically methionine, lysine, and threonine³¹). Novel almond milks have also been evaluated using this method. Vanga et al.⁴⁴ used a static *in vitro* method to study the impact of pulsed ultrasonication on almond milk digestibility, among other properties. Ultrasonication can induce significant changes to protein structure, but the authors found no difference in digestibility between ultrasonicated samples and a control untreated sample. Another *in vitro* method used in some digestibility studies is the dynamic human gastric simulator (HGS), which aims to more closely mimic the process of human digestion through mechanical peristalsis of a "stomach" along with secretion of gastric fluids and controlled emptying⁶³. Wang et al.⁶⁴ utilized this method to investigate the structural and physicochemical changes of proteins and lipids in digestion of

almond milk made by soaking, wet grinding, and then filtration through a sieve. The authors found that the digestion of almond proteins by pepsin led to disruption of almond oil bodies followed by oil coalescence and creaming. Though this slowed the rate of oil delivery to the small intestine, it did not impact the rate of protein delivery. Ultimately, almond milk digestibility has been found to be relatively high in these studies, though the low level of protein in these almond milks presents an opportunity for investigating the impact of a higher protein content on overall protein digestibility.

1.5 Optimizing almond milk's nutritional profile: future directions in protein enrichment and quality

Using aqueous extraction processing (AEP) and enzyme-assisted aqueous extraction processing (EAEP), both lipids and proteins are simultaneously extracted⁶⁵⁻⁶⁷, enabling the removal of insoluble fiber components. This has the potential to overcome the issue of low protein concentration in the almond milk while maintaining an opaque, fully liquid product, as all components are either solubilized (carbohydrates and protein) or extracted as droplets in a fine emulsion (lipids). AEP is a flammable solvent-free method that simultaneously extracts proteins, carbohydrates, and fats from foods using water and mechanical agitation. This process often requires the use of upstream processing techniques such as flaking, grinding, or other cellular wall disruption^{65,66,68,69} to enhance the overall process extractability.

Traditional solvent extraction of oil relies upon the high chemical affinity the oil has for the solvent, causing the former to dissolve in and be extracted easily by the latter. AEP, however, does not employ direct oil solvation in this way as the molecular interactions between water and nonpolar molecules like triglycerides are weak, and the energy penalty for lipid-water solvation is too great to overcome⁶⁶. These interactions cannot overcome the hydrogen bonds in the aqueous

media; thus, the lipid portion of the extracted plant material preferentially associates with itself and remains separate from the aqueous phase. Instead, the AEP relies on the solubility of other components which do associate with and are solvated in the water, facilitating the release of previously bound oil bodies into the extraction medium⁶⁶. Extraction efficiency is typically quantified by total oil and total protein extractability (TOE and TPE, respectively). These metrics are calculated as the ratio of total proximate in the extract over the total proximate in the starting material, expressed as a percentage.

The EAEP is performed in the same manner as the AEP but with the addition of enzymes during the extraction step. A variety of enzymes have been used in EAEP, including enzymes that primarily hydrolyze cell walls (cellulase, hemicellulose, pectinase) and proteases⁶⁶. Proteases hydrolyze proteins, cutting them into smaller peptides with potentially increased solubility compared to their native state⁶⁷. The hydrolyzed proteins and peptides solubilize in the aqueous medium, which disrupts the internal cellular matrix, freeing oil to be washed into the slurry⁶⁷. The selection of enzyme used impacts the extractability and physicochemical properties of the recovered proteins. The selection of enzyme concentration and effective pH range for enzyme activity also plays a key role on protein extraction efficiency. Most EAEP studies utilize proteases that are active in neutral to alkaline pH ranges, which is beneficial for protein extractability as this is far from the isoelectric point of plant proteins^{66,68}, thereby improving protein solubility.

In AEP/EAEP of oil-containing seeds, extraction products can be divided into four major fractions: a protein-rich skim, oil-rich cream emulsion, free oil, and fiber-rich insoluble fraction⁶⁹. Frequently these fractions are separated to recover individual components such as protein or oil. Almond milk presents a unique opportunity for AEP/EAEP in that the liquid phases of skim, cream, and free oil may be combined to form one nutrient-rich milk product. Additionally, these

phases could be further separated and re-combined to almond milk processors' specifications with the aims of creating tailored milks with precisely formulated levels of protein and lipids.

1.6 Bridging the knowledge gaps: enhancing almond milk through focused research

The production of almond milk that meets desired nutritional, functional and sensory properties requires addressing existing research gaps. The following section highlights targeted research areas essential for enhancing protein extractability and digestibility, optimizing the extraction process, and improving consumer acceptance. Addressing these areas is crucial for developing a more competitive and appealing product within the plant-based milk market.

1.6.1. Impact of roasting conditions on protein extractability and digestibility

Roasting of nuts prior to size reduction is a common process, as it leads to changes in texture, flavor, and numerous other properties, as well as facilitating grinding⁷⁰. This is typically performed via convective heat transfer through hot air roasting as opposed to conductive heat transfer through oil roasting, either through continuous or batch processes⁷¹. Numerous chemical and physical reactions occur during roasting, notably the Maillard reaction, which contributes to the development of the typical roasted nut flavor and darkening of the nut's surface. Internally, roasting significantly changes the microstructure of nuts⁷². Dehydration and browning occur during roasting depending upon temperature and roasting time. During the roasting of almonds, the moisture content is decreased to 2% or less⁷¹ and the oleosomes may burst. Protein bodies become deformed due to denaturation, and the endoplasmic network keeping these in place is damaged. Additionally, cell walls may burst due to water vapor buildup as well as alteration of pectic cell contents during heating⁷³. As the cells begin to separate, cell porosity increases, and oil is released to flow intracellularly, leading to coalescence of oil droplets. Oxidation increases due to this change in microstructure; thus, roasting must be performed carefully.

Roasting nuts not only changes the color of the finished product but also imparts the desirable typical “nutty” flavors⁷² that enhance their sensory appeal. This transformation is the result of various chemical reactions, including the Maillard reaction, which involves the association between amino acids and reducing sugars and leads to the formation of brown pigments and flavor compounds.

Roasting can increase acceptability of nut products so long as over-roasting or burning is avoided⁷⁴. Extensive roasting of almonds can also lead to the formation of acrylamide, a potential human carcinogen⁷⁵. Thus, roasting must be done with attention to time and temperature to prevent burning and acrylamide formation.

Thermal processing of proteins often leads to conformational changes, beginning with the initial unfolding of the protein structure. This unfolding often leads to changes or losses in the secondary structure and the formation of aggregates through protein-protein interactions⁷⁶. These changes can significantly influence the physicochemical and biological properties of proteins, yielding positive and negative effects. For example, the degree of roasting can impact TOE and TPE in complex ways. Roasting can increase oil and protein yields because it facilitates grinding, thereby contributing to a reduction in particle size and subsequent increase in extractability. This effect, however, is impeded by prolonged roasting⁷⁷. Li et al.⁷⁷ found that protein content in the insoluble fraction (i.e., unextracted fraction) increased from 8.71% to 43.74% when increasing peanut roast temperature from 130 to 210 °C for 20 min, indicating a decrease in protein extraction.

IVPD can be improved by mild denaturation and unfolding of proteins caused by roasting, as this exposes more attack sites for enzyme hydrolysis. However, extensive denaturation could lead to protein aggregation and reduced IVPD⁷⁸. Zhou et al.⁷⁹ reported that peanuts roasted for 10 min at 170 °C led to an increase in digestibility compared to unroasted peanuts. Similarly, Embaby⁸⁰

found that boiling, microwave heating, autoclaving, and roasting all increased IVPD of peanuts. To investigate the impact of roasting on these and other properties, the degree of almond roast will be investigated as an upstream processing variable in Chapter 2.

1.6.2. Impact of particle size on extraction of protein and lipids

Some particle size reduction is necessary to expose the intracellular compounds to the aqueous medium in AEP and EAEP⁶⁶. Typically, reduced particle size is beneficial, as higher levels of cell wall breakdown facilitates the solubilization and diffusion of water-soluble compounds. This process facilitates the release of oil through the porous structure left behind. Additionally, smaller particle size also enhances the effectiveness of enzyme hydrolysis in the EAEP by increasing the surface area and accessibility for enzymes. Grinding is, however, an energy-intense unit operation, thus a balanced approach to determining the degree of particle size reduction is essential in process development. The impact of particle size on extractability will be investigated in Chapter 2.

1.6.3. Impact of solids-to-liquids ratio on extractability and sensory properties

The ratio of starting material to water, or solids-to-liquids ratio (SLR), in solid-liquid extractions significantly impact the concentration gradient, affecting the total solids content and the extractability of both soluble components as well as insoluble lipids⁶⁶. Typically, a richer product can be produced with a higher SLR; however, the efficiency of the reaction is decreased considering the concentration gradient between the material and the water has been decreased. Although a higher SLR has more starting material in its slurry and thus may yield a product with more protein and oil, the starting material will be less exhausted than that from a low SLR extraction. Both outcomes are likely to impact consumer perception and preference of the finished almond milk. To investigate the impact of SLR on extraction yield and acceptability, this parameter will be investigated in Chapter 3.

1.6.4. Impact of pH on extractability and protein physicochemical properties

The pH of extraction is of great importance in AEP and EAEP. Proteins are more soluble in water at pH values away from their IEP as discussed in Section 7.3. Lowest oil and protein extraction yields in AEP and EAEP typically occur at pH values near the IEP of the protein, where the protein solubility is minimum. Conversely, extractions carried out at pH values far from the IEP, either more acidic or alkaline, result in higher efficiency, as proteins become more soluble and available for diffusion into the extraction medium⁶⁶. For EAEP, the selection of the extraction pH is critical to the activity of the enzyme selected. De Almeida et al.⁶⁹ reported that setting extraction pH at 9.0 was equally beneficial to EAEP with a neutral protease, and AEP in terms of total yield of oil and protein due to the inherent solubility of the proteins at this alkaline pH. Conversely, in that study, a lower pH (6.5) led to a significantly higher yield in EAEP due to the lower inherent solubility of the protein in AEP closer to the IEP.

Other factors, such as the functionality of the extracted proteins, should also be considered when selecting the extraction pH. Although proteins extracted via the EAEP at alkaline pH may exhibit higher solubility at lower pH values than their AEP counterparts, likely due to the release of peptides with increased solubility^{69,81,82}. Exposure of protein hydrophobic sites due to proteolysis and protein-protein interactions can also promote aggregation, ultimately reducing solubility⁸³. These interactions and potential impacts of functionality underscore the importance of selecting extraction pH to optimize both extraction efficiency and protein functionality in the final products. To further investigate the impact of pH on these factors, the impact of extraction pH will be systematically investigated in Chapter 3.

1.6.5. The role of enzymes in optimizing total protein and oil extractability

Current research has demonstrated that the use of enzymes during the extraction can have profound impacts on TOE and TPE due to the factors outlined above (Section 6). Work from our group has demonstrated that EAEP increased TOE to 75% compared to 70% in AEP, as well as TPE to 72% compared to 65% in AEP using full-fat almond flour. The use of protease during the extraction thus offers a great potential for increased efficiency and a more enriched product. Increased oil extraction would lead to a product with a higher oil content, which could lead to a richer product, but could also cause a weaker emulsion due to a higher oil load for the emulsifier and proteins in solution. Similarly, a higher protein extractability could lead to a richer product with higher protein content. This higher protein content could improve the emulsion stability, but the proteolysis could negatively impact this (see section 7.7). The impact of enzyme use on TOE and TPE will be investigated in Chapters 2-4.

1.6.6. Harnessing proteolysis for enhanced control of protein zeta-potential

The zeta-potential (ξ -potential) of a protein is a measure of its electrical potential near its surface, and can indicate its stability in solution⁸⁴. A high total charge on the surface of proteins leads to electrostatic repulsion, reducing protein-protein interactions and sedimentation. Dias & de Moura Bell²⁷ found that the use of a neutral protease in EAEP increased the absolute zeta potential of almond flour proteins compared to AEP proteins at pH above the proteins' isoelectric point (5-9). Similarly, de Souza et al.⁸⁵ found that alkaline protease in EAEP resulted in higher absolute zeta-potential of proteins extracted from almond cake compared to AEP at alkaline pH, attributing this to the increased exposure of ionizable moieties previously hidden within the native protein. The addition of bromelain, a thiol endopeptidase, to cashew milk made by the standard blended method, also reflected this trend⁸⁶. This release of ionizable groups may improve protein solubility due to a reduction in interactions between proteins. This could be beneficial in almond milk as it

would lead to increased stability of the proteins in solution. Chapter 2 will investigate the impact of enzyme use on this metric.

1.6.7. Tailoring enzyme use for enhanced emulsion stability

Emulsion stability over time is of critical importance to almond milk, especially with the expected higher oil content in AEP and EAEP milks in this study. The destabilization of the emulsified almond milk could lead to creaming and a texture that is not acceptable to consumers. Extraction method (AEP or EAEP) could have a significant impact on this metric. Protein hydrolysis can increase the emulsifying capacity of proteins; however, extensive hydrolysis can also destabilize emulsions⁸⁷. The cleavage of native proteins into potentially more soluble peptides can negatively impact emulsion stability away from the isoelectric point, as charged native proteins are more likely to exhibit steric hindrance of oil droplet coalescence⁸⁸. Near the isoelectric point, protein hydrolysates can have higher emulsion capacities and stabilities than native protein due to the reduction in protein-protein repulsion^{27,34,85}. This negative impact of hydrolysis on emulsion stability away from the isoelectric point, with an improvement of emulsion stability near the isoelectric point, has been observed on almond oil press cake proteins⁸⁵, almond flour²⁷, and flaxseed⁸⁹. Therefore, in addition to determining and optimizing the use of enzyme from an overall extractability perspective, this variable should also be explored for its potential impact on the stability of almond milk, which is an oil in water emulsion (evaluated in Chapter 4).

1.6.8. Reducing almond protein immunoreactivity with proteolysis

During hydrolysis, proteins are broken down into smaller peptides through enzymatic action. This process alters the sequences and conformation of epitopes, potentially leading to significantly reduction in their immunoreactivity⁹⁰. Such an approach offers an opportunity to produce reduced-allergenic or hypoallergenic versions of these proteins. This is of importance in

almond milk in that almonds are a major allergen, with many people having life-threatening reactions to consuming almond proteins⁹¹. Amandin, the major protein in almonds, is a 11S globulin, comprising 65% of total almond protein³¹. Previous work in our group has shown that EAEP can reduce immunoreactivity of amandin by 75%⁹². Similar hydrolysis-induced reductions in antigenicity have been observed in lentil, peanut, and chickpea proteins^{90,93,94}. Chapters 3 and 4 will investigate the impact of enzyme use during the extraction of almond paste on almond milk immunoreactivity.

1.6.9. Impact of enzymatic extraction on protein digestibility

Enzyme use during the extraction can have a pronounced impact on *in vitro* protein digestibility (IVPD). The mechanism and extent of hydrolysis, along with the method of analysis, can vary widely, leading to some variations regarding its impact on digestibility.

Dias et al.⁹² found that proteins extracted via EAEP from blanched almond flour were more digestible (89%) than those extracted via AEP (79%); however, de Souza et al.⁹⁵ found that the use of enzyme during the extraction decreased IVPD of proteins extracted from a partially defatted almond cake from 73 to 64%. In the former study, a neutral protease was used at 0.5% (w/w) of the extraction, while the later utilized an alkaline protease at 0.85% (w/w). The difference between the two studies could be attributed to differences in the starting material and enzymes used. Additionally, increased hydrolysis can leave fewer protein sites available for digestion by the enzymes used in these assays, thus decreasing this value despite the high bioavailability of more highly digested proteins. Furthermore, in de Souza et al.'s study, the undigested protein content was calculated using only the proteinaceous nitrogen present, which ignores the hydrolyzed peptides present in the EAEP extract prior to digestion. This can lead to an underestimation of IVPD.

Betancur-Acona et al.⁹⁶ found that hydrolysis of lima bean protein isolate via Alcalase (an alkaline protease) yielded 72% IVPD, while Flavourzyme (an exopeptidase-endoprotease mix) hydrolyzed isolate IVPD was 70%: both were lower than the unhydrolyzed isolate's 79% digestibility. Similarly, Chan and Ma⁹⁷ reported that soymilk okara hydrolyzed with trypsin was reported as having a lower IVPD (73-75%) than the unhydrolyzed okara protein isolate (78%). Digestibility of extracted proteins in AEP and EAEP are investigated in Chapters 2 and 4 of this work.

1.6.10. Exploring the impact of proteolysis on the release of bitter peptides

Though the use of enzymes can improve extractability and some functional and biological properties of proteins, the production of hydrolysates can lead to bitter flavors^{98,99}. Low molecular weight peptides with high concentrations of hydrophobic and basic amino acids can be perceived as very bitter, limiting the use of hydrolysates in food products¹⁰⁰. Alkaline protease, for example, is especially prone to the release of bitter peptides⁹⁸. This leads to the potential incompatibility of enzyme use in almond milk production for human consumption, as bitterness is an undesirable quality in plant-based milks. Bitterness perception varies widely within humans due to variations in sensitivity, impeding the use of analytical techniques to determine perceived degree of bitterness in consumers. Chapter 3 will detail our investigation into the impact of enzyme hydrolysis on perceived almond milk bitterness.

1.6.11. Other extraction conditions

Other extraction conditions such as temperature, extraction time, centrifugation speed/duration, agitation speed, etc. also have pronounced impacts on extraction yield. Given the complex nature and sheer quantity of these factors, in this study we have chosen to select

conditions previously validated on almond flour in our group^{69,101}: 1 hour of extraction at 50 °C with agitation speed of 120 rpm, followed by centrifugation at 4 °C at 3000 x g for 30 minutes.

6.12. Potential challenges

Though the proposed methods present many potential benefits, given the difference in processing between AEP and EAEP and a conventional suspended solids almond milk, there are also potential challenges. As outlined in Section 6.10, the release of peptides by enzyme extraction could lead to bitter flavors, potentially decreasing consumer acceptance of this milk. The expected increase of oil in these milks due to the extraction method could lead to an oily mouthfeel in the milk, which could be difficult to fully emulsify and lead to creaming and separation. To address these, the impact of processing parameters on sensory perception of the milks will be investigated in Chapter 3, along with their physiochemical composition, while the impact of processing scale-up and UHT processing/homogenization will be studied in Chapter 4.

6.13. Potential impact

The proposed method presents an opportunity for almond milk processors to produce a product that is richer in nutrients than currently available milks on the retail market. By exploring the relationship between preprocessing (degree of roast and grind), extraction parameters, and scale-up on the yield and physicochemical properties of the milks, we aim to demonstrate the feasibility and value of this method to the almond milk industry.

1.7 Conclusions

Almond milk is a popular non-dairy beverage that is low in nutrients compared to bovine milk, especially protein and lipids. Though some research has been performed on almond milk, much of it has been on processing methods on the finished milk, and not focused on the improvement on the nutritional quality of the milk itself (i.e., on the production method itself).

This review highlights some recent work in this field; however, there is notable lack of research focused on developing innovative processing strategies to improve the nutritional quality of almond milk. This gap presents an opportunity to explore completely novel methods for producing almond milk with enhanced nutritional and sensory properties. The use of flammable solvent-free processes, such as AEP and EAEP, offers various opportunities to further optimize key extraction conditions, which could also enhance the functional and biological properties of almond milk. This review detailed the mechanism of these processes, as well as the potential impact of various pre-processing and extraction parameters on the extracted milk.

1.8 Objectives

The work performed for this dissertation has the following objectives:

1. Determine the impact of degree of roast and grind on the extractability of oil and protein from almonds as well as physicochemical properties of the extracted protein to select an optimized extraction starting material for AEP/EAEP;
2. Investigate the impact of extraction parameters (SLR, pH, enzyme level) on sensory and physicochemical outcomes of AEP/EAEP to select an optimized extraction process;
3. Perform scaled-up extractions of the milks produced by the methods selected in Objective 2 to then pasteurize and homogenize at a pilot scale and investigate the stability of the milks over their shelf-life.

1.9 References

1. Springmann, M. *et al.* Options for keeping the food system within environmental limits. *Nature* **562**, 519–525 (2018).
2. Poore, J. & Nemecek, T. Reducing food's environmental impacts through producers and consumers. *Science* **360**, 987–992 (2018).
3. McClements, D. J., Newman, E. & McClements, I. F. Plant-based Milks: A Review of the Science Underpinning Their Design, Fabrication, and Performance. *Comprehensive Reviews in Food Science and Food Safety* **18**, 2047–2067 (2019).
4. McCarthy, K. S., Parker, M., Ameerally, A., Drake, S. L. & Drake, M. A. Drivers of choice for fluid milk versus plant-based alternatives: What are consumer perceptions of fluid milk? *Journal of Dairy Science* **100**, 6125–6138 (2017).
5. Franklin-Wallis, O. White gold: the unstoppable rise of alternative milks | Food | The Guardian. *The Guardian* <https://www.theguardian.com/news/2019/jan/29/white-gold-the-unstoppable-rise-of-alternative-milks-oat-soy-rice-coconut-plant> (2019).
6. Mordor Intelligence. Almond Milk Market Size & Share Analysis - Industry Research Report - Growth Trends. <https://www.mordorintelligence.com/industry-reports/global-almond-milk-market> (2024).
7. Grant, C. A. & Hicks, A. L. Comparative life cycle assessment of milk and plant-based alternatives. *Environmental Engineering Science* **35**, 1235–1247 (2018).
8. Collard, K. M. & McCormick, D. P. A Nutritional Comparison of Cow's Milk and Alternative Milk Products. *Academic Pediatrics* **21**, 1067–1069 (2021).
9. Vanga, S. K. & Raghavan, V. How well do plant based alternatives fare nutritionally compared to cow's milk? *Journal of Food Science and Technology* **55**, 10–20 (2018).
10. Bridges, M. Moo-ove over, cow's milk: The rise of plant-based dairy alternatives. *Practical Gastroenterology* **42**, 20–27 (2018).
11. Jeske, S., Zannini, E. & Arendt, E. K. Past, present and future: The strength of plant-based dairy substitutes based on gluten-free raw materials. *Food Research International* **110**, 42–51 (2018).
12. Mäkinen, O. E., Wanhalinna, V., Zannini, E. & Arendt, E. K. Foods for Special Dietary Needs: Non-dairy Plant-based Milk Substitutes and Fermented Dairy-type Products. *Critical Reviews in Food Science and Nutrition* **56**, 339–349 (2016).
13. McClements, D. J., Newman, E. & McClements, I. F. Plant-based Milks: A Review of the Science Underpinning Their Design, Fabrication, and Performance. *Comprehensive Reviews in Food Science and Food Safety* **18**, 2047–2067 (2019).
14. Reyes-Jurado, F. *et al.* Plant-Based Milk Alternatives: Types, Processes, Benefits, and Characteristics. *Food Reviews International* **39**, 2320–2351 (2023).
15. Sethi, S., Tyagi, S. K. & Anurag, R. K. Plant-based milk alternatives an emerging segment of functional beverages: a review. *Journal of Food Science and Technology* **53**, 3408–3423 (2016).
16. Silva, A. R. A., Silva, M. M. N. & Ribeiro, B. D. Health issues and technological aspects of plant-based alternative milk. *Food Research International* **131**, 108972 (2020).
17. King, J. C., Blumberg, J., Ingwersen, L., Jenab, M. & Tucker, K. L. *Tree Nuts and Peanuts as Components of a Healthy Diet 1,2. Nuts and Health Symposium J. Nutr* vol. 138 1736–1740 www.ars.usda.gov/foodsurvey (2007).

18. Yada, S., Lapsley, K. & Huang, G. A review of composition studies of cultivated almonds: Macronutrients and micronutrients. *Journal of Food Composition and Analysis* **24**, 469–480 (2011).
19. Tomishima, H., Luo, K. & Mitchell, A. E. The Almond (*Prunus dulcis*): Chemical Properties, Utilization, and Valorization of Coproducts. *Annu. Rev. Food Sci. Technol.* **13**, 145–166 (2022).
20. House, J. D., Hill, K., Neufeld, J., Franczyk, A. & Nosworthy, M. G. Determination of the protein quality of almonds (*Prunus dulcis* L.) as assessed by in vitro and in vivo methodologies. *Food Science & Nutrition* **7**, 2932–2938 (2019).
21. Yada, S., Huang, G. & Lapsley, K. Natural variability in the nutrient composition of California-grown almonds. *Journal of Food Composition and Analysis* **30**, 80–85 (2013).
22. Sathe, S. K., Seeram, N. P., Kshirsagar, H. H., Heber, D. & Lapsley, K. A. Fatty Acid Composition of California Grown Almonds. *Journal of Food Science* **73**, (2008).
23. Schaafsma, G. The Protein Digestibility-Corrected Amino Acid Score (PDCAAS)—A Concept for Describing Protein Quality in Foods and Food Ingredients: A Critical Review. *Journal of AOAC INTERNATIONAL* **88**, 988–994 (2005).
24. Ahrens, S., Venkatachalam, M., Mistry, A. M., Lapsley, K. & Sathe, S. K. Almond (*Prunus dulcis* L.) protein quality. *Plant Foods for Human Nutrition* **60**, 123–128 (2005).
25. Young, C. T., Schadel, W. E., Pattee, H. E. & Sanders, T. H. The micro structure of almond (*Prunus dulcis* (Mill.) D.A. Webb cv. 'Nonpareil') cotyledon. *LWT - Food Science and Technology* **37**, 317–322 (2004).
26. Wolf, W. J. & Sathe, S. K. *Ultracentrifugal and Polyacrylamide Gel Electrophoretic Studies of Extractability and Stability of Almond Meal Proteins*. *J Sci Food Agric* vol. 78 (1998).
27. Dias, F. F. G. & de Moura Bell, J. M. L. N. Understanding the impact of enzyme-assisted aqueous extraction on the structural, physicochemical, and functional properties of protein extracts from full-fat almond flour. *Food Hydrocolloids* **127**, 107534 (2022).
28. Venkatachalan, M. & Sathe, S. K. Chemical composition of selected edible nut seeds. *Journal of Agricultural and Food Chemistry* **54**, 4705–4714 (2006).
29. Kshirsagar, H. H., Fajer, P., Sharma, G. M., Roux, K. H. & Sathe, S. K. Biochemical and spectroscopic characterization of almond and cashew nut seed 11S legumins, amandin and anacardein. *Journal of Agricultural and Food Chemistry* **59**, 386–393 (2011).
30. Albillos, S. M., Menhart, N. & Fu, T. J. Structural stability of amandin, a major allergen from almond (*prunus dulcis*), and its acidic and basic polypeptides. *Journal of Agricultural and Food Chemistry* **57**, 4698–4705 (2009).
31. Sathe, S. K. *et al.* Biochemical characterization of amandin, the major storage protein in almond (*Prunus Dulcis* L.). *Journal of Agricultural and Food Chemistry* **50**, 4333–4341 (2002).
32. Beisson, F., Ferte, N., Voultour, R. & Arondel, V. Large scale purification of an almond oleosin using an organic solvent procedure. *Plant Physiology and Biochemistry* **39**, 623–630 (2001).
33. Sharma, G. M., Su, M., Joshi, A. U., Roux, K. H. & Sathe, S. K. Functional properties of select edible oilseed proteins. *Journal of Agricultural and Food Chemistry* **58**, 5457–5464 (2010).
34. Amirshaghghi, Z., Rezaei, K. & Habibi Rezaei, M. Characterization and functional properties of protein isolates from wild almond. *Journal of Food Measurement and Characterization* **11**, 1725–1733 (2017).

35. Sze-Tao, K. W. C. & Sathe, S. K. Functional properties and in vitro digestibility of almond (*Prunus dulcis* L.) protein isolate. *Food Chemistry* **69**, 153–160 (2000).
36. Sathe, S. K. & Sze, K. W. C. Thermal aggregation of almond protein isolate. *Food Chemistry* **59**, 95–99 (1997).
37. Dias, F. F. G. Leveraging the use of oxylipin measurements to elucidate the integrated impact of greener extraction and recovery methods on almond oil quality. (2023).
38. Ahmad, Z. The uses and properties of almond oil. *Complementary Therapies in Clinical Practice* **16**, 10–12 (2010).
39. Mathai, J. K., Liu, Y. & Stein, H. H. Values for digestible indispensable amino acid scores (DIAAS) for some dairy and plant proteins may better describe protein quality than values calculated using the concept for protein digestibility-corrected amino acid scores (PDCAAS). *British Journal of Nutrition* **117**, 490–499 (2017).
40. Deeth, H. C. Heat Treatment of Milk: Extended Shelf-Life (ESL) and Ultra-High Temperature (UHT) Treatments. in *Encyclopedia of Dairy Sciences* 618–631 (Elsevier, 2022). doi:10.1016/B978-0-12-818766-1.00140-9.
41. Browning, E., Lewis, M. & Macdougall, D. Predicting safety and quality parameters for UHT-processed milks. *Int J of Dairy Tech* **54**, 111–120 (2001).
42. Sarangapany, A. K., Murugesan, A., Annamalai, A. S., Balasubramanian, A. & Shanmugam, A. An overview on ultrasonically treated plant-based milk and its properties – A Review. *Applied Food Research* **2**, 100130 (2022).
43. Maghsoudlou, Y., Alami, M., Mashkour, M. & Shahraki, M. H. Optimization of Ultrasound-Assisted Stabilization and Formulation of Almond Milk. *Journal of Food Processing and Preservation* **40**, 828–839 (2016).
44. Vanga, S. K., Wang, J., Orsat, V. & Raghavan, V. Effect of pulsed ultrasound, a green food processing technique, on the secondary structure and in-vitro digestibility of almond milk protein. *Food Research International* **137**, 109523 (2020).
45. Manzoor, M. F. *et al.* Thermosonication effect on bioactive compounds, enzymes activity, particle size, microbial load, and sensory properties of almond (*Prunus dulcis*) milk. *Ultrasonics Sonochemistry* **78**, 105705 (2021).
46. Faraloni, C. *et al.* New Route to the Production of Almond Beverages Using Hydrodynamic Cavitation. *Foods* **12**, 935 (2023).
47. Campbell, J. R. *Dairy Production and Processing: The Science of Milk and Milk Products*. (Waveland Press, Inc., Long Grove, Illinois, 2016).
48. Briviba, K., Gräf, V., Walz, E., Guamis, B. & Butz, P. Ultra high pressure homogenization of almond milk: Physico-chemical and physiological effects. *Food Chemistry* **192**, 82–89 (2016).
49. Bernat, N., Cháfer, M., Rodríguez-García, J., Chiralt, A. & González-Martínez, C. Effect of high pressure homogenisation and heat treatment on physical properties and stability of almond and hazelnut milks. *LWT - Food Science and Technology* **62**, 488–496 (2015).
50. Valencia-Flores, D. C., Hernández-Herrero, M., Guamis, B. & Ferragut, V. Comparing the Effects of Ultra-High-Pressure Homogenization and Conventional Thermal Treatments on the Microbiological, Physical, and Chemical Quality of Almond Beverages. *Journal of Food Science* **78**, 199–205 (2013).
51. Iorio, M. C. *et al.* A case study on the use of ultrasound for the inhibition of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in almond milk. *Ultrasonics Sonochemistry* **52**, 477–483 (2019).

52. Munekata, P. E. S. S. *et al.* Effect of innovative food processing technologies on the physicochemical and nutritional properties and quality of non-dairy plant-based beverages. *Foods* **9**, 1–16 (2020).
53. Ghoshal, G. Comprehensive review on pulsed electric field in food preservation: gaps in current studies for potential future research. *Heliyon* **9**, e17532 (2023).
54. Manzoor, M. F. *et al.* Impact of pulsed electric field on rheological, structural, and physicochemical properties of almond milk. *J Food Process Engineering* **42**, e13299 (2019).
55. Manzoor, M. F. *et al.* Effect of pulsed electric field and thermal treatments on the bioactive compounds, enzymes, microbial, and physical stability of almond milk during storage. *J Food Process Preserv* **44**, (2020).
56. Dhakal, S. *et al.* Effect of high pressure processing on the immunoreactivity of almond milk. *Food Research International* **62**, 215–222 (2014).
57. Bernat, N., Cháfer, M., Chiralt, A. & González-Martínez, C. Development of a non-dairy probiotic fermented product based on almond milk and inulin. *Food Science and Technology International* **21**, 440–453 (2015).
58. Al-Zahrani, A. J. & Shori, A. B. Viability of probiotics and antioxidant activity of soy and almond milk fermented with selected strains of probiotic *Lactobacillus* spp. *LWT* **176**, 114531 (2023).
59. Lipan, L. *et al.* Spray drying and storage of probiotic-enriched almond milk: probiotic survival and physicochemical properties. *J Sci Food Agric* **100**, 3697–3708 (2020).
60. Minekus, M. *et al.* A standardised static in vitro digestion method suitable for food-an international consensus. *Food and Function* **5**, 1113–1124 (2014).
61. Martínez-Padilla, E. *et al.* In vitro protein digestibility and fatty acid profile of commercial plant-based milk alternatives. *Foods* **9**, 1–17 (2020).
62. Komatsu, Y. *et al.* Nutritional Evaluation of Milk-, Plant-, and Insect-Based Protein Materials by Protein Digestibility Using the INFOGEST Digestion Method. *J. Agric. Food Chem.* **71**, 2503–2513 (2023).
63. Kong, F. & Singh, R. P. A Human Gastric Simulator (HGS) to Study Food Digestion in Human Stomach. *Journal of Food Science* **75**, (2010).
64. Wang, X., Ye, A. & Singh, H. Structural and physicochemical changes in almond milk during: In vitro gastric digestion: Impact on the delivery of protein and lipids. *Food and Function* **11**, 4314–4326 (2020).
65. Rosenthal, A., Pyle, D. L. & Niranjana, K. Simultaneous aqueous extraction of oil and protein from soybean: Mechanisms for process design. *Food and Bioproducts Processing: Transactions of the Institution of Chemical Engineers, Part C* **76**, 224–230 (1998).
66. Rosenthal, A., Pyle, D. L. & Niranjana, K. Aqueous and enzymatic processes for edible oil extraction. *Enzyme and Microbial Technology* **19**, 402–420 (1996).
67. Campbell, K. A. *et al.* Advances in aqueous extraction processing of soybeans. *JAOCs, Journal of the American Oil Chemists' Society* **88**, 449–465 (2011).
68. Rosenthal, A., Pyle, D. L., Niranjana, K., Gilmour, S. & Trinca, L. Combined effect of operational variables and enzyme activity on aqueous enzymatic extraction of oil and protein from soybean. *Enzyme* **28**, 499–509 (2001).
69. de Almeida, N. M., Dias, F. F. G., Rodrigues, M. I. & Bell, J. M. L. N. de M. Effects of processing conditions on the simultaneous extraction and distribution of oil and protein from almond flour. *Processes* **7**, (2019).

70. Gorrepati, K., Balasubramanian, S. & Chandra, P. Plant based butters. *Journal of Food Science and Technology* **52**, 3965–3976 (2015).
71. Huang, G. *Hot Air Roasting of Almonds*. 1–5 (2014).
72. Perren, R. & Escher, F. E. Impact of roasting on nut quality. in *Improving the Safety and Quality of Nuts* 173–197 (Elsevier, 2013). doi:10.1533/9780857097484.2.173.
73. Altan, A., McCarthy, K. L., Tikekar, R., McCarthy, M. J. & Nitin, N. Image Analysis of Microstructural Changes in Almond Cotyledon as a Result of Processing. *Journal of Food Science* **76**, 212–221 (2011).
74. de Freitas Floriano, R., Gräbin, K., Rossi, R. C., Ferreira, C. D. & Ziegler, V. Impact of roasting conditions on the quality and acceptance of the peanut paste. *Journal of Texture Studies* **51**, 841–848 (2020).
75. Zhang, G., Huang, G., Xiao, L., Seiber, J. & Mitchell, A. E. Acrylamide formation in almonds (*Prunus dulcis*): Influences of roasting time and temperature, precursors, varietal selection, and storage. *Journal of Agricultural and Food Chemistry* **59**, 8225–8232 (2011).
76. Yu, P. Protein Molecular Structures, Protein SubFractions, and Protein Availability Affected by Heat Processing: A Review. *American J. of Biochemistry and Biotechnology* **3**, 66–86 (2007).
77. Li, P. *et al.* Effects of roasting temperatures and grinding type on the yields of oil and protein obtained by aqueous extraction processing. *Journal of Food Engineering* **173**, 15–24 (2016).
78. Orlie, V. *et al.* Effect of processing on *in vitro* digestibility (IVPD) of food proteins. *Critical Reviews in Food Science and Nutrition* **63**, 2790–2839 (2023).
79. Zhou, H. *et al.* The effect of roasting on peanut allergens' digestibility, allergenicity, and structure. *Food Bioscience* **44**, 101454 (2021).
80. Embaby, H. E.-S. Effect of Heat Treatments on Certain Antinutrients and *in vitro* Protein Digestibility of Peanut and Sesame Seeds. *FSTR* **17**, 31–38 (2010).
81. Sari, Y. W., Bruins, M. E. & Sanders, J. P. M. Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. *Industrial Crops & Products* **43**, 78–83 (2013).
82. Jarpa-Parra, M. *et al.* Optimization of lentil protein extraction and the influence of process pH on protein structure and functionality. *LWT - Food Science and Technology* **57**, 461–469 (2014).
83. Zhang, Y. *et al.* Soy peptide aggregates formed during hydrolysis reduced protein extraction without decreasing their nutritional value. *Food Funct.* **8**, 4384–4395 (2017).
84. Cano-Sarmiento, C. *et al.* Zeta Potential of Food Matrices. *Food Engineering Reviews* **10**, 113–138 (2018).
85. De Souza, T. S. P., Dias, F. F. G., Koblit, M. G. B. & De Moura Bell, J. M. L. N. Effects of enzymatic extraction of oil and protein from almond cake on the physicochemical and functional properties of protein extracts. *Food and Bioprocess Technology* **122**, 280–290 (2020).
86. Pan, W., Zheng, Z., Li, P., Ai, Z. & Liu, Y. Effects of enzymatic modification on the stability of cashew-based milk. *Food Funct.* **14**, 7946–7958 (2023).
87. Damodaran, S. Protein Stabilization of Emulsions and Foams. *Journal of Food Science* **70**, R54–R66 (2006).
88. McClements, D. J. *Food Emulsions: Principles, Practices, and Techniques, Third Edition*. (CRC Press, Boca Raton, 2015). doi:10.1201/b18868.
89. Tirgar, M., Silcock, P., Carne, A. & Birch, E. J. Effect of extraction method on functional properties of flaxseed protein concentrates. *Food Chemistry* **215**, 417–424 (2017).

90. Cabanillas, B. *et al.* Influence of Enzymatic Hydrolysis on the Allergenicity of Roasted Peanut Protein Extract. *Int Arch Allergy Immunol* **157**, 41–50 (2012).
91. Mandalari, G. & Mackie, A. R. Almond Allergy: An Overview on Prevalence, Thresholds, Regulations and Allergen Detection. *Nutrients* **10**, 1706 (2018).
92. Furlan Goncalves Dias, F. *et al.* Effects of protease-assisted aqueous extraction on almond protein profile, digestibility, and antigenicity. *Current Research in Food Science* **6**, 100488 (2023).
93. Clemente, A., Vioque, J., Sanchez-Vioque, R., Pedroche, J. & Millán, F. Production of Extensive Chickpea (*Cicer arietinum* L.) Protein Hydrolysates with Reduced Antigenic Activity. *J. Agric. Food Chem.* **47**, 3776–3781 (1999).
94. Cabanillas, B. *et al.* Effects of enzymatic hydrolysis on lentil allergenicity. *Molecular Nutrition Food Res* **54**, 1266–1272 (2010).
95. de Souza, T. S. P., Dias, F. F. G., Oliveira, J. P. S., de Moura Bell, J. M. L. N. & Koblitiz, M. G. B. Biological properties of almond proteins produced by aqueous and enzyme-assisted aqueous extraction processes from almond cake. *Scientific Reports* **10**, 1–12 (2020).
96. Betancur-Ancona, D. *et al.* Functional properties of hydrolysates from Phaseolus lunatus seeds. *International Journal of Food Science and Technology* **44**, 128–137 (2009).
97. Chan, W. -M. & Ma, C. -Y. Modification of Proteins from Soymilk Residue (Okara) by Trypsin. *Journal of Food Science* **64**, 781–786 (1999).
98. Liu, B. *et al.* Review on the release mechanism and debittering technology of bitter peptides from protein hydrolysates. *Comp Rev Food Sci Food Safe* **21**, 5153–5170 (2022).
99. Yan, J. & Tong, H. An overview of bitter compounds in foodstuffs: Classifications, evaluation methods for sensory contribution, separation and identification techniques, and mechanism of bitter taste transduction. *Comp Rev Food Sci Food Safe* **22**, 187–232 (2023).
100. Mireku-Gyimah, N. A. & Jibira, Y. Sensory Perception of Bioactive Peptides and Debittering Techniques Employed for Taste Improvement. in *Bioactive Peptides* (eds. Onuh, J. O., Selvamuthukumar, M. & Pathak, Y. V.) 281–296 (CRC Press, First edition. | Boca Raton : CRC Press, 2021. | Series:, 2021). doi:10.1201/9781003052777-13.
101. Dias, F. F. G., Taha, A. Y. & Leite Nobrega De Moura Bell, J. Effects of enzymatic extraction on the simultaneous extraction of oil and protein from full-fat almond flour, insoluble microstructure, emulsion stability and functionality. *Future Foods* **5**, 100151 (2022).

Chapter 2: From solid dispersion to the simultaneous extraction of lipids and proteins: a bio-guided strategy to improve the nutritional and biological properties of almond milk

Abstract: Almond milk consumption has increased as an alternative to dairy milk; however, its low nutrient density has negatively impacted consumer perception. Unroasted, light, and dark roasted almonds were ground to different particle sizes (flour, butter, or paste) and subjected to aqueous (AEP) and enzyme-assisted extraction processes (EAEP) to produce more nutrient-dense and functional milks. Although dark roasting increased oil extractability from flour (D(4,3) diameter of 300 μm) to 79%, particle size reduction from flour to butter (35 μm) or paste (20 μm) increased oil extractability up to 96% for both processes. Roasting enhanced grinding and protein extractability from flour (46% for unroasted vs. 62% for dark), but reduced extractability from butter and paste (81 to 71% in the AEP), likely due to protein denaturation. Regardless of roast and grind, EAEP increased protein extractability up to 91.7%. Confocal laser scanning microscopy revealed that roasting led to bursting and coalescence of oil bodies, increased cellular disruption, and protein aggregation. Light roasted butter led to an almond milk with improved nutrient density (1.63% protein and 4.68% oil for AEP and 1.87% protein and 5.16% oil for EAEP) and was selected for subsequent nutritional and physicochemical characterization. *In vitro* protein digestibility was high across all samples, ranging from 84-88.1%. Proteolysis and roasting decreased the α -helix content but increased the unordered structures of dark roasted butter milk proteins. Surface hydrophobicity was reduced for unroasted and light roasted EAEP milks but increased for dark roasted milk. EAEP milk proteins exhibited higher absolute zeta potential values than AEP milk proteins, regardless of roast. These findings highlight how roasting and grinding affect milk composition and physicochemical properties and underscore the potential of AEP and EAEP as alternative methods for producing almond milks with increased nutritional and functional properties.

Keywords: almond milk, protein, aqueous extraction processing, enzyme-assisted extraction processing, roasting, particle size

2.1 Introduction

Almond milk has emerged as a popular alternative to dairy milk, with an expected global market value of \$31.3B by 2028¹. Non-dairy milks in general are growing in popularity, with almond milk as the category leader in the United States². However, its nutritional composition lacks in comparison to dairy milk, with most containing ~0.5% protein and ~1.25% fat³. Most commercial almond milk production involves dispersing finely ground roasted almond paste in water under high shear mixing⁴ (Fig 2.1a). Emulsifiers and/or gums may be added to keep the solids in suspension, along with sweeteners and/or flavorants⁵. This method limits the quantity of almond paste that can be used in the product, as high solid loadings would result in particle sedimentation and unpleasant gritty textures. Because of this, the protein and fat content of almond milk produced using solid dispersion methods are also limited. Most almond milks on the retail market in the United States are roughly 2% almonds, leading to a final protein concentration of 0.5 g/100 g based on a market survey. Compared to bovine milk's roughly 3.3 g protein and 3.7 g fat per 100 g, this disparity precludes almond milk from serving as a true nutritional analog to bovine milk⁶.

Almonds are intrinsically a nutrient-dense food, with relatively high levels of protein (~20%) and fat (~50-55%) by weight^{7,8}. Therefore, almond milk has great potential for nutritional improvement. Although some protein- and fat-rich almond milks (up to 2.1% protein and 4.6% fat, based on market survey) are commercially available, the use of more almond paste or larger particle size ground almonds in their manufacturing process often leads to sedimentation and an unpleasant grittiness on the tongue.

In the most popular almond milks, emulsifiers and gums such as lecithin, gellan gum, and xanthan gum are used to both create and stabilize the emulsion in the milk. These additives keep

the insoluble almond solids in suspension and impart a desirable creamy mouthfeel. Though more research is needed in this area, food additives within this category have been linked to potential gut health issues such as increased inflammation and potential carcinogenesis, feeding negative publicity and consumer wariness of almond and other plant-based milks^{9–12}. These ingredients can also contribute to equipment fouling (i.e., deposition of unwanted material on heat exchangers) during thermal treatment^{13,14}, which can lead to increased equipment downtime.

To overcome the limitation in nutrient content of almond milk while maintaining a fluid texture without sedimentation, a new processing approach is necessary. Aqueous-based extractions present a valuable alternative to the current approach of commercial almond milk production. In aqueous (AEP) and enzyme-assisted aqueous extraction processes (EAEP), lipids and proteins are simultaneously extracted into the aqueous phase, while the fiber-rich insoluble fraction is removed through centrifugation. Therefore, a soluble protein- and lipid-enriched extract (i.e., almond milk) can be obtained^{15–17}. By tailoring the upstream processing conditions for the raw materials, i.e., degrees of roast and grind, as well as the extraction conditions (pH, temperature, extraction time, degree of hydrolysis), manufacturers can optimize the extraction step to meet their product specifications. Exploiting the natural emulsifying and gelation capacities of the proteins within the almonds can also potentially reduce the need of emulsifiers and/or hydrocolloids.

The protein bodies and oleosomes (oil bodies) contained within almond cells are bound by an intracellular cytoplasmic network¹⁸. When almonds are ground, this network is destroyed, the protein and oil bodies rupture, and oil becomes a continuous phase¹⁹. Roasting can also lead to oleosome rupture and protein body distortion²⁰. Due to these and other desirable sensory changes incurred in roasting (i.e., enhanced flavor and color²¹), almonds are commonly roasted before grinding, as it facilitates the size reduction process. The pastes used in the almond milk

manufacturing process are roasted almonds that have been very finely ground, making them suitable candidates for oil and protein extraction. AEP and EAEP rely on this type of upstream disruption of plant cell walls along with selection of extraction medium pH to favor protein solubilization and diffusion out of the cellular matrix, further facilitating oil body or free oil release¹⁶.

Although roasting can improve extraction yields, it also has the potential to denature proteins, leading to aggregation and change in functionality that may impact their solubility and/or extractability^{20,22,23}. Similarly, reducing particle size can improve extractability by increasing the surface area to volume ratio^{16,22}. However, depending on the magnitude of increase in extractability, this may not justify the additional energy input. Furthermore, the addition of proteolytic enzymes to assist the extraction can increase oil and protein extractability by hydrolyzing proteins into smaller and more soluble peptides and disrupting the oil body membrane^{24,25}. However, proteolysis has the potential to modulate protein functionality depending upon the enzyme's activity^{26,27}, necessitating careful processing optimization to generate proteins with desired functional, biological, and nutritional properties for specific applications. To date, sustainable extraction approaches such as AEP and EAEP, along with the impact of key extraction parameters on the overall extractability and protein structural modifications that influence their functional and biological properties have yet to be evaluated.

Given the significant influence of the described unit operations on extraction yields and structural composition of the extracted compounds, this study was undertaken to elucidate the combined effects of roasting intensity, grinding methods, and use of enzymes (aqueous vs. enzyme assisted aqueous processes) on oil and protein extractability from almonds and the structure of the spent-fiber rich byproduct via confocal laser scanning microscopy. Importantly, this study

investigated how these factors collectively influenced the nutritional composition, physicochemical properties (including secondary structure via circular dichroism, surface hydrophobicity, zeta-potential) and *in vitro* protein digestibility of almond milks produced via AEP and EAEP. By exploring how the structural and compositional modifications resulting from the upstream processing conditions impact the final product, a bio-guided process can be developed to produce milks with tailored nutritional composition and desired functional and biological properties. This innovative approach could offer almond milk producers with a valuable tool for enhancing product quality and meeting consumer preferences.

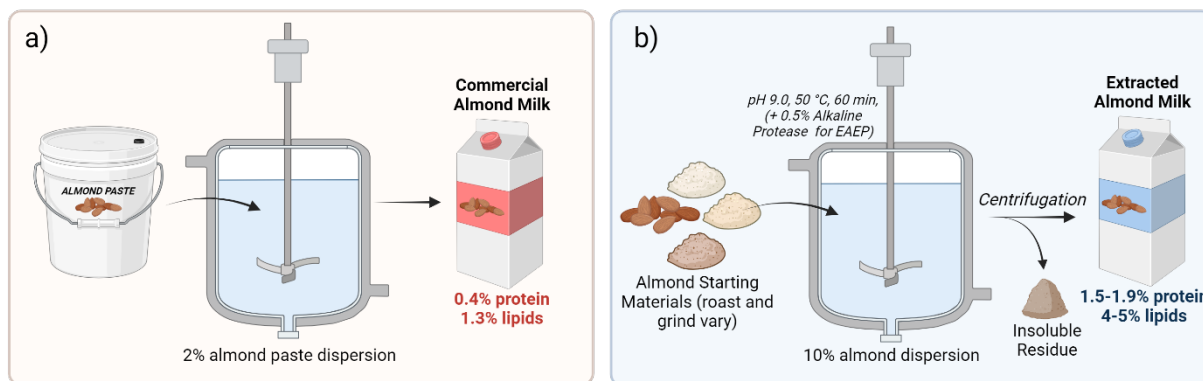


Figure 2.1. a) Conventional almond milk processing: dispersion of almond paste in water at 1:50, yielding almond milk low in protein and fat. b) Aqueous and enzyme-assisted aqueous extraction processes yielding almond milk with increased protein and fat levels. Created with BioRender.com.

2.2 Materials and methods

2.2.1 Almond processing

Blanched deskinmed sweet almonds (*Prunus dulcis*) were generously provided by Blue Diamond Growers (Sacramento, CA). Almonds were divided into three sets of 12 kg each and either left unroasted or roasted to “light” or “dark” color. Roasting was performed in aluminum sheet pans on in a Turbofan convection oven (Moffat, Winston-Salem, NC) for 18 min at either

138 °C (“light” roast) or 148 °C (“dark” roast) according to industry guidelines²¹, with almonds spread into a single layer on the sheet to ensure even roasting. Almonds were then ground to “flour” (large grind), “butter” (medium grind), and “paste” (fine grind) consistency on a Comitrol 1700 nut grinder (Urschel Laboratories, Chesterton, IN) that is commonly used for industrial almond paste manufacturing (Fig. 2.2).

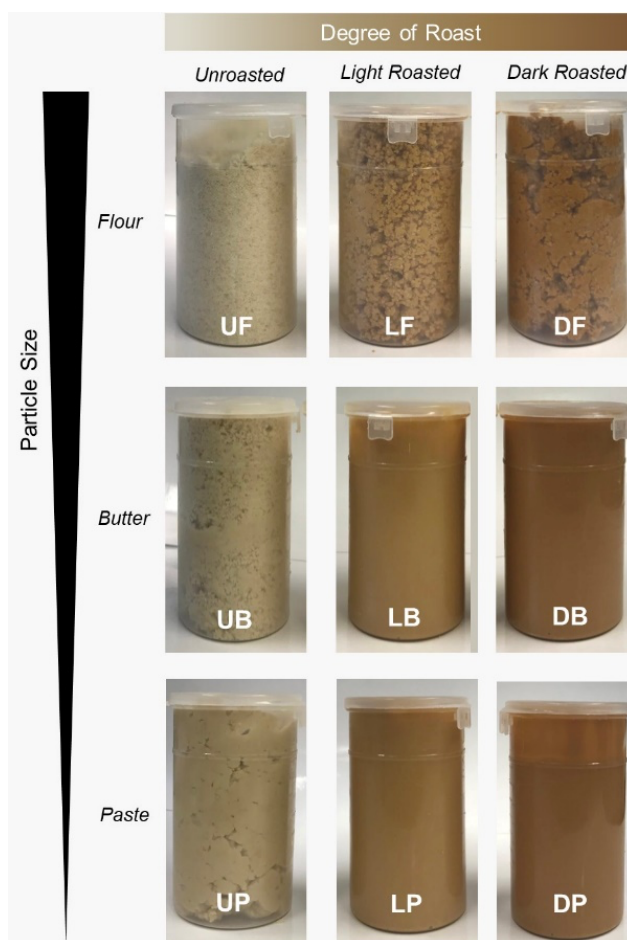


Figure 2.2. Almond roasting and grinding conditions to produce AEP and EAEP milks. U = unroasted, L = light roasted, D = dark roasted: F = flour, B = butter, P = paste.

2.2.2 Characterization of almond starting materials

2.2.2.1 Particle size analysis

The particle size distribution of almond butters and pastes was determined using laser diffraction with a Mastersizer 3000E particle analyzer (Malvern Panalytical, Malvern, United Kingdom) using isopropanol (99.9% pure, Fisher Chemical, Waltham, MA) as a dispersant. This selection was made due to the large amount of free oil, and it aligns with previous methods described for almond butter, which has a refractive index of 1.59²⁸. Each type of raw material (i.e., unroasted flour, light roasted flour, etc.) was prepared in triplicate (three separate samples), and each sample was analyzed 10 times, resulting in an average value based on 30 measurements per sample. Particle size was reported as the De Brouckere [4,3] volume-weighted mean diameter using Mie theory calculations, as well as d10, d50, and d90 percentiles. Due to their larger particle size, almond flours were measured using the same method on a Mastersizer 3000 (generously provided by Malvern Panalytical).

2.2.2.2 Starting material proximate analyses

The total oil content of the starting materials was determined using the acid hydrolysis method (AOAC Method 922.06 for Fat in Flour)²⁹. Total protein content (%) was quantified using the Dumas method (Vario Max Cube, Elementar America, Ronkonkoma, NY, USA), and calculated as total nitrogen (%) $\times$ conversion factor of 5.18²⁶. Total solids were quantified by drying the samples in a vacuum oven at 65 °C until a constant weight was achieved³⁰. Ash was determined via combustion in muffle furnace for 4 h at 600 °C³¹. Each sample replicate was analyzed in triplicate.

2.2.3. Almond milk extraction using the AEP and EAEP

For each roasting and grinding condition, almonds were processed using AEP and EAEP at a 1:10 solids-to-liquid ratio with DI water (50 g ground almond:500 g water) at pH 9.0 for 60 min. Extractions were carried out in 1 L glass beakers in a 50 °C recirculating water bath under

constant stirring of 120 rpm (Fig. 2.2 b). For the EAEP, the same extraction conditions as the AEP were employed, with the addition of 0.5% w/w (weight of enzyme/weight of ground almonds) FoodPro® alkaline protease (Danisco, Palo Alto, CA, USA). Following extraction, the slurry was centrifuged at 25 °C for 30 min at $3,280 \times g$ and the supernatant (“milk”) was decanted to separate it from the remaining insoluble pellet fraction (“insolubles”). All extractions were performed in triplicate.

2.2.4 Quantifying oil and protein extractability

Oil and protein extraction was measured using the methods described in section 2.2.2. Total protein and oil extractability (TPE/TOE), which quantify the total amount of all protein/oil extracted from the original starting material, were calculated according to Equations (1) and (2), respectively:

$$TPE (\%) = \left[1 - \left(\frac{\text{Protein (g) in the insoluble fraction}}{\text{Protein (g) in the almond material}} \right) \right] \times 100 \quad (1)$$

$$TOE (\%) = \left[1 - \left(\frac{\text{Oil (g) in the insoluble fraction}}{\text{Oil (g) in the almond material}} \right) \right] \times 100 \quad (2)$$

2.2.5 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) was employed to image the cellular structure of starting materials and insoluble fractions, providing insights into pre- and post-extraction morphology. Fresh extractions were performed for each starting material to prevent cellular damage from freezing of insolubles. Samples were applied to slides immediately following extraction and centrifugation. For slide preparation, small amounts of the starting material or

insolubles were placed in the well of a concave depression slide, then stained with fluorophores. Proteins were stained with fluorescein isothiocyanate (FITC, Sigma-Aldrich [St. Louis, MO], 1 mg/mL in acetone, diluted 1:100 v/v in deionized water, with 20 μ L pipetted onto slide). Lipids were labeled with Nile red (Sigma-Aldrich, 1 mg/mL in dimethylsulfoxide [Alfa Aesar, Ward Hill, MA], further diluted to 1:100 v/v in deionized water, with 20 μ L pipetted onto slide). Cell walls were stained with Calcofluor white (Sigma-Aldrich), with 20 μ L pipetted directly onto slide. After staining, the slide was sealed with a fluoroprotectant (ProLong Gold Antifade, Thermo Fisher Scientific, Waltham, MA), and a glass coverslip was applied. The slides were allowed to cure in the dark at ambient temperature for 24 h. Subsequently, the edges of the coverslips were sealed with clear nail polish. Slides were stored at 4 °C until imaging. Imaging was performed using an Olympus Fluoview FV1000 laser scanning confocal microscope (Olympus Optical Co., Tokyo, Japan) equipped with excitation lasers in the following lines: 488 nm for FITC, 515 nm for Nile red, and 405 nm for Calcofluor white using a 40x magnification oil immersion objective lens.

2.2.6 Starting material selection and almond milk compositional, nutritional, and physicochemical characterization

Due to its comparable protein and oil extractability to the paste, along with less demanding grinding requirements, the butter was selected as the starting material for producing milks for subsequent comprehensive compositional, nutritional, and physicochemical characterization. These almond milks were analyzed in triplicate for protein, oil, moisture, and ash composition using methods described in section 2.3. The carbohydrate content was calculated by difference.

2.2.6.1 Impact of almond butter roasting on color of AEP and EAEP milks

The impact of roasting on color was assessed using *CIE L*a*b** colorimetry. Triplicate measurements were performed on a Hunter 1-UV-Vis colorimeter (HunterLab, Reston, VA) for

both the almond butter starting materials (UB, LB, DB) and the corresponding milks produced with them.

2.2.6.2 Influence of almond butter roasting and extraction methods on in vitro protein digestibility of almond milk

The *in vitro* protein digestibility (IVPD) of AEP and EAEP milks produced with UB, LB, and DB was determined using the INFOGEST 2.0 method³² with a modified pancreatin loading as described by Yang et al (in press as of 2024). Enzyme activities were determined and simulated digestion fluids were prepared according to the INFOGEST protocol. Briefly, 2.5 mL of each milk extracted from “butter” materials was subjected to simulated salivary, gastric (2,000 U/mL of pepsin), and intestinal (20 TAME U/mL of pancreatin) digestion. Immediately after digestion, 24% trichloroacetic acid solution was added in a 1:1 (v/v) proportion to stop digestion and the samples were incubated at 4 °C overnight to promote protein precipitation. Samples were then centrifuged at $3,280 \times g$ for 30 min at 4 °C. The supernatant was decanted, and the pellet was washed with 10 mL cold acetone (Thermo Fisher Scientific), followed by incubation at –20 °C for 60 min. The tubes were again centrifuged ($3,280 \times g$, 4 °C, 30 min), the supernatant decanted, and the residual acetone was evaporated in a fume hood overnight. The remaining pellets were then quantified for total nitrogen by the Dumas method as described in section 2.3.

An enzyme blank was prepared by performing an empty digest with 2.5 mL of RO water instead of sample. IVPD was calculated based on a mass balance of nitrogen using Eq. (3):

$$IVPD\% = \frac{N_{milk} - (N_{pellet} - N_{blank})}{N_{milk}} \times 100 \quad (3)$$

Where N_{milk} is the mass of nitrogen (mg) of the milk prior to digestion, N_{pellet} is the mass of nitrogen (mg) of the pellet after digestion, and N_{blank} is the mass of nitrogen (mg) of the pellet of the enzyme blank.

2.2.6.3 Almond milk protein characterization via SDS-PAGE

To characterize changes in protein molecular weights between extraction methods, AEP and EAEP milks derived from UB, LB, and DB were analyzed for molecular weight via SDS-PAGE. The protein profile was determined using the method described by Laemmli³³ with some modifications. Fifty μg of each of the proteins were loaded onto a precast 12% acrylamide Criterion TGX Precast gel, along with a low range SDS-PAGE standard (14.4-97.4 kDa) as a molecular mass marker. Following staining with Coomassie blue dye, gels were imaged using a Gel Doc EZ Imager system and Image Lab software.

2.2.6.4 Defatting of almond milk

Almond milk produced from the AEP and EAEP was refrigerated overnight and then centrifuged at $3280 \times g$ for 30 min at 4 °C to separate the milk (aqueous + cream fraction) from the solid fraction. The cream layer (oil-rich fraction) was manually removed via transfer pipet to isolate the defatted milk, which was subsequently freeze-dried for circular dichroism spectroscopy and physicochemical analyses.

2.2.6.5 Circular dichroism (CD) spectra

CD spectroscopy was utilized to determine the secondary structure of the almond milk proteins obtained from UB, LB, and DB. Freeze-dried defatted milk samples were diluted to a final protein concentration of 0.5 mg/mL in 0.005 M sodium hydroxide (pH 11) and filtered with a 22 μm paper filter to reduce scattering. The same buffer was used as a blank. The CD spectra were

scanned at the far-UV range (190-250 nm) with a CD spectropolarimeter (Jasco J-715, Jasco Corp., Tokyo, Japan) in an 0.1 cm quartz CD cuvette (Hellma, Muellheim, Baden, Germany) at 25 °C. The scanning speed, response, and bandwidth used were 10 nm/min, 8 s, and 1.0 nm, respectively. Two scans were used and averaged to produce one spectrum. The CD data were expressed as delta epsilon ($\Delta\epsilon$) and the proportions of the secondary structures present were interpreted using the BeStSel online software³⁴ with a scale factor of 0.05. The secondary structures quantified were α -helix, β -sheet, β -turns, and unordered structures. Each defatted milk sample was measured in triplicate.

2.2.6.7 Surface hydrophobicity (H_0) of defatted almond milk proteins

The H_0 of the proteins in the milks was determined with the ANS (1-anilino-8-naphthalenesulfonate) fluorescence probe method described by Zhang et al.³⁵ and Dias et al.³⁶ with some modifications. Freeze-dried defatted almond milk samples were suspended in a sodium phosphate buffer (PBS, 100 mM, pH 7.0) to achieve protein concentrations from 0.02-0.70 mg/mL. A 250 μ L aliquot of this sample and 1.25 mL of ANS solution (8.0 mM in 100 mM PBS, pH 7.0) were then pipetted into a 96-well black plate and shaken at 300 rpm for 30 min. Fluorescence intensity was measured in a SpectraMax M5 plate reader (Molecular Devices, San Jose, CA, USA) using an excitation wavelength of 390 nm and emission wavelength of 470 nm. The hydrophobicity of the protein sample was defined as the slope of the fluorescence intensity versus the protein concentration curve as determined by linear regression. Each extraction replicate (n=3) was analyzed in duplicate and the H_0 was calculated as the average of six measurements for each condition (roasting and extraction method).

2.2.6.8 Zeta potential of defatted almond milk proteins

To assess the impact of roast and grind on surface charge of proteins, the zeta potential of the almond milk proteins was measured using dynamic light scattering with a Zetasizer Nano (Malvern Instruments Ltd., United Kingdom). Freeze-dried defatted almond milk samples were diluted to 0.05% (w/v) with 0.01 M pH 7 phosphate buffer to achieve a mean count rate over 100 kcps. Each extraction replicate (n=3) was analyzed in triplicate at 25 °C.

2.2.7 Statistical analysis

Results are presented as means $\pm$ one standard deviation. Statistical analyses were conducted using two-way ANOVA for extractability and proximate composition followed by one-way ANOVA and Tukey's tests to discriminate between means, and one-way ANOVA for all other experiments. ANOVA was followed by Tukey's tests for mean differences using a significance of $p < 0.05$ using Statistica™ Software (TIBCO Software Inc, Palo Alto, CA, US).

2.3. Results

2.3.1 Impact of roasting and grinding on particle size

The mean [4,3] volume-weighted diameter of all almond starting materials was reported along with the d10, d50, and d90 values (Table 2.2). The mean diameter of flours exhibited a wide standard deviation among all roasts, indicating a broad range of particle sizes. Though the standard deviation of the mean particle size of the light roasted flour was too large to be significantly different from that of unroasted flour, both the light and dark roasted flours tended to be smaller in diameter than the unroasted flour. This is likely due to the release of oil during roasting, along with the shrinking and hardening of cell walls, which contribute to easier grinding during processing¹⁹.

Table 2.1. Influence of roasting and grinding on almond particle size distribution as measured by dynamic laser scattering.

Starting Material	d10 (μm)	d50 (μm)	d90 (μm)	d[4,3] (μm)
UF	20.93 ± 6.03 ^a	453.65 ± 60.15 ^a	849.35 ± 109.00 ^a	440.15 ± 62.88 ^a
LF	5.55 ± 0.23 ^{bc}	380.0 ± 106.51 ^b	382.7 ± 38.43 ^c	270.5 ± 145.49 ^b
DF	7.38 ± 0.27 ^b	318.75 ± 21.20 ^c	556.4 ± 106.62 ^b	250.75 ± 56.51 ^b
UB	5.58 ± 0.05 ^{bc}	23.33 ± 00.83 ^d	103.33 ± 10.45 ^d	39.11 ± 2.44 ^c
LB	4.80 ± 0.10 ^c	12.96 ± 0.71 ^d	69.31 ± 37.91 ^{de}	25.09 ± 2.00 ^c
DB	4.88 ± 0.13 ^c	12.70 ± 0.82 ^d	54.75 ± 15.91 ^{de}	22.83 ± 1.75 ^c
UP	5.10 ± 0.03 ^c	17.96 ± 0.26 ^d	52.96 ± 2.22 ^{de}	24.08 ± 0.68 ^c
LP	4.75 ± 0.02 ^c	11.95 ± 0.58 ^d	38.69 ± 9.84 ^c	18.54 ± 0.83 ^c
DP	4.63 ± 0.05 ^c	11.13 ± 0.32 ^d	33.78 ± 3.04 ^c	18.27 ± 1.24 ^c

Almond starting material particle size measurements for unroasted (U), light (L), and dark (D) almond flour (F), butter (B), and paste (P), as measured by dynamic laser scattering. Different letters within each column indicate statistical significance by two-way ANOVA followed by one-way ANOVA and Tukey's test for HSD ($p > 0.05$) to discriminate means.

2.3.2 Impact of roasting, grinding, and extraction methods on oil and protein

extractability

AEP and EAEP were used to produce almond milks with nine different almond starting materials with varying degree of roast and grind (UF, LF, DF, UB, LB, DB, UP, LP, and DP). The effects of upstream roasting and particle size reduction during grinding on total oil (TOE) and protein extraction (TPE), which are crucial to control the nutrient profile of the final milk, are shown in Fig. 2.3a and b. In general, both degree of roasting and particle size significantly impacted total oil extraction (TOE). Oil extractability from the flour was favored by higher levels of roasting in the AEP, although no statistically significant differences were observed between TOE of dark and light roasted flours. However, for the more finely ground butter and paste, TOE was similar or slightly lower than that of unroasted flour. The increase of oil extraction with roast in flour can be attributed to the release of intracellular oil in roasting, but the lack of increase or even the small reduction observed in TOE for the roasted butters and pastes imply that this is not the case with finer particle size. This result is in agreement with the findings by Li et al²², who

reported that AEP oil extraction from crushed peanut pastes decreased with increased temperature of roasting. The reduction in oil extractability is likely due to the distortion of intracellular protein bodies brought on by roasting²⁰. During roasting, the denaturation and aggregation of proteins may lead to oil entrapment within the cell wall matrix, thereby reducing oil and protein extraction yields. This phenomenon is evident when comparing the lower oil extractability from the dark roasted paste, which was significantly lower than the that of the unroasted (89.42% vs. 96.39%), highlighting the substantial impact of roasting. These effects, combined with the fine particle size of the paste, can lead to particle aggregation and emulsion formation within the extraction slurry. This could further hinder the separation of the extracted oil into the liquid phase during centrifugation, thus decreasing oil yield³⁷.

With the addition of protease in the EAEP, small but significant increments in oil extractability were observed in some roast/grind conditions (light/dark butter and paste) compared to the AEP. This underscores the potential role of the endoprotease in hydrolyzing the denatured protein bodies into more soluble protein fragments and disrupting the oil body membrane, which could in turn favor the release of more oil^{26,24}. Notable exceptions were observed for the unroasted flour (65.08% for AEP vs. 53.36% for EAEP), unroasted butter (94.36% for AEP vs. 92.06% for EAEP), and the EAEP unroasted and light roasted pastes (95.97 and 95.92%, respectively), whose values were not statistically different. The fine particle size of the butter and paste and lower roasting intensity may explain the high levels of oil extraction in both AEP and EAEP. However, at higher levels of roasting and grinding (i.e., dark butter and paste) the observed reduction in oil extractability due to roasting was overcome with the addition of enzyme. This is consistent with results reported by Zhang et al.³⁸ for roasted peanut pastes; however, no particle size measurements were taken in that study.

When assessing the impact of roasting and grinding on protein extractability, a decrease in protein extractability was evident when moving from unroasted to dark roasted butter and paste in the AEP (Fig. 3b). TPE for both light and dark roasted butter and paste was significantly lower (70.40-75.14%) than that for unroasted butter and paste (80.99-81.99%). Heat treatment significantly impacts the secondary and tertiary structures of proteins³⁹⁻⁴¹, inducing conformational changes such as unfolding, structural loss and rearrangement into new conformations. Almond proteins have been found to denature when exposed to temperatures as low as 96 °C^{42,43}, well below the roasting temperatures (138 °C for light and 148 °C for dark roasting) employed in this study. This denaturation and aggregation could explain the observed decrease in protein extractability for dark roasted butter and paste compared with their unroasted counterparts.

Similar to what was observed for TOE, the use of protease in the EAEP significantly enhanced protein extractability from all almond starting materials compared to the AEP. For butter and paste, protein extraction in the EAEP was statistically similar among all roasts (88.84-91.76%), but higher than all their AEP counterparts (maximum TPE in the AEP was 81.99% for unroasted paste). This is consistent with previous research that demonstrated increased TPE with protease-assisted extraction for almond flour and almond oil press cake^{24,26,44,45}. Proposed mechanisms for higher extraction yields in the EAEP include proteases' ability to disrupt and loosen the protein bodies from the cellular matrix, releasing polypeptides that are more soluble in the extraction medium than native proteins¹⁷.

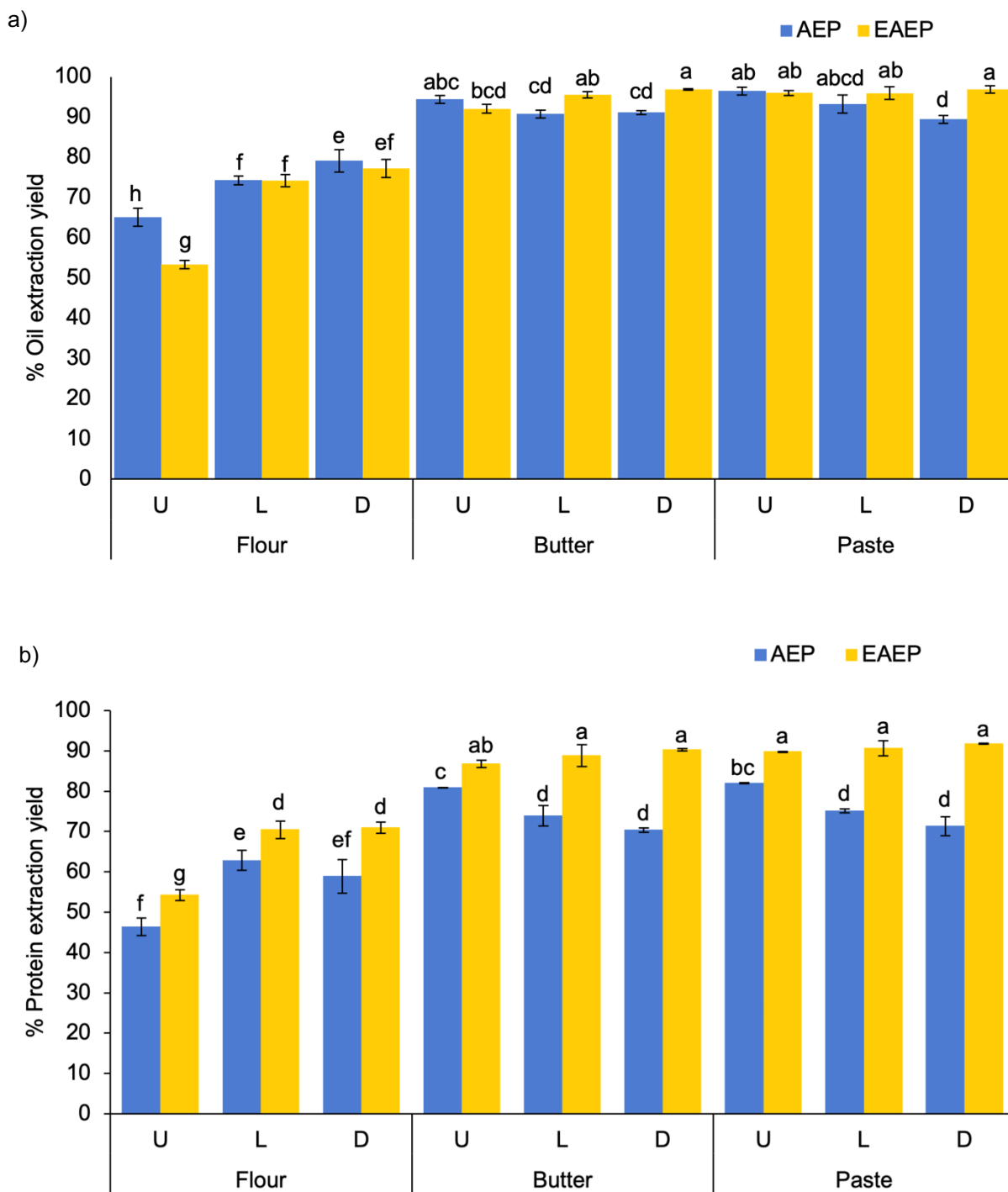


Figure 2.3. Impact of roasting, particle size, and extraction methods (AEP vs. EAEP) on almond oil a) and protein extractability. Unroasted (U), light roasted (L), and dark roasted (D) almond flour, butter, and paste. Different letters indicate statistically significant difference between means by two-way ANOVA followed by one-way ANOVA with Tukey's test for HSD ($p < 0.05$) to discriminate means.

2.3.3 Confocal laser scanning microscopy (CLSM)

The microstructure of the almond starting materials and insoluble fractions (extraction byproduct) were illustrated using CLSM (Fig. 4-6). The fluorescence dyes (red, green, and blue colors indicate lipids, protein bodies, and cell walls/background light, respectively) displayed the effects of grinding and roasting on the release of free oil as well as extractability of oil and protein components. Fig. 4a shows the microstructure of unroasted almond flour, in which intact cells are visible with entrapped oil and lipid bodies. Greater cell disruption and distortion of some cell walls were also observed in roasted flours (Fig. 4b and 4c). Upon roasting (Fig. 4b), oil bodies were found to have burst and coalesced into a continuous oil phase both outside and within intact cells. Changes in oil body size and distribution after roasting were also observed by Altan et al.⁴⁶ and Li et al.²² using CLSM. AEP and EAEP insoluble fractions of the unroasted almond flour (Fig. 4d, g) demonstrate the extraction of a portion of the oil and protein from the almond flour, although a significant amount remained entrapped within the cells. The oil bodies appear to have ruptured in the EAEP insolubles (Fig. 4g-i), underscoring the hydrolysis of oleosins in the oil body membrane by the protease used. As the degree of roast increased from unroasted to dark roasted flour (Fig. 4a-c), there was an increase in free oil release in the starting materials, leading to higher oil extraction as depicted in the insoluble fractions (Fig. 4e, f, h, and j). Intact cells, however, still prevented the release of contained oil and protein bodies. Finer grinds such as butter (Fig. 5) and paste (Fig. 6) led to even greater cellular disruption, with increased coalescence of oil bodies and aggregation of protein bodies. The insoluble fractions (Fig. 4 and 5e, f, h, and j) highlight how grinding and proteolysis favor the removal of lipids and proteins, in agreement with the TOE and TPE results. The paste grind (Fig. 6) left virtually no cells intact, and EAEP insolubles of pastes

exhibited extremely few protein and oil bodies remaining (Fig. 6g-i). This indicates that physical disruption is a critical upstream processing step in AEP/EAEP.

2.3.4 Starting material selection and almond milk compositional, nutritional, and physicochemical characterization

Given that butter and paste demonstrated similar TOE and TPE, butter was chosen as the starting material to produce milks for further analyses. By not grinding materials as finely as a paste, this method can potentially reduce energy consumption during upstream processing for almond milk production. The milks extracted from the almond UB, LB, and DB were analyzed for proximate composition (Table 2.2). No significant difference in oil content was found between AEP and EAEP milks regardless of roast (average 4.84%) however, EAEP milks tended to have higher protein content than AEP (1.90% vs. 1.63%), which is consistent with the TOE and TPE.

Table 2.2 Impact of roasting and extraction methods on the macronutrient composition of almond milks produced via AEP and EAEP compared to an average commercial almond milk.

Extraction	Lipids (%)	Protein (%)	Ash (%)	Moisture (%)	Carbohydrate (%)
AEP UB	4.61 ± 0.36 ^a	1.74 ± 0.07 ^{bc}	0.12 ± 0.01 ^a	92.29 ± 0.25 ^{ab}	1.24 ± 0.22 ^a
AEP LB	4.68 ± 0.29 ^a	1.63 ± 0.04 ^{cd}	0.13 ± 0.01 ^a	92.36 ± 0.14 ^{ab}	1.20 ± 0.08 ^a
AEP DB	4.45 ± 0.61 ^a	1.51 ± 0.08 ^d	0.12 ± 0.01 ^a	92.53 ± 0.55 ^a	1.38 ± 0.13 ^a
EAEP UB	5.42 ± 0.60 ^a	1.88 ± 0.02 ^{ab}	0.20 ± 0.01 ^b	91.51 ± 0.43 ^b	1.00 ± 0.24 ^a
EAEP LB	5.16 ± 0.45 ^a	1.87 ± 0.06 ^a	0.19 ± 0.01 ^b	91.67 ± 0.44 ^{ab}	1.09 ± 0.62 ^a
EAEP DB	4.73 ± 0.30 ^a	1.91 ± 0.01 ^a	0.21 ± 0.02 ^b	92.20 ± 0.23 ^{ab}	0.96 ± 0.06 ^a
Commercial Almond Milk	1.25	0.42	-----	-----	0.83

Unroasted (U), light roasted (L) and dark roasted (D) almond butter compared with commercial almond milk (nutrients calculated from Nutrition Facts panel). Different superscript letters within the same column indicate statistically significant difference of means ($p < 0.05$).

The ash content was significantly higher in EAEP milks than in AEP milks due to the increased NaOH added during extraction to keep the pH elevated, since pH decreases as proteolysis occurs due to the exposure of acidic end groups. All milks were significantly higher in oil and protein content than a leading commercial almond milk (calculated from its Nutrition Facts panel). As most commercial almond milks are considerably lower in oil and protein than dairy milk^{5,47}, this provides a promising method for the development of a nutritionally superior almond milk.

2.3.5 Impact of almond butter roasting on color of AEP and EAEP milks

The colorimetry data indicated lower L^* values for the roasted almond butters compared to the unroasted almond butter, which was expected due to Maillard browning occurring during roasting²¹ (Table 2.3). The a^* and b^* values also increased, which is consistent with previous studies^{48,49}. Some degree of roast is desirable due to the aforementioned improvement of flavor and color qualities of almonds, but extensive roasting can lead to the formation of dangerous acrylamide products, thus a moderate roast is safer for consumers^{48,50}. More intense roasting also requires higher energy inputs and can lead to extensive denaturation of proteins, resulting in decreased protein in solubility and extractability^{37,51}.

Table 2.3. Impact of almond butter roasting on color ($CIE L^*a^*b^*$ values) of almond starting materials

Starting Material	L^*	a^*	b^*
UB	70.36 ± 0.14^a	2.20 ± 0.04^c	25.90 ± 0.14^c
LB	58.47 ± 0.02^b	10.56 ± 0.01^b	34.79 ± 0.04^b
DB	52.48 ± 0.08^c	13.81 ± 0.03^a	36.53 ± 0.02^a

Unroasted (UB), light roasted (LB), and dark roasted (DB) almond butter materials. Different superscript letters within the same column indicate statistically significant difference of means ($p < 0.05$).

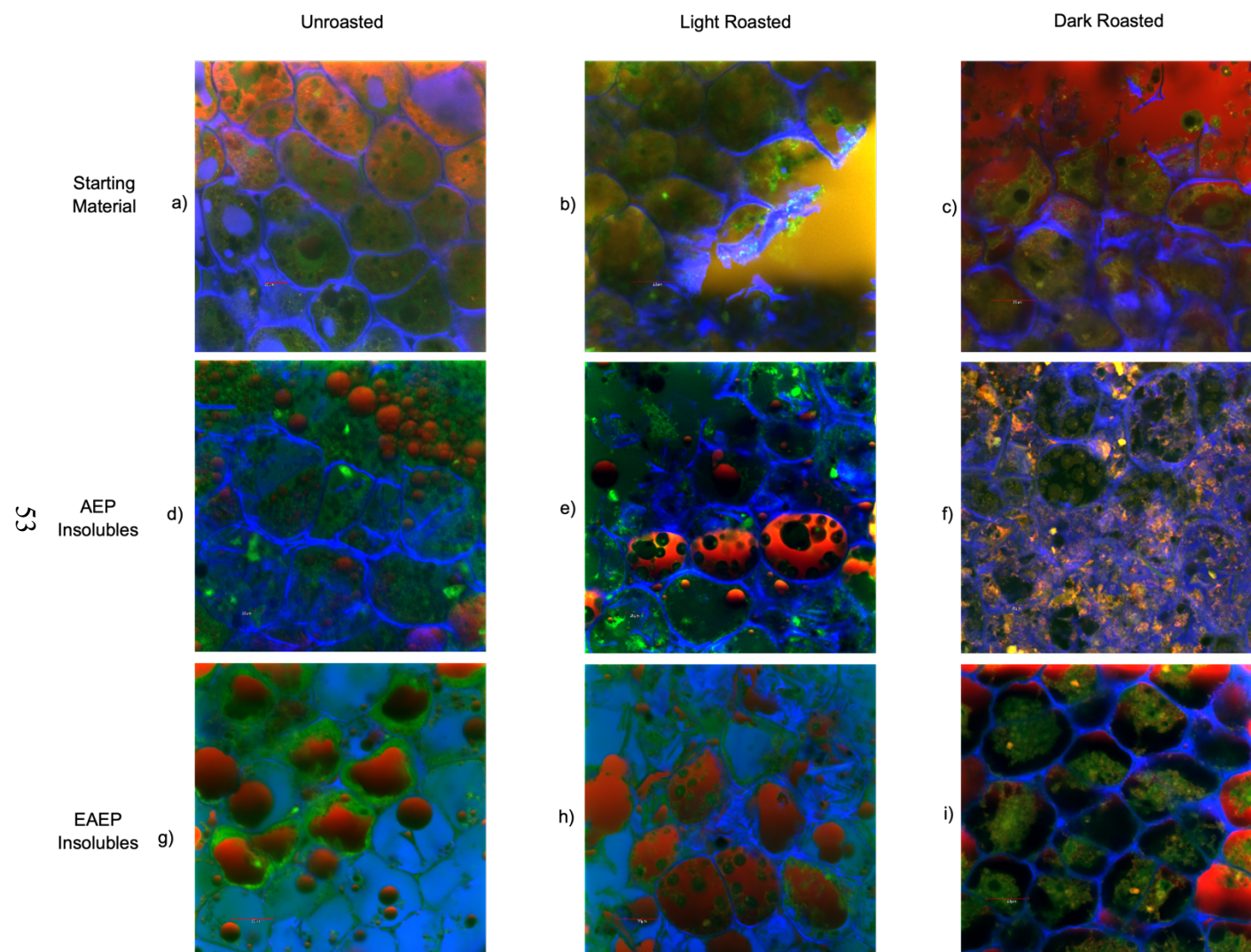


Figure 2.4. Confocal laser scanning microscopy images of almond flour materials, AEP insolubles, and EAEP insolubles. Red stains indicate lipids, green indicate protein bodies, and blue indicate cell walls or background light. Images were captured using a 40x oil immersion lens.

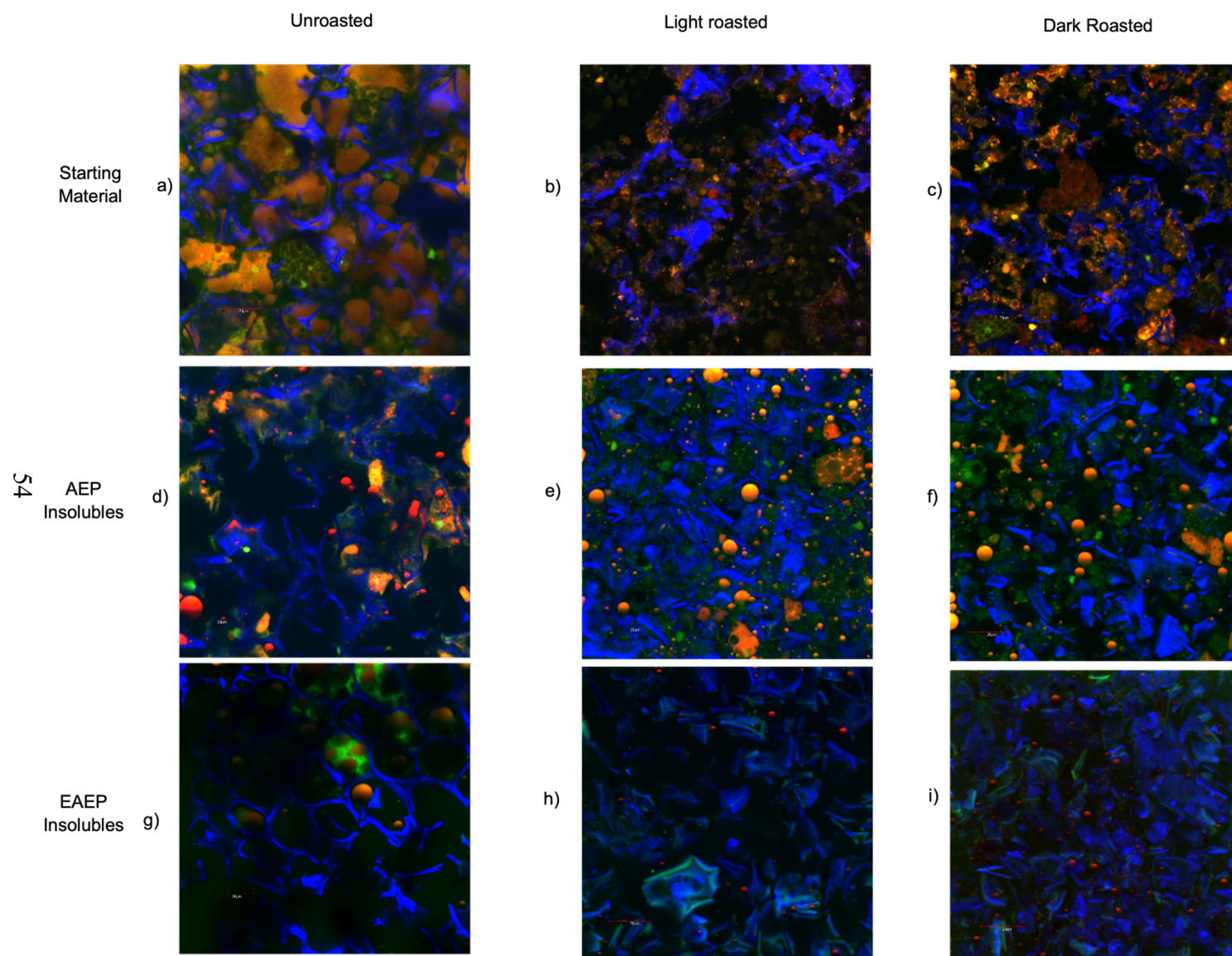


Figure 2.5. Confocal laser scanning microscopy images of almond butter starting materials, AEP insolubles, and EAEP insolubles. Red stains indicate lipids, green indicate protein bodies, and blue indicate cell walls or background light. Images were captured using a 40x oil immersion lens.

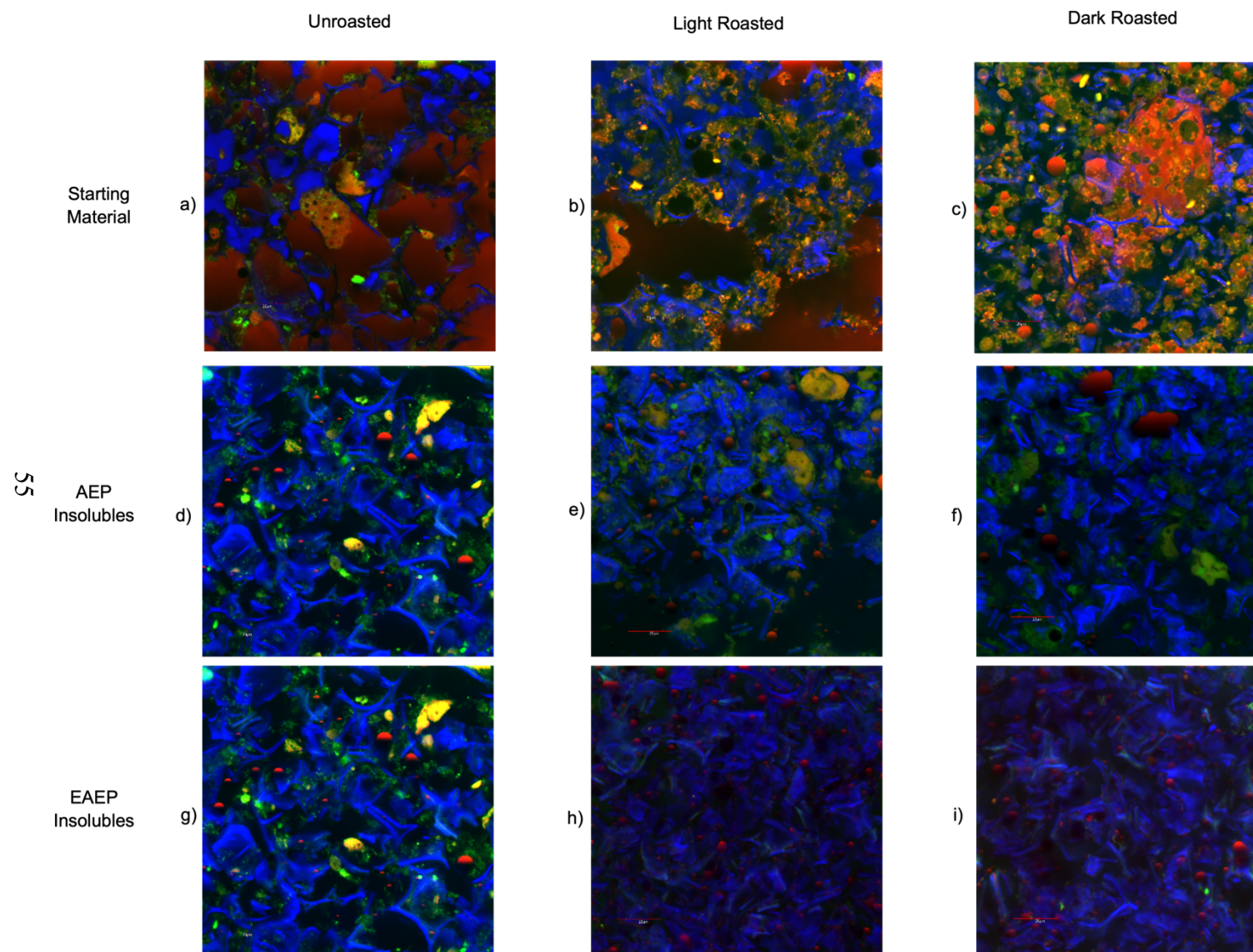


Figure 2.6. Confocal laser scanning microscopy images of almond paste starting materials, AEP insolubles, and EAEP insolubles. Red stains indicate lipids, green indicate protein bodies, and blue indicate cell walls or background light. Images were captured using a 40x oil immersion lens.

*2.3.6 Influence of almond butter roasting and extraction methods on *in vitro* protein digestibility of almond milk*

Overall, the *in vitro* protein digestibility (IVPD) of all milks was very high (84.4-88.1%) (Fig. 2.7), with no significant impact from roasting conditions employed or extraction processes. The overall *in vitro* protein digestibility values in this study were higher than those previously reported in almond milk protein digestibility studies. Martinez-Padilla et al.⁵² reported 25.19% IVPD in a commercial almond drink using a similar static IVPD assay, and Vanga et al.⁵³ found 75.53% IVPD in a milk made from whole almonds blended with water using a two-stage pepsin and pancreatin digestion assay. Particle size was not reported in those studies. Previous studies^{39,40} on peanut and tree nut protein digestibility have found that protein digestibility increased with roasting; however, this could be due to differences in roast time/temperature utilized (170 °C for 10 min/50-75 °C for 15-45 min, respectively) and the specific proteins present in the matrix. However, these changes in protein digestibility of the starting materials with roasting were not reflected in the milk proteins produced by the AEP. Variations in unit operations employed for almond milk production, including those applied to the starting material (such as the common practice of blending blanched unroasted almonds with water with or without straining of solids), can significantly influence the digestibility of the final milk. Consequently, direct comparisons of our data with the existing literature may be limited due to process-related differences.

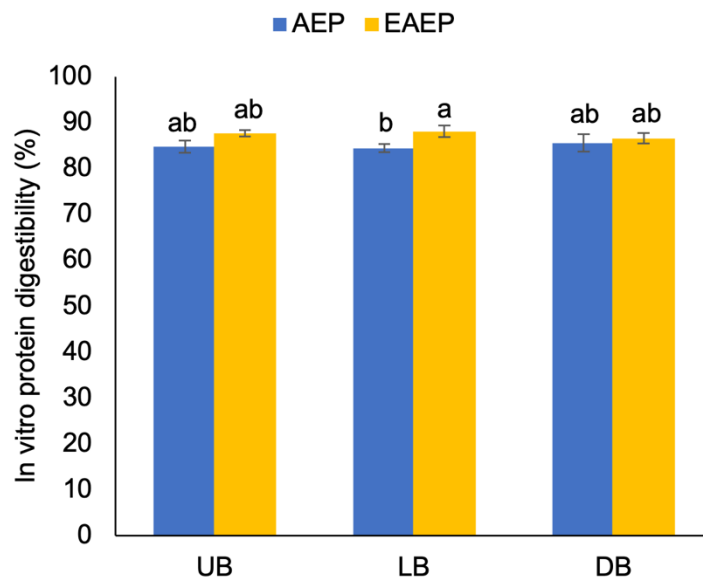


Figure 2.7. *In vitro* protein digestibility (%) of almond milks produced by different roasting and extraction methods (AEP/EAEP) using the INFOGEST method. Unroasted, light roasted, and dark roasted almond butter-extracted milks. Different superscripts indicate statistically significant difference between means by one-way ANOVA followed by Tukey's test ($p < 0.05$).

2.3.7 Almond milk protein characterization via SDS-PAGE

SDS-PAGE (Fig. 8) revealed a protein profile for the almond milks characterized by prominent bands between 31.0 and 45.0 kDa and 21.5 and 31.0 kDa, corresponding to the acidic (42-46 kDa) and basic (20-22 kDa) subunits of amandin protein⁵⁴, respectively. Additional protein bands were observed within a broader molecular weight range for the AEP samples. Lanes 3 and 4 (light and dark roasted AEP) appear to show a higher concentration of high-molecular weight proteins near the top of the gel compared to lane 2 (unroasted AEP). This suggests denaturation and aggregation in the roasted butters, which could have impacted TPE as discussed in section 3.2. EAEP samples exhibited significantly lower molecular weight proteins/polypeptides compared with the AEP. Although some bands were observed between 21.5 and 31.0 kDa, the majority of the protein fragments was below 14.4 kDa, indicating significant hydrolysis of proteins. This is in agreement with Dias et al.'s finding that proteolysis did not affect the 22 kDa acidic subunit of

amandin⁵⁵. Despite the higher protein content in EAEP milks, lanes 5-7 are not demonstrably denser than those of the AEP milks; perhaps indicating that a great deal of proteins in the EAEP were so hydrolyzed as to have washed out of the gel.

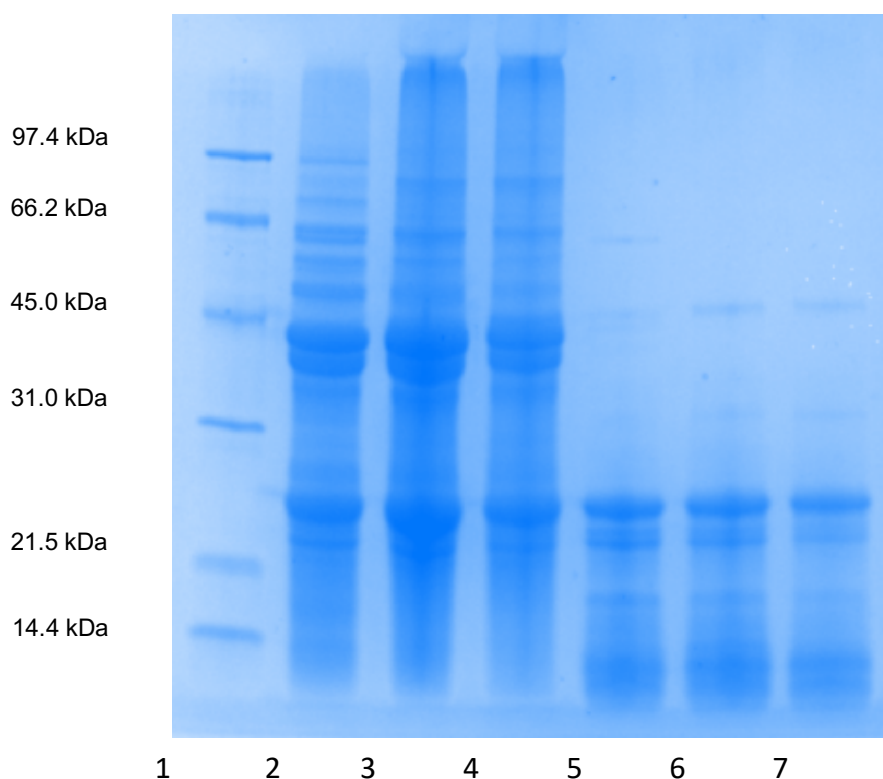


Figure 2.8. Almond milk protein molecular weight by SDS-PAGE (12% Criterion TGX Precast Gels) analysis on AEP and EAEP almond butter milks. Lane (1): molecular mass marker standards (14.4-97.4 kDa); lane (2) AEP unroasted butter milk, lane (3) AEP light roasted butter milk, lane (4) AEP dark roasted butter milk, lane (5) EAEP unroasted butter milk, lane (6) EAEP light roasted butter milk, lane (7) EAEP dark roasted butter milk.

2.3.8 Circular dichroism (CD) spectra

CD was used to estimate the secondary structure of the AEP and EAEP almond milks (Table 2.4). The relative proportions of these four structures (α -helix, β -sheet, β -turn, and unordered structures) remained stable in all AEP milk skims regardless of roast. These findings align with previous research on extracting whole proteins from unroasted almond products (i.e. flour and milk)^{24,56}. While Albillos et al.⁵⁷ found that native amandin secondary structure was

relatively heat-stable, while ordered structures in amandin's acidic polypeptide decreased with increased temperature during CD measurements, indicating the importance of covalent links in stabilizing the native protein. Ma et al.⁵⁸ found higher processing temperatures (150 and 180 °C for 5-30 min) actually increased α -helix structures in sesame seed proteins as opposed to 120 °C for unhydrolyzed proteins.

Table 2.4. Secondary structure composition of almond “butter” milk proteins as determined by circular dichroism in the far UV region (190-250 nm).

Extraction	Roast	α -helix (%)	β -sheet (%)	β -turns (%)	Unordered (%)
AEP	UB	7.67 $\pm$ 0.40 ^a	27.93 $\pm$ 0.15 ^{bc}	16.97 $\pm$ 1.69 ^a	47.43 $\pm$ 1.69 ^b
	LB	7.77 $\pm$ 0.42 ^a	27.03 $\pm$ 0.76 ^c	16.33 $\pm$ 0.42 ^{ab}	48.87 $\pm$ 0.76 ^{ab}
	DB	7.87 $\pm$ 1.08 ^a	28.47 $\pm$ 1.10 ^{abc}	15.87 $\pm$ 0.38 ^{ab}	47.83 $\pm$ 0.21 ^b
EAEP	UB	1.47 $\pm$ 0.38 ^c	34.00 $\pm$ 1.93 ^{ab}	16.20 $\pm$ 0.36 ^{ab}	47.37 $\pm$ 2.15 ^b
	LB	1.50 $\pm$ 0.44 ^c	35.33 $\pm$ 5.28 ^a	15.93 $\pm$ 0.97 ^{ab}	47.20 $\pm$ 4.78 ^b
	DB	3.87 $\pm$ 0.47 ^b	25.20 $\pm$ 1.25 ^c	15.17 $\pm$ 0.32 ^b	54.97 $\pm$ 2.25 ^a

Different superscript letters within the same column indicate statistically significant difference of means ($p < 0.05$).

In contrast, the structural composition of the EAEP proteins differed significantly from that of the AEP and among the various roasting conditions within the EAEP, highlighting the combined effects of both roasting and proteolysis in the EAEP protein structure. The α -helix content of the EAEP proteins was reduced to 1.50% for the unroasted and light roast milks and to 4% for the dark roasted milk, compared with 7.8% for the AEP proteins. Albillos et al.⁵⁷ theorized that the instability of α -helices in almond protein in the absence of tertiary structures contributed to an observed decrease with thermal processing, as released polypeptides were no longer available to stabilize these structures, and helices unwound. Perhaps this instability was further impacted by hydrolysis. The decrease in the α -helix content of the EAEP proteins was accompanied by an increment of the β -sheet content for the unroasted and light roast milk proteins, while it decreased

for dark roast milk proteins. The β -turns content was relatively unchanged and unordered structures of the EAEP milk proteins were higher for the dark roast milk proteins.

Overall, despite no change in the α -helix content of the AEP milk proteins across roasting conditions, our data suggests that the protein modifications (i.e., denaturation and/or aggregation) induced by darker roasting, in alignment with reduced protein extractability at these conditions (e.g. 80.96% TPE for UB vs. 73.97 and 70.40% TPE for LB and DB, respectively), may have enhanced the enzyme's ability to alter these structures. This becomes evident from the reduced α -helix and β -sheet contents and increased proportion of unordered structures of dark roasted milk proteins compared to lighter roasts and unroasted almond butters. Additionally, the unordered structures of the EAEP proteins were similar to that of AEP but were significantly higher in the dark roast, suggesting that the higher degree of roasting caused conformational changes that enhanced the protease activity. The observed decrease in α -helix content and increase in unordered structures in EAEP proteins, compared to AEP, align with previous work in almond flour and peanuts, indicating that proteolysis disrupts these structures^{24,59}.

2.3.9 Surface hydrophobicity and zeta-potential of almond butter milk proteins

Roast intensity had a significant effect on the H_0 of extracted proteins in AEP-extracted milks (Fig. 9a). Overall hydrophobicity decreased as roast intensity increased, indicating that protein denaturation and aggregation induced by roasting hid the protein's hydrophobic sites. Roasting has been demonstrated to reduce H_0 of proteins in the presence of carbohydrates, leading to hydrophilic sugar molecules forming aggregates and polymers with hydrophobic amino acids, thus reducing detectable H_0 ⁶⁰. These results were similar to those of Rao et al.⁶¹ in boiled peanuts. Interestingly, Li et al.²² inferred from confocal scanning microscopy that roasting led to protein unfolding and exposure of hydrophobic amino acid groups in AEP-extracted peanut insolubles due

to their increased oil-holding capacity. The interaction between these exposed proteins can lead to aggregation, reducing functional hydrophobicity^{62,63}. The proteins extracted in the milk likely differ from those remaining in the insoluble fraction, which could explain this discrepancy.

The H_0 of proteins in EAEP unroasted and light roasted milks was lower than their AEP milks, but the H_0 of the EAEP DB milk was higher than that of AEP DB milk. Previous work in almond flour and almond oil press cake has shown a similar trend in AEP versus EAEP protein H_0 ^{24,64}. Proteolytic enzymes can cleave the extracted proteins and expose sites that otherwise would be hidden by roasting as in AEP. The higher H_0 of the EAEP DB milk compared with the EAEP LB milk highlights the extensive conformational changes brought about by the more intense roasting, which may have exposed sites that favored proteolysis, thereby increasing the exposure of hydrophobic sites⁶² compared with other EAEP. This is corroborated by the secondary structure results described in Table 4, specifically the increase in unordered structures observed for the EAEP DB milk. Such changes in secondary structure have been correlated with variations in H_0 ^{41,51,65}.

The overall surface charge of all butter milk proteins was negative at pH 7.0 (Fig. 9b), which is expected, as the isoelectric point of most almond proteins is 5.0⁶⁶. The surface charge of all EAEP milk proteins exhibited higher absolute values (more highly negative) than those of AEP milk proteins, regardless of roast. This increase of net charge after proteolysis is consistent with previous work with EAEP almond proteins^{24,64} and highlights the role of enzyme in protein surface properties, which could affect functionality (e.g., solubility, foaming, emulsification). The higher negative zeta potential of the EAEP milk proteins indicates stronger electrostatic repulsive force between proteins, likely caused by the exposure of areas more prone to ionization (i.e. hydrophobic sites) through hydrolysis^{24,67}.

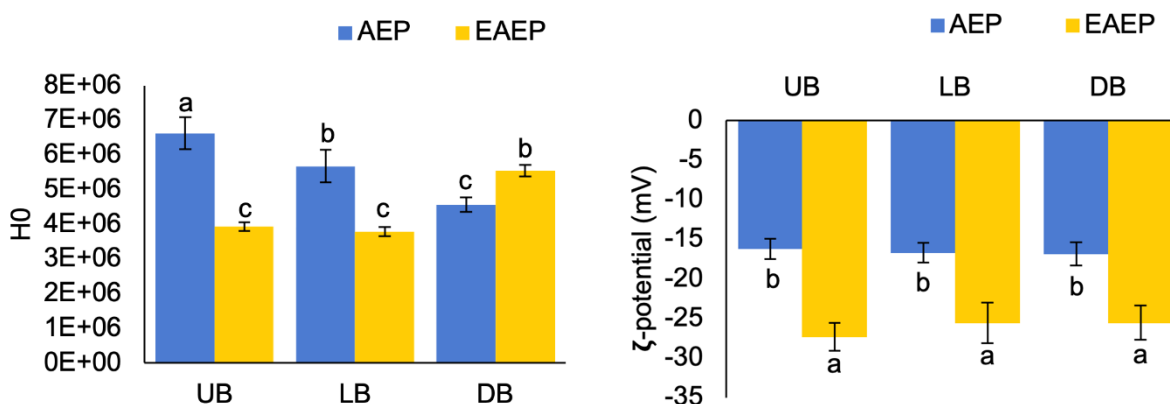


Figure 2.9. a) Impact of roasting and extraction methods on surface hydrophobicity a) and zeta-potential (mV) of butter milk defatted surface proteins at pH 7.0. Different letters within each graph indicant significant difference of means by one-way ANOVA followed by Tukey's test ($p < 0.05$).

2.4. Conclusions

This study evaluated how various degrees of roasting and grinding affect the extraction of almond proteins and lipids as well as the nutritional and physicochemical characteristics of almond milks produced by the aqueous and enzyme-assisted aqueous extraction processes. Particle size reduction led to increased overall extractability, whereas the effect of roasting on extractability varied. Increased roasting intensity reduced oil and protein extraction in the AEP, likely due to protein denaturation and aggregation, despite potential benefits to flavor and grindability. This hurdle was overcome using an alkaline endoprotease in the EAEP, with similar extraction yields (86.76-91.76%) achieved for butter and paste grinds regardless of roast. Confocal laser scanning microscopy illustrated that roasting led to bursting and coalescence of oil bodies and increased cellular disruption and protein aggregation, while increased grinding improved overall extractability. Butter was selected as the starting material for milk production and further characterization due to its reduced energy requirement and high extractability. Protein and lipid contents of the UB, LB, and DB milks ranged from 1.51-1.91% to 4.61-5.42%, respectively, higher than most commercial almond milks. This represents a notable improvement in nutritional

composition over commercial almond milks, and further formula optimization could increase nutrient density even further. Although the secondary structure of the AEP milk proteins was unaffected by roast, the combined impact of roasting and proteolysis resulted in fewer α -helix and β -sheet structures and a higher proportion of unordered structures in the DB milk proteins, which could affect their stability and functionality. Increased roast intensity decreased H_o in AEP proteins, likely due to protein unfolding and exposure of hydrophilic sites that enhanced protein-water interactions. Proteolysis also led to reduced protein H_o , except for the DB milk protein, where enhanced denaturation followed by proteolysis might have enhanced exposure of hydrophobic sites. Similarly, EAEP proteins exhibited lower ζ -potential compared to AEP, likely due to increased hydrolysis and subsequent exposure of additional ionizable groups. Proteolysis increased *in vitro* digestibility of LB milk proteins only, though digestibility was high across all samples. Overall, the results presented in this study demonstrated that roasting and proteolysis significantly influenced protein properties as well as the final almond milk composition. Further research to optimize the impact of additional extraction parameters with respect to composition, stability, and consumer acceptance is warranted to develop a more nutritious almond milk and demonstrate its potential for commercial adoption.

2.5 Acknowledgements

Funding for publication was made possible by the U. S. Department of Agriculture's (USDA) Agricultural Marketing Service through grant 21-0001-024-SF. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the USDA. The authors wish to thank Blue Diamond Almond Growers for their donation of almonds for this study, as well as Urschel Laboratories for the use of their pilot-scale almond grinder. The Olympus FV1000 confocal used in this study was purchased using NIH Shared Instrumentation Grant 1S10RR019266-01. We thank the MCB Light Microscopy Imaging Facility, which is a UC Davis Campus Core Research Facility, for the use of this microscope.

2.6 References

1. Global Almond Milk Market to Reach \$2.68 Billion with 15.44% CAGR Forecast to 2023. *Digital Journal* <http://www.digitaljournal.com/pr/3621716> (2018).
2. Poinski, M. Oat milk surges to second most popular in plant-based dairy | Food Dive. <https://www.fooddive.com/news/oat-milk-surges-to-second-most-popular-in-plant-based-dairy/586010/>.
3. Singhal, S., Baker, R. D. & Baker, S. S. A Comparison of the Nutritional Value of Cow's Milk and Nondairy Beverages. *Journal of Pediatric Gastroenterology and Nutrition* **64**, 799–805 (2017).
4. Grant, C. A. & Hicks, A. L. Comparative life cycle assessment of milk and plant-based alternatives. *Environmental Engineering Science* **35**, 1235–1247 (2018).
5. McClements, D. J., Newman, E. & McClements, I. F. Plant-based Milks: A Review of the Science Underpinning Their Design, Fabrication, and Performance. *Comprehensive Reviews in Food Science and Food Safety* **18**, 2047–2067 (2019).
6. Vanga, S. K. & Raghavan, V. How well do plant based alternatives fare nutritionally compared to cow's milk? *Journal of Food Science and Technology* **55**, 10–20 (2018).
7. Yada, S., Lapsley, K. & Huang, G. A review of composition studies of cultivated almonds: Macronutrients and micronutrients. *Journal of Food Composition and Analysis* **24**, 469–480 (2011).
8. Yada, S., Huang, G. & Lapsley, K. Natural variability in the nutrient composition of california-grown almonds. *Journal of Food Composition and Analysis* **30**, 80–85 (2013).
9. Viennois, E., Merlin, D., Gewirtz, A. T. & Chassaing, B. Dietary emulsifier-induced low-grade inflammation promotes colon carcinogenesis. *Cancer Research* **77**, 27–40 (2017).
10. Chassaing, B. *et al.* Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature* **519**, 92–96 (2015).

11. Partridge, D. *et al.* Food additives: Assessing the impact of exposure to permitted emulsifiers on bowel and metabolic health – introducing the FADiets study. *Nutrition Bulletin* **44**, 329–349 (2019).
12. Cani, P. D. Metabolism: Dietary emulsifiers-sweepers of the gut lining? *Nature Reviews Endocrinology* **11**, p319 (2015).
13. Huang, K. & Goddard, J. M. Influence of fluid milk product composition on fouling and cleaning of Ni-PTFE modified stainless steel heat exchanger surfaces. *Journal of Food Engineering* **158**, 22–29 (2015).
14. Prakash, S., Huppertz, T., Karvchuk, O. & Deeth, H. Ultra-high-temperature processing of chocolate flavoured milk. *Journal of Food Engineering* **96**, 179–184 (2010).
15. Rosenthal, A., Pyle, D. L. & Niranjana, K. Simultaneous aqueous extraction of oil and protein from soybean: Mechanisms for process design. *Food and Bioprocess Technology: Transactions of the Institution of Chemical Engineers, Part C* **76**, 224–230 (1998).
16. Rosenthal, A., Pyle, D. L. & Niranjana, K. Aqueous and enzymatic processes for edible oil extraction. *Enzyme and Microbial Technology* **19**, 402–420 (1996).
17. Campbell, K. A. *et al.* Advances in aqueous extraction processing of soybeans. *JAOCs, Journal of the American Oil Chemists' Society* **88**, 449–465 (2011).
18. M. J. Pascual-Albero, I. P.-M. y M. A. L. Cotyledon structure of raw, soaked and roasted almond (*Prunus amygdalus* L.). *Food Science and Technology International* **4**, 189–197 (1998).
19. Gorrepati, K., Balasubramanian, S. & Chandra, P. Plant based butters. *Journal of Food Science and Technology* **52**, 3965–3976 (2015).
20. Perren, R. & Escher, F. E. Impact of roasting on nut quality. in *Improving the Safety and Quality of Nuts* 173–197 (Elsevier, 2013). doi:10.1533/9780857097484.2.173.
21. Huang, G. *Hot Air Roasting of Almonds*. 1–5 (2014).
22. Li, P. *et al.* Effects of roasting temperatures and grinding type on the yields of oil and protein obtained by aqueous extraction processing. *Journal of Food Engineering* **173**, 15–24 (2016).
23. Liu, J. J., Gasmalla, M. A. A., Li, P. & Yang, R. Enzyme-assisted extraction processing from oilseeds: Principle, processing and application. *Innovative Food Science and Emerging Technologies* **35**, 184–193 (2016).
24. Dias, F. F. G. & de Moura Bell, J. M. L. N. Understanding the impact of enzyme-assisted aqueous extraction on the structural, physicochemical, and functional properties of protein extracts from full-fat almond flour. *Food Hydrocolloids* **127**, 107534 (2022).
25. Campbell, K. A., Vaca-Medina, G., Glatz, C. E. & Pontalier, P. Y. Parameters affecting enzyme-assisted aqueous extraction of extruded sunflower meal. *Food Chemistry* **208**, 245–251 (2016).
26. Souza, T. S. P., Dias, F. F. G., Koblitz, M. G. B. & M. L. N. de M. Bell, J. Aqueous and Enzymatic Extraction of Oil and Protein from Almond Cake: A Comparative Study. *Processes* **7**, 472 (2019).
27. Campbell, K. A. & Glatz, C. E. Mechanisms of aqueous extraction of soybean oil. *Journal of Agricultural and Food Chemistry* **57**, 10904–10912 (2009).
28. Gebauer, S. K., Novotny, J. A., Bornhorst, G. M. & Baer, D. J. Food processing and structure impact the metabolizable energy of almonds. *Food and Function* **7**, 4231–4238 (2016).
29. Min, D. B. & Ellefson, W. C. Fat Analysis. in *Food Analysis* (ed. Nielsen, S. S.) 602 (New York, 2010).

30. AACC Approved Methods of Analysis, 11th Edition - AACC Method 44-40.01. Moisture -- Modified Vacuum-Oven Method. <http://methods.aaccnet.org/summaries/44-40-01.aspx>.
31. American Oil Chemists' Society. *Official Methods and Recommended Practices of the AOCS*. (2017).
32. Brodkorb, A. *et al.* INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols* **14**, 991–1014 (2019).
33. Laemmli, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **227**, 680–685 (1970).
34. Micsonai, A. *et al.* BeStSel: A web server for accurate protein secondary structure prediction and fold recognition from the circular dichroism spectra. *Nucleic Acids Research* **46**, W315–W322 (2018).
35. Zhang, Y. *et al.* Preparation and functional properties of protein from heat-denatured soybean meal assisted by steam flash-explosion with dilute acid soaking. *Journal of Food Engineering* **119**, 56–64 (2013).
36. Dias, F. F. G., de Almeida, N. M., de Souza, T. S. P., Taha, A. Y. & de Moura Bell, J. M. L. N. Characterization and Demulsification of the Oil-Rich Emulsion from the Aqueous Extraction Process of Almond Flour. *Processes* **8**, 1–18 (2020).
37. De Moura, J. M. L. N., Maurer, D., Jung, S. & Johnson, L. A. Integrated countercurrent two-stage extraction and cream demulsification in enzyme-assisted aqueous extraction of soybeans. *JAOCs, Journal of the American Oil Chemists' Society* **88**, 1045–1051 (2011).
38. Zhang, S. B., Lu, Q. Y., Yang, H., Li, Y. & Wang, S. Aqueous enzymatic extraction of oil and protein hydrolysates from roasted peanut seeds. *JAOCs, Journal of the American Oil Chemists' Society* **88**, 727–732 (2011).
39. Vanga, S. K. *et al.* Effect of Thermal and High Electric Fields on Secondary Structure of Peanut Protein. *International Journal of Food Properties* **19**, 1259–1271 (2016).
40. Zhou, H. *et al.* The effect of roasting on peanut allergens' digestibility, allergenicity, and structure. *Food Bioscience* **44**, 101454 (2021).
41. Rahaman, T., Vasiljevic, T. & Ramchandran, L. Effect of processing on conformational changes of food proteins related to allergenicity. *Trends in Food Science and Technology* **49**, 24–34 (2016).
42. Amirshaghghi, Z., Rezaei, K. & Habibi Rezaei, M. Characterization and functional properties of protein isolates from wild almond. *Journal of Food Measurement and Characterization* **11**, 1725–1733 (2017).
43. Zhang, Y., Zhang, J., Sheng, W., Wang, S. & Fu, T. J. Effects of heat and high-pressure treatments on the solubility and immunoreactivity of almond proteins. *Food Chemistry* **199**, 856–861 (2016).
44. Niu, R., Zhou, L., Chen, F., Zhu, T. & Duan, X. Effect of Enzyme on the Demulsification of Emulsion during aqueous enzymatic Extraction and the corresponding mechanism. *Cereal Chemistry* 1–10 (2021) doi:10.1002/cche.10401.
45. de Almeida, N. M., Dias, F. F. G., Rodrigues, M. I. & Bell, J. M. L. N. de M. Effects of processing conditions on the simultaneous extraction and distribution of oil and protein from almond flour. *Processes* **7**, (2019).
46. Altan, A., McCarthy, K. L., Tikekar, R., McCarthy, M. J. & Nitin, N. Image Analysis of Microstructural Changes in Almond Cotyledon as a Result of Processing. *Journal of Food Science* **76**, 212–221 (2011).

47. McCarthy, K. S., Parker, M., Ameerally, A., Drake, S. L. & Drake, M. A. Drivers of choice for fluid milk versus plant-based alternatives: What are consumer perceptions of fluid milk? *Journal of Dairy Science* **100**, 6125–6138 (2017).
48. Zhang, G., Huang, G., Xiao, L., Seiber, J. & Mitchell, A. E. Acrylamide formation in almonds (*Prunus dulcis*): Influences of roasting time and temperature, precursors, varietal selection, and storage. *Journal of Agricultural and Food Chemistry* **59**, 8225–8232 (2011).
49. Ünver, A. Effect of Roasting Conditions and Storage on Acrylamide Content and Colour of Almonds. *Academic Food Journal* **14**, 8–14 (2016).
50. Amrein, T. M. *et al.* Acrylamide in roasted almonds and hazelnuts. *Journal of Agricultural and Food Chemistry* **53**, 7819–7825 (2005).
51. Yan, X., Wang, Y., Chen, Y., Xie, J. & Yu, Q. Effect of roasting duration on the solubility, structure, and IgE-binding capacity of cashew nut proteins. *Innovative Food Science and Emerging Technologies* **68**, 102635 (2021).
52. Martínez-Padilla, E. *et al.* In vitro protein digestibility and fatty acid profile of commercial plant-based milk alternatives. *Foods* **9**, 1–17 (2020).
53. Vanga, S. K., Wang, J., Orsat, V. & Raghavan, V. Effect of pulsed ultrasound, a green food processing technique, on the secondary structure and in-vitro digestibility of almond milk protein. *Food Research International* **137**, 109523 (2020).
54. Sathe, S. K. *et al.* Biochemical characterization of amandin, the major storage protein in almond (*Prunus Dulcis* L.). *Journal of Agricultural and Food Chemistry* **50**, 4333–4341 (2002).
55. Furlan Goncalves Dias, F. *et al.* Effects of protease-assisted aqueous extraction on almond protein profile, digestibility, and antigenicity. *Current Research in Food Science* **6**, 100488 (2023).
56. Devnani, B., Ong, L., Kentish, S. & Gras, S. L. Structure and functionality of almond proteins as a function of pH. *Food Structure* **30**, 100229 (2021).
57. Albillos, S. M., Menhart, N. & Fu, T. J. Structural stability of amandin, a major allergen from almond (*prunus dulcis*), and its acidic and basic polypeptides. *Journal of Agricultural and Food Chemistry* **57**, 4698–4705 (2009).
58. Ma, X. *et al.* Effect of Roasting on the Conformational Structure and IgE Binding of Sesame Allergens. *Journal of Agricultural and Food Chemistry* **70**, 9442–9450 (2022).
59. Li, P. *et al.* Characterization and demulsification of cream emulsion from aqueous extraction of peanut. *Journal of Food Engineering* **185**, 62–71 (2016).
60. Hiller, B. & Lorenzen, P. Chr. Surface Hydrophobicity of Physicochemically and Enzymatically Treated Milk Proteins in Relation to Techno-functional Properties. *J. Agric. Food Chem.* **56**, 461–468 (2008).
61. Rao, H. *et al.* Key factors affecting the immunoreactivity of roasted and boiled peanuts: Temperature and water. *LWT - Food Science and Technology* **72**, 492–500 (2016).
62. Jung, S., Murphy, P. A. & Johnson, L. A. Physicochemical and Functional Properties of Soy Protein Substrates Modified by Low Levels of Protease Hydrolysis. *Journal of Food Science* **70**, C180–C187 (2005).
63. Yang, J. S., Dias, F. F. G., Pham, T. T. K., Barile, D. & De Moura Bell, J. M. L. N. A sequential fractionation approach to understanding the physicochemical and functional properties of aqueous and enzyme-assisted aqueous extracted black bean proteins. *Food Hydrocolloids* **146**, 109250 (2024).

64. de Souza, T. S. P., Dias, F. F. G., Oliveira, J. P. S., de Moura Bell, J. M. L. N. & Koblitiz, M. G. B. Biological properties of almond proteins produced by aqueous and enzyme-assisted aqueous extraction processes from almond cake. *Scientific Reports* **10**, 1–12 (2020).
65. Hemung, B. O., Li-Chan, E. C. Y. & Yongsawatdigul, J. Thermal stability of fish natural actomyosin affects reactivity to cross-linking by microbial and fish transglutaminases. *Food Chemistry* **111**, 439–446 (2008).
66. Kshirsagar, H. H., Fajer, P., Sharma, G. M., Roux, K. H. & Sathe, S. K. Biochemical and spectroscopic characterization of almond and cashew nut seed 11S legumins, amandin and anacardein. *Journal of Agricultural and Food Chemistry* **59**, 386–393 (2011).
67. Dias, F. F. G., de Almeida, N. M., de Souza, T. S. P., Taha, A. Y. & de Moura Bell, J. M. L. N. Characterization and Demulsification of the Oil-Rich Emulsion from the Aqueous Extraction Process of Almond Flour. *Processes* **8**, 1–18 (2020).

Chapter 3: Optimizing the nutritional profile and sensory attributes of almond milks using aqueous and enzymatic extraction methods

Abstract:

Although almond milk is typically low in nutritional content, sustainable aqueous (AEP) and enzyme-assisted extraction processes (EAEP) can produce milks with improved nutritional quality. The role of solids-to-liquid ratio (SLR) (1:10 to 1:6) and extraction pH (7 to 9) in the AEP and enzyme use (0.25 to 0.75%) in the EAEP was evaluated with respect to the nutritional, physicochemical, and sensory properties of the milks. While extraction pH had minimal impact on the composition and properties of AEP milks, high SLR increased protein content but reduced overall protein extractability in both milks. Proteolysis significantly increased oil and protein extractability, oil droplet diameter, and significantly reduced allergenicity in EAEP milks. Descriptive analysis revealed significant differences between AEP and EAEP milks, notably bitterness caused by proteolysis. Six milks described as the most different from each other were selected for hedonic analyses, which revealed that milks produced with higher SLR (1:6) were preferred. Following consumer hedonic testing, both processes were reformulated to final conditions of 1:6 SLR and pH 8 for AEP and 0.01% protease and pH 9.0 for EAEP. AEP and EAEP milks had final protein contents of 2.7 and 3.3%, respectively, with hedonic scores of 6.0 and 4.9 on 9-point scale, indicating a high degree of liking and potential consumer acceptability for these improved almond milks.

Keywords: almond milk, nutritional and sensory properties, aqueous extraction process, enzyme-assisted aqueous extraction process

3.1 Introduction

Almonds are a vital crop for California, ranking just behind dairy and grapes as the top valued commodities in the state¹. California is the world's main producer of almonds, accounting for 2.9 billion pounds, roughly 78% of the global almond crop yield in 2022¹. One end-use for these almonds is almond milk, which contributes to the global plant-based milk market, valued at \$13.24B in 2022 and projected to reach \$30.79B by 2031². The increasing consumer purchase of plant-based milks can be attributed to a variety of reasons, including lactose intolerance, sustainability/animal welfare concerns, and health perception of these milks³. Of the many commercially available plant-based milks in the United States, almond milk is currently the most popular, with an estimated market value of \$1.5B in 2022. Despite its rising popularity, currently available retail almond milks are nutritionally inferior to dairy milk, with most containing 1 g or less protein per 250 mL serving compared to dairy milk's ~7.5-8 g/ 250 mL⁴. Almond milks are also low in lipids, with only 2.5 g per serving compared to 8 g per serving in dairy milk. While almond kernels are a nutritionally dense food, containing ~21% protein, 52% lipids, and 21% carbohydrates⁵, the poor nutritive quality of almond milk can be explained by inherent limitations of the production method employed. To produce almond milk, roasted blanched almonds are ground into a fine paste and then suspended in water at approximately 2% of the total formula to prevent sedimentation of almond particles. In many formulations, hydrocolloids such as gellan gum and emulsifiers such as sunflower lecithin are added to provide mouthfeel, emulsify the lipid and water phases in the milk and stabilize the suspended solids⁶. This approach enhances the product's texture, prevents the separation of solids and liquids, and ensures a uniform and consistent product that is more pleasant to drink. As the concentration of almond paste is relatively low, the protein content of the final milk is typically less than 0.5%. While this results in an almond

milk with appealing sensory properties, it is not a viable nutritional substitute for dairy milk for consumers that cannot or choose not to drink dairy milk.

To enhance the nutritional composition of almond milk, an innovative extraction-based approach should be utilized rather than the traditional solid dispersion method. We have demonstrated in Chapter 2 that aqueous (AEP) and enzyme-assisted aqueous extraction processes (EAEP), strategies that allow for proteins and lipids to be simultaneously extracted from plant materials and separated from the insoluble fibers that might cause unpleasant textures or mouthfeel⁷⁻¹⁰, result in a nutritionally enhanced almond milk. In AEP and EAEP, the starting material is dispersed into water and the slurry pH adjusted to values that favor protein solubility into the extraction medium and/or enzyme performance. When proteins are solubilized in the surrounding aqueous medium, they leave behind pores in the cellular material, allowing for the release of lipids in the medium as well. Following the extraction, the slurry is centrifuged, separating the aqueous phase, containing the soluble proteins and released lipids, from the fiber-rich fraction, also known as “insolubles”. By using AEP and EAEP to produce almond milk, it becomes possible to increase the concentration of nutrients in the milk, such as proteins and lipids, while minimizing issues related to grittiness and solids sedimentation. Importantly, tailoring extraction methods and conditions affects not only the nutritional composition but also the physicochemical and sensory properties of the milk^{9,11}, which in turn impacts the consumer acceptability of the milk.

Previous work exploring the integrated impacts of degree of roast (unroasted, light roast and dark roast) and almond particle size (flour, butter, and paste) on the nutritional and physicochemical properties of almond milks produced via AEP and EAEP demonstrated that light roasted butter was a suitable starting material for almond milk production due to its fine particle

size and mild roast, both of which favored extraction compared to other degrees of roast and grind. When evaluating milk with selected light roasted butter, AEP produced milk with 1.63% protein and 4.58% lipids, while the EAEP yielded milk with 1.87% protein and 5.16% lipids. However, the impact of specific extraction conditions on the nutritional, physicochemical and sensory properties has yet to be optimized.

Several parameters in AEP and EAEP, including solid-to-liquid ratio (SLR), extraction pH, and enzyme concentration and type, can significantly impact protein extractability, ultimately dictating the protein content of the extracted milk. Additionally, these parameters can also alter the physicochemical, functional, and sensory properties of the extracted proteins^{11–13}. The SLR determines the concentration gradient of proteins in the water, driving their diffusion out of the almond cells^{7,8}. This impacts the final nutritional composition of the milk, and consequently, its flavor and mouthfeel. An extraction pH far from the protein's isoelectric point increases protein solubility, thus improving protein extraction efficiency^{14,15}. However, the impact of alkaline pH on flavor and its potential effect on consumer acceptance of the milk remains unknown. Similarly, the type of enzyme selected and the level of its addition are critical factors for the EAEP. Proteases are useful for improving protein extractability as well as improving their functional and biological properties such as protein solubility, immunoreactivity and antioxidant capacity^{11,12,16}. Proteases, however, can lead to the release of small peptides with relatively high hydrophobic amino acid content, which can be perceived as bitter¹⁷. When utilizing proteases, it is critical to find a suitable concentration that will enhance protein yields without diminishing consumer acceptance. Each of these parameters may be manipulated to produce a finished almond milk product with tailored extractability, nutritional, and sensory properties.

To accomplish this goal, this study evaluated the impact of a) SLR and extraction pH in the AEP and extraction pH and enzyme concentration in the EAEP with respect to total protein and oil extractability, oil droplet diameter and viscosity of milks, as well as immunoreactivity, antioxidant activity, zeta potential, and degree of hydrolysis of the milk proteins; b) performed a descriptive sensory analysis with AEP and EAEP milks from all conditions and compared them with retail almond milks; and c) selected the best AEP and EAEP conditions based on sensory feedback for subsequent hedonic sensory analysis and reformulation of AEP and EAEP conditions to produce milks with improved nutritional composition, functional properties, and consumer acceptance. The results presented in this work provide a fundamental understanding of how extraction methods and conditions impact the nutritional composition and physicochemical properties of almond milks, and, consequently, consumer acceptance. This knowledge is critical for advancing the production and formulation of almond milks towards the development of more viable and attractive options in the plant-based milk market.

3.2. Materials and methods

3.2.1. Materials

Commercial food-grade blanched light roasted almond paste was purchased from a local supplier (Harris-Woolf, Coalinga, CA, USA). The enzyme used for the EAEP was generously donated by Danisco (FoodPro® Alkaline Protease from *Bacillus licheniformis*, Danisco, NY, USA). Gum Arabic was purchased from Modernist Pantry (Eliot, ME, USA) and sunflower lecithin from NOW Foods (Bloomington, IL, USA). Criterion Pre-Cast 12% acrylamide low-range protein gels, low-range protein standard, tris buffer, β -mercaptoethanol, Laemmli sample buffer, and Coomassie Blue G250 were purchased from Bio-Rad (Hercules, CA, USA).

3.2.2. Characterization of almond paste

Particle size of the almond paste was measured using laser diffraction with a particle size analyzer (Mastersizer 3000E—Malvern Panalytical Inc. Westborough, MA, USA) using a refractive index of 1.59¹⁸ and calculated according to Mie theory. The D [4,3] was calculated as 35.7 μm , while the D (10), D (50), and D (90) were 6.08, 24.0, and 81.3 μm , respectively. The almond paste contained $1.98 \pm 0.01\%$ moisture, $51.5 \pm 0.4\%$ oil, $21.5 \pm 0.1\%$ protein, $2.89 \pm 0.01\%$ ash, and $22.1 \pm 0.3\%$ carbohydrates. Moisture was determined by the AOAC method 925.09, lipids by AOAC method 989.05, protein by Dumas combustion (Vario Max Cube, Elementar Americas, Ronkonkoma, NY, USA) using a nitrogen conversion factor of 5.18, ash by the AOAC method 920.125¹⁹, and carbohydrates by difference.

3.2.3. Leveraging experimental design tools to elucidate the impact of extraction methods on almond milk properties

A central composite design (CCD) was used to assess the impacts of SLR and extraction pH in the AEP and SLR and enzyme concentration in the EAEP on milk composition, physicochemical properties, and consumer preference. Extraction replicates are represented by the central point condition, which was performed in triplicate (AEP 5, 6, 7, and EAEP 5, 6, 7). Extraction conditions for AEP and EAEP milks are shown in Tables 1 and 2, respectively.

3.2.4 Extraction of almond milk using the AEP and EAEP

Almond milk extractions were performed as described in Chapter 2, with almond paste purchased from a manufacturer to produce food-grade milks. This product was chosen as it was the closest to the light roasted almond butter chosen as the best starting material in Chapter 2, as there was little difference observed in extraction yields between butter and paste grinds. Light roasted blanched almond paste was mixed with filtered water at 50 °C in a 1000 mL glass beaker at SLR values described in Tables 3.1 and 3.2. The beaker was placed in a recirculating water bath

at 50 °C and agitated with a 1” magnetic stir bar at approximately 120 RPM. For the EAEP, the appropriate amount of food-grade alkaline protease (Table 3.2) was added to the extraction slurry.

Table 3.1. Solids-to-liquid ratio and extraction pH levels evaluated in the production of AEP milks.

Experiment	Solids-to-Liquid Ratio (x_1)		pH (x_2)	
	Coded Value	Real Value	Coded Value	Real Value
AEP1	-1	1:10	-1	7
AEP2	1	1:6	-1	7
AEP3	-1	1:10	1	9
AEP4	1	1:6	1	9
AEP5	0	1:7.5	0	8
AEP6	0	1:7.5	0	8
AEP7	0	1:7.5	0	8

Table 3.2. Solids-to-liquid ratio and extraction pH levels evaluated in the production of EAEP milks.

Experiment #	Solids-to-Liquid Ratio (x_1)		Enzyme Concentration (x_2) (w/w)	
	Coded Value	Real Value	Coded Value	Real Value
EAEP1	-1	1:10	-1	0.25%
EAEP2	1	1:6	-1	0.25%
EAEP3	-1	1:10	1	0.75%
EAEP4	1	1:6	1	0.75%
EAEP5	0	1:7.5	0	0.50%
EAEP6	0	1:7.5	0	0.50%
EAEP7	0	1:7.5	0	0.50%

The pH was then adjusted to the desired value using food-grade solutions of 1 M HCl/1 M NaOH in water and maintained for the duration of the extraction (Tables 3.1 and 3.2). After one hour of

extraction, the solution was centrifuged for 30 min at 4 °C and 3,280 x g, then the supernatant (“milk”) was separated from the fiber-rich fraction (“insolubles”). The milk was then mixed with sunflower lecithin (1% w/w, NOW Foods, Bloomingdale, IL, USA) and gum Arabic (0.5% w/w, Modernist Pantry, Eliot, ME, USA) in a kitchen blender at high speed for 2 minutes to provide a mouthfeel similar to conventional almond milk. The milk was then transferred to sealed glass jars and batch pasteurized in a heated circulating water bath at 63 °C for 30 min with constant stirring at 120 rpm. After pasteurization, the milk was refrigerated until ready to serve or frozen for further analysis.

3.2.5. Proximate analyses

The protein content of the milks (%), as well as the total protein extraction (TPE, %) from the starting material was quantified using the methods outlined in section 2.2. TPE was calculated according to Equation (1):

$$TPE (\%) = [1 - (\frac{Protein (g) \text{ in the insoluble fraction}}{Protein (g) \text{ in the almond paste}})] \times 100 \quad (1)$$

The oil content of the milks (%) was determined via acid hydrolysis method (AOAC Method 922.06 for Fat in Flour)²⁰. Total oil extraction (TOE, %) was determined according to Equation (2):

$$TOE (\%) = [1 - (\frac{Oil (g) \text{ in the insoluble fraction}}{Oil (g) \text{ in the almond paste}})] \times 100 \quad (2)$$

Total solids and moisture content were determined by drying the samples in a vacuum oven at 65 °C until they reached constant weight. Ash was quantified AOAC method 920.125¹⁹. All measurements were performed in triplicate on each milk.

3.2.6. Oil Droplet Diameter of AEP and EAEP milks

The oil droplet diameter of AEP and EAEP milks was measured within 24 h of production using laser diffraction (Mastersizer 3000E, Malvern Panalytical, Malvern, United Kingdom) with a refractive index of 1.466 for almond oil²¹ and distilled water as a dispersant. Samples were added to dispersant until an obscuration of 8-12% was reached and 10 measurements were immediately taken for each sample. Measurements were repeated in triplicate for each extraction, resulting in a total of 30 measurements per sample. Mean droplet diameter was reported as the De Brouckere [4,3] volume-weighted mean diameter using Mie theory calculations.

3.2.7. Viscosity of AEP and EAEP milks

Viscosity of milks was measured within 24 h of production using an MCR 302e rheometer (Anton Parr, Graz, Austria) with a CC27 cup and bob attachment. Viscosity was measured at 25 °C with data taken every 3 s for 120 points total from 0.5-10000 RPM. Data was interpreted via RheoCompass software and reported as viscosity at a shear rate of 50 s⁻¹²². Measurements were performed in triplicate for all milks.

3.2.8. Molecular weight of AEP and EAEP milk proteins

Milk protein molecular weights were analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a method as described by Laemmli²³ with some modifications. An aliquot of 50 µg of protein was loaded into the lanes of 12% acrylamide Criterion TGX Precast gels, alongside a low range protein standard ladder (14.4-97.4 kDa). Gels were stained with Coomassie blue dye and imaged using the Gel Doc EZ Imager system with Image Lab software.

3.2.9. Defatted AEP and EAEP Milk Protein Analyses

Following whole milk analyses, milks were frozen and thawed to separate the cream from the defatted milk to avoid lipid interference. Defatted milks were then analyzed in triplicate for

degree of hydrolysis, zeta potential, molecular weight, immunoreactivity, and total antioxidant capacity (ABTS).

3.2.9.1. Degree of hydrolysis and zeta potential of defatted AEP and EAEP milk proteins

Degree of hydrolysis of skim proteins was determined using the o-phthaldialdehyde (OPA) method²⁴. An aliquot of 3 mL of freshly prepared OPA reagent was added to 400 µL of defatted milk at 1% w/v in water. After vortexing and incubating at room temperature for 2 min, the absorbance of the solution was then measured at 340 nm using a SpectraMax iD5 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA) and compared against an L-serine solution standard. DH was calculated as Equation (3):

$$DH(\%) = \frac{h}{h_{tot}} \times 100 \quad (3)$$

in which h is the number of bonds that have been hydrolyzed and h_{tot} is the total peptide bonds per protein equivalent (for almond protein, 7.58 eq/kg¹¹).

The zeta potential of the milks was determined using dynamic light scattering (Zetasizer Plus, Malvern Instruments Ltd., United Kingdom). Defatted milk samples were freeze-dried and diluted to 0.05% (w/v) in 0.01 M pH 7 phosphate buffer. Each sample was read at a mean count rate of at least 100 kcps and measurements were performed in triplicate at 25 °C.

3.2.9.2. Immunoreactivity of AEP and EAEP milk proteins

The immunoreactivity of defatted AEP and EAEP milk proteins was determined by the use of a Veratox for Almond sandwich enzyme-linked immunosorbent assay (S-ELISA) kits (Neogen, Lansing, MA), as described by Dias et. al¹⁶. Briefly, a standard curve ($r^2 = 0.996$) was created based on absorbance readings of supplied standards at 0, 2.5, 5, 10, and 25 ppm of Amandin, the major allergenic protein in almonds, at 650 nm on a SpectraMax iD5 Multi-Mode plate reader (Molecular devices, San Jose, CA). Defatted milk samples were extracted using the supplied

10mM PBS extraction solvent and diluted with this solvent until final absorbance readings fell within the standard curve. Samples were added to the almond antibody-coated wells, along with antibody conjugates, substrates, and a stop solution. Following the procedure, the concentration of almond proteins in the defatted milk was calculated in ppm by comparing the absorbance readings to the standard curve. Each milk sample was analyzed in triplicate.

3.2.9.3. Antioxidant capacity of AEP and EAEP milks

The antioxidant capacity of the milks was analyzed using the ABTS assay as described by de Souza et al¹² with some modifications. Defatted milk samples were diluted in distilled water to concentrations ranging from 0.5 to 5 mL/mg. Then, 20 μ L of this dilution was added to 200 μ L of diluted ABTS reagent [2,20-Azino-bis (3-ethylbenzothiazoline-6-sulfphonic acid)] in a 96-well plate. After 6 minutes of shaking at 300 rpm at room temperature, the absorbance of the plate was recorded at 730 nm in a microplate reader at 25 °C. The antioxidant capacity was then calculated against a standard curve of Trolox (Sigma-Aldrich, St. Louis, USA) ranging from 0 to 340 μ M ($R^2 = 0.996$) and expressed as μ mol of Trolox equivalent per mL of defatted milk. Each milk sample was analyzed in triplicate.

3.2.10. Statistical analysis of physicochemical assays

Experimental results were analyzed using Protimiza Experimental Design Software (Protimiza Experimental Design, Campinas, Brazil). The significance of the linear regression models was assessed by the coefficient of determination R^2 and the F test, with a significance level set at $p < 0.05$. JMP statistical software (JMP Statistical Discovery LLC, Cary, NC, USA) was utilized to analyze the differences between the proximate composition of the final milk formulas with a one-way ANOVA followed by Tukey's post hoc test at the level of confidence of $p < 0.05$.

3.2.11. Sensory analysis of AEP and EAEP milks

All sensory panels were approved by the University of California, Davis Institutional Review Board (1900526-1). Oral consent was obtained from each panelist prior to their participation in the study, and they were all compensated for their time.

3.2.11.1. Descriptive analysis

A quantitative descriptive analysis study was first conducted on all AEP and EAEP milks (Tables 3.1 and 3.2) to understand how the sensory properties of the different formulations differed from each other. Fourteen panelists were trained to evaluate the sensory attributes of the 14 formulated samples (7 AEP and 7 EAEP), along with 12 commercial almond milks. While the full descriptive analysis was reported in a previous study, this study focuses solely on the results related to the AEP and EAEP samples. Each panelist completed 10 hours of training which included attribute generation and training on individual attributes with references. The abridged list of attributes pertaining to the formulated samples is shown in Table 3.3. Panelists evaluated the samples on a continuous line scale, assessing 8 samples per session and performing three replicates for each milk. All responses were recorded on a computer using Compusense20 software (Compusense Inc., Guelph, Canada).

Table 3.3. Sensory attributes, techniques, and definitions of almond milks, as evaluated by trained panelists.

Type	Attribute	Definition	Procedure	Anchor low	Anchor high
Appearance	Oily Coating	How much oil separates from the sample	Observe before shaking, observe under light, look for oily sheen	No oil	Very oily
	Brown Color	Color of the sample	Shake the sample, then observe the color under direct light	White	Light brown
	Particle size	Size of particulate in the sample	Swirl in cup, look for particulates on the edge of the cup	Smooth	Chunky
Odor	Grainy smell	Smells like oats, rice, wheat, wood, hay	Swirl the sample, then smell the sample	No grainy smell	High grainy smell
Taste	Salty	Tastes salty	Drink water, shake the sample, plug nose, taste it, swirl it around the mouth	Not salty	Salty
	Sweet	Tastes sweet		Not sweet	Sweet
	Bitter	Tastes bitter		Not Bitter	Bitter
Texture	Mouthcoating	How much the sample sticks to your palate, tongue, and cheeks	Drink water, swirl the sample in the mouth, swallow slowly	Not sticky	Very sticky
	Creamy/not diluted	Feels like cream, smooth, fatty, slightly thick, not thin	Drink water, swirl sample in the mouth and evaluate while it is in the mouth	Watery	Creamy
	Oily aftertaste	Oiliness in the mouth and on the lips, fattiness	Shake sample, drink water, swirl the sample in the mouth, evaluate in the mouth and after swallowing	No oily aftertaste	Very oily aftertaste
	Astringent	Drying sensation in the mouth	Drink water, swirl the sample in the mouth, swallow sample	Not astringent	Astringent
Flavor	Nutty flavor	Flavor of nuts		No nutty flavor	High nutty flavor
	Roasted flavor	Flavor of roasted almonds		No roasted flavor	High roasted flavor
	Grainy flavor	Flavor of grains (oats, wheat, bread, rice)	Shake sample, drink water, swirl the sample in the mouth, evaluate flavor	No grainy flavor	High grainy flavor
	Beany flavor	Flavor of soybeans, umami, savory, vegetable oil		No beany flavor	High beany flavor

3.2.11.2. Consumer tests

Based on the results of the descriptive analysis, 4 AEP milks (AEP1: pH 7, SLR 1:10; AEP 2: pH 7, SLR 1:6; AEP3: pH 9, SLR 1:10; and AEP4: pH 9, SLR 1:6) and 2 EAEP milks (EAEP3: 0.75% enzyme and 1:10 SLR, and EAEP4: 0.75% enzyme and 1:6 SLR) were chosen for an initial consumer testing. These samples were chosen due to their significantly different ratings from the descriptive analysis. For the first consumer study, panelists were recruited from the UC Davis and the Davis community through emails and posters. Interested participants were asked to complete a pre-screening survey which assessed their eligibility through information like gender, diet, and frequency of consumption of milk. In total, 121 panelists were selected to take part in the first consumer study and were asked to rate their liking on a 9-point hedonic scale. In addition, panelists were asked to complete a check-all-that-apply (CATA) question that included the 12 attributes of the sensory descriptors generated from the trained panel. Samples were presented in a monadic sequential design.

Following the initial consumer test, additional samples were selected for further consumer testing. AEP2 and AEP4 were chosen for additional tests due to their high scores in the initial consumer testing. External preference mapping analysis revealed that a sample obtained under extraction conditions between those of AEP2 and AEP4 milks might have higher preference. Additionally, a new aqueous formulation with 1:6 SLR ratio and pH of 8, designated as AEP8, was included in the testing. Because the EAEP milks had lower scores during the first consumer test, it was hypothesized that lowering the enzyme use during the extraction might increase the liking scores. Thus, EAEP2 (0.75% enzyme, 1:6 SLR) was added, and additional EAEP milk produced with 0.1% enzyme and 1:6 SLR ratio was also included (EAEP8). These 5 samples were tested in a second consumer test with 129 panelists. Like the first consumer test, panelists were

asked to rate their liking on a 9-point hedonic scale and they were also asked to complete a CATA question.

3.2.11.3. Statistical analysis of sensory data

Analyses were performed using R (version 4.2.1). For the descriptive analysis, a three-way MANOVA with a two-way interaction was performed on AEP and EAEP almond milks. Univariate three-way ANOVAs with two-way interactions were conducted on each of the 24 attributes and a pseudo-mixed model was used to test the product effect of any attributes that contained both a significant product effect and a significant judge*product and/or replication*product interaction. A Canonical Variate Analysis (CVA) as performed on the MANOVA using the package “candisc” but attributes that were found to have no significant product effect were excluded from the model. Confidence ellipses were created using bootstrap resampling with 1000 replicates. The unstandardized scores from the CVA were used to create the score plot and the loadings plot was generated based on the top five sensory attributes with the longest vectors.

For the consumer studies, one-way ANOVAs were conducted on the liking data with product as the main effect, and Tukey’s post hoc test was used to test for differences in groups using the package “agricolae”. External preference mapping was conducted using the package “SensoMineR” by overlaying liking scores over a Principal Component Analysis (PCA) plot of the descriptive data (correlation matrix). The data from the check-all-that-apply was analyzed using correspondence analysis with the package “FactoMineR”.

3.3 Results

3.3.1. Impact of extraction conditions on the composition of AEP and EAEP milks and their effect on oil and protein extractability

The impact of extraction methods (AEP vs. EAEP) and conditions (SLR, enzyme concentration, and pH) on the protein, lipid, moisture, ash, and carbohydrate contents of the milks is shown in Table 3, while their effects on overall oil and protein extractability are illustrated in Fig. 1.

3.3.1.1. Protein content of AEP and EAEP milks and total protein extractability

A major objective of this study was to produce almond milk with higher protein content than that found in currently available retail almond milks. This goal was achieved in all extractions, resulting in AEP and EAEP milks with a protein content ranging from 1.65-2.64% and 2.02-3.08%, respectively (Table 3.4). The protein content of AEP milks increased with an increase in SLR, with the highest protein content associated with the highest SLR (1:6). Higher SLR tends to lead to higher protein content in the milk as there is more protein to be solubilized than in a lower SLR extraction⁹. Similarly, pH had a minor but significant impact ($p < 0.05$) on the milk protein content (Table 4). Almond proteins are more soluble at higher pH values (i.e., more alkaline), away from their isoelectric point^{25,26}; thus pH can greatly impact the protein content of the milks. AEP1 (pH 7.0, 1:10 SLR) and AEP 3 (pH 9.0, 1:10 SLR) milks had a protein content of ~1.7%, whereas AEP2 (pH 7.0, 1:6 SLR), and AEP 4 (pH 9.0, 1:6 SLR), milks had a protein content of ~2.6%, highlighting the stronger impact of SLR on the protein content of the milks.

Table 3.4. Impact of extraction conditions on the macronutrient composition of AEP and EAEP almond milks.

Exp.	Moisture (%)	Protein (%)	Oil (%)	Ash (%)	Carbohydrate (%)
AEP1	90.87 ± 0.04	1.65 ± 0.03	5.50 ± 0.11	0.19 ± 0.00	1.79 ± 0.10
AEP2	86.27 ± 0.0	2.60 ± 0.01	8.54 ± 0.21	0.23 ± 0.01	2.36 ± 0.19
AEP3	90.98 ± 0.04	1.76 ± 0.04	5.49 ± 0.08	0.18 ± 0.01	1.60 ± 0.16
AEP4	86.37 ± 0.04	2.64 ± 0.01	8.39 ± 0.49	0.22 ± 0.02	2.39 ± 0.48
AEP5	88.22 ± 0.04	2.15 ± 0.03	6.98 ± 0.16	0.22 ± 0.00	2.44 ± 0.15
AEP6	88.41 ± 0.04	2.16 ± 0.03	6.99 ± 0.08	0.22 ± 0.01	2.22 ± 0.16
AEP7	88.06 ± 0.08	2.16 ± 0.03	7.29 ± 0.11	0.23 ± 0.01	2.26 ± 0.19
EAEP1	90.66 ± 0.06	1.98 ± 0.02	5.08 ± 0.10	0.29 ± 0.01	1.99 ± 0.04
EAEP2	85.87 ± 0.14	3.07 ± 0.06	8.08 ± 0.09	0.30 ± 0.01	2.68 ± 0.27
EAEP3	90.39 ± 0.11	2.02 ± 0.04	5.37 ± 0.04	0.24 ± 0.01	1.97 ± 0.12
EAEP4	85.75 ± 0.05	3.08 ± 0.02	7.98 ± 0.10	0.38 ± 0.01	2.81 ± 0.08
EAEP5	88.50 ± 0.02	2.55 ± 0.02	6.45 ± 0.16	0.28 ± 0.01	2.21 ± 0.18
EAEP6	88.16 ± 0.01	2.58 ± 0.02	6.68 ± 0.07	0.31 ± 0.01	2.27 ± 0.07
EAEP7	88.27 ± 0.07	2.57 ± 0.02	6.67 ± 0.10	0.32 ± 0.01	2.17 ± 0.13

Experimental conditions for AEP (# 1: pH 7.0, 1:10 SLR; #2: pH 7.0, 1:6 SLR; #3: pH 9.0, 1:10 SLR; #4: pH 9.0, 1:6 SLR; #5-7: pH 8.0, 1:7.5 SLR) and EAEP (#1: 0.25% enzyme, 1:10 SLR; #2: 0.25% enzyme, 1:6 SLR; #3: 0.75% enzyme, 1:10 SLR; #4: 0.75% enzyme, 1:6 SLR; #5-7: 0.50% enzyme, 1:7.5 SLR). AEP and EAEP results were analyzed as separate groups.

According to the regression model for the protein content (Table 4), maximum protein content in the AEP milk (2.64%) can be achieved when SLR of 1:6 (x_1) and pH values of 9.0 (x_2) are used. The protein content of EAEP milks also increased with higher extraction SLR, whereas enzyme level was not significant within the range evaluated. Overall, at the same SLR, the protein content of EAEP milks was slightly higher than that of AEP milks, underscoring the benefits of using proteases under alkaline pH to aid the extraction process. For example, the protein content

of the EAEP 2 milk, produced using a 1:6 SLR and 0.25% w/w protease at pH 9.0, was 3.1% compared with 2.6% for AEP 2 (1:6 SLR and pH 7) and AEP 4 (1:6 SLR and pH 9). Regression analysis of the data revealed that a 3.9% maximum protein content in EAEP milks can be achieved by using the highest SLR of 1:6 (Table 5). The enzyme used in EAEP extractions was an alkaline endoprotease which has been demonstrated as an effective aid in protein extraction^{27,28}. A high SLR increases the concentration of enzyme and starting material, thus more protein can be expected in an EAEP milk with a high SLR²⁹.

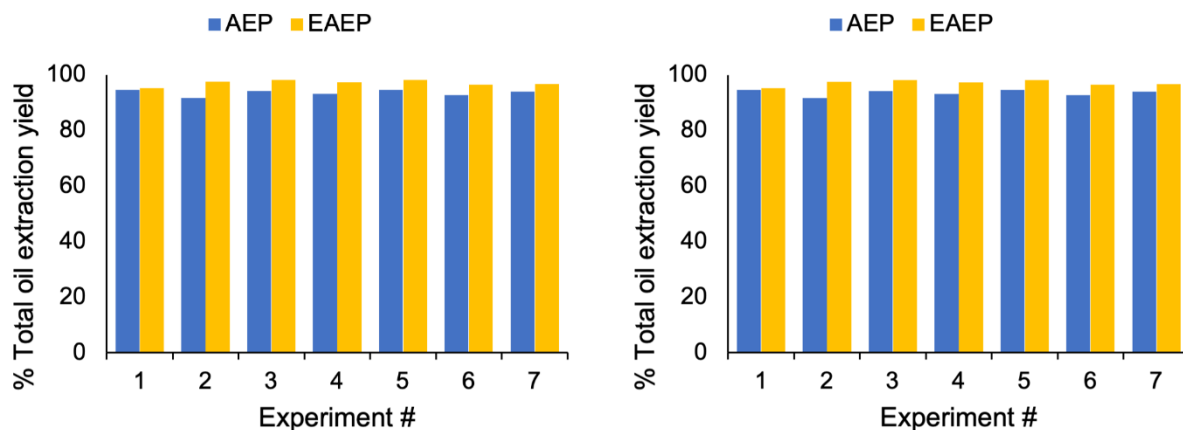
However, when considering overall protein extractability, the total amount of protein extracted from the almond paste (i.e., starting material) decreased with the use of higher SLR (Fig. 1a). At pH 9.0, the TPE (%) in the AEP decreased from 73.5% to 69.8% as SLR increased from 1:10 to 1:6. This is in agreement with previous studies^{9,30,31}, which have shown that using a lower SLR (i.e., more diluted slurries) increases the concentration gradient between solids and the aqueous medium, thereby enhancing extraction efficiency by facilitating the diffusion of proteins out of the cellular material⁷. Regression analysis of the AEP experimental data (Table 3.4) revealed that TPE was enhanced by lower SLR (X_1) ($Y_2 = 71.34 - 1.25 x_1$), with pH not being statically significant within the range evaluated ($p < 0.1$). A similar trend was observed for the EAEP milk, where the use of lower SLR (x_1) and higher enzyme concentration (x_2) favored TPE (Table 3.5). For both AEP and EAEP, the central points of the CCD showed low relative standard deviation (1.7 and 0.4%, respectively), indicating high experimental data reproducibility.

Table 3.5. Two-way analysis of variance (ANOVA) of the estimated regression models AEP and EAEP milk physicochemical properties.

Estimated Regression Models	R^2	F_{cal}	F_{tab}
<i>AEP</i> (x_1 = solids-to-liquids ratio, x_2 = pH)			
Protein content in the milk (%) = $2.16 + 0.46 x_1 + 0.04 x_2 - 0.02 x_1 x_2$	0.99	6752.6	9.277
Protein extraction (%) = $71.34 - 1.25 x_1^*$	0.54	5.8	4.06
Oil content in the milk (%) = $7.03 + 1.49 x_1$	0.99	474.9	6.608
Ash (%) = $0.21 + 0.02 x_1$	0.68	10.8	6.608
Solids (%) = $11.5 + 2.30 x_1$	0.98	315.1	6.608
Carbohydrates (%) = $2.15 + 0.34 x_1$	0.73	13.4	6.608
Viscosity (mPa s at 50 s^{-1}) = $1.97 + 0.20 x_1$	0.84	25.7	6.608
Degree of Hydrolysis (%) = $1.80 - 0.79 x_1 - 1.11 x_2 + 0.40 x_1 x_2$	0.98	48.1	10.128
Antioxidant Activity (mmol Trolox Equivalent/mL milk) = $65352.83 + 14692.03 x_1$	0.68	10.9	6.608
<i>EAEP</i> (x_1 = solids-to-liquids ratio, x_2 = % enzyme)			
Protein content in the milk (%) = $2.55 + 0.54 x_1$	1.00	2082.2	6.608
Protein Extraction (%) = $90.71 - 0.46 x_1 + 0.42 x_2 - 0.47 x_1 x_2$	0.92	11.6	9.277
Oil content in the milk (%) = $6.62 + 1.40 x_1$	0.99	478.9	6.608
% Ash = $0.30 + 0.04 x_1 + 0.03 x_1 x_2$	0.90	18.0	6.944
% Solids = $11.77 + 2.36 x_1$	0.99	801.6	6.608
% Carbohydrates = $2.30 + 0.38 x_1$	0.92	58.3	6.608
D(4,3) Oil Droplet Diameter (μm) = $4.92 + 0.43 x_1 + 0.35 x_2$	0.91	20.6	6.944
Viscosity (mPa s at 50 s^{-1}) = $1.71 + 0.17 x_1$	0.99	327.3	6.608
Degree of Hydrolysis (%) $13.96 + 2.32 x_2$	0.87	32.1	6.608
Antioxidant activity (mmol Trolox Equivalent/mL defatted milk) $402461.99 + 46966.38 x_1 - 18211.98 x_1 x_2$	0.95	35.7	6.944

Significance of variables presented at $p < 0.05$, except where noted with * $p < 0.10$.

Figure 3.1. Impact of extraction conditions on total protein a) and oil b) extraction yields of AEP and EAEP milks. Experimental conditions for AEP (# 1: pH 7.0, 1:10 SLR; #2: pH 7.0, 1:6 SLR; #3: pH 9.0, 1:10 SLR; #4: pH 9.0, 1:6 SLR; #5-7: pH 8.0, 1:7.5 SLR) and EAEP (#1: 0.25% enzyme, 1:10 SLR; #2: 0.25% enzyme, 1:6 SLR; #3: 0.75% enzyme, 1:10 SLR; #4: 0.75% enzyme, 1:6 SLR; #5-7: 0.50% enzyme, 1:7.5 SLR). AEP and EAEP results were analyzed as separate groups.



3.3.1.2. Oil content of AEP and EAEP milks and total oil extractability

While increased SLR lead to a significantly increased oil content in all milks, pH and enzyme levels did not impact the oil content of the milks ($y = 7.03 + 1.49 x_1$ for AEP, $y = 6.62 + 1.40 x_1$ for EAEP, Tables 3-4). Again, this is intuitive as there is more almond butter, and thus more oil, present in the extraction, and more oil to be washed into the milk phase. As in protein extraction (Section 3.1.1), the use of higher SLR decreased the concentration gradient and thus negatively impacted TOE (%) in AEP milks, while pH had no significant impact ($\alpha < 0.10$; Fig. 3.1b). In EAEP milks neither SLR nor enzyme level impacted TOE, though EAEP milks were all higher in TOE (%) than AEP milks at the same SLR. Similar to TPE, TOE at the central points of the CCDs demonstrated low relative standard deviation (1.0% for AEP and 0.8% for EAEP), demonstrating low variability of the results around the central point conditions.

3.3.1.3. Carbohydrate and ash content of AEP and EAEP milks

Ash contents of milks was minimally affected by SLR in AEP milks, whereas in EAEP milks the interaction between SLR and enzyme level (but not enzyme alone) was also impactful (Table 5). Overall ash content of all EAEP milks was higher than AEP milks, likely due to the addition of larger amounts of NaOH to maintain the extraction pH constant.

Similarly, the overall carbohydrate content was only minimally impacted by SLR in both AEP and EAEP milks. The carbohydrate content around the central points showed low relative standard deviation for both AEP and EAEP experiments (4.9% and 2.1%, respectively), again indicating the significance of these results. In the case of both ash and carbohydrate, a higher quantity of starting material in the extractions (i.e., the use of higher SLR) led to a higher quantity of these components in the final milk, as observed for proteins and lipids.

3.3.2 Impact of extraction conditions on oil droplet diameter and milk viscosity

The mean diameter of oil droplets in the milks as measured 24 h after pasteurization was found to be larger in EAEP milks than those of AEP (4.11-5.68 vs. 2.94-4.18 μm ; Fig. 3.2a). In AEP milks, neither pH nor SLR significantly affected oil droplet size. In contrast, for EAEP milks, both SLR and pH had a mildly positive impact on this parameter (Table 3.4). The larger oil droplet size in EAEP milks, despite relatively similar total oil content compared to AEP milks, indicates a weaker emulsion⁶. This could be attributed to proteolysis of the oleosin, a lipid body protein that normally stabilize the surface of oil bodies^{32,33} and helps prevent lipid coalescence. Therefore, lipid coalescence due to oleosin proteolysis could contribute to increased oil droplet size as observed for EAEP milks⁸. Another potential explanation is that the shorter hydrolyzed proteins do not extend as far into the aqueous phase as native proteins, impeding steric interactions that keep oil droplets from coalescing³⁴. The dynamic viscosities of the EAEP milks were lower than that of the AEP milks (1.5-1.9 vs. 1.8-2.1 mPa s), also implying a weaker emulsion⁶.

SLR had a minor but statistically significant impact on the viscosity of AEP milks, with increasing SLR being associated with increased viscosity (Table 3.4). This effect was consistent regardless of the extraction pH, which did not have a statistically significant impact. The impact of SLR on viscosity observed in AEP milks was also seen in EAEP milks ($p < 0.05$). The higher lipid content in the milks produced at higher SLR likely contributes to their higher viscosity, as can be seen in the difference in viscosity between skim milk and heavy cream⁶. Although one would expect the higher protein content of EAEP milks to contribute to higher viscosity of the EAEP milks, an opposite trend was observed. That discrepancy could likely be attributed to the effect of proteolysis when compared with the AEP. Protein fragmentation and reduced emulsion stability of EAEP milks could explain the reduced viscosity of EAEP milks³⁵. However, enzyme level did not show a significant impact on EAEP milk viscosity. These results align with the literature, which shows that enzymatic hydrolysis reduces the viscosity of protein dispersions³⁶.

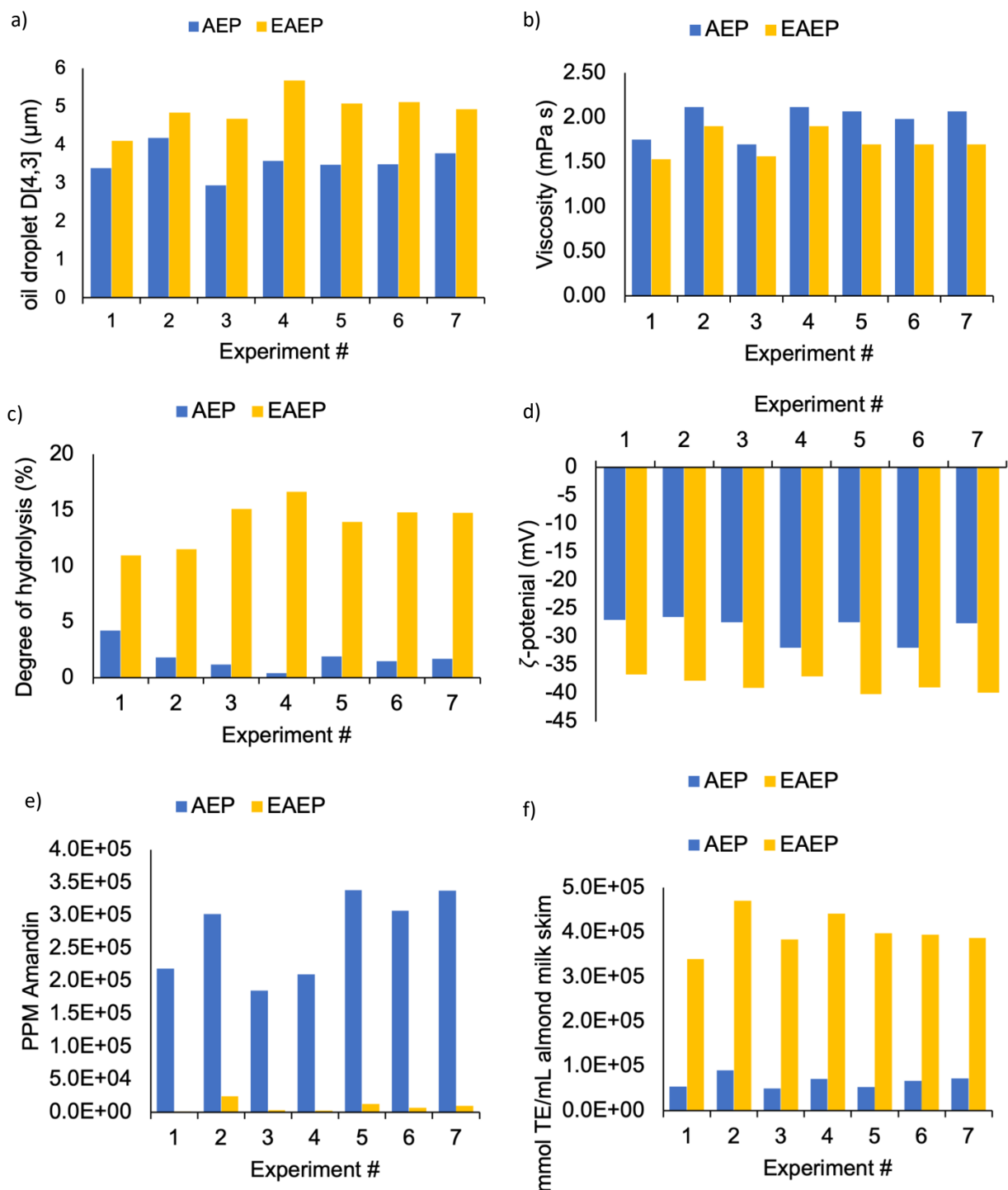


Figure 3.2. Characterization of AEP and EAEP milks at different experimental conditions: oil droplet diameter (μm) (a), viscosity at 50 s^{-1} (mPa s) (b), degree of hydrolysis of milk proteins (%) (c), ζ -potential of milk proteins (mV) (d), PPM ammandin in defatted milk, e) and mmol Trolox equivalents/mL milk proteins (f). Experimental conditions for AEP (# 1: pH 7.0, 1:10 SLR; #2: pH 7.0, 1:6 SL; #3: pH 9.0, 1:10 SLR; #4: pH 9.0, 1:6 SLR; #5-7: pH 8.0, 1:7.5 SLR) and EAEP (#1: 0.25% enzyme, 1:10 SLR; #2: 0.25% enzyme, 1:6 SLR; #3: 0.75% enzyme, 1:10 SLR; #4: 0.75% enzyme, 1:6 SLR; #5-7: 0.50% enzyme, 1:7.5 SLR). AEP and EAEP results were analyzed as separate groups.

3.3.3. Impact of extraction conditions on degree of hydrolysis

The degree of hydrolysis (DH), as measured by the OPA method, was significantly higher in EAEP milk proteins (10.9-16.6%) compared to AEP milk proteins (0.38-4.2%), as expected due to the proteolysis that occurs during enzymatic extractions (Fig. 3.2b). Extraction pH and SLR impacted DH in AEP milks. As described by the regression model in Table 4, DH decreases as SLR and pH increase from -1 to 1 levels. The use of higher SLR can reduce the concentration gradient between solids and liquids, and that could lead to less efficient water-solids interaction compared with milks produced by the use of lower SLR⁸. When examining the impact of SLR on both total protein extractability (TPE) and protein content of the milks (Table 3.4 and Fig. 3.1), it was observed that increasing the SLR from 1:10 to 1:6 did not significantly reduce the total protein extractability for AEP milks. However, it resulted in milks with a higher protein content, which may contribute to the reduced DH observed for AEP milks produced using higher SLR. Typically, hydrolysis is not quantified on native (unhydrolyzed) samples with a DH below 10%, thus conclusions are difficult to make on AEP milk samples regarding the degree of and impact of hydrolysis on the extracted almond proteins, as the efficacy of the OPA method is reduced at low levels of hydrolysis³⁷. However, the reduction in DH could possibly be attributed to the potential interference with OPA measurements caused by factors such as dilution of free amino acids due to the higher protein content, protein aggregation, and matrix effects³⁷. Similar to the effect of SLR, increasing extraction pH from 7.0 to 9.0 also resulted in a small, but significant at $p < 0.05$, reduction in the DH of AEP milks. The decrease in DH with increasing pH values (1.81% for AEP 2 at pH 7 compared with 0.38% for AEP 4 at pH 9) was somewhat surprising as it is expected that proteins, when away from their isoelectric point, would carry increased charge density and

enhanced protein solubility, which in turn would lead to increased protein-water interactions, and these proteins would have undergone further hydrolysis. Such behavior could be explained by the fact that the use of more alkaline pH can lead to protein destabilization, which may result in protein aggregation and precipitation³⁸. These changes can also lead to conformational changes that reduce the accessibility of amino groups, ultimately leading to lower readings of the DH when measured by the OPA method³⁹.

In EAEP milks, higher levels of enzyme usage led to a DH of up to 16.6% (Fig. 3.2c). Enzyme level was significantly impactful on DH ($p < 0.05$, Table 3.5), while SLR did not have a significant impact. This is intuitive in that higher enzyme use typically leads to higher proteolysis, and is in agreement with previous work^{16,40}. As proteolysis can produce bitter peptides and undesirable flavors, this was a concern for sensory trials of the milk.

3.3.4. SDS-PAGE

SDS-PAGE of the extracted milk skims illustrated significant proteolysis in EAEP milks compared to native proteins (Figure 3.3). In AEP milk proteins, strong bands are visible at ~38 kDa and ~22 kDa, corresponding to the basic β - and acidic α -subunits of amandin⁴¹. Notably, no difference is evident between the molecular weights of AEP proteins at different pH levels despite the calculated differences in the DH. The running buffer effectively forces all of the proteins loaded within the gel to be at the same pH, and perhaps this masks any differences in aggregation that could have explained the reduction in DH at higher pH (section 3.3.5); or this aggregation is reversible.

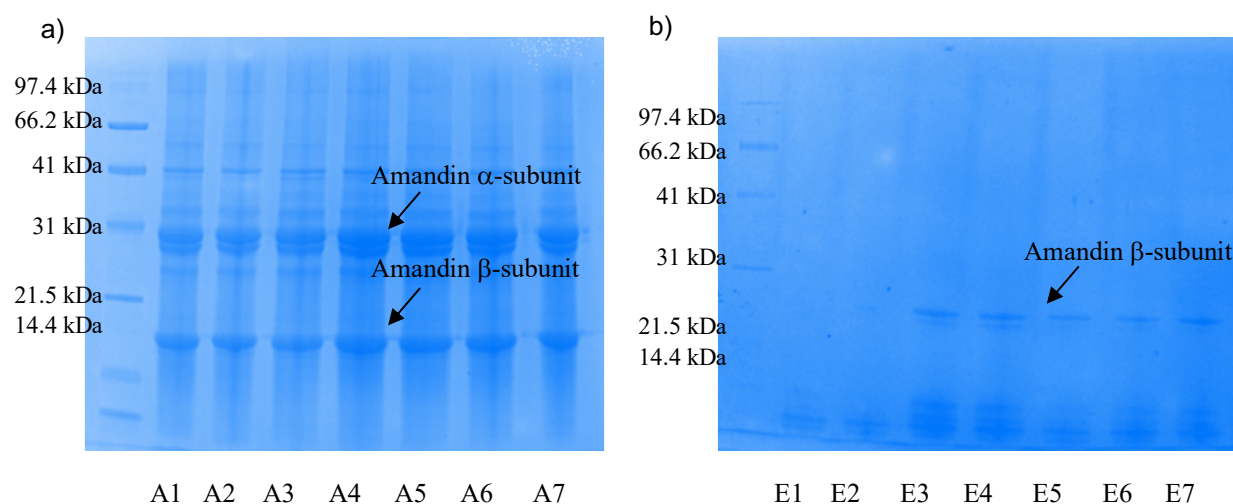


Figure 3.3. SDS-PAGE protein gels for AEP (a) and EAEP (b) milk proteins. Experimental conditions for AEP (# 1: pH 7.0, 1:10 SLR; #2: pH 7.0, 1:6 SLR; #3: pH 9.0, 1:10 SLR; #4: pH 9.0, 1:6 SLR; #5-7: pH 8.0, 1:7.5 SLR) and EAEP (#1: 0.25% enzyme, 1:10 SLR; #2: 0.25% enzyme, 1:6 SLR; #3: 0.75% enzyme, 1:10 SLR; #4: 0.75% enzyme, 1:6 SLR; #5-7: 0.50% enzyme, 1:7.5 SLR). AEP and EAEP results were analyzed as separate groups.

EAEP milks were nearly completely hydrolyzed, with most protein bands visible at or below ~22 kDa. This is consistent with previous work by the authors as well as others in our group evaluating the impact of proteolysis on almond flour protein extractability, in that the basic subunit of amandin is susceptible to proteolytic hydrolysis whereas the acidic subunit is more stable¹¹. Interestingly, in lanes 1 and 2, this acidic subunit is not visible, indicating that the higher enzyme level (0.75% w/w) has hydrolyzed this subunit. Few protein bands are visible at all in these lanes, indicating that most hydrolysates were too small to be capture in the gel.

3.3.5. Zeta-potential

Zeta-potential (ξ -potential) is a measure of the net electrical potential of the surface of proteins in solution and can impact their stability. Zeta-potential values of all milk proteins were highly negative, ranging from -26.5 to -31.9 mV for AEP and -36.7 to -40.1 mV for EAEP (Figure

2d). EAEP milk proteins ξ -potential values were more highly negative than AEP, indicating stronger electrostatic repulsion between hydrolyzed proteins⁴². This stronger repulsion could lead to greater stability in the milk in terms of protein aggregation⁴². Within each extraction method, neither SLR, pH, nor enzyme levels were found to have statistically significant impacts on the measured zeta-potential (Tables 4 & 5).

3.3.6. Immunoreactivity by ELISA sandwich assay

Almond milk immunoreactivity, evaluated based on the detection of amandin, the major allergenic protein in almonds³⁸, was not significantly impacted by pH, SLR, or enzyme levels within the same extraction method (Tables 3.4-3.5). However, a notable difference in the amount of amandin detected in the milk samples was observed between the two extraction methods (Figure 2e). Detected amandin concentration in AEP milks ranged from $1.8\text{--}3.4 \times 10^5$ ppm, while in EAEP milks it ranged from $1.0 \times 10^3\text{--}2.4 \times 10^4$ ppm, a 93% reduction in milk immunoreactivity potential despite the higher concentration of proteins in EAEP milks. Typical concentration of amandin in commercial almond milk has been measured as approximately $3.3\text{--}4.8 \times 10^4$ ppm using this kit⁴³, and the results obtained in this work are consistent with these values, considering the increased amount of almond paste used and total protein within the milks. A more comprehensive analysis can be made by comparing detected mg of amandin per kg of protein in the milk. By this metric, AEP milks contained $7.35 \times 10^6\text{--}1.18 \times 10^7$ mg amandin/kg protein, whereas EAEP milks contained $2.34 \times 10^4\text{--}8.07 \times 10^5$ mg amandin/kg protein. This reduction is indicative of the protease's potential ability to reduce the immunoreactivity of the protein, though the presence of intact immunoreactive epitopes could still pose a threat to those with almond allergies and should be more extensively evaluated.

The enzyme used in this study is derived from *Bacillus licheniformis*, and is primarily composed of subtilisin, a serine endoprotease. As the protease hydrolyzes the protein, the protein is cleaved into smaller peptides that may not have the same immunoreactivity as native proteins⁴⁴. The significant decrease observed in detected amandin immunoreactivity associated with the use of protease in extraction agrees with previous work from our group¹⁶, in which immunoreactivity of protein extracted from almond flour was reduced by 75% through the use of enzymatic extraction over AEP with a neutral subtilisin endoprotease. In their work, EAEP sample peptides (< 5kDa) were found to not contain any intact IgE-reactive epitopes in their complete sequences¹⁶. This protein cleavage and molecular weight reduction is confirmed in the results of the SDS-PAGE analysis above, in that the 38 kDa band, corresponding to the acidic subunit of amandin⁴¹ (Fig. 33.) was completely hydrolyzed. Some epitopes, however, can be as small as <5 kDa, which likely persist despite hydrolysis as demonstrated by SDS-PAGE in Chapter 2¹⁶. Hydrolysis with this type of enzyme has been demonstrated to be effective at significantly reducing immunoreactivity in protein sources such as peanuts^{45,46}, soybeans⁴⁷, and chickpeas⁴⁸. This phenomenon has been observed in human sera via Western blotting as well^{16,49}. Additional research is needed to further investigate the feasibility of using enzyme hydrolysis to reduce the allergenicity of almond proteins for those with allergies.

3.3.7. Total antioxidant activity

Overall, the use of enzyme to assist the extraction was associated with an increase in the total antioxidant capacity of EAEP almond milks, as measured by the ABTS radical scavenging method (Figure 3.2f). EAEP milks demonstrated $3.4\text{--}4.7 \times 10^5$ μmol Trolox equivalent/mL of milk whereas AEP milks ranged from $4.9\text{--}9.1 \times 10^4$ μmol Trolox equivalent (TE)/mL. Previous work from our group has suggested that enzymatic hydrolysis can lead to higher extraction of phenolic

compounds as well as cleave the native protein into peptides that may exhibit antioxidant activity, suggesting the use of alkaline protease as an effective tool for almond products¹². The reducing power of proteins and their hydrolysates are typically concentration-dependent^{40,50,51}. A higher SLR was associated with a significant increase in total antioxidant capacity of AEP and EAEP milks ($p < 0.05$) (Table 3.4), which is intuitive as higher SLR increased protein content of the milks (Section 3.3.1). pH and enzyme concentration, however, did not demonstrate this relationship and were not impactful within the range evaluated. However, the interaction between enzyme concentration and SLR was found to be significant for EAEP milks ($p < 0.05$), likely due to the increased total protein available for hydrolysis and extraction in higher SLR and enzyme extractions.

3.3.8. Descriptive analyses

Descriptive analyses of the milks revealed that AEP almond milks differed significantly from EAEP milks with respect to their sensory characteristics (Fig. 3.4). The EAEP milks were perceived as bitter, with a darker brown color and more pronounced roasted flavor, with AEP milks perceived as sweeter and creamier than AEP. AEP 1, AEP 2, AEP 3, and AEP 4 milks were perceived as significantly different from each other in terms of sensory characteristics such as taste and flavor. In contrast, AEP 5, AEP 6, and AEP 7 milks, which were the central points with identical extraction conditions, were not significantly different from one other, showing good repeatability of the process. On the other hand, there was less separation amongst the milks produced using the enzyme assisted extraction process. EAEP 2 and EAEP 4 milks were not significantly different from each other, but both were significantly different from the EAEP 3 milk. For the AEP milks, higher SLRs resulted in increased attributes of sweetness and creaminess, likely due to the increase in fat and protein and thus viscosity in these formulas. For the EAEP

milks, higher SLRs increased the roasted flavor and brown color, which could be attributed to increased total extracted protein in solution, and overall higher degree of hydrolysis (Fig. 3.2c). Protein hydrolysis has been associated with the release of phenolic compounds¹², which can contribute to browning via Maillard reaction⁵². This increase in browning can lead to an increase of flavors associated with roasted nuts⁵³.

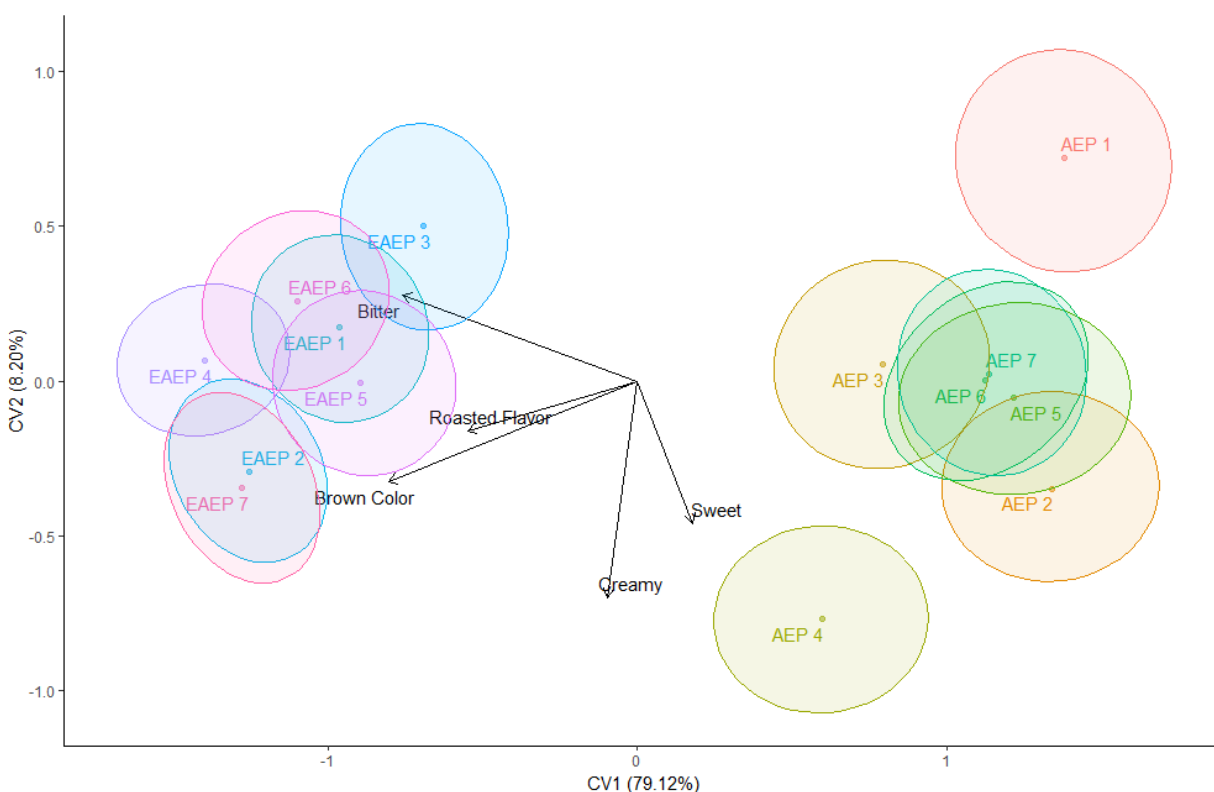


Figure 3.4. CVA score plot of formulated almond milks with top 5 contributing attributes. Experimental conditions for AEP (# 1: pH 7.0, 1:10 SLR; #2: pH 7.0, 1:6 SLR; #3: pH 9.0, 1:10 SLR; #4: pH 9.0, 1:6 SLR; #5-7: pH 8.0, 1:7.5 SLR) and EAEP (#1: 0.25% enzyme, 1:10 SLR; #2: 0.25% enzyme, 1:6 SLR; #3: 0.75% enzyme, 1:10 SLR; #4: 0.75% enzyme, 1:6 SLR; #5-7: 0.50% enzyme, 1:7.5 SLR). AEP and EAEP results were analyzed as separate groups.

3.3.9. Leveraging hedonic analyses to guide AEP and EAEP milk formulations

Assessment of consumer preferences and liking for AEP and EAEP milk formulations revealed that AEP2 and AEP4 milks had significantly higher liking scores (5.89 and 6.10,

respectively) than AEP 1 and AEP 3 milks (5.20 and 5.07, respectively), as well as the two EAEP milks (4.61 for EAEP 3 and 4.21 in EAEP 4), indicating the consumers preferred AEP milks produced with the use of a high SLR (Fig. 3.5a). The milks produced with a higher SLR had higher protein and lipid content (Table 3.3), as well as higher viscosity, leading to richer, more flavorful milks that consumers preferred to the milks with lower SLR.

Based on the CATA results, AEP 2 and AEP 4 milks (1:6 SLR) were sweeter and creamier than AEP1 and AEP 3 milks (1:10 SLR), and EAEP 3 and EAEP 4 milks (0.75% enzyme) were more bitter than AEP milks (Fig. 3.5b). EAEP 3 and EAEP 4 milks were produced by using the highest levels of protease, a known factor that can lead to the formation of bitter peptides^{17,54,55}. As the degree of hydrolysis of EAEP milks was higher than all AEP milks (Fig. 3.2c), one might expect that the former could be perceived as more bitter than the latter.

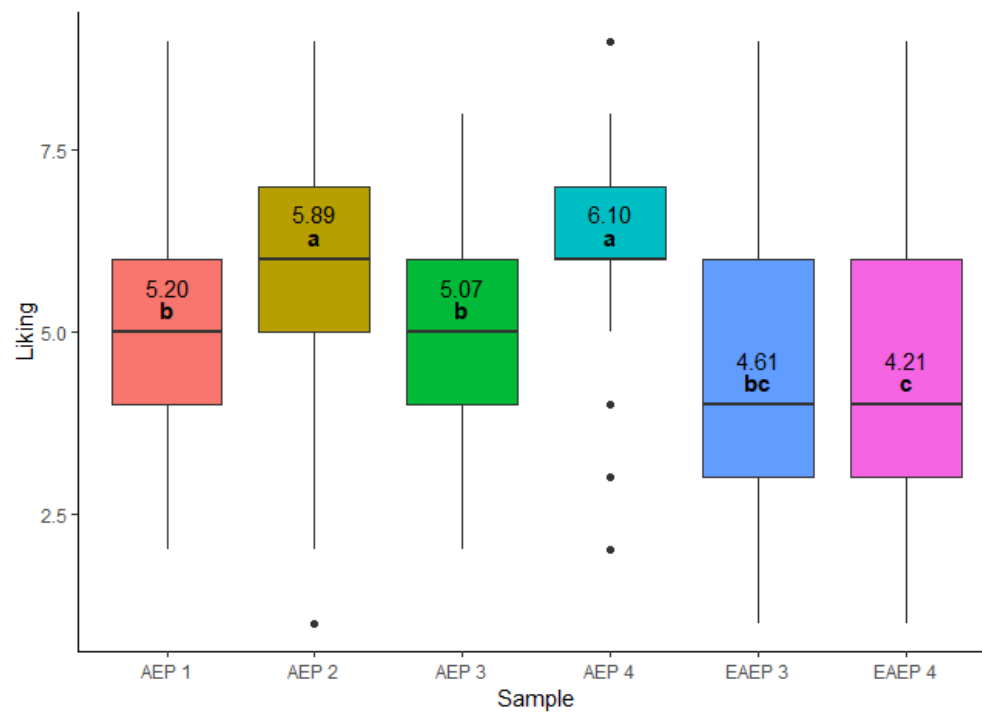
Based on the external preference mapping analysis, most consumers preferred samples with high sweetness and creaminess, while few consumers preferred the EAEP milks which were characterized by high bitterness and brown color (Fig. 3.5c). In the initial hedonic sensory trials, AEP almond milks produced with high SLR of 1:6 were preferred the most, with minor significant differences observed between milks produced at pH 7 and pH 9. For the EAEP milks, no significant differences in the liking scores were observed for milks produced when using enzyme concentrations of 0.25% and 0.75%, though both milks were scored lower than AEP milks.

To identify the optimum extraction conditions that would lead to almond milks well acceptable by consumers and suitable for scale-up, a second round of hedonic testing was performed with reformulated milks (Fig. 3.6a-b). This round included AEP milks produced at higher SLR (1:6 SLR), this time at pH 7 and 9 as well as a midpoint, pH 8. Though panelists reported slightly higher liking of pH 9 AEP milk, the difference between pH 8 and pH 9 was not

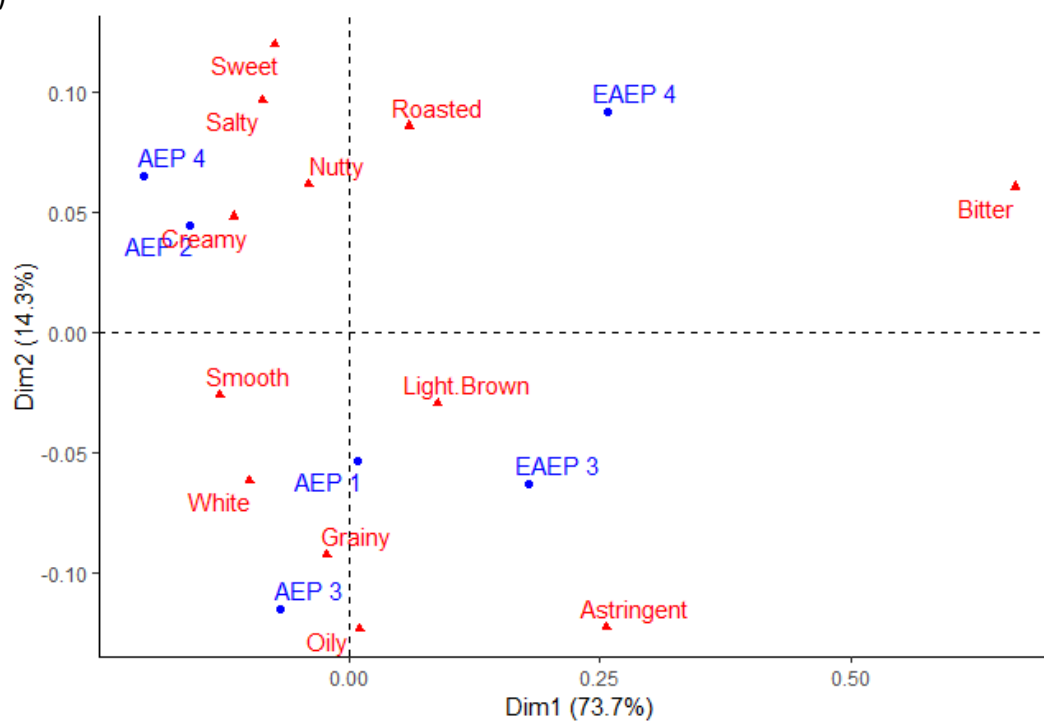
significant (Fig. 3.6a). Notably, over prolonged storage periods, the AEP milk produced pH 9 AEP began to exhibit a greenish color, which could likely be attributed to the chlorogenic acid content in whole almonds, which is typically around 1 mg/100 g almond⁵⁶. At alkaline conditions, such as pH 9, this color shift becomes particularly noticeable. Under alkaline conditions, chlorogenic acid can bind with proteins, leading to the formation of a green pigment⁵⁷. pH 8 and pH 7 AEP milks did not exhibit this color shift and thus pH 8 was selected as the final AEP formula (referred to as AEP 8). Remarkably, none of the EAEP milks, which were also extracted at pH 9, demonstrated a color shift over storage of 25 days. One potential explanation is that the peptides released with enzyme hydrolysis of the almond proteins could be less likely to bind with the chlorogenic acid and form green pigments. More research is necessary to investigate this phenomenon.

With respect to enzyme levels used in the extraction, the EAEP milk with 0.1% protease was preferred the most among the EAEP milks and it was thus selected as the final EAEP formula (EAEP 8). According to the CATA results of the second consumer test, EAEP 8 milk was slightly less bitter and astringent than EAEP 2 (Fig. 3.9). Consumers rated AEP 8 and EAEP 8 milks with scores of 6.03 and 4.91 on a nine-point hedonic scale, respectively, indicating a high degree of liking for both formulas. Considering neither formula was optimized for salt, flavor, etc., this is extremely promising for future development.

a)



b)



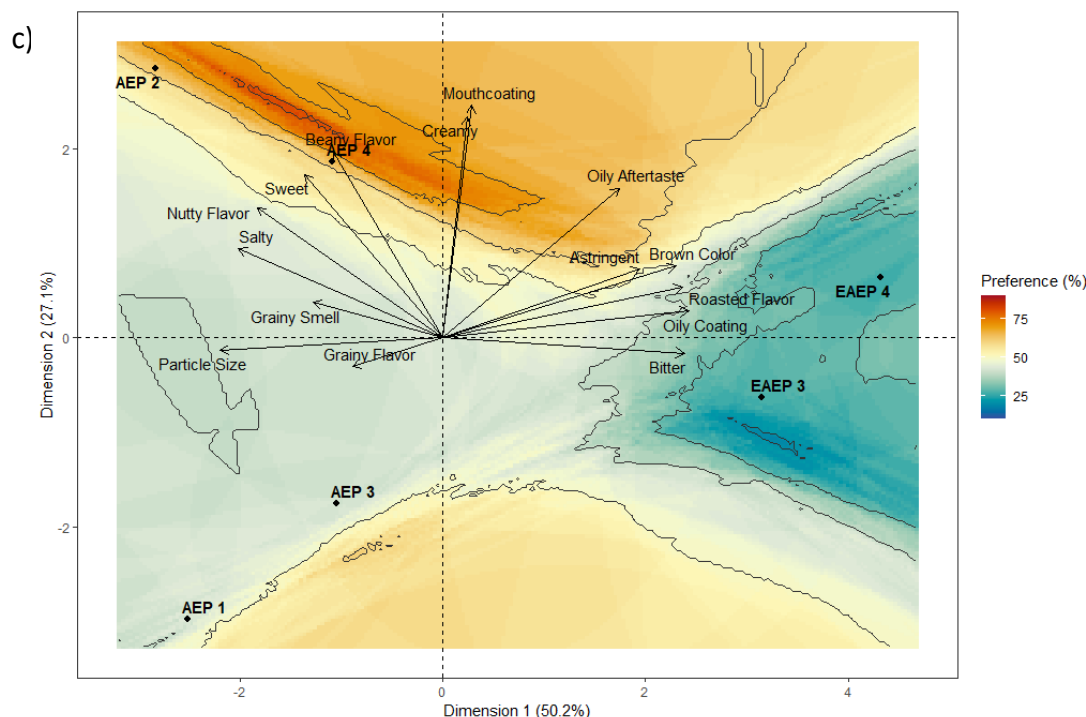


Figure 3.5a-c. Results of first consumer tests. a) Means of liking scores of selected AEP and EAEP milks. Samples with different letters were significantly different from each other as determined by Tukey's HSD. b) Correspondence analysis of CATA attributes for selected AEP and EAEP milks. c.) External preference mapping of liking scores of selected AEP and EAEP milks.

3.3.10. Composition and physicochemical characteristics of optimized AEP and EAEP milks

The nutritional composition of the final AEP 8 and EAEP 8 milks were markedly higher in protein and lipids than most commercial almond milks (Table 6). AEP 8 milk contained 2.71% protein, or 6.8 g protein/serving, while EAEP 8 milk contained 3.25% protein, or 8.1 g/serving. This increase compared to 0.42% protein or 1 g/serving of commercial almond milk is significant, and in the case of the EAEP 8 milk it matches the protein content of dairy milk. Notably, the protein content of the EAEP 8 milk was higher than all other EAEP milks despite using a lower enzyme concentration. This can be attributed to the fact that the reduced hydrolysis of proteins

compared to enzyme levels in the original CCD required less NaOH addition over the extraction period to maintain pH at 9, thus the milk was diluted less than the other EAEP milks. Though the total protein extractability with 0.1% enzyme use was slightly lower (87.3%) than the other EAEP milks (~90%), it was still significantly higher than all AEP formulas (69.5-72.8%). TOE for AEP 8 was similar to that of the other AEP milks (92.15% vs. 91.68-94.66%, respectively). The oil content of the AEP 8 milk was 7.6% (19 g fat/serving) while that of the EAEP 8 milk was 8.7% (22 g fat/serving), a significant increase over commercial almond milk (1% fat, 2.5 g/serving). The oil content of the EAEP 8 milk was higher (8.77%) than other EAEP milks, likely also due to this concentrating effect of less NaOH added during extraction. The degree of hydrolysis of EAEP 8 milk was significantly lower than all other EAEP milks (7.64%), consistent with the expectation that a lower enzyme concentration would reduce hydrolysis overall. This finding indicates that extensive hydrolysis is not necessary to achieve high protein content and total protein extraction in almond milk production. Therefore, processors could utilize lower levels of enzyme, reducing costs associated with this expensive ingredient while still producing a product that consumers will enjoy. The allergenic potential of the EAEP 8 milk, assessed by the amount of amandin present in the milk, was also similarly significantly reduced compared to all AEP milks (1.00×10^5 vs. at least 7.35×10^6 mg amandin/kg protein), indicating that minimal hydrolysis is necessary to hydrolyze the major allergenic protein in almonds, which could lead to an overall reduction in the immunoreactivity of the EAEP milk. Total antioxidant capacity of the EAEP 8 milk was lower compared to other EAEP milks, likely due to its lower enzyme level; however, it was still significantly higher than all AEP milks (2.47×10^5 for EAEP 8 vs. a maximum of 9.06×10^4 mmol TE/mL almond milk skim for AEP). Given that the total antioxidant capacity was still within the same order of magnitude as other EAEP milks, the use of even minimal amounts of protease is

beneficial to the production of peptides with antioxidant activity while maintaining a flavor profile that consumers will prefer to a more hydrolyzed beverage.

Table 3.6. Extraction conditions and proximate composition of final AEP and EAEP milks.

	AEP	EAEP
SLR	1:6	1:6
pH/Enzyme Level	9	0.10%
Protein (%)	2.71 ± 0.05 ^b	3.25 ± 0.06 ^a
TPE (%)	74.34 ± 1.50 ^b	87.29 ± 0.11 ^a
Oil (%)	7.63 ± 0.54 ^b	8.77 ± 0.23 ^a
TOE (%)	92.15 ± 0.39 ^b	97.19 ± 0.12 ^a
Moisture (%)	88.06 ± 0.08 ^a	85.16 ± 0.16 ^b
Ash (%)	0.22 ± 0.01 ^b	0.28 ± 0.01 ^a
Carbohydrate (%)	2.96 ± 0.57	2.55 ± 0.11

Proximate composition, total protein (TPE) and oil extractability (TOE), and carbohydrate content (CHO). Different letters within each column denote a statistically significant difference via one-way ANOVA followed by Tukey's HSD.

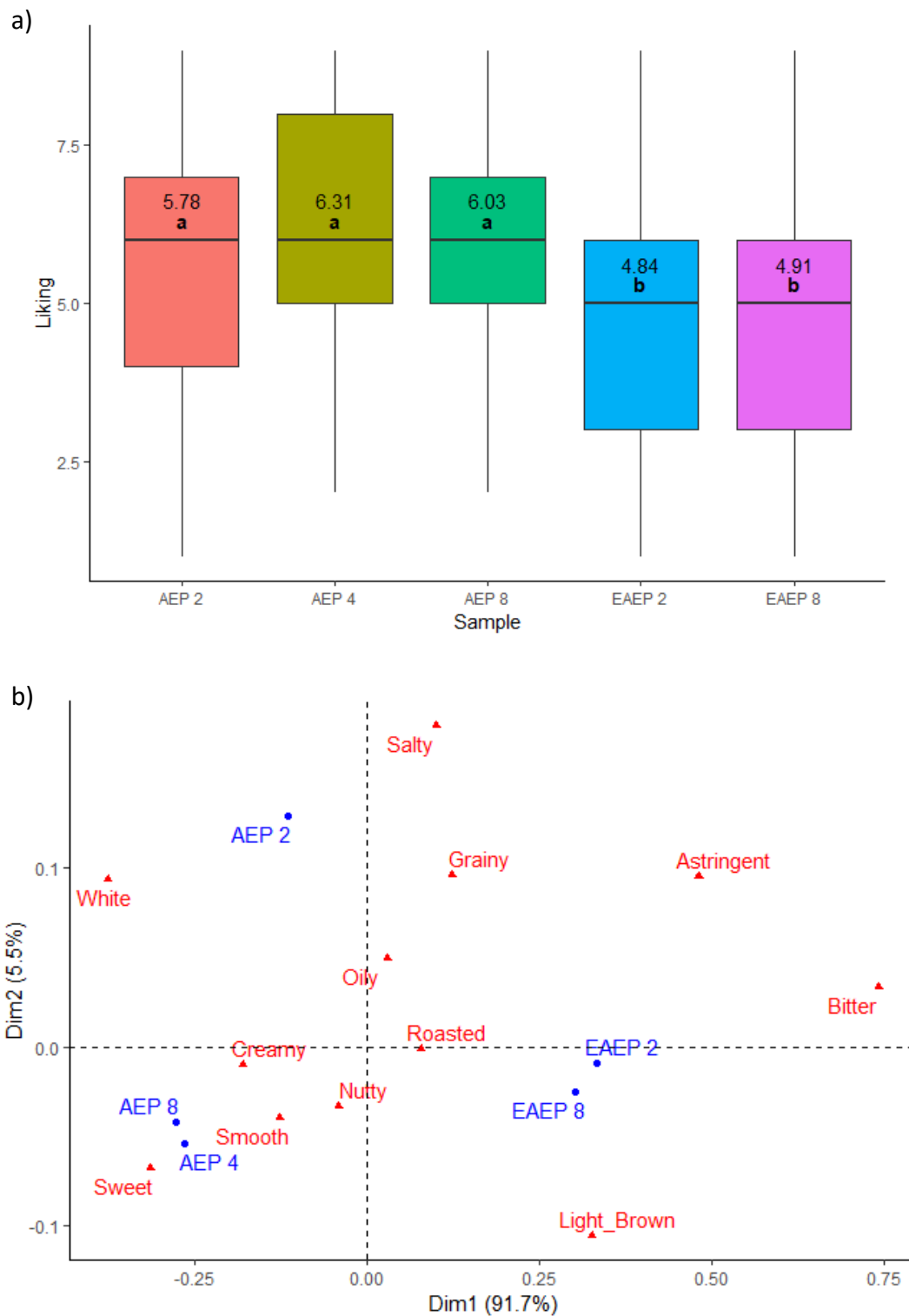


Figure 3.6. a) Means of liking scores of samples from consumer test 2. Samples with different letters were significantly different from each other as determined by Tukey's HSD. b) Correspondence analysis of CATA attributes from consumer test 2.

3.4 Conclusions

This study integrated consumer and descriptive sensory analyses with physicochemical and biological analyses to guide the development of a more nutritious almond milk that received high ratings from consumers. This unique, feedback-driven strategy to optimize a sustainable and effective processing method proved effective in producing two milk formulations of a beverage with great potential for commercial development. Higher SLR resulted in AEP and EAEP milks with higher protein content and increased viscosity, which were rated higher by consumers in sensory testing. Extraction pH had little effect on any property of AEP milks. Enzyme use in the EAEP significantly impacted the composition, physicochemical and sensorial qualities of EAEP milks. EAEP led to milks with higher TPE and total antioxidant capacity and reduced concentration of the allergenic protein amandin. Descriptive testing provided valuable insights to the perceived sweetness, creaminess, and other positive aspects of the almond milks, as well as perceived bitterness in enzyme-extracted milks. These sensory characteristics were closely associated with consumer preferences observed in the subsequent hedonic tests. Hedonic testing guided the reformulation of milks with optimized pH and enzyme levels, leading to the selection of one formula for each of AEP and EAEP that is suitable for scale-up. These reformulated milks were liked by consumers, with mean consumer liking scores of 6.03 and 4.91 on nine-point hedonic scale for AEP 8 and EAEP 8, respectively. Though further work is needed to determine the feasibility of scaling up this process to a pilot or commercial scale, almond milks that were liked by consumers and had improved nutritional properties were successfully produced. Importantly, the findings of this study emphasize the importance of including sensory and physicochemical analyses to guide the selection of processing conditions for development of nutritional and desirable food products.

3.5 Acknowledgements

Funding for publication was made possible by the U. S. Department of Agriculture's (USDA) Agricultural Marketing Service through grant 21-0001-024-SF. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the USDA.

3.6 References

1. Almond Board of California. 2022 *Almond Almanac*. 48
https://www.almonds.com/sites/default/files/2022-12/2022_Almanac.pdf (2022).
2. University of California, Davis Innovation Institute for Food and Health. *The Evolving Market for Plant-Based Milk: Alfalfa and Other Sources*.
<https://foodaghealth.solutions/plant-based-milk-market-report/> (2022).
3. Rombach, M., Dean, D. L. & Bitsch, V. “Got Milk Alternatives?” Understanding Key Factors Determining U.S. Consumers’ Willingness to Pay for Plant-Based Milk Alternatives. *Foods* **12**, 1277 (2023).
4. Chalupa-Krebsdak, S., Long, C. J. & Bohrer, B. M. Nutrient density and nutritional value of milk and plant-based milk alternatives. *Int. Dairy J.* **87**, 84–92 (2018).
5. Tomishima, H., Luo, K. & Mitchell, A. E. The Almond (*Prunus dulcis*): Chemical Properties, Utilization, and Valorization of Coproducts. *Annu. Rev. Food Sci. Technol.* **13**, 145–166 (2022).
6. McClements, D. J., Newman, E. & McClements, I. F. Plant-based Milks: A Review of the Science Underpinning Their Design, Fabrication, and Performance. *Compr. Rev. Food Sci. Food Saf.* **18**, 2047–2067 (2019).
7. Campbell, K. A. & Glatz, C. E. Mechanisms of aqueous extraction of soybean oil. *J. Agric. Food Chem.* **57**, 10904–10912 (2009).
8. Rosenthal, A., Pyle, D. L. & Niranjana, K. Aqueous and enzymatic processes for edible oil extraction. *Enzyme Microb. Technol.* **19**, 402–420 (1996).
9. de Almeida, N. M., Dias, F. F. G., Rodrigues, M. I. & Bell, J. M. L. N. de M. Effects of processing conditions on the simultaneous extraction and distribution of oil and protein from almond flour. *Processes* **7**, (2019).
10. De Moura, J. M. L. N., De Almeida, N. M., Jung, S. & Johnson, L. A. Flaking as a pretreatment for enzyme-assisted aqueous extraction processing of soybeans. *JAOCs J. Am. Oil Chem. Soc.* **87**, 1507–1515 (2010).
11. Dias, F. F. G. & de Moura Bell, J. M. L. N. Understanding the impact of enzyme-assisted aqueous extraction on the structural, physicochemical, and functional properties of protein extracts from full-fat almond flour. *Food Hydrocoll.* **127**, 107534 (2022).
12. de Souza, T. S. P., Dias, F. F. G., Oliveira, J. P. S., de Moura Bell, J. M. L. N. & Koblitiz, M. G. B. Biological properties of almond proteins produced by aqueous and enzyme-assisted aqueous extraction processes from almond cake. *Sci. Rep.* **10**, 1–12 (2020).
13. García Arteaga, V., Apéstegui Guardia, M., Muranyi, I., Eisner, P. & Schweiggert-Weisz, U. Effect of enzymatic hydrolysis on molecular weight distribution, techno-functional properties and sensory perception of pea protein isolates. *Innov. Food Sci. Emerg. Technol.* **65**, 102449 (2020).

14. Wu, L. *et al.* Effect of extraction pH on functional properties, structural properties, and in vitro gastrointestinal digestion of tartary buckwheat protein isolates. *J. Cereal Sci.* **101**, 103314 (2021).
15. Kheto, A., Sehrawat, R., Gul, K. & Kumar, L. Effect of extraction pH on amino acids, nutritional, in-vitro protein digestibility, intermolecular interactions, and functional properties of guar germ proteins. *Food Chem.* **444**, 138628 (2024).
16. Furlan Goncalves Dias, F. *et al.* Effects of protease-assisted aqueous extraction on almond protein profile, digestibility, and antigenicity. *Curr. Res. Food Sci.* **6**, 100488 (2023).
17. Liu, B. *et al.* Review on the release mechanism and debittering technology of bitter peptides from protein hydrolysates. *Compr. Rev. Food Sci. Food Saf.* **21**, 5153–5170 (2022).
18. Gebauer, S. K., Novotny, J. A., Bornhorst, G. M. & Baer, D. J. Food processing and structure impact the metabolizable energy of almonds. *Food Funct.* **7**, 4231–4238 (2016).
19. American Oil Chemists' Society. *Official Methods and Recommended Practices of the AOCS.* (2017).
20. Min, D. B. & Ellefson, W. C. Fat Analysis. in *Food Analysis* (ed. Nielsen, S. S.) 602 (New York, 2010).
21. Gallier, S. & Singh, H. Behavior of almond oil bodies during in vitro gastric and intestinal digestion. *Food Funct.* **3**, 547–555 (2012).
22. Ong, J. J.-X., Steele, C. M. & Duizer, L. M. Challenges to assumptions regarding oral shear rate during oral processing and swallowing based on sensory testing with thickened liquids. *Food Hydrocoll.* **84**, 173–180 (2018).
23. Laemmli, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **227**, 680–685 (1970).
24. Petersen, D., Nielsen, P. M. & Dambmann, C. Determination of the degree of hydrolysis (DH) based on OPA reaction. *Novo Nord. AS* 1–7 (1995).
25. Sathe, S. K. Solubilization, electrophoretic characterization and in vitro digestibility of almond (*Prunus amygdalus*) proteins. *J. Food Biochem.* **16**, 249–264 (1993).
26. Amirshaghghi, Z., Rezaei, K. & Habibi Rezaei, M. Characterization and functional properties of protein isolates from wild almond. *J. Food Meas. Charact.* **11**, 1725–1733 (2017).
27. Sari, Y. W., Bruins, M. E. & Sanders, J. P. M. Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. *Ind. Crops Prod.* **43**, 78–83 (2013).
28. Yang, J. S., Dias, F. F. G., Pham, T. T. K., Barile, D. & De Moura Bell, J. M. L. N. A sequential fractionation approach to understanding the physicochemical and functional properties of aqueous and enzyme-assisted aqueous extracted black bean proteins. *Food Hydrocoll.* **146**, 109250 (2024).
29. Gao, Y., Ding, Z., Liu, Y. & Xu, Y.-J. Aqueous enzymatic extraction: A green, environmentally friendly and sustainable oil extraction technology. *Trends Food Sci. Technol.* **144**, 104315 (2024).
30. Souza, T. S. P., Dias, F. F. G., Koblitz, M. G. B. & M. L. N. de M. Bell, J. Aqueous and Enzymatic Extraction of Oil and Protein from Almond Cake: A Comparative Study. *Processes* **7**, 472 (2019).
31. Rosenthal, A., Pyle, D. L., Niranjana, K., Gilmour, S. & Trinca, L. Combined effect of operational variables and enzyme activity on aqueous enzymatic extraction of oil and protein from soybean. *Enzyme* **28**, 499–509 (2001).

32. Tzen, J. T. C., Cao, Y. Z., Laurent, P., Ratnayake, C. & Huang, A. H. C. Lipids, proteins, and structure of seed oil bodies from diverse species. *Plant Physiol.* **101**, 267–276 (1993).
33. Huang, A. H. C. Oil bodies and oleosins in seeds. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 177–200 (1992).
34. McClements, D. J. *Food Emulsions: Principles, Practices, and Techniques, Third Edition*. (CRC Press, Boca Raton, 2015). doi:10.1201/b18868.
35. Jung, S., Murphy, P. A. & Johnson, L. A. Physicochemical and Functional Properties of Soy Protein Substrates Modified by Low Levels of Protease Hydrolysis. *J. Food Sci.* **70**, C180–C187 (2005).
36. Vogelsang-O'Dwyer, M., Sahin, A. W., Arendt, E. K. & Zannini, E. Enzymatic Hydrolysis of Pulse Proteins as a Tool to Improve Techno-Functional Properties. *Foods* **11**, 1307 (2022).
37. Nielsen, P. M., Petersen, D. & Dambmann, C. Improved Method for Determining Food Protein Degree of Hydrolysis. *J. Food Sci.* **66**, 642–646 (2001).
38. Zhu, Z. *et al.* Food protein aggregation and its application. *Food Res. Int.* **160**, 111725 (2022).
39. Mulcahy, E. M., Fargier-Lagrange, M., Mulvihill, D. M. & O'Mahony, J. A. Characterisation of heat-induced protein aggregation in whey protein isolate and the influence of aggregation on the availability of amino groups as measured by the ortho-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods. *Food Chem.* **229**, 66–74 (2017).
40. Islam, M. *et al.* Influence of the Degree of Hydrolysis on Functional Properties and Antioxidant Activity of Enzymatic Soybean Protein Hydrolysates. *Molecules* **27**, 6110 (2022).
41. Albillos, S. M., Menhart, N. & Fu, T. J. Structural stability of amandin, a major allergen from almond (*prunus dulcis*), and its acidic and basic polypeptides. *J. Agric. Food Chem.* **57**, 4698–4705 (2009).
42. Cano-Sarmiento, C. *et al.* Zeta Potential of Food Matrices. *Food Eng. Rev.* **10**, 113–138 (2018).
43. Slotwinski, E. *et al.* Development and Validation of a Quantitative ELISA for the Detection of Almond Residues in Foods. *J. AOAC Int.* **101**, 118–123 (2018).
44. Cabanillas, B. *et al.* Influence of Enzymatic Hydrolysis on the Allergenicity of Roasted Peanut Protein Extract. *Int. Arch. Allergy Immunol.* **157**, 41–50 (2012).
45. Mikiashvili, N. & Yu, J. Changes in immunoreactivity of allergen-reduced peanuts due to post-enzyme treatment roasting. *Food Chem.* **256**, 188–194 (2018).
46. Yu, J. & Mikiashvili, N. Effectiveness of different proteases in reducing allergen content and IgE-binding of raw peanuts. *Food Chem.* **307**, 125565 (2020).
47. Yin, H., Zhang, X. & Huang, J. Study on enzymatic hydrolysis of soybean β -conglycinin using alkaline protease from *Bacillus subtilis* ACCC 01746 and antigenicity of its hydrolysates. *Grain Oil Sci. Technol.* **4**, 18–25 (2021).
48. Clemente, A., Vioque, J., Sanchez-Vioque, R., Pedroche, J. & Millán, F. Production of Extensive Chickpea (*Cicer arietinum* L.) Protein Hydrolysates with Reduced Antigenic Activity. *J. Agric. Food Chem.* **47**, 3776–3781 (1999).
49. Calcinai, L. *et al.* Effectiveness of enzymatic hydrolysis for reducing the allergenic potential of legume by-products. *Sci. Rep.* **12**, 16902 (2022).

50. Li, X. *et al.* Antioxidant activities and functional properties of enzymatic protein hydrolysates from defatted *Camellia oleifera* seed cake. *J. Food Sci. Technol.* **52**, 5681–5690 (2015).
51. Elias, R. J., Kellerby, S. S. & Decker, E. A. Antioxidant Activity of Proteins and Peptides. *Crit. Rev. Food Sci. Nutr.* **48**, 430–441 (2008).
52. Srichamnong, W. & Srzednicki, G. Internal discoloration of various varieties of Macadamia nuts as influenced by enzymatic browning and Maillard reaction. *Sci. Hortic.* **192**, 180–186 (2015).
53. Perren, R. & Escher, F. E. Impact of roasting on nut quality. in *Improving the Safety and Quality of Nuts* 173–197 (Elsevier, 2013). doi:10.1533/9780857097484.2.173.
54. Ryland, D., Goldberg, E., Aliani, M. & Methods, S. Sensory Evaluation Techniques for Detecting and Quantifying Bitterness in Food and Beverages. in *Bitterness: Perception, Chemistry and Food Processing* (eds. Aliani, M. & Eskin, M. N. A.) 133–160 (John Wiley & Sons Ltd. and The Institute of Food Technologists, 2017).
55. Seo, W. H., Lee, H. G. & Baek, H. H. Evaluation of bitterness in enzymatic hydrolysates of soy protein isolate by taste dilution analysis. *J. Food Sci.* **73**, (2008).
56. Bolling, B. W. Almond Polyphenols: Methods of Analysis, Contribution to Food Quality, and Health Promotion: Almond polyphenols.... *Compr. Rev. Food Sci. Food Saf.* **16**, 346–368 (2017).
57. Wildermuth, S. R., Young, E. E. & Were, L. M. Chlorogenic Acid Oxidation and Its Reaction with Sunflower Proteins to Form Green-Colored Complexes. *Compr. Rev. Food Sci. Food Saf.* **15**, 829–843 (2016).

Chapter 4: Scaling aqueous and enzyme-assisted extraction for enhanced almond milk production

Abstract

Despite its popularity with consumers, almond milk is nutritionally inferior to bovine milk. To address this limitation, protein- and lipid-rich almond milks were previously produced using aqueous (AEP) and enzyme-assisted extraction processes (EAEP) optimized for enhanced nutritional composition, physicochemical and sensory properties. AEP and EAEP processes were scaled up to 30 L batches and milks were pasteurized and homogenized to mimic retail almond milk processing conditions, in parallel with a conventional “commercial” formula, and evaluated for quality (emulsion and oxidative stability) over 16 weeks of refrigerated storage. Protein extractability was 74.8% for AEP and 89.0% for EAEP, while oil extractability reached 89.4% and 96.4%, respectively. The resulting AEP and EAEP milks contained 2.64-2.66% protein and 6.65-7.82% oil, compared to 0.5% protein and 1.75% oil for the commercial formula. Enzymatic extraction significantly reduced the immunoreactivity of the EAEP milk, as measured by ELISA, and increased its antioxidant activity. Although lipid oxidation remained low throughout the shelf-life for all three formulas, as indicated by peroxide and TBARs values; oxylipin levels were measured to detect minute variations within milk samples and over the course of storage. Despite initial emulsion creaming, all milks were stable in terms of oil droplet size, emulsion creaming index, viscosity, and color throughout their shelf-life. *In vitro* digestibility of both AEP and EAEP milks was high (92-93%) compared to the commercial formula (78.2%). This proof-of-concept study demonstrates the feasibility of utilizing these sustainable methods to make nutritionally and qualitatively improved almond milk at scale.

Key Words: Almond milk, protein, aqueous extraction, enzyme-assisted extraction, UHT pasteurization, shelf-life, immunoreactivity, lipid oxidation

4.1 Introduction

Consumers are increasingly turning to non-dairy milk alternatives for a variety of reasons, whether due to health concerns such as lactose intolerance, dietary practices such as veganism, or environmental reasons^{1,2}. The leading variety of non-dairy milks is almond milk, with a current estimated global market value of \$5.49B (2024), and a projected market value of \$9.61B in 2029³. Despite its popularity, almond milk is nutritionally inferior to bovine milk in terms of protein and lipid content⁴. Most currently available retail almond milks in the United States contain less than 0.5% protein and 1% lipids, compared with dairy milk's 3% and 3.25%, respectively⁵. Due to this deficiency of macronutrients compared to dairy milk, almond milk is considered unsuitable as a substitute to cow's milk, especially for vulnerable populations such as children or the elderly^{6,7}.

Previous work by our group has demonstrated the feasibility of extracting nutrients from almond materials using environmentally-friendly extraction methods such as aqueous (AEP) and enzyme-assisted extraction processing (EAEP)⁸⁻¹⁰. By adding enzymes to the extraction process – typically a protease – yields can be greatly improved through increased solubility and diffusion of water soluble compounds in the extraction medium, along with the “washing out” of water insoluble compounds as lipids, often with significant modifications to the properties and functionality of the extracted proteins⁸. In the case of almonds, this aqueous extract, often split into “cream” (oil-rich) and “skim” (protein-rich) fractions in AEP and EAEP, offers a promising opportunity for making these processes a selected choice to produce almond milks with desired nutritional and functional properties. Previous work (Chapters 2-3) demonstrated the effectiveness of such processes for producing nutritionally improved almond milks (AEP milk containing 2.71% protein and 7.6% oil, and EAEP containing 3.25% protein and 8.77% oil) that consumers rated highly (4.5-6 on a nine-point scale) in hedonic testing.

To confirm the feasibility of almond milk production using AEP and EAEP on an industrial scale, a pilot scale proof-of-concept study was necessary to evaluate the impact of industrial unit operations such as continuous pasteurization/sterilization and homogenization. Conventional almond milk is produced by blending finely ground roasted almond butter in water along with emulsifiers such as lecithin to keep lipids suspended, and hydrocolloid thickeners to provide mouthfeel¹. They are then processed in a similar manner to dairy milk products in terms of pasteurization and homogenization¹. The most common form of almond milk on the retail market is “extended shelf-life” (ESL) milks, which are pasteurized at or near ultra-high temperature (UHT) conditions but not packaged in an aseptic manner, necessitating refrigerated storage to prevent spoilage^{11,12}. ESL products have a typical commercial life span of 90 days. The combination of high temperature (120-145 °C) and short time (2-4 s) provides an effective bactericidal step while preserving flavor¹³. This is accomplished either directly (through steam injection/infusion) or indirectly through a plate or tubular heat exchanger¹¹. Previous work in our group has been performed at the bench scale (0.5 L extractions), blended with countertop appliances, and batch pasteurized (63 °C for 30 min). This is significantly different from the conventional production methods, and the combination of large-scale extraction with pilot-scale thermal treatments and homogenization could yield a final product with different properties and present unforeseen difficulties such as a different product texture or emulsion. Importantly, conventional almond milk is significantly lower in protein and fats than the AEP and EAEP milks, and thus it likely has very different properties in terms of potential shelf-life (oil oxidation, emulsion stability, etc.) and nutritional, biological, and functional properties (composition, physicochemical properties, protein immunoreactivity, antioxidant activity, and digestibility).

With this in mind, the objectives of the present study were: (1) to scale-up the extraction of AEP and EAEP milks previously optimized for upstream processing from 0.5 to 30 L; (2) to pasteurize and homogenize the milks alongside a “commercial” formula prepared in the conventional suspended-solids manner, and (3) compare these three products in terms of extractability, nutrient content, biological properties such as immunoreactivity, antioxidant capacity, *in vitro* protein digestibility, and quality parameters such as oil oxidation, emulsion stability, and viscosity. These analyses will assess the stability of AEP and EAEP milks compared to the current method, demonstrating their feasibility for future adoption by the almond milk processing industry.

4.2 Materials and Methods

4.2.1 Materials

Blanched light roasted almond paste was purchased from Harris-Woolf Almonds (Ballico, CA). The almond paste was characterized for proximate composition as described in section 2.1 of Chapter 2 (Materials) and contained $51.45 \pm 0.4\%$ oil, $21.5 \pm 0.1\%$ protein, $2.89 \pm 0.0\%$ ash, $1.98 \pm 0.0\%$ moisture, and $22.13 \pm 0.3\%$ carbohydrates. Moisture and lipid content were quantified by AOAC method 925.09 (oven drying) and 989.05 (Mojonnier method)¹⁴. Protein content was determined using Dumas combustion (Vario Max Cube, Elementar Americas, Ronkonkoma, NY, USA) and a nitrogen conversion factor of 5.18. Ash was determined using AOAC method 920.125 and carbohydrates via difference.

FoodPro® Alkaline Protease from *Bacillus licheniformis* was generously supplied by Danisco (Palo Alto, CA, USA). Gum Arabic was purchased from Modernist Pantry (Eliot, ME, USA) and sunflower lecithin was purchased from NOW Foods (Bloomington, IL, USA). 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), gallic acid, 2,2'-Azobis (2-

methylpropionamidine) dihydrochloride (AAPH), Trolox, thiobarbituric acid (TBA), propyl 3,4,5-trihydroxy-benzoate (PG), butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid disodium (2 Na-EDTA), triphenylphosphene (TPP) and glacial acetic acid (AcOH) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). NaOH and ethylenediaminetetraacetic acid disodium salt (EDTA) were purchased from Spectrum Chemical (New Brunswick, NJ, USA) and chloroform and KCl were purchased from Fisher Scientific (Hampton, NH, USA). Oxylypin surrogate solutions were purchased from Cayman Chemical (Ann Arbor, MI, USA).

4.2.2 Almond milk extractions and commercial milk formulation

AEP and EAEP milks were performed as follows: 2.5 kg of almond paste were added to 15 L of deionized water at 50 °C to achieve a 1:6 solids-to-liquid ratio (SLR) in a 20 L jacketed glass reactor. For the AEP, the slurry pH was maintained at 8.0 with addition of 2 M NaOH and kept under constant stirring at 120 RPM for 1 hour. For the EAEP, 0.1% (weight of enzyme/weight of paste) FoodPro Alkaline Protease was added to the slurry prior to extraction and the pH was maintained at 9.0 under constant stirring at 120 RPM for 1 hour. After the extraction, the slurry was then centrifuged at 3280 x g for 30 min at 4 °C to separate the insoluble fraction (i.e., solids) from the milk (liquid fraction). The insoluble fraction was weighed and retained for proximate analysis. A “commercial” milk sample (C), meant to mimic retail almond milk, was prepared by mixing 96.5% water, 2% almond paste, 1.0% sunflower lecithin, and 0.5% gum Arabic in a sanitary tank while blending with a handheld industrial immersion blender for 2 min at high speed. The same ratios of sunflower lecithin and gum Arabic were similarly added with high shear to the AEP and EAEP milks prior to pasteurization and homogenization. Six extraction replicates were performed for both AEP and EAEP milks, as well as for the commercial milk sample. Two 15 L

extraction replicates were combined to create three 30 L batches, which were then refrigerated overnight prior to pasteurization and homogenization.

4.2.3 Pasteurization and homogenization

Ultra-High Temperature (UHT) pasteurization of the milks was performed using a MicroThermics 25 pilot tube-in-shell indirect heat exchanger (Raleigh, NC, USA). The milk was pumped into the system at a constant flow rate of 2 L/min. The preheat section was set to 115 °C, with the final heating stage set to 138 °C. The holding tube kept the milk at this final temperature for 3 s, after which it was chilled to 20 °C, homogenized at 200 bar, and filled in a sanitary hood into 16 oz PET bottles (Fig. 4.1). The bottles were immediately sealed and stored in opaque containers at 4 °C for 16 weeks.



Fig. 4.1: Pasteurized and homogenized milks. Left to right: Commercial, AEP, and EAEP milks.

4.2.4 Proximate analyses

The proximate composition of the milks and insolubles was determined as described in section

4.2.1.

4.2.5 Total protein and oil extraction

Total protein extraction (TPE) was calculated according to Eq.1:

$$TPE (\%) = \left[1 - \left(\frac{\text{Protein (g) in the insoluble fraction}}{\text{Protein (g) in the almond paste}} \right) \right] \times 100 \quad (2)$$

Similarly, total oil extraction (TOE) was calculated according to Eq. 2.:

$$TOE (\%) = \left[1 - \left(\frac{\text{Oil (g) in the insoluble fraction}}{\text{Oil (g) in the almond paste}} \right) \right] \times 100 \quad (2)$$

4.2.6 Antioxidant capacity of AEP, EAEP, and commercial milks via ABTS and ORAC assays

To determine the antioxidant capacity of the milks, ABTS and ORAC assays were employed. The ABTS assay quantifies the capacity of the antioxidant to transfer electrons, while the ORAC assay measures the capacity of the antioxidant to transfer hydrogen atoms¹⁵. The ABTS assay was performed as described by Ngoh and Gan¹⁶ with some modifications. Samples were diluted to 1/10 (v/v) in ethanol. A 20 uL aliquot of the diluted sample was then added to 200 uL of diluted (to obtain an absorbance of 0.700 ± 0.005 at 730 nm) ABTS reagent within a microplate well. The plate was shaken at ambient temperature for 6 min at 300 rpm, and then the absorbance read at 730 nm in a microplate reader (SpectraMax iD5 Multi-Mode, Molecular devices, San Jose, CA) at 25 °C. The antioxidant activity was compared to a standard Trolox curve ranging from 0 to 340 μM ($R^2 = 0.992$) and calculated as μmol Trolox equivalent/mL of milk. Each extraction sample (n=3) was analyzed in triplicate, resulting in a total of n = 9 per condition.

The oxygen radical antioxidant capacity (ORAC) assay was performed as described by Zulueta et. al¹⁵ with some modifications. Milk samples were diluted at 1:100 (v/v) in PBS and 50 µL of this dilution was added to a microplate well, followed by 50 µL of fluorescein (78 nM in 75 mM PBS). Following 15 min of shaking at 300 rpm at 37 °C, 25 µL of AAPH solution (221 mM in PBS) was added and the plate shaken for 1 min. The fluorescence was then read once every 5 minutes for 60 min (excitation 485 nm; emission 535 nm). Samples were compared to a calibration curve of Trolox solution ranging from 20-100 µM and the antioxidant activity expressed as µmol of Trolox equivalent (TE) per mL of sample using Eq. 3.

$$ORAC \left(\frac{\mu\text{mol TE}}{\text{mL sample}} \right) = \frac{C_{\text{Trolox}} \times (AUC_{\text{Sample}} - AUC_{\text{Blank}}) \times DF}{AUC_{\text{Trolox}} - AUC_{\text{Blank}}} \quad (3)$$

Where C_{Trolox} is the concentration of the standard (20 µM), DF is the sample dilution factor, and AUC is the area below the fluorescence decay curve. Each extraction sample (n=3) was analyzed in triplicate, resulting in a total of n = 9 per condition.

4.2.7 Allergenicity potential of AEP and EAEP milks by sandwich ELISA

The potential allergenicity of almond proteins was assessed by quantifying the total amount (ppm) of amandin, the major almond protein, using the Veratox sandwich enzyme-linked immunosorbent kit (S-ELISA) (Neogen, Lansing, MA) for almond. This kit works through the addition of the sample to almond antibody-coated wells, followed by the addition of the substrate, conjugate, and stop solution. By comparing the absorbance of the sample to that of a standard curve of amandin protein, an estimate of the amount of total detectable amandin present can be calculated¹⁷. The assay was performed as described by Dias et. al¹⁸, with milk samples prepared according to the manufacturer's guidelines. A standard curve ($r^2 = 0.989$) was prepared using supplied amandin standards to achieve concentrations of 0, 2.25, 5, 10, and 25 ppm in PBS. Milk samples were diluted in the supplied PBS to fall within this curve, then added to rabbit antibody-

coated wells along with the conjugate, substrate, and stop solution. Absorbance was read at 650 nm on a microplate reader, then compared to the standard curve to calculate total almond protein in milks in ppm. Each extraction sample (n=3) was analyzed in triplicate, resulting in a total of n = 9 per condition.

4.2.8 In vitro protein digestibility of AEP and EAEP milks

In vitro milk protein digestibility was quantified using the INFOGEST 2.0 method¹⁹ with some modifications as described by Yang et al.²⁰. INFOGEST protocols were used to determine enzyme activities and prepare the simulated salivary, gastric (2,000 U/mL pepsin), and intestinal (20 TAME U/mL pancreatin, reduced from original INFOGEST method) digestive fluids. These fluids were used to digest 2.5 mL of each milk, followed by the addition of 24 % trichloroacetic acid (TCA) solution (1:1 v/v) into the digested solutions to halt digestion. Milk samples were stored at 4 °C overnight to facilitate protein precipitation, and subsequently centrifuged at 3,280 x g at 4 °C for 30 min. Following decanting of the supernatant, 10 mL of chilled acetone was added to the pellet and incubated at -20 °C for 60 min to wash any residual TCA that might damage the Dumas combustion elemental analyzer. Following a second centrifugation (3,280 x g at 4 °C for 30 min) and decanting of the supernatant, the pellets were left in a fume hood overnight to evaporate remaining acetone. The total nitrogen content of the pellets was then determined using the Dumas method as described in section 4.2.1. A blank consisting of the digestive enzymes and 2.5 mL RO water was used to account for the nitrogen contribution of the digestive enzymes and fluid components. *In vitro* protein digestibility (IVPD) was calculated using Eq. 4:

$$IVPD\% = \frac{N_{milk} - (N_{pellet} - N_{blank})}{N_{milk}} \times 100 \quad (4)$$

in which N_{milk} is the nitrogen content (mg) of the milk prior to digestion, N_{pellet} is the nitrogen content (mg) of the pellet after digestion, and N_{blank} is the nitrogen content (mg) of the pellet of the enzyme blank. Each extraction sample (n=3) was analyzed in duplicate.

4.2.9 Impact of production method, pasteurization, and homogenization on milk shelf-life

To evaluate the impact of production methods (suspended solids or AEP/EAEP), pasteurization, and homogenization on almond milk quality (primary and secondary oxidation markers..., emulsion stability, and viscosity) the milks were analyzed over their shelf-life. For the following analyses, all milks were analyzed at 0, 1, 2, 3, 4, 6, 8, 10, 12, 14, and 16 weeks after processing to mimic the 90-day shelf-life, plus an additional 20%, of commercial ESL almond milks. All milks were stored at 4 °C in sealed bottles within opaque containers.

4.2.9.1 Peroxide value

Lipid oxidation is a critical determinant of food quality and acceptability. Primary oxidation products, which indicate early-stage oxidation²¹, were analyzed by determining the peroxide values of the milks using the ferrous oxidation-xylenol orange (FOX II) method with Pierce Quantitative Peroxide Assay-Lipid compatible kits (Thermo Fisher, Waltnam, MA, USA). Due to the low level of detected peroxides, the quantity of milk used in the assay was doubled from 90 to 180 µL and the hydrogen peroxide standard curve ranged from 1.95 to 1000 µmol/L (prepared in methanol). All measurements were performed in triplicate for each extraction sample (9 total measurements) and are presented as mean ± standard deviation.

4.2.9.2. Secondary oxidation markers via TBARS

Primary oxidation products are inherently unstable and susceptible to degradation into secondary oxidation markers such as aldehydes, ketones, alcohols, among others. Therefore, these

oxidation products serve as indicators of ongoing oxidation²². Secondary oxidation markers were determined via the thiobarbituric acid reactive substances (TBARS) assay as described by Papastergiadis et al. with some modifications²³. A 3 mL almond milk aliquot was frozen in a 15 mL centrifuge tube overnight to break the emulsion, then defrosted the following day for analysis. Upon analysis, 6 mL of a homogenizing solution containing 7.5% TCA, 0.1% PG, and 0.1% EDTA in deionized water was then added to the 3 mL of milk and vortexed for 30 s. Five mL of this mixture were then added to a 10 mL glass test tube and incubated with 5 mL of a 46 mM solution of TBA in deionized water. The tubes were incubated in a boiling water bath for 1 h then immediately chilled in an ice water bath and centrifuged at 3000 x g for 30 min at 4 °C. The resulting supernatant was decanted and its absorbance read at 532 nm in a spectrophotometer (Thermo Scientific, GENESYS 10S UV-Vis, USA). The results were compared to a standard curve of 1,1,3,3-tetra-ethoxypropane (TEP) ($R^2 = 0.995$) in deionized water ranging from 0.2-8.8 µg/g. All measurements were performed in triplicate for each extraction sample (9 total measurements) and are presented as mean ± standard deviation.

4.2.9.3 Oxylipin analysis

Peroxides and secondary oxidation products in foods are often difficult to quantify as most analysis methods lack the sensitivity needed to detect them. To that end, ultra-high pressure liquid chromatography tandem mass (UPLC-MS/MS) has been utilized to detect oxylipins, or the end products of lipid peroxyl and alkyl radicals with fatty acids, indicating early stages of oxidation with greater sensitivity than traditional oxidation markers^{24,25}.

The evolution of early lipid oxidation as measured by conventional oxidation markers such as peroxides and TBARS is challenging due to the dynamic nature of lipid oxidation and rapid conversion of primary oxidation compounds into secondary ones that might not be adequately

measured used by the methods employed, which might not offer the sensitivity needed. To that end, ultra-high pressure liquid chromatography tandem mass (UPLC-MS/MS) has been utilized to detect oxylipins, or the end products of lipid peroxy and alkyl radicals with fatty acids. This method offers greater sensitivity for indicating early stages of oxidation compared with traditional oxidation markers^{24,25}.

4.2.9.3.1 Sample preparation

To determine total oxylipin content in milks at the beginning and end of shelf-life, spectrometry (UPLC-MS/MS) was employed. Milks from beginning and end of shelf-life were stored at -80 °C until being thawed on wet ice for analysis. A 200 µL almond milk aliquot was pipetted into test tubes along with 3 mL of a 2:1 chloroform:methanol mix and 550 µL of a 1 mM 2Na-EDTA and 0.9% (w/v) KCl solution in MilliQ water. This solution was then centrifuged at 1,437 x g at 0 °C for 15 min and the supernatant discarded. The liquid pellet layer was then transferred to a new test tube and dried under nitrogen. This was then reconstituted in 1 mL of 2:1 chloroform:methanol. An aliquot of this reconstituted mixture was measured to contain no more than 1 mg of total lipids, and the solvents evaporated under nitrogen. An aliquot of a 200 µL extraction solution containing 0.1% (w/v) BHT and 0.1% (v/v) AcOH in methanol was then added, followed by 10 µL of a 0.2 mg/mL of an antioxidant solution composed of BHT, EDTA and TPP in 1:1 water:methanol. Ten µL of a surrogate standard mix containing 2 µM of the surrogate solutions described in section 2.1 in methanol. 200 µL of 0.4 M NaOH in 1:1 methanol/water was then added to hydrolyze the solution and the samples vortexed. Samples were heated for 30 min at 60 °C, then cooled to room temperature. Forty µL of acetic acid and 1560 µL of Milli-Q water was added to neutralize the acid and bring final methanol concentration to approximately 15%.

Samples were then loaded onto solid phase extraction (SPE) columns (Oasis HLB 3 cc Vac Cartridge, 60 mg sorbent, 30 µm particle size, Waters Corporation, Milford, CA, USA). The columns were washed with ethyl acetate and methanol, then pre-conditioned with SPE buffer (5% methanol, 0.1% acetic acid in Milli-Q water). Samples were decanted into the columns and rinsed with SPE buffer twice, then dried under vacuum for 20 min. Oxylipins were then eluted with 0.5 mL methanol and 1.5 mL ethyl acetate. Samples were then dried under nitrogen and reconstituted in 100 µL methanol (LC-MS grade). Samples were centrifuged in Ultrafree-MC VV centrifugal filters (0.1 µm; Millipore Sigma, Burlington, MA, USA) for 2 min at 0 °C at 13000 x g. The filtered samples were then transferred to amber LC-MS vials and stored at -80 °C until LC-MS analysis.

4.2.9.3.2 Ultra-high-pressure-liquid chromatography mass-spectrometry analysis

Almond milk oxylipin content was analyzed on an Agilent 1290 Infinity II UHPLC (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 6460 Triple Quadrupole MS (Agilent Technologies). Species were separated on an Agilent ZORBAX Eclipse Plus C18 column (Agilent Technologies) using a binary gradient of two phases: solvent A (0.1% AcOH in Milli-Q water) and solvent B (80:15 acetonitrile:methanol with 0.1% AcOH). The autosampler temperature was set to 4 °C and column temperature set to 45 °C. Solvent B concentration was adjusted throughout the run according to the gradient program, starting at 35%, up to 100%, before returning to 35%. Oxylipins were detected using negative ESI mode under optimized settings for temperatures of drying and sheath gas, nebulizer pressure, and flow rates. All measurements were performed in duplicate for each extraction (n = 6) and are presented as mean ± standard deviation.

4.2.9.4 Emulsion stability over shelf-life: creaming index, oil droplet diameter, and viscosity

To determine impact of production methods (AEP/EAEP vs. commercial formula) and industrial practices such as pasteurization and homogenization on milk stability over its shelf life, emulsion stability was analyzed. Each milk was transferred to 50 mL graduated plastic centrifuge tubes and stored at 4 °C. Emulsion stability was calculated via creaming index (CI) : the height of the serum layer at the bottom of the tube (h_s) was divided by the total height of the emulsion (h_e) and expressed as a percentage as in Eq. (5):

$$CI (\%) = \left(\frac{h_s}{h_e} \right) \times 100 \quad (5)$$

Milks were stored and evaluated in triplicates for each extraction (n=9).

Oil droplet diameter is another indicator of emulsion stability, and thus milk quality. The De Brouckere (D[4,3]) mean diameter of the oil droplets within the milks was measured using a laser diffraction particle size analyzer (Mastersizer 3000E, Malvern Panalytical, Malvern, UK) and calculated using Mie theory, with deionized water as a dispersant and a refractive index set to 1.46²⁶. To analyze the particle size, milks were transferred to 50 mL centrifuge tubes, shaken and vortexed thoroughly, and ~1 mL was added to the beaker of dispersant with stirring until laser obscuration was between 8-13%. Each processing replicate (n=3 for AEP, EAEP, and commercial formula) was subjected to ten measurements in triplicate, totaling 30 measurements for each milk sample.

The viscosity of the milks was also evaluated over time to determine the impact of production methods, pasteurization, and homogenization on this parameter to provide further insights about the emulsion stability and overall product quality. Milk viscosity (Pa s) was recorded using an MCR 702e rheometer with a cup and bob concentric cylinder geometry (Anton Paar, Graz, Austria) at 25 °C using a recirculating glycol bath and a shear stress of 50 s⁻¹, within the

laminar range of the viscosity curve for a hydrocolloid-thickened beverage²⁷. Results were calculated using Rheocompass software.

4.2.10 Statistical analyses

Experimental results were analyzed for statistical significance using JMP® Pro 17.0.0 statistical software (JMP Statistical Discovery LLC, Cary, NC, USA). Difference between means of each extraction method were analyzed with two-way ANOVA, followed by one-way ANOVA with Tukey's post hoc test to discriminate means at the level of confidence of $p < 0.05$, or Student's t-test ($p < 0.05$).

4.3. Results

4.3.1 Proximate composition of AEP, EAEP, and commercial almond milks

Proximate compositions of the pasteurized milks are provided in Table 1. Both AEP and EAEP milks were significantly higher in oil, protein, and carbohydrates than the commercial formula. The commercial formula contained 1.75% oil and 0.54% protein, which is similar to estimates of leading retail almond milks⁴. AEP milk contained over 300% more oil (7.82%) than the commercial formula, while EAEP milk was over 200% higher (6.65%). Both AEP (2.64%) and EAEP (2.66%) milks contained nearly 5 times more protein than the commercial formula, which was expected based on previous research on this product (Chapters 2-3). Previous work (Chapter 3) using finely ground roasted almond paste as starting materials produced AEP milks with 2.71% protein and 7.63% oil, and EAEP milks with 3.25% protein and 8.77% oil. Interestingly, in the present study, EAEP milk protein content was very similar to that of AEP milk (~2.6%), indicating a dilution effect in the EAEP brought on by the addition of NaOH during extraction. Protein hydrolysis can occur even during aqueous extraction, with its rate influenced by the extraction pH²⁸; therefore, some alkali must be added to maintain the slurry pH. In the case

of enzyme-assisted extraction, which often enhances protein extractability, a larger quantity of alkaline addition is typically required to maintain the desired extraction pH. In future experiments, a more concentrated NaOH solution (above 2 M) could be employed to avoid this effect when scaling up to larger extraction volumes. Similarly, ash content was higher in EAEP milks, resulting from the addition of NaOH throughout the extraction process. These data demonstrate the capacity for the AEP and EAEP methods to produce almond milk at scale with improved nutritional quality compared to a commercial “suspended solids” almond milk formula.

Table 4.1. Proximate composition of AEP, EAEP, and Commercial Milks.

Formula	Oil (%)	Protein (%)	Ash (%)	Moisture (%)	Carbohydrates (%)
Commercial	1.75 ± 0.07 ^c	0.54 ± 0.05 ^b	0.10 ± 0.01 ^c	96.65 ± 0.06 ^a	1.91 ± 0.37 ^b
AEP	7.82 ± 0.20 ^a	2.64 ± 0.07 ^a	0.20 ± 0.01 ^b	87.27 ± 0.33 ^b	2.35 ± 0.28 ^a
EAEP	6.65 ± 0.21 ^b	2.66 ± 0.25 ^a	0.25 ± 0.01 ^a	87.82 ± 0.54 ^c	2.31 ± 0.34 ^a

Different superscript letters within columns denote statistically significant difference of means within each column via one-way ANOVA ($p < 0.05$).

4.3.2 Total protein and oil extraction yields

TPE from AEP and EAEP is presented in Fig. 4.2a. Protein extraction was significantly higher in EAEP milks (89.0%) compared to AEP (74.8%). This is consistent with previous work on this product as well as with a variety of EAEP research in myriad matrices such as almond, soy, rapeseed, and other materials using alkaline protease^{29–31}. Proteases generally improve extraction through hydrolysis of proteins, thereby releasing peptides and increasing their solubility in the aqueous medium⁸. These results agree with previous work (Chapter 3) at bench scale (500 mL) with the same extraction conditions, in which TPE was and 87.3% for EAEP and 74.0% for AEP.

TOE for both AEP and EAEP is shown in Fig. 4.2b. Though both achieved high TOE, EAEP showed a statistically significant higher extractability (96.4% vs 89.4% for AEP). These results agree with those of the previous bench scale experiments (97.2% for EAEP and 92.2% for

AEP, Chapter 3). The oil in the almond paste used in this study is readily extractable in that it is extremely finely ground to the degree that nearly no cells remain intact (Chapter 2), and the roasting of the almonds prior to grinding facilitates the release of oil into the extraction medium^{32–35}. In general, the mechanism of oil extraction is functionally different though related to those of protein extraction: protein extraction relies on the solubility of the proteins in the aqueous medium while oil is insoluble and is instead washed out of the plant cellular matrix by the dissolution of soluble components into the water⁸. By increasing the solubility of the proteins in the extraction, the motility of the oil out of the almond matrix and into the “milk” is thus simultaneously improved.

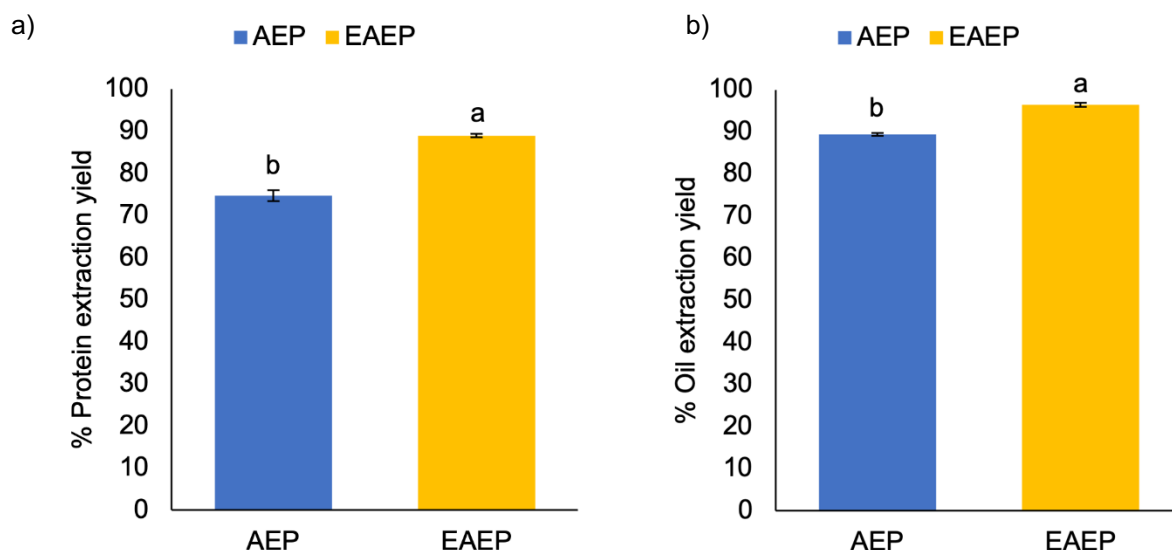


Figure 4.2. a) Total protein extraction (TPE, %) of AEP and EAEP almond milks. B) Total oil extraction (TOE, %) of AEP and EAEP almond milks. Different letters indicate significant difference ($p < 0.05$) between the extraction methods. Data represent the mean $\pm$ one standard deviation of extraction triplicates.

4.3.3 Antioxidant activity of the milks by ABTS and ORAC assays

Total antioxidant capacity of the milks was analyzed via both ABTS and ORAC assays (Fig. 4.3a-b). The commercial milk formula demonstrated the lowest antioxidant capacity by both ABTS ($1.55 \times 10^4 \mu\text{mol/mL}$) and ORAC ($2.47 \times 10^3 \mu\text{mol/mL}$), likely due to its more dilute nature

(less almond material). AEP milk exhibited 3.28×10^4 $\mu\text{mol/mL}$ for ABTS and 6.88×10^3 $\mu\text{mol/mL}$ by ORAC, while EAEP milks had the highest antioxidant activities (9.83×10^4 $\mu\text{mol/mL}$ by ABTS; 8.06×10^3 $\mu\text{mol/mL}$ by ORAC). Overall, the results suggest that all of the milks, regardless of processing method, exhibited higher antioxidant capacity through the ABTS mechanism compared to ORAC. The ORAC method has been found to have a greater sensitivity to a wider variety of antioxidant compounds than ABTS, thus it may be detecting differences that the latter assay does not¹⁵. Previous work from our group²⁹ investigated the antioxidant activity of proteins extracted from almond cake, the solid material that remains after mechanical expression of oil from almonds. In that study, the ABTS assay resulted in higher $\mu\text{mol TE/g}$ dry sample (222 $\mu\text{mol TE/g}$ for AEP and 650 $\mu\text{mol TE/g}$ for EAEP) than those of ORAC (109 $\mu\text{mol TE/g}$ for AEP and 397 $\mu\text{mol TE/g}$ for EAEP). It is difficult to compare these results directly to those of the present study due to differences in starting material and processing methods, as well as the different mechanisms of the ABTS and ORAC assays. The ABTS assay measures the electron transfer capacity of an antioxidant while the ORAC assay quantifies the capacity of the antioxidant to transfer hydrogen atoms^{21,36}. These qualities could differ within the same sample, let alone within samples extracted from different starting materials and with different extraction methods. Given the complex food matrices of these almond milks, the discrepancy between ABTS and ORAC methods can likely be explained by this difference in analysis mechanisms.

A likely explanation for increased antioxidant activity in the EAEP milk is due to the hydrolysis of the extracted proteins into peptides that may exhibit antioxidant properties^{29,37–39}. Though total phenolic compounds were not investigated in this work, De Souza et al.'s work demonstrated a significant increase in the concentration of phenolic compounds in the EAEP extract from the press cake, which could also be the case in the present study²⁹. In the present

study, AEP and EAEP milks were processed using the same SLR (1:6) and pH (8), thus the difference between the two can be explained by the addition of enzyme in the EAEP. The major finding from these assays in both the previous and current studies was that enzyme usage greatly increased total antioxidant capacity of the extracts and, this case, of the milks.

4.3.4 Allergenicity of the milks via S-ELISA

To determine impact of extraction methods (AEP vs. EAEP) on allergenicity, the potential immunoreactivity of the milks was evaluated by a sandwich ELISA assay able to quantify amandin, the major allergenic protein in almonds. Previous work with almond products¹⁸ investigated the impact of a neutral protease on proteins extracted from almond flour and found that the enzyme greatly reduced the allergenicity of the proteins. In that study, the protein content of AEP and EAEP extracts was very similar (57.3 and 59.2%, respectively, dry basis), and amandin detection was reduced by 75% in the EAEP extract. In the present study, both extracts had similar protein levels and again amandin detection was reduced by over 75% (1.7×10^5 ppm for AEP milks and 4.07×10^4 ppm for EAEP milks). Both AEP and EAEP milks had significantly higher concentration of detected amandin than the commercial formula (9.37×10^3 ppm), likely due to the higher protein content of AEP and EAEP milks compared with that of the commercial sample, which is more than 75% lower than that of AEP and EAEP milks (Table 4.1). To provide a more accurate comparison for all milk samples, the amount of amandin detected (mg) is reported per kg of total protein (Fig. 3d). By this metric, EAEP milks contained similar amandin (1.55×10^6 mg almond protein/kg total protein) than the commercial formula (1.66×10^6 mg almond protein/kg total protein) despite containing more than 4 times more total protein. This reduction in detected allergenicity is due to the hydrolysis of the proteins, suggesting that the structure of the protein is being altered in a manner that reduces their detectable antibody-binding capacity. This effect on

ELISA-detectable protein hydrolysates has been observed in chickpea, lentil and peanut proteins as well^{40–42}. These results indicate promise for producing a reduce allergenicity almond milk through enzymatic hydrolysis, though further work is needed to evaluate the allergenic potential of the hydrolysates produced.

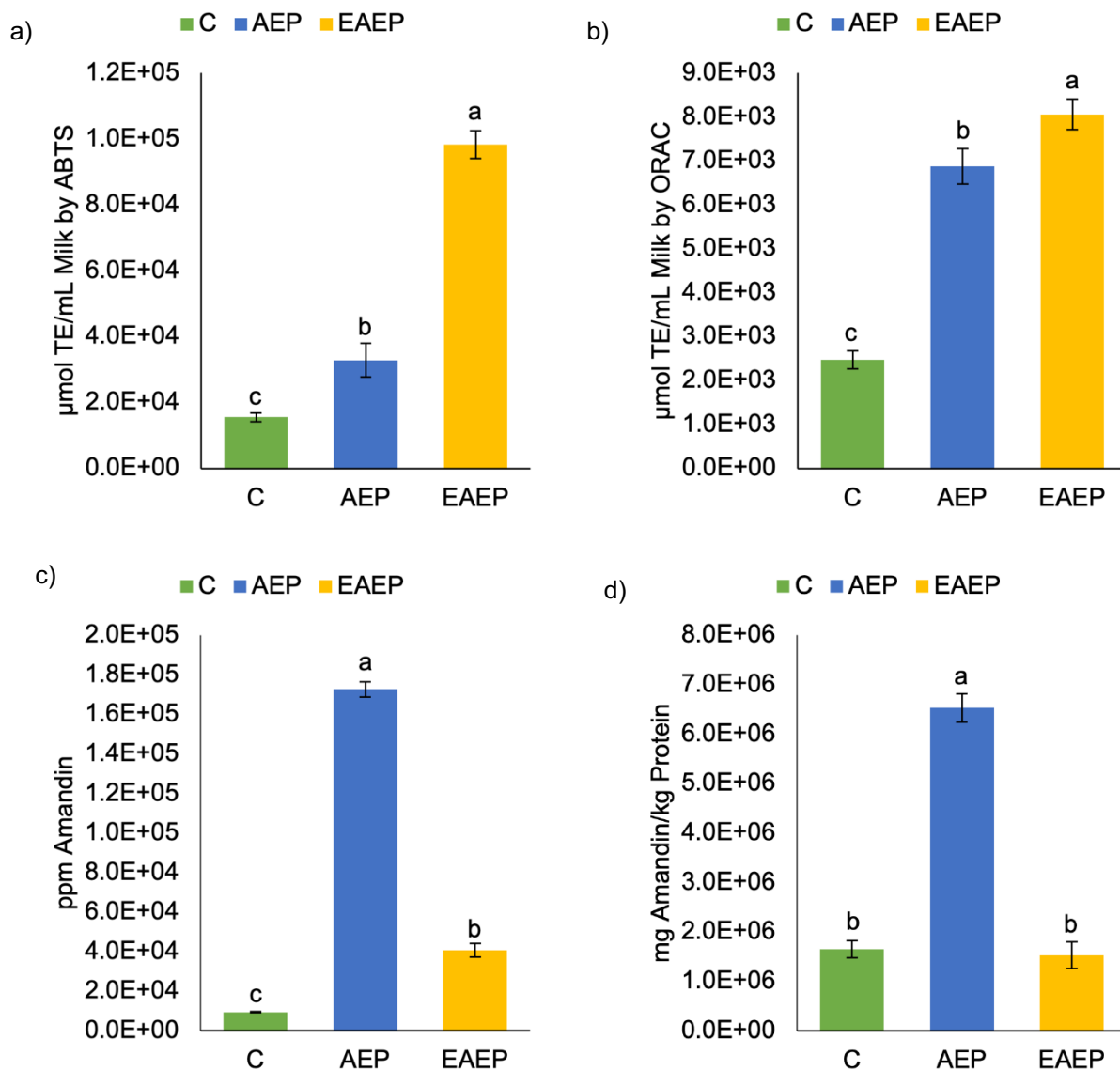


Figure 4.3. Total antioxidant activity of milks as determined by a) ABTS and b) ORAC assays; immunoreactivity by S-ELISA as measured by c) ppm amandin and d) mg amandin detected/kg protein in milk. Different letters indicate significant difference ($p < 0.05$) between the extractions.

Data represent the mean $\pm$ one standard deviation of triplicates. Different lowercase letters indicate significant difference of means ($p < 0.05$).

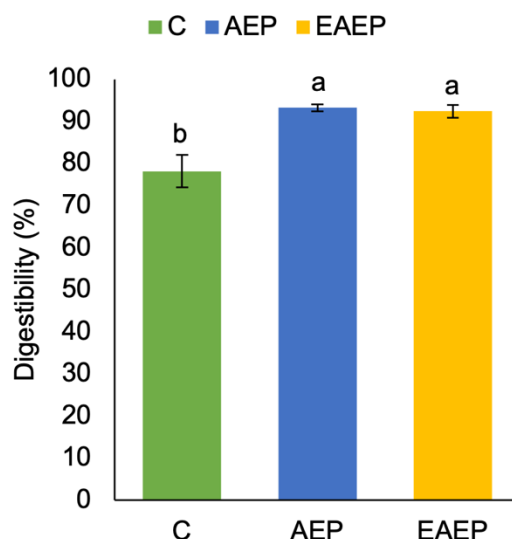


Figure 4.4 *In vitro* protein digestibility of commercial (C), AEP, and EAEP almond milk formulas. Data represent the mean $\pm$ one standard deviation of triplicates. Different lowercase letters indicate significant difference of means ($p < 0.05$).

4.3.5 *In vitro* protein digestibility of AEP and EAEP milks

To assess the impact of extraction on protein digestibility, a static *in vitro* digestibility protocol was employed on the three milk formulas (Fig. 4). Overall, IVPD of the AEP and EAEP milks was significantly higher (93.2 and 92.4%, respectively) than that of the commercial formula (78.2%). Almond protein IVPD has previously been reported as relatively high^{43,44}. Previous work from our group on almond press cake²⁹ demonstrated that EAEP with alkaline protease led to slightly lower IVPD (64%) than AEP (73%). The authors theorized the high degree of hydrolysis in the EAEP milk (23%) limited the number of sites for the digestive enzymes to act upon, artificially lowering estimated IVPD. Conversely, work from our group on full fat almond flour¹⁸ demonstrated that the use of a neutral protease during the extraction increased protein digestibility from 79.1% for AEP milk to 88.5% for EAEP milk. In the latter study, the EAEP milk protein had a degree of hydrolysis (DH) of 7%, lower than the previous work. The present study differs from

the previous two mentioned in that degree of hydrolysis of the protein in the EAEP milk formula using alkaline protease selected in Chapter 3 was 7.6% (unlike the 23% DH noted above), along with a different starting material for extraction (full fat almond flour vs. roasted almond paste). In Chapter 3, the EAEP milk IVPD was slightly higher (86.6-88.1%) than that of AEP milks (84.4-85.6%). Ultimately, this difference was not statistically significant at $p < 0.05$, which agrees with the findings in the present work. The IVPD of both AEP and EAEP milks being higher in the scaled-up samples could perhaps be attributed to the more intense heat treatment they were exposed to, possibly causing some partial denaturation of the proteins, facilitating attack by digestive enzymes⁴⁵.

The differences in IVPD of AEP and EAEP milks and the commercial formula are large (14-15% higher for AEP/EAEP milks). This can potentially be explained by the hydrolysis of proteins in both formulas due to the extraction process and enzyme use. Though the degree of hydrolysis in native proteins is difficult to measure (see Chapter 3), AEP milk proteins are hydrolyzed/unfolded to a certain degree during the extraction due to the alkaline conditions (pH 8). AEP milk #8, the formula selected in Chapter 3 (pH 8, SLR of 1:6), exhibited a DH of 3.3%, while EAEP milk #8 had a DH of 7.6% (pH 9, SLR of 1:6, 0.10% alkaline protease). Given the low concentration of enzyme used in the EAEP and the lack of enzyme use in the AEP and the similar DH of both milks, it is reasonable that the digestibility was similar between the two. The commercial almond milk, however, does not go through a prolonged extraction process or pH adjustment. Therefore, soluble and insoluble proteins, as well as fibers and other non-protein components remain in a conventional almond milk, potentially acting as a barrier to digestive hydrolysis⁴⁶. Thus, the proteins in the commercial milk formula likely have different structures and profile from those extracted through AEP and EAEP. Ultimately, the high digestibility of the

proteins in the AEP and EAEP milks is another advantage of this processing method. The increase in protein content, along with improved protein digestibility, makes these milks more nutritious products.

4.3.6. Understanding the impact of extraction and processing methods on milk shelf-life and quality

4.3.6.1. Primary and secondary oxidation markers

The oxidation of the milks was of concern in this study, especially due to the expected higher lipid content of AEP and EAEP milks compared to the commercial formula. Primary lipid oxidation of all milks, measured by peroxide value, remained low across storage time, with all milks below the limit of detection ($1.95 \mu\text{mol/L}$) at nearly all data points (data not shown). Similarly, secondary lipid oxidation measured via the TBARS assay was low, with all milks at the beginning and end of shelf-life having less than $1 \mu\text{mol MDA/L}$ (Fig. 4.5). Compared with a previous study from our group on bovine milk, which found $15 \mu\text{g MDA/kg}$ in UHT-processed bovine milk²⁵, this value is quite low. A slight increase in $\mu\text{g MDA/L}$ was observed in weeks 1-3 for some samples, but this is likely an artifact considering the consistently low values observed for peroxides. The low amount of primary and secondary markers in the milks is consistent with previous studies on UHT-treated milk products^{24,25} subjected to refrigerated storage conditions⁴⁷.

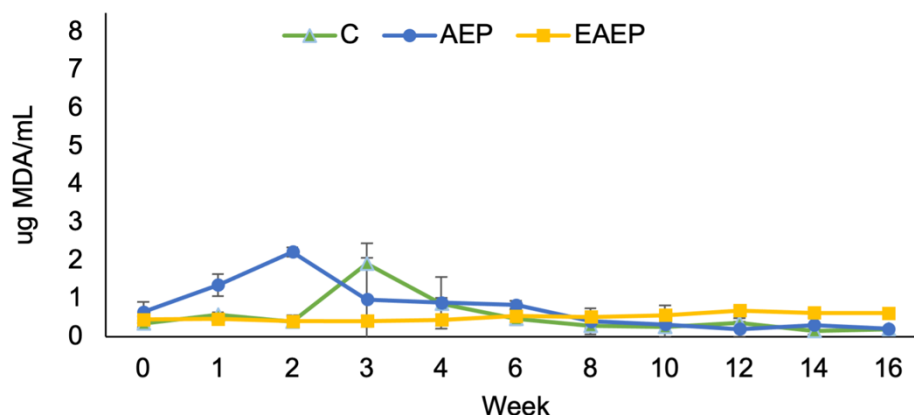


Figure 4.5. Secondary lipid oxidation products via TBARS assay over shelf-life of commercial (C), AEP, and EAEP milks. Data represent the mean $\pm$ one standard deviation of triplicates. Different lowercase letters indicate significant difference of means ($p < 0.05$). MDA: malondialdehyde

4.3.6.2 Oxylipin content of milk through shelf-life

Oxylipins are primary oxidation products resulting from the oxidation of polyunsaturated fatty acids⁴⁸. However, due to the dynamic nature of lipid oxidation, markers such as hydroperoxides and secondary markers such as TBARs can be present at low concentrations, making it challenging to fully understand the oxidation process. Moreover, relying on only a few analytical methods may fail to capture the wide range of compounds that can be formed during oxidation, underscoring the need for employing multiple analytical techniques to better assess lipid oxidation. Based on previous research from our group^{24,25} that demonstrated that quantifying oxylipins via LC-MS/MS is a more sensitive method for detecting oxidation at earlier stages, we assessed the impact of storage on the oxylipin content of all three milk formulas (AEP, EAEP, commercial) as measured at both the start (T_0) and end (T_{end}) of their shelf-life using LC-MS/MS.

The total oxylipin concentration in the almond milk samples is shown in Table 2. The most prevalent oxylipin detected in the milks was 9,12,13-trihydroxy-10-octadecanoic acid (9,12,13-TriHOME), a metabolite of linoleic acid, with concentrations ranging from 65059-120920

fmol/ μ L at T_0 to 61200-122421 pmol/mL at T_{end} . Overall, 9,12,13-TriHOME concentrations were higher for AEP (138887 pmol/mL) and EAEP (120920 pmol/mL) milks at T_0 compared to the commercial sample, which might indicate the impact of the extraction conditions employed (dilution, solids-to-liquid ratio, temperature, time), which can affect both lipid oxidation rates and final composition of the milks. The commercial formula contained the lowest concentration of lipids (1.8%) compared to that of AEP (7.8%) and EAEP (6.7%) milks, which may explain the higher oxidation values per mL in AEP and EAEP milks, as these milks have more lipids available to undergo oxidation. AEP and EAEP milks underwent a 1-hour extraction period at elevated temperature (50 °C), potentially promoting lipid oxidation, while commercial milks were mixed immediately before pasteurization and homogenization. However, a small decrease in concentration was observed from T_0 to T_f for the commercial and EAEP milk samples. A similar trend was observed for the next most abundant oxylipin, 12,13-epoxyoctadecenoic acid (12 (13)-EpOME), which is also derived from linoleic acid, with the commercial formula containing the lowest concentration at T_0 (5501 pmol/mL) and a statistically significant ($p < 0.05$) increase at T_{end} (6931 pmol/mL). When evaluating the concentration of oxylipins in the milk samples, it is important to consider the extraction methods employed for both processes and their impact on the proximate composition of the AEP and EAEP milks, which differ significantly from the commercial formula, and lipid oxidation.

Table 4.2. Oxylin concentration (pmol/mL) in commercial (C), AEP (A), and EAEP (E) milks at beginning (T₀) and end (T_{end}) of shelf-life.

Oxylin	Formula	T ₀	T _{end}	Change
9,12,13-TriHOME	C	65059.0 ± 14818.5 ^b	61200.4 ± 9091.3 ^c	-
	A	138887.9 ± 8643.4 ^a	122421.2 ± 4205.0 ^a	Decrease*-
	E	120920.2 ± 25987.5 ^a	86288.9 ± 7091.7 ^b	Decrease
9,10,13-TriHOME	C	0 ^a	0 ^a	-
	A	0 ^a	0 ^a	-
	E	0 ^a	0 ^a	-
12,13-DiHOME	C	2781.9 ± 848.6 ^a	3021.1 ± 784.1 ^a	-
	A	3563.9 ± 100.4 ^a	3260.1 ± 124.6 ^a	Decrease
	E	3745.5 ± 667.5 ^a	2635.1 ± 626.6 ^a	-
9,10-DiHOME	C	2115.2 ± 1022.4 ^a	2103.1 ± 666.7 ^a	-
	A	1354.8 ± 87.5 ^a	1375.5 ± 229.4 ^{ab}	-
	E	1146.5 ± 301.9 ^a	901.2 ± 420.1 ^b	-
9-HOTrE	C	100.4 ± 18.6 ^a	148.2 ± 8.9 ^a	Increase
	A	97.3 ± 24.9 ^a	122.3 ± 16.3 ^{ab}	-
	E	164.6 ± 35.1 ^a	71.2 ± 33.5 ^b	Decrease
13-HOTrE	C	0 ^a	0 ^a	-
	A	0 ^a	0 ^a	-
	E	0 ^a	47.8 ± 82.8 ^a	-
9-HODE	C	3923.4 ± 1561.2 ^a	7023.5 ± 4236.1 ^b	-
	A	4001.8 ± 926.0 ^a	3266.6 ± 602.1 ^b	-
	E	4440.0 ± 811.1 ^a	5270.8 ± 3080.3 ^a	-
13-HODE	C	0 ^a	0 ^a	-
	A	0 ^a	0 ^a	-
	E	0 ^a	0 ^a	-
9-oxo-ODE	C	0 ^a	0 ^a	-
	A	0 ^a	0 ^a	-
	E	0 ^a	0 ^a	-
13-oxo-ODE	C	456.7 ± 63.3 ^b	821.1 ± 52.6 ^a	Increase
	A	570.6 ± 82.9 ^b	890.8 ± 22.7 ^a	Increase
	E	788.5 ± 84.5 ^a	947.6 ± 217.2 ^a	-
12(13)-EpOME	C	5501.4 ± 413.2 ^b	6931.6 ± 451.3 ^a	Increase
	A	9162.2 ± 473.2 ^a	8277.6 ± 534.1 ^b	Increase*
	E	9452.4 ± 1025.7 ^a	8631.3 ± 643.5 ^{ab}	-
9(10)-EpOME	C	2042.7 ± 523.4 ^a	2449.0 ± 547.0 ^a	-
	A	2150.9 ± 188.9 ^a	2000.3 ± 39.1 ^a	-
	E	2317.9 ± 264.8 ^a	2033.4 ± 225.3 ^a	-

Different letters within columns of each oxylin represent a statistically significant ($p < 0.05$) difference between those formulas via one-way ANOVA followed by Tukey's post hoc test for HSD. Statistically significant ($p < 0.05$ as measured by t-test) change over time of each formula is denoted by "Increase" or "Decrease" while no significant change is denoted with "-". An asterisk indicates that the change was significant at $p < 0.10$.

Dias et al. (in press as of October 2024) found similar ratios of oxylipins in oil extracted via AEP and EAEP (using a neutral protease) from blanched almond flour in the only work to date to analyze oxylipins in almond oil. The majority of oxylipins found in that work were derived from linoleic and alpha-linoleic acid, and our results are in agreement. However, the present study found no concentration of 13-hydroxyoctadecadienoic acid (13-HODE), 9,10,13-trihydroxyoctadecadienoic acid (9,10,13-TriHOME), while Dias et al. did find these metabolites in almond oil. Similarly, Dias et al. did not find any evidence of 13-oxo-octadecadienoic acid (13-oxo-ODE), 9(10)-epoxyoctadecanoic acid (9(10)Ep-OME), 9,10-dihydroxyoctadecenoic acid (9,10-DiHOME), 12,13-dihydroxyoctadecenoic acid (12,13-DiHOME), or 9-hydroxyoctadecatrienoic acid (9-HOTrE), while the present study found concentrations of all of these in all three milk formulas. The present work differs from Dias et al.'s in a variety of ways, including the starting almond material (roasted finely ground almond butter), ESL pasteurization, enzyme used in EAEP extraction, and nature of the product examined (extracted and isolated oil vs. whole aqueous extract from AEP and EAEP and whole almond butter in water in the commercial formula), which could have contributed to the differences in measured oxylipins.

Our results corroborate with previous research indicating that oxylipin analysis provides a more sensitive method to assess early lipid oxidation than conventional oxidations markers such as peroxide values and secondary oxidation markers via TBARS, as the latter analyses showed little to no evidence of oxidation at any stage of the study. In EAEP milk formulas, no increase in any oxylipin marker was observed, while in AEP formulas 9,12,13-TriHOME, 12,13-DiHOME, and 12(13)-EpHOME decreased while 13-oxo-ODE increased (Table 2). The commercial formula tended to have more increases in oxylipin concentration over storage time (9-HOTrE, 13-oxo-ODE, 12(13)-EpOME). An observed increase in levels of an oxylipin could indicate oxidation,

while an observed decrease could indicate that that metabolite was degraded over time, or perhaps converted into a secondary volatile compound⁴⁹.

Given the dynamic nature of lipid oxidation, additional studies are needed to further explore the causes of the increase or decrease in oxylipins observed. Suggested further research could include lipidomic analyses of the starting almond paste material prior to extraction and suspension, milk prior to heat treatment and homogenization, and to investigate whether these formulas would fare similarly in aseptic packaging at ambient temperatures as many almond milks are sold at retail.

4.3.6.3. Emulsion stability of the milks via oil droplet size and creaming index

The emulsion stability of the milks was measured via oil droplet size by dynamic light scattering and creaming index over the 16-week shelf-life period. As oil droplet size changed very little throughout the shelf-life, only start and end points are shown in Table 3. All milk emulsions showed some separation after week 2 (Fig. 6a) but were stabilized after this point. It is likely that the bulk of the emulsion separation occurred in the first days after processing and the milks remained stable after this. AEP milk demonstrated a peak in creaming index at week 3-4, likely caused by the initial formation of a three-layer (upper “cream,” middle intermediate “emulsion” layer, and lower “serum”) system that often forms in the beginning stages of creaming before separating into more distinct cream and serum layers⁵⁰. Notably, the commercial and AEP milks had relatively similar oil droplet sizes, despite the AEP milk having considerably more oil than the commercial, and the same amount of lecithin. This could be due to the presence of native proteins in the milk associating with the surfaces of oil droplets and providing steric stabilization of the emulsion due to protein-protein repulsion, preventing oil droplet coalescence⁵¹. A stable emulsion is often observed during aqueous extractions of oil-bearing seeds, indicating the presence of

emulsifying proteins^{8,52–54}. Interestingly, the AEP milk exhibited a larger relative standard deviation in oil droplet size at the end of shelf-life, indicating significant variation between the triplicate extractions at this point. The low creaming index of the commercial milk is likely explained by the relatively low quantity of total fat in the formula (1.8% vs. 6.7% for AEP and 7.8% for EAEP), which was effectively emulsified through the combined use of homogenization and lecithin.

Table 4.3. D (4,3) oil droplet mean diameter (μm) of almond milks over shelf-life.

	Milk		
	Commercial	AEP	EAEP
T ₀	14.2 $\pm$ 2.4 ^a	12.4 $\pm$ 1.4 ^{ab}	6.0 $\pm$ 3.5 ^b
T _{End}	15.4 $\pm$ 1.3 ^a	13.3 $\pm$ 6.3 ^a	6.5 $\pm$ 3.5 ^a

Droplet size (μm) of commercial, AEP, and EAEP milks at beginning (T₀) and end (T_{End}) of shelf-life testing. Different superscript letters represent a statistically significant ($p < 0.05$) difference of means of the three formulas via one-way ANOVA within each time point (T₀ or T_{End}). No significant difference was found between T₀ and T_{End} for each individual formula.

Conversely, despite its similarly high oil content in comparison to the commercial formula, EAEP milk demonstrated a smaller oil droplet than both commercial and AEP milks (Table 4.2), though this difference was not significant by the end of storage. This could potentially be explained by the presence of protein hydrolysates released during extraction, which have different conformations than in their native state, potentially exposing hydrophobic domains previously hidden within the protein core, allowing for easier adsorption at the oil/water interface⁵⁵. In the pasteurization and homogenization process, free and suspended oil droplets are forced through a small gap, shearing them into smaller droplets that become emulsified in the aqueous phase¹. Despite the seeming stabilization of oil droplets, it appears that the sheer volume of oil overcame the emulsifying capacity of the proteins and lecithin used, thus a higher quantity of lecithin or a different hydrocolloid stabilizer could be investigated for further study. In Chapter 2, EAEP milk

formulas exhibited a higher mean oil droplet diameter than AEP milk (4.1-5.7 μm vs. 2.9-4.2 μm). The mixing process in that study was accomplished by using a kitchen blender and no high-pressure homogenization occurred, indicating that high pressure homogenization was an effective tool in reducing oil droplet size for EAEP milk. This difference could have been overcome by the end of storage, at which time EAEP milk oil droplets were not significantly smaller than commercial or AEP droplets due to high variation within replicates. The hydrolysis of native proteins due to enzyme use in extraction could have impacted the oleosins (lipid membrane proteins), further destabilizing oil droplets in emulsions, leading to coalescence.

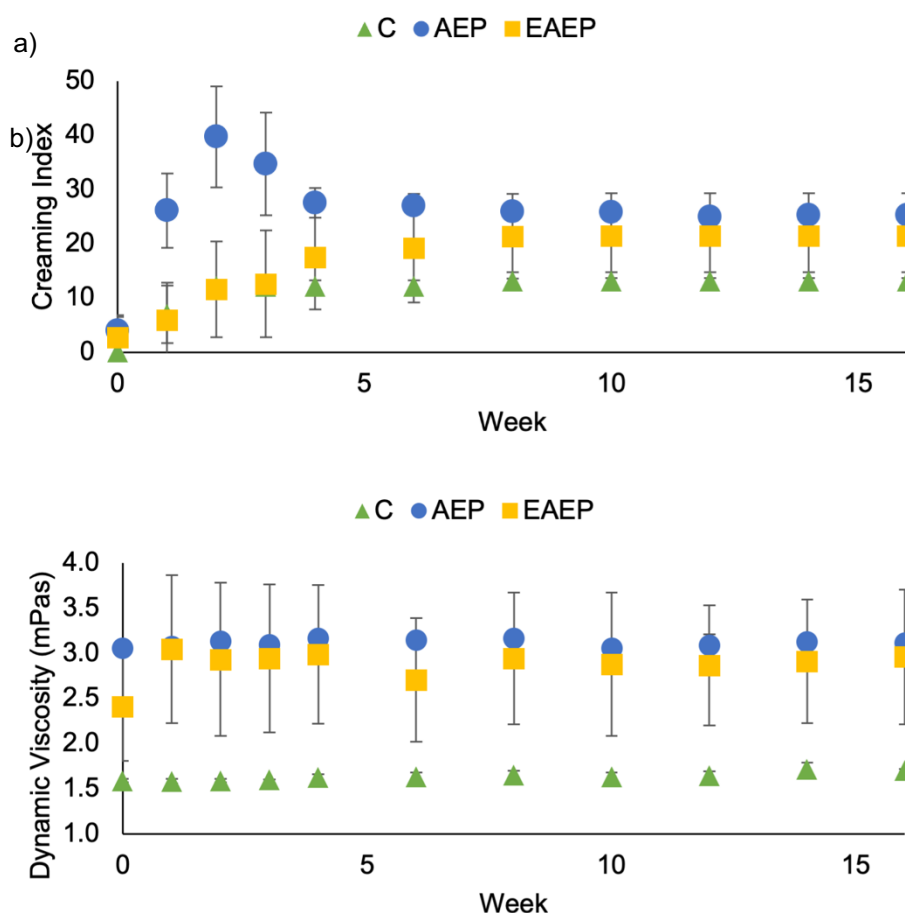


Figure 4.6. Creaming index (a) and dynamic viscosity (mPa s) at 50 s^{-1} (b) of milks over shelf-life of commercial (C), AEP and EAEP milks. Data represent the mean $\pm$ one standard deviation of extraction triplicates.

4.3.6.4. *Viscosity of milks*

Viscosity of milks was also relatively stable throughout storage (Fig. 4.6b), with the commercial milk demonstrating the lowest viscosity. EAEP and AEP milks were relatively similar in viscosity, with EAEP trending slightly lower. In a study on soybean oleosins, reduced viscosity was observed in emulsions prepared with hydrolyzed lipid membrane proteins oleosins compared to native⁵⁶, indicating the impact of protein structure on this parameter. Interestingly, the viscosities of AEP and EAEP milks were higher in this study (2.4-3.2 mPa s) than those of Chapter 2 (1.5-2.1 mPa s). This could be due to the high-pressure homogenization the milk in this study underwent. The higher viscosity of AEP and EAEP milks relative to the commercial milk is likely due to their higher overall solid content (SLR of 1:6 for AEP/EAEP and 1:50 for commercial formula). The viscosity of a colloidal suspension such as almond milk increases with an increase in particle concentration^{50,57}. This can be seen in conventional dairy milk, with skim milk having a lower viscosity than whole or heavy cream. Previous sensory testing with these milk formulas associated higher viscosity with increased liking (Chapter 3), indicating that these milks will be perceived as creamier and likely have greater consumer acceptance than a conventionally produced almond milk.

EAEP milk exhibited a larger increase in viscosity after week 1 than AEP or commercial milks (Fig. 54.b). This could be due to flocculation (aggregation of droplets), which is difficult to measure from D [4,3] alone. A dilute emulsion containing flocs of oil in water will have a higher viscosity than those with more sequestered droplets, as these droplets can immobilize the surrounding solvent phase⁵⁸. The polydispersity index (PDI, calculated as the D [4,3]/D50 of emulsion droplet size) of EAEP milk did not change significantly over weeks 0-4 (data not shown), indicating that there was not a major change in the distribution of small or large particles, but as

light scattering methods rely on the assumption that oil droplets are isolated, the underlying theories governing the interpretation of this data can no longer be assumed⁵⁰. Further study into the creaming behavior of this milk is warranted. Additionally, adjusting the levels of emulsifiers or stabilizers in the formulation could potentially address this issue, as well as adjusting parameters such as homogenization pressure.

4.7 Conclusions

In this study, the AEP and EAEP methods were used to scale the production of previously optimized almond milk formulas from 0.5 to 15 L batch size, which were compared with a conventionally made commercial almond milk formula. Following UHT pasteurization and homogenization, a shelf-life study was performed to assess the changes in the physicochemical properties of the milks over time in refrigerated conditions. The use of AEP and EAEP produced milks with higher protein and oil content than the commercial formula. Enzyme use in the extraction led to higher protein extraction as well as increased antioxidant activity and reduced almond protein immunoreactivity of EAEP milks. In addition to their higher protein content, digestibility of AEP and EAEP milks was higher than that of the commercial formula. Conventional oxidation markers, such as peroxide values and TBARs, showed low oxidation across all milks throughout the storage period. In contrast, LC-MS/MS analysis detected small changes in specific oxylipins (increase or decrease), highlighting the sensitivity of this method for identifying early-stage oxidation. Overall, the milks exhibited good oxidative stability over their shelf-life. Emulsion stability varied between the milks, with all three formulas demonstrating creaming after two weeks of storage. The AEP and EAEP milk formulas had the highest creaming indices, likely due to their higher total fat concentration compared to the commercial formula. The creaming stability of AEP and EAEP milks was similar to that of the commercial milk formula,

indicating that these milks would be as stable as conventional almond milk if produced at scale. This work demonstrates that almond milk with improved protein and lipid content can be produced at scale utilizing environmentally friendly AEP and EAEP. The extracted milks contained significantly higher nutrient content than a commercial formula, while maintaining quality. The use of EAEP offers manufacturers an opportunity to improve biological properties such as antioxidant activity and reduced allergenicity. These findings validate the feasibility of these methods for producing a value-added almond milk for the retail market.

4.8 Acknowledgements

Funding for publication was made possible by the U. S. Department of Agriculture's (USDA) Agricultural Marketing Service through grant 21-0001-024-SF. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the USDA.

4.9 References

1. McClements, D. J., Newman, E. & McClements, I. F. Plant-based Milks: A Review of the Science Underpinning Their Design, Fabrication, and Performance. *Compr. Rev. Food Sci. Food Saf.* **18**, 2047–2067 (2019).
2. Haas, R., Schnepps, A., Pichler, A. & Meixner, O. Cow milk versus plant-based milk substitutes: A comparison of product image and motivational structure of consumption. *Sustain. Switz.* **11**, (2019).
3. Mordor Intelligence. Almond Milk Market Size & Share Analysis - Industry Research Report - Growth Trends. <https://www.mordorintelligence.com/industry-reports/global-almond-milk-market> (2024).
4. Singhal, S., Baker, R. D. & Baker, S. S. A Comparison of the Nutritional Value of Cow's Milk and Nondairy Beverages. *J. Pediatr. Gastroenterol. Nutr.* **64**, 799–805 (2017).
5. Chalupa-Krebsdak, S., Long, C. J. & Bohrer, B. M. Nutrient density and nutritional value of milk and plant-based milk alternatives. *Int. Dairy J.* **87**, 84–92 (2018).
6. Craig, W. J. & Fresán, U. International Analysis of the Nutritional Content and a Review of Health Benefits of Non-Dairy Plant-Based Beverages. *Nutrients* **13**, 842 (2021).
7. Scholz-Ahrens, K. E., Ahrens, F. & Barth, C. A. Nutritional and health attributes of milk and milk imitations. *Eur. J. Nutr.* **59**, 19–34 (2020).
8. Rosenthal, A., Pyle, D. L. & Niranjana, K. Aqueous and enzymatic processes for edible oil extraction. *Enzyme Microb. Technol.* **19**, 402–420 (1996).
9. Campbell, K. A. & Glatz, C. E. Mechanisms of aqueous extraction of soybean oil. *J. Agric. Food Chem.* **57**, 10904–10912 (2009).
10. Campbell, K. A. *et al.* Advances in aqueous extraction processing of soybeans. *JAOCs J. Am. Oil Chem. Soc.* **88**, 449–465 (2011).
11. Deeth, H. C. Heat Treatment of Milk: Extended Shelf-Life (ESL) and Ultra-High Temperature (UHT) Treatments. in *Encyclopedia of Dairy Sciences* 618–631 (Elsevier, 2022). doi:10.1016/B978-0-12-818766-1.00140-9.
12. eCFR :: 21 CFR Part 131 Subpart A -- General Provisions. <https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-131/subpart-A>.
13. Deeth, H. Optimum Thermal Processing for Extended Shelf-Life (ESL) Milk. *Foods* **6**, 102 (2017).
14. AOAC Official Method. 5–6 (2000).
15. Zulueta, A., Esteve, M. J. & Frígola, A. ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food Chem.* **114**, 310–316 (2009).

16. Ngoh, Y.-Y. & Gan, C.-Y. Enzyme-assisted extraction and identification of antioxidative and α -amylase inhibitory peptides from Pinto beans (*Phaseolus vulgaris* cv. Pinto). *Food Chem.* **190**, 331–337 (2016).
17. Slotwinski, E. *et al.* Development and Validation of a Quantitative ELISA for the Detection of Almond Residues in Foods. *J. AOAC Int.* **101**, 118–123 (2018).
18. Furlan Goncalves Dias, F. *et al.* Effects of protease-assisted aqueous extraction on almond protein profile, digestibility, and antigenicity. *Curr. Res. Food Sci.* **6**, 100488 (2023).
19. Brodkorb, A. *et al.* INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nat. Protoc.* **14**, 991–1014 (2019).
20. Yang, J. S., Dias, F. F. G. & De Moura Bell, J. M. L. N. Optimizing alkaline and enzymatic extraction of black bean proteins: a comparative study of kinetics, functionality, and nutritional properties. *Sustain. Food Technol.* 10.1039.D4FB00163J (2024)
doi:10.1039/D4FB00163J.
21. Abeyrathne, E. D. N. S., Nam, K. & Ahn, D. U. Analytical Methods for Lipid Oxidation and Antioxidant Capacity in Food Systems. *Antioxidants* **10**, 1587 (2021).
22. Shahidi, F. & Zhong, Hy. J. Methods for Measuring Lipid Oxidation. in *Bailey's Industrial Oil and Fat Products* (ed. Shahidi, F.) 1–27 (Wiley, 2020).
doi:10.1002/047167849X.bio050.pub2.
23. Papastergiadis, A., Mubiru, E., Van Langenhove, H. & De Meulenaer, B. Malondialdehyde Measurement in Oxidized Foods: Evaluation of the Spectrophotometric Thiobarbituric Acid Reactive Substances (TBARS) Test in Various Foods. *J. Agric. Food Chem.* **60**, 9589–9594 (2012).
24. Ferraz Teixeira, B., Furlan Gonçalves Dias, F., Ferreira de Souza Vieira, T. M., Y. Taha, A. & Leite Nobrega de Moura Bell, J. M. Early detection of lipid oxidation in infant milk formula by measuring free oxylipins—Comparison with hydroperoxide value and thiobarbituric acid reactive substance methods. *J. Food Sci.* **87**, 5252–5262 (2022).
25. Dias, F. F. G. *et al.* Effects of industrial heat treatments on bovine milk oxylipins and conventional markers of lipid oxidation. *Prostaglandins Leukot. Essent. Fatty Acids* **152**, 102040 (2020).
26. Moayedi, A., Rezaei, K., Moini, S. & Keshavarz, B. Chemical Compositions of Oils from Several Wild Almond Species. *J. Am. Oil Chem. Soc.* **88**, 503–508 (2011).
27. Ong, J. J.-X., Steele, C. M. & Duizer, L. M. Challenges to assumptions regarding oral shear rate during oral processing and swallowing based on sensory testing with thickened liquids. *Food Hydrocoll.* **84**, 173–180 (2018).
28. Almeida, F. S., Dias, F. F. G., Sato, A. C. K. & De Moura Bell, J. M. L. N. Scaling up the Two-Stage Countercurrent Extraction of Oil and Protein from Green Coffee Beans: Impact of Proteolysis on Extractability, Protein Functionality, and Oil Recovery. *Food Bioprocess Technol.* **15**, 1794–1809 (2022).
29. de Souza, T. S. P., Dias, F. F. G., Oliveira, J. P. S., de Moura Bell, J. M. L. N. & Koblitiz, M. G. B. Biological properties of almond proteins produced by aqueous and enzyme-assisted aqueous extraction processes from almond cake. *Sci. Rep.* **10**, 1–12 (2020).
30. Sari, Y. W., Bruins, M. E. & Sanders, J. P. M. Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. *Ind. Crops Prod.* **43**, 78–83 (2013).
31. De Moura, J. M. L. N., Campbell, K., De Almeida, N. M., Glatz, C. E. & Johnson, L. A. Protein extraction and membrane recovery in enzyme-assisted aqueous extraction processing of soybeans. *JAOCs J. Am. Oil Chem. Soc.* **88**, 877–889 (2011).

32. Gorrepati, K., Balasubramanian, S. & Chandra, P. Plant based butters. *J. Food Sci. Technol.* **52**, 3965–3976 (2015).
33. Perren, R. & Escher, F. E. Impact of roasting on nut quality. in *Improving the Safety and Quality of Nuts* 173–197 (Elsevier, 2013). doi:10.1533/9780857097484.2.173.
34. Zhang, S. B., Lu, Q. Y., Yang, H., Li, Y. & Wang, S. Aqueous enzymatic extraction of oil and protein hydrolysates from roasted peanut seeds. *JAOCs J. Am. Oil Chem. Soc.* **88**, 727–732 (2011).
35. Altan, A., McCarthy, K. L., Tikekar, R., McCarthy, M. J. & Nitin, N. Image Analysis of Microstructural Changes in Almond Cotyledon as a Result of Processing. *J. Food Sci.* **76**, 212–221 (2011).
36. Bibi Sadeer, N., Montesano, D., Albrizio, S., Zengin, G. & Mahomoodally, M. F. The Versatility of Antioxidant Assays in Food Science and Safety—Chemistry, Applications, Strengths, and Limitations. *Antioxidants* **9**, 709 (2020).
37. Jamdar, S. N. *et al.* Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food Chem.* **121**, 178–184 (2010).
38. De Castro, R. J. S. & Sato, H. H. Synergistic actions of proteolytic enzymes for production of soy protein hydrolysates with antioxidant activities: An approach based on enzymes specificities. *Biocatal. Agric. Biotechnol.* **4**, 694–702 (2015).
39. Bolling, B. W. Almond Polyphenols: Methods of Analysis, Contribution to Food Quality, and Health Promotion: Almond polyphenols.... *Compr. Rev. Food Sci. Food Saf.* **16**, 346–368 (2017).
40. Clemente, A., Vioque, J., Sanchez-Vioque, R., Pedroche, J. & Millán, F. Production of Extensive Chickpea (*Cicer arietinum* L.) Protein Hydrolysates with Reduced Antigenic Activity. *J. Agric. Food Chem.* **47**, 3776–3781 (1999).
41. Cabanillas, B. *et al.* Effects of enzymatic hydrolysis on lentil allergenicity. *Mol. Nutr. Food Res.* **54**, 1266–1272 (2010).
42. Cabanillas, B. *et al.* Influence of Enzymatic Hydrolysis on the Allergenicity of Roasted Peanut Protein Extract. *Int. Arch. Allergy Immunol.* **157**, 41–50 (2012).
43. Sathe, S. K. Solubilization, electrophoretic characterization and in vitro digestibility of almond (*Prunus amygdalus*) proteins. *J. Food Biochem.* **16**, 249–264 (1993).
44. Sze-Tao, K. W. C. & Sathe, S. K. Functional properties and in vitro digestibility of almond (*Prunus dulcis* L.) protein isolate. *Food Chem.* **69**, 153–160 (2000).
45. Embaby, H. E.-S. Effect of Heat Treatments on Certain Antinutrients and in vitro Protein Digestibility of Peanut and Sesame Seeds. *Food Sci. Technol. Res.* **17**, 31–38 (2010).
46. Barbana, C. & Boye, J. I. In vitro protein digestibility and physico-chemical properties of flours and protein concentrates from two varieties of lentil (*Lens culinaris*). *Food Funct* **4**, 310–321 (2013).
47. Lu, J., Langton, M., Sampels, S. & Pickova, J. Lipolysis and Oxidation in Ultra-High Temperature Milk Depend on Sampling Month, Storage Duration, and Temperature. *J. Food Sci.* **84**, 1045–1053 (2019).
48. Mosblech, A., Feussner, I. & Heilmann, I. Oxylipins: Structurally diverse metabolites from fatty acid oxidation. *Plant Physiol. Biochem.* **47**, 511–517 (2009).
49. Thum, C. *et al.* Concentration of milk oxylipins after heat and homogenization treatments. *Front. Food Sci. Technol.* **3**, 1027418 (2023).

50. McClements, D. J. *Food Emulsions: Principles, Practices, and Techniques, Third Edition*. (CRC Press, Boca Raton, 2015). doi:10.1201/b18868.
51. Damodaran, S. Protein Stabilization of Emulsions and Foams. *J. Food Sci.* **70**, R54–R66 (2006).
52. Dias, F. F. G., de Almeida, N. M., de Souza, T. S. P., Taha, A. Y. & de Moura Bell, J. M. L. N. Characterization and Demulsification of the Oil-Rich Emulsion from the Aqueous Extraction Process of Almond Flour. *Processes* **8**, 1–18 (2020).
53. Li, P. *et al.* Characterization and demulsification of cream emulsion from aqueous extraction of peanut. *J. Food Eng.* **185**, 62–71 (2016).
54. Li, P. *et al.* Demulsification of oil-rich emulsion and characterization of protein hydrolysates from peanut cream emulsion of aqueous extraction processing. *J. Food Eng.* **204**, 64–72 (2017).
55. Lacou, L., Léonil, J. & Gagnaire, V. Functional properties of peptides: From single peptide solutions to a mixture of peptides in food products. *Food Hydrocoll.* **57**, 187–199 (2016).
56. Maurer, S. *et al.* The role of intact oleosin for stabilization and function of oleosomes. *J. Phys. Chem. B* **117**, 13872–13883 (2013).
57. Genovese, D. B., Lozano, J. E. & Rao, M. A. The Rheology of Colloidal and Noncolloidal Food Dispersions. *J. Food Sci.* **72**, (2007).
58. Berli, C. L. A., Quemada, D. & Parker, A. Modelling the viscosity of depletion flocculated emulsions. *Colloids Surf. Physicochem. Eng. Asp.* **203**, 11–20 (2002).