Topic: Effect of protein type on the loss of flavor compounds in protein bars

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Abstract

A problem with high-protein foods is that flavor compounds added are “lost” and no longer perceptible by the consumers over time, due to the flavor compounds undergoing various binding interactions with the protein. Knowing which flavors interact more extensively with which proteins can help manufacturers decide if a certain flavor should be added in excess in order for the flavor to still be perceptible by the time consumers consume it. Gaining an understanding of which proteins work as a better carrier for which flavor compounds allows for better formulations that holds up well over time without degradation in sensory quality.

The aim of the project was to investigate the effects of using different types of protein isolate (whey, soy, rice and pea) on the rate of loss of nine different flavors compounds (allyl sulfide, isoamyl acetate, furfuryl mercaptan, benzaldehyde, methyl salicylate, menthol, D-carvone, γ-nonalactone and trans-α-ionone) in a protein bar matrix system.

The nine flavor compounds were added a protein bar model system and stored at 45°C over four weeks for an accelerated shelf-life study. Samples were taken at 0, 1, 2 and 4 week

s of storage time. Flavor compounds were extracted from each sample by Stir Bar Sorptive Extraction and thermally desorbed in the injection port of a gas chromatography. The amount of detectable flavor compounds (assumed to not be bound to the protein) was determined semi-quantitatively using the peak areas of each flavor compound on the chromatogram and corrected with an internal standard. The percentage loss of each flavor compound was then calculated at each time point, with Week 0 as a baseline of 100%.

Soy protein showed the least binding with flavor compounds initially, especially with compounds of lower molecular weight. However, soy protein had a very high rate of reactions with flavor compounds over one month of storage. Rice protein the slowest rate of binding interactions with flavor compounds, and was the most unreactive with low molecular weights flavor compounds such as allyl sulfide, isoamyl acetate and benzaldehyde. Pea and whey protein were both rather reactive with flavor compounds, but pea protein showed better performance with flavor compounds of higher molecular weights.

Unlike whey and soy protein, rice and pea protein are not as commonly used in food products due to their lack of functionality. However, as a flavor carrier, rice protein shows much potential due to its lack of interaction with flavor compounds. In addition, the emerging trend of plant proteins might increase consumer demand and make these proteins more cost-effective to produce too.

Introduction

A common problem that the protein bar industry faces is that while many protein bars start out with desirable flavors, as the bar is stored over time, the flavor disappears and undesirable flavors emerge. Proteins play a significant role in the change of flavor. While proteins have little flavor on their own, they can interact with flavor compounds, decreasing the perception of the flavor compounds by consumers (O' Neill, 1996). Much of perceived flavor in food is due to aroma. Identifying what proteins are efficient at carrying these aromas for an extended period of time can help businesses maintain consumer acceptance even after long storage times. The amount of flavor compound that can bind to protein depends on the type, amount and amino acid composition of the protein tested, as well as the food matrix, such as the presence of lipids (O' Neill, 1996).

Whereas whey and soy proteins are commonly used as protein sources in protein bars, their allergenicity poses a problem for many. Soy proteins closely resembles proteins from related plants like peanuts, making cross-reactivity an issue (Kattan and others 2012). Plant proteins such as pea and rice protein can be a useful alternative due to their lack of allergic response.

The use of plant proteins has been increasing in popularity over the past few years, with plant protein projected to make up 50% of the alternative protein market by 2054 and named the biggest trend at the 2016 IFT Annual Meeting & Food Expo (Tarver 2016). Plant proteins not only appeal to vegetarians and vegans, but also consumers with dietary restrictions due to increasing food allergies (Sicherer and others 2009). These proteins also appeal to a growing mass of consumers who demand for more sustainability protein sources.

Plant proteins is an umbrella term for a wide range of proteins, such as algal protein, pulse protein, grain protein and seed proteins. For this project, pea protein was chosen as it has great potential for market growth and can be easily sourced. A recent survey conducted by Global Food Forums Inc showed that 88% of R&D professionals predict an increase in products made with pea protein, compared to 74% predicting an increasing in products made with other legumes (Tarver 2016).

Rice protein was also chosen, due to its availability as a staple cereal consumed around the world. It is also one of the grain proteins with the highest protein content (Tarver 2016). The organic rice protein market alone was worth USD 34.3 million in 2015 ("Organic Rice Market Analysis by products" 2016) and projected to reach USD 96.5 million in 2021 (Markets and markets 2016). The sports & energy nutrition already dominated over 85% of the organic rice protein market in 2015 ("Organic Rice Market Analysis by products" 2016), with rice protein is already being used in protein bars.

In this paper, when flavor compounds are said to be "lost", the author means that the flavor compounds are no longer detectable with the extraction method used. This can be due to either flavor compounds undergoing reactions with the protein bar, flavor compounds undergoing other reactions such as dimerization or oxidation, or volatilization of the flavor compounds during storage.

Background

Flavor Compounds

Nine flavor compounds were chosen to represent a range of functional groups, including an alcohol, thiol, ionone, aldehyde, lactone, sulfide and two ketones and two esters. These compounds were: allyl sulfide, isoamyl acetate, furfuryl mercaptan, benzaldehyde, methyl salicyclate, menthol, D-carvone, γ-nonalactone and trans-α-ionone). Each of the flavor compounds chosen is a key chemical in a given flavor. For instance, furfuryl mercaptan is the flavor compound primarily responsible for the character of coffee flavor. Benzaldehyde is the key component in both almond and cherry, as is menthol in mint. The flavor compounds used in this study are not just used in certain flavors, they are essential to these flavors.

Protein Structure

Whey Protein

Whey protein is typically made up of ~65% B-lactoglobulin, ~25% alpha-lactalbumin, ~8% bovine serum albumin (Haug and others 2007). However, the BiPro whey protein used in this experiment is mainly made up of B-lactoglobulin as it is produced using ion-exchange technology instead of the typical membrane method. Numerous studies have been done on B-lactoglobulin, especially on its 3D structure and its interactions with flavor compounds (O'Neill 1996). B-lactoglobulin has a hydrophobic core which appears to be the binding site for a range of nonpolar molecules including alkanes, ketones, 2-nonanone, free fatty acids, triglycerides, retinol, aromatic hydrocarbons and other structurally similar flavor compounds (Hansen 1996, O'Neill 1996). Little information is available on the types of residues and mechanisms of binding that can occur. However, X-ray crystallographic data indicates that there is a tryptophan residue in the interior of B-lactoglobulin molecule which can allow for nonpolar flavor molecules to bind (Papiz and others 1986). Besides tryptophan, aromatic amino acids such as phenylalanine and tyrosine can also bind with non-polar molecules. Whey protein typically undergoes the least amount of denaturation during the production process, unlike other proteins used in this experiment. Thus, the globular form of B-lactoglobulin and alpha-lactalbumin is quite retained.

Soy Protein

The major components of soy protein are the globulins are 11S (glycinin) and 7S (B-conglycinin) (Kinsella and others 1985) which makes up more than 80% of soy protein (Nishinari and others 2014). Although the amino acid compositions of glycinin and B-conglycinin has been analyzed, crystallization of the proteins is challenging which makes it difficult to exactly visualize their three-dimensional structures (Nishinari and others 2014). Although there is relatively less research done on flavor-soy protein interactions compared to flavor-whey protein interactions, some work has been done on soy proteins on their reversible and irreversible binding with various types of polar and non-polar flavor compounds; the types of interactions involved, and how relatively humidity affects binding (Gremlin 1974, Zhou and

others 2006). Most commercial soy protein isolates are denatured, as heating is a necessary step to inactivate trypsin inhibitors.

Pea Protein

Vicilin mainly of 11S legumin and 7S vicilin (Gharsallaoui and others 2010). Legumin is hexameric protein with polypeptides that have an acidic and basic subunit associated by disulfide bridges. Vicilin is a trimeric glycoprotein composed of polypeptides of various sizes (Yin and others 2015). Some research has been done on the interaction of homologous series of aldehyde and ketone compounds with PPIs, with much work done with the use of differential scanning calorimetry to detect the degree of unfolding and retention different aldehydes and ketones have on the protein. The effect of heat treatment on protein-flavor compound interactions have also been studied (Heng and others 2004). Most commercial pea proteins are denatured as a result of the isolation process and thus, have low solubility in water.

Rice Protein

Glutelins makes up about 75% of the protein in rice, whereas globulins made up about 15% followed by smaller amounts of albumin and prolamin (Agboola and others 2005). There is little literature available on the interaction of flavor compounds with rice proteins. Rice protein tend to aggregate very easily and is very insoluble in water. Its low functionality has made is unpopular for use in the industry.

Methods

Protein Bar Formulation

To simulate flavor-protein interactions in a protein bar, a protein bar matrix model had to be first created. The matrix model should be similar to a real protein bar in terms of water activity and in ingredients typically found in a protein bar, but without interfering ingredients like chocolate chips and peanut butter which can affect the reactivity of the flavor compounds. The goal was to observe the loss of flavor compounds due to their interactions with the protein elements of the bar, and not the interactions of flavor compounds with other ingredients in the bar.

Table 1. Protein Bar Matrix Formulation

Ingredient	**Percentage by weight (%)**
Protein Isolate (Whey, Soy, Pea or Rice)	47.0
Isomalto-oligosaccharide syrup	33.0
Triacetin	9.9
Water	9.0
Potassium Sorbate	1.0
Flavor Compound Mixture	0.1

The approximate proportion of ingredients simulates a real protein bar and is consistent with previous studies done on protein bar model systems (Zhou Peng et al., 2013).

Protein Isolates/Powders. Four different types of protein were chosen. BiPro Whey protein isolate was obtained from Davisco Foods International Inc (now Agropur, Le Sueur MN, USA). Soy Protein isolate, Pea Protein powder, and Brown Rice powder were obtained from NOW Foods (Bloomingdale, IL, USA).

Flavor Compounds. The nine flavor compounds were chosen based on their functional groups to showcase a range of types of organic compounds. Flavor compounds chosen include ketones, esters, a cyclic ester, an aldehyde, alcohol, thiol, and a sulfide. The flavor compounds were allyl sulfide, isoamyl acetate, furfuryl mercaptan, benzaldehyde, menthol, nonalactone, trans-α-ionone obtained from Sigma-Aldrich (St Louis, MO, USA), methyl salicylate obtained from Robertet Flavors Inc. (Piscataway, NJ, USA) and D-carvone obtained from Consolidated Chemical Solvents LLC (Quakertown, PA, USA). Each protein bar (35g) was formulated to contain 2.08 x 10^{-5} moles of each flavor compound, or about 90 ppm of the protein bar by mass.

Bar Ingredients. Isomalto-oligosaccharide syrup (IMO, Vitafiber ™) was obtained from BioNeutra North America Inc. (Edmonton, Alberta, Canada). IMO is typically used in protein bars as a binder to bind all the ingredients together. Other binders such as glycerol were also considered, but given glycerol's reactivity with flavor compounds, IMO was considered to be less reactive and a better choice.

Triacetin was used as an organic solvent to dissolve the flavor compound mixture in, so that the flavor compounds could be evenly dispersed throughout the bar. An alternative solvent, medium-chain triglycerides, was considered but its potential thermal degradation in the injection port or column during gas chromatography would be a problem. Triacetin, however, does not thermally degrade in the column and is also unreactive with flavor compounds, making it a more suitable solvent. Triacetin also elutes within the same time frame as the flavor compounds during gas chromatography, making it a viable internal standard to track the loss of flavor compounds over time.

Water was used to dissolve the potassium sorbate before it was dispersed, and to adjust the water activity to the desired level. Potassium sorbate was added as an antimicrobial agent, since mold growth proved to be a problem in earlier trials of the experiment. Lastly, the flavor compound mixture contained equal molar amounts of the 9 flavor compounds.

Making the bar

The flavor compound mixture was made by adding solid flavor compounds to triacetin and stirring till they dissolve. Liquid flavor compounds were added directly to the triacetin using a micropipette. To this flavor compound mixture, IMO was also added. Potassium sorbate was then dissolved in the deionized water, and this mixture was also added. This mixture was stirred with a stir bar on the highest setting to disperse the ingredients evenly.

Four beakers were set up with the protein isolate of choice. The mixture prepared above was poured into the beaker until it reached the target weight of 35 g per bar. The resulting mass was

stirred to evenly distribute the liquid within the solid matrix. Once mixed, the mass was transferred to an air-tight glass jar where it was stored, and the jar is placed in an incubator at 45°C until analysis.

Sampling and Extraction

Samples were taken at weeks 0, 1, 2 and 4. Assuming a Q10 of 2 at an elevated temperature of 45C, the samples correspond to storage at ambient temperature (25C) at 0, 1, 2 and 4 months.

The flavor compounds were extracted using Stir Bar Sorptive Extraction (SBSE). SBSE is a method of sample preparation and extraction of organic compounds from aqueous matrices, making it an excellence choice for extraction of flavor compounds which are organic compounds. SBSE is based on sorptive extraction, whereby the solute (flavor compound) is partitioned between the food matrix (protein bar) and the polymer coating on the magnetic stir bar (David & Sandra 2007).

Although Solid Phase Micro-extraction (SPME) is more commonly used to isolate flavor compounds from food matrices, SBSE was chosen as the extraction method of choice because it has a much larger extraction phase mass than SPME, giving a larger amount of sorptive extraction phase and thus a much higher sensitivity. Previous studies done on both methods showed that SBSE had a capacity that was 50 to 250 times larger than SPME, and was able to detect more types of volatiles such as terpenes and fatty acids compared to SPME (Benet et al., 2014). A high capacity is important for this study as multiple flavor compounds must be extracted. Thus, a higher mass of extracting phase will reduce competition between flavor compounds as they bind to the polymer coating on the Twister™, allowing more of each compound to be extracted and thereby increasing sensitivity.

At least ten samples were taken from various positions of the protein bar model (top and bottom, diagonally, sides) to form a composite sample of 1 g, which was then placed into a 25 mL Erlenmeyer flask. Ten mL of deionized water is added to disperse the protein bar. A commercial stir bar (Twister™, a magnetic stirring rod enclosed in a glass jacket and coated with PDMS) with a length of 10 mm and thickness of 0.5 mm was used to stir the mixture. The stir bar was obtained from GERSTEL (Linthicum, MD, USA). Extraction was performed at 1000 rpm on a hot plate stirrer PC-351 (Corning, Corning, NY) for 10 mins.

The same Twister was used for samples analyzed for Week 0, Week 1 and Week 2, while a different Twister was used for samples analyzed for Week 4 because the operator broke the first Twister. The different Twister was also used to analyze whey samples in Week 2. It is generally recommended that the same Twister be used throughout the experiment to maintain the same extraction efficiency, so this might have affected the data obtained.

During method development, several concentrations of protein bar: water and amount of time stirred was studied, and these extraction parameters were found to give the best results in terms of sensitivity in the GC in a relatively short extraction time. The 10 mm stir bar used was most suitable for stirring sample volumes from 10 – 50 mL, and typical stirring times for equilibration are between 30 and 60 min (Baltussen, 1999).

After extraction, the stir bar was removed, rinsed with deionized water three times to remove any residue from the protein bar (which can degrade in the GC), dried with a lint-free tissue, and thermally desorbed into a gas chromatograph.

Thermal Desorption and GC-FID Analysis

Analyses were performed with a Hewlett Packard HP 5890 Series II (Hewlett Packard, Palo Alto, CA) gas chromatograph coupled to a flame-ionization detector from the same company. Separation of flavor compounds was done using a DB-5 capillary column (30 m × 0.32 mm × 1 μm) made by Agilent (Santa Clara, CA), with helium as a carrier gas. The oven was programmed with an initial temperature of 40 °C, and a rate of 10°C/min up to 240 °C.

Mass Spectrometry

Although pure standards were ran on GC-FID to confirm the identity of peaks in the protein bars, GC-MS was also performed once on a soy protein bar sample to cross-check identity of the peaks. The protein bar sample was extracted the same way using SBSE, but the Twister bar underwent a cryofocusing step right before thermal desorption.

Data Analysis

The amount of each flavor compound was tracked over a period of 4 weeks, and any loss of the flavor compound was assumed to be due to the interaction between the flavor compound and the proteins in the protein bar. Peak areas of each flavor compound (from GC) was corrected with triacetin as an internal standard, to correct for small differences in injection volumes and extraction efficiency during different repetitions. Peak areas that have been corrected give the **A-values** (see calculation example in Table 2). Using A-values from Week 0 has a reference, the percentage of flavor compound remaining at subsequent weeks were calculated to give the **B-values** (see calculation example in Table 2).

Table 2. Example calculation for isoamyl acetate in pea protein bar

	Obtained from GC		**Calculations**	
Pea Protein Bar Sample #1	**Isoamyl acetate peak area**	**Triacetin peak area**	**A-value = (Ratio of flavor compound peak area/triacetin peak area) * 100**	**B-value = Percentage of original amount remaining**
Week 0	548497	2422020	(548497 / 2422020)*100 = 22.64	100%
Week 1	328402	2009490	(328402 / 2009490)*100 = 16.34	(16.34 / 22.64)*100 = 72.16 %
Week 2	177135	2481582	(177135 / 2481582)*100 = 7.13	(7.13 / 22.64)*100 = 67.64 %

A-values were used to compare the free amount of each aroma compound in soy, pea, and rice protein. Unfortunately, triacetin levels drastically decreased over storage for whey protein bars suggesting that reactions have taken place between triacetin the whey protein bars. Without

triacetin as an internal standard for whey protein, **B-values** had to be used to compare the relative rates of loss between whey protein and the other three proteins.

Results

Notes: A different stir bar was used at Week 4 for all protein bars due to a crack in the original stir bar used. For Week 2's whey protein samples, a cracked stir bar was used which gave falsely high values and thus omitted from analysis. Using a different stir bar might have altered the extraction efficiency of the stir bar, and caused what appears to be an increase in the amount of some flavor compounds instead of a decrease in various trials.

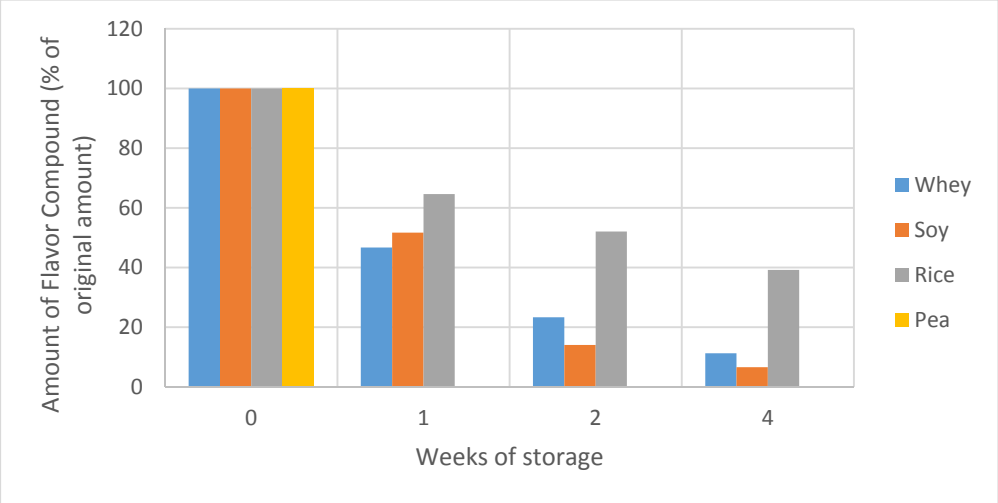


Fig. 1. Loss of allyl sulfide over 4 weeks of storage at 45 C

Allyl sulfide showed high percentage loss for all four protein bars, but the loss was much faster for pea protein (100% loss by Week 1). Soy protein showed the highest detectable amounts of allyl sulfide initially, but had a much higher rate of loss compared to rice protein. Rice protein initially had lower levels of detectable allyl sulfide, but the rate of loss was much slower. Although soy protein started out with the highest level of allyl sulfide, by Week 4, rice protein had the highest level of allyl sulfide. Thus, rice protein appeared to have the lowest rate of loss of allyl sulfide. Whey protein had a rate of loss that was slightly lower than soy protein. However, without the corrected peak area of allyl sulfide for whey protein, it is difficult to evaluate the initial amounts of allyl sulfide present at Week 0.

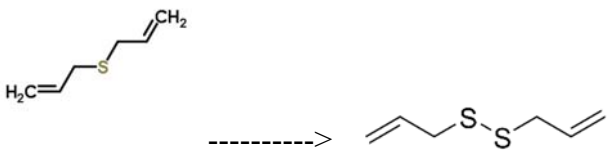


Fig 2. Allyl sulfide's oxidation into diallyl sulfide

Allyl sulfide could also oxidize into diallyl disulfide (Fig 2), as some diallyl disulfide was discovered in the sample through GC-MS. Allyl sulfide, due to its sulfur group, could also react

with methionine or cysteine in the protein bar through disulfide exchange which would reduce the amount detectable by SBSE-GC. However, pea protein actually has the least cysteine and methionine content out of all four proteins (Table 3), with soy protein having twice the amount of cysteine as pea protein and brown rice protein having almost triple the amount of methionine compared to pea protein. It is possible that a great deal of allyl sulfide-pea protein interaction is due to hydrophobic interactions instead of through the binding of the sulfur group.

Table 3. Amino acid composition of whey, soy, pea and brown rice protein used

Amino Acid	**BiPro ™ Whey Protein Isolate**		**NOW Foods™ Soy Protein Isolate**		**NOW Foods™ Pea Protein Isolate**		**NOW Foods™ Sprouted Brown Rice Concentrate**	
	mg/100g	% Total AA	mg/100g	% Total AA	mg/100g	% Total AA	mg/100g	% Total AA
Alanine	4561	4.9	3521	4.0	2952	4.1	5880	5.9
Arginine	1955	2.1	6621	7.5	6152	8.5	8320	8.3
Aspartic acid	10611	11.4	10308	11.7	8552	11.8	8480	8.5
Cysteine	2606	2.8	2642	3.0	1085	1.5	1760	1.8
Glutamic acid	14986	16.1	16521	18.8	12988	17.9	18460	18.4
Glycine	1582	1.7	3392	3.9	2988	4.1	4140	4.1
Histidine	1862	2.0	2692	3.1	1788	2.5	2320	2.3
Isoleucine	5212	5.6	3458	3.9	3315	4.6	4120	4.1
Leucine	11821	12.7	7521	8.6	6088	8.4	8740	8.7
Lysine	9494	10.2	5429	6.2	5448	7.5	3680	3.7
Methionine	2141	2.3	1258	1.4	639	0.9	2740	2.7
Phenylalanine	3258	3.5	3871	4.4	4006	5.5	5500	5.5
Proline	4375	4.7	4442	5.1	3158	4.3	4880	4.9
Serine	3072	3.3	4550	5.2	3782	5.2	5100	5.1
Threonine	4375	4.7	2879	3.3	2836	3.9	3720	3.7
Tryptophan	2699	2.9	1500	1.7	639	0.9	1100	1.1
Tyrosine	3351	3.6	3150	3.6	2712	3.7	5640	5.6
Valine	5026	5.4	4058	4.6	3585	4.9	5520	5.5

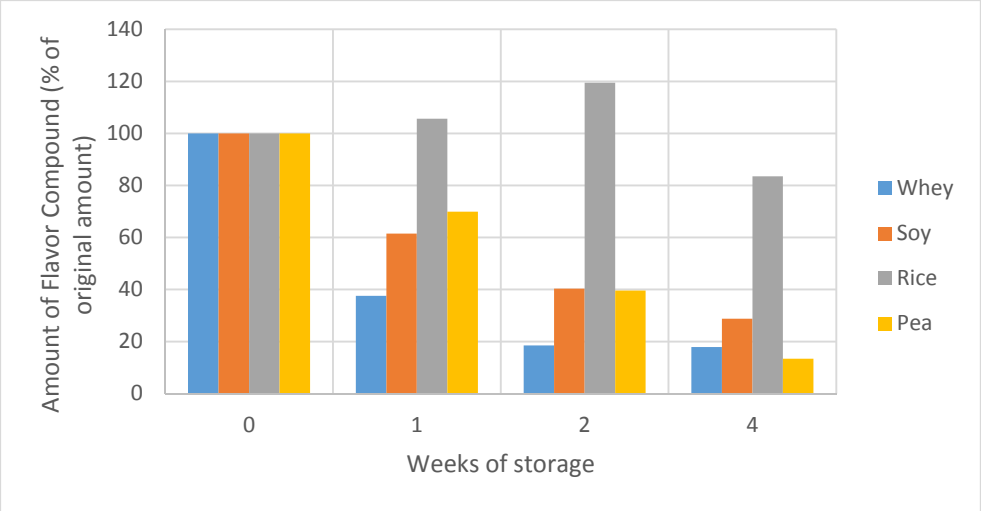


Fig. 3. Loss of isoamyl acetate over 4 weeks of storage at 45 C

Although soy protein started with the highest level of isoamyl acetate, rice protein had the highest level of isoamyl acetate by Week 4. Isoamyl acetate showed the highest rate of loss in whey protein, comparable rates of losses in soy and pea protein, and no loss in rice protein. This suggests that isoamyl acetate has low binding affinity for both soy and rice protein. Pea protein had a rate of loss similar to soy protein, but started out with a lower initial level of allyl sulfide. Whey protein showed the highest rate of loss of isoamyl acetate, suggesting that there is a high degree of interactions between whey protein and isoamyl acetate.

Overall, the degree of loss of isoamyl acetate was unanticipated, and unless conditions favored its hydrolysis into acetic acid and isoamyl acetate (Fig. 4), it is unlikely to occur. Hydrolysis could occur if the pH of the protein bar was low or high enough, and if there are viable lipases in the system.

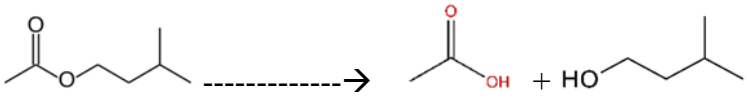


Fig. 4. Hydrolysis of isoamyl acetate into acetic acid and isoamyl alcohol

Both isoamyl acetate and allyl sulfide were predicted to be relatively stable flavor compounds. Thus, the relative decreases in detectability of allyl sulfide and isoamyl acetate were unexpected. This might be attributed to the vaporization of the compounds into the headspace of the glass jars during storage, and then the loss of that flavor compound into the atmosphere as the container is opened. This is especially the case with allyl sulfide and isoamyl acetate, both of which have the lowest boiling points out of all the flavor compounds tested. There could also be unintended interactions between the two flavor compounds and other flavor compounds.

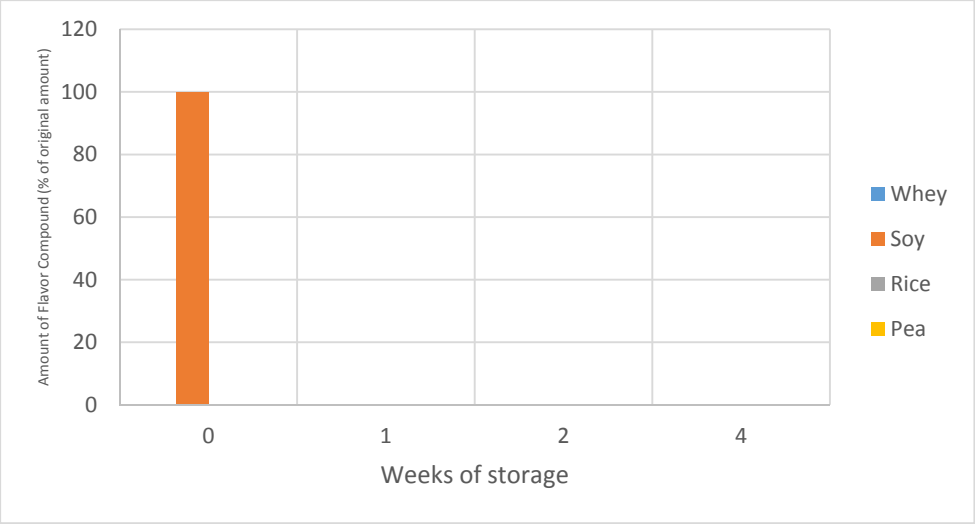


Fig. 5. Loss of furfuryl mercaptan over 4 weeks of storage at 45 C

As early about 4 hours after making the protein bars and when GC was performed, no furfuryl mercaptan was detectable in any of the four protein bars except for soy protein, in which it was no longer detectable by Week 1.

It is possible that the rate of reaction between furfuryl mercaptan and the proteins could be so fast that in the few hours in between the preparation of the bar and the first GC run, furfuryl mercaptan could have been lost. However, it is also likely that furfuryl mercaptan could have undergone dimerization with itself to form compounds such as difurfuryl disulfide through oxidation and radical reaction (Fig. 6.), since difurfuryl mercaptan was detected in small amounts through GC-MS. If the main reason for loss was due to dimerization, the amount of difurfuryl mercaptan should be equivalent to the amount of monomer lost.

Fig. 6. Dimerization of furfuryl mercaptan into difurfuryl disulfide

The hydrogen atom of the thiol group can be easily abstracted, and they are easily oxidized to disulfide (Hofmann and others 1996) via Fenton-type reactions, which can happen within 1 day at 6°C and would increase at increased temperatures (Weerawatanakorn 2015). A separate study showed that 20% of furfuryl mercaptan in aqueous solution was lost in 1 hour at room temperature, whereas ~90% was lost in 1 hour at 37°C (Blank and others 2002), signifying the role that temperature plays in the loss of furfuryl mercaptan, especially since the storage study was conducted at 45°C for this project. The major degradation products of such reactions were difurfuryl disulfide, followed by furfuryl and furfuryl alcohol (Weerawatanakorn 2015).

A similar compound, 2-methyl-5-methyltihofuran (Fig. 7) was also detected in GC-MS in miniscule amounts but it is unclear what the mechanism would be for furfuryl mercaptan to

transform into this compound, and in fact, 2-methyl-5-methylthiofuran could have been an impurity in the vial of furfuryl mercaptan used.

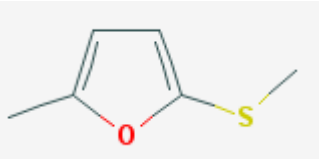


Fig. 7. Molecular structure of 2-methyl-5-methylthiofuran

It is hard to determine if the loss of furfuryl mercaptan was due to self-dimerization or because of reaction with amino acids such as cysteine and methionine, both of which also have a thiol group. If the latter occurs at a significant enough amount, then proteins such as soy protein which has a significantly higher percentage of cysteine in its amino acid profile should show a higher affinity for furfuryl mercaptan and thus a higher loss of furfuryl mercaptan compared to other proteins. The thiol groups from cysteine or methionine could form disulfide bridges with furfuryl mercaptan, rendering them unavailable for detection. However, the opposite was actually true, with soy protein showing the lowest loss of furfuryl mercaptan. Furfuryl mercaptan was detected in soy protein but not the other proteins, however this was at a minute amount (about 100-fold less than other flavor compounds), and might not be significant enough.

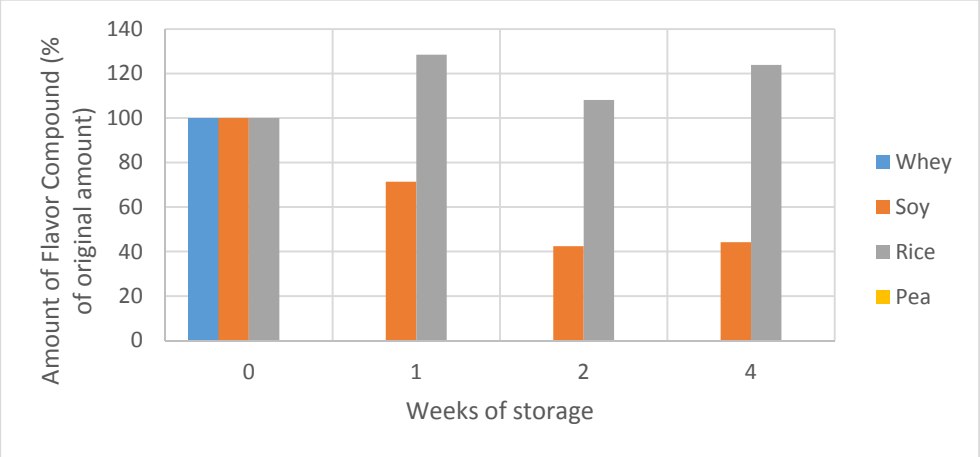


Fig. 8. Loss of benzaldehyde over 4 weeks of storage at 45 C

Benzaldehyde could not be detected in pea protein at all from Week 0 onwards, and showed 100% loss for whey protein by week 1. It decreased by about 50% in soy protein by week 2, but didn't seem to show much decrease after, possibly due to all reactive sites being saturated. Benzaldehyde showed an increase in rice protein. The increase can be attributed to a high degree of error and also due to a change in stir bar efficiency, as a different stir bar was used in Week 2 and Week 4. It can be assumed that very little loss of detectable benzaldehyde occurred in rice protein.

Rice protein and soy protein showed significantly less loss of benzaldehyde, suggesting that the two proteins have lower binding affinities for benzaldehyde. This could be due to the structural differences in the proteins compared to whey and pea, or it could be due to the amino acid makeup. For instance, rice and soy protein has significantly less lysine compared to the other two proteins, with rice protein having only 3.7% lysine but whey protein having 9.7% lysine (Table 3). Lysine has an ε-amino group which can be very reactive through Maillard reaction.

Pea had significantly less methionine compared to the other three proteins, which should result in less protein-flavor interaction assuming thiol-aldehyde interactions occur. The proposed lack of binding interactions should have led to more detectable benzaldehyde, which unfortunately was not the case here.

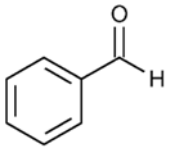


Fig. 9. Molecular structure of benzaldehyde

Although aliphatic aldehydes typically associate with protein amino groups by binding covalently (Stapelfeldt and others 1994), Marin and others (2000) discovered through tryptophan spectrofluorimetry that B-lactoglobulin and benzaldehyde associates through a non-covalent binding interaction instead. Andriot and others (1999) observed through HPLC with radiometric detection that benzaldehyde binds strongly to B-lactoglobulin, but the use of Raman, IR and electro-spray MS indicates that these were not covalent linkages (Relkin and others 2000).

Based on these previous studies, it could be possible that benzaldehyde-protein interaction is mostly through non-covalent interactions such as hydrophobic interactions, unlike typical covalent interactions that aldehydes and proteins usually go through. Thus, protein such as B-lactoglobulin (in whey protein) which can bind hydrophobic compounds in its central calix (Monaco and others 1987, Papiz and others 1986) could show higher affinity for benzaldehyde. Similarly, amino acid profiles with more hydrophobic amino acids might also promote the binding of benzaldehyde.

A sensory test showed that benzaldehyde showed a significant drop in flavor intensity as WPC concentration increased from 0 to 0.5%, whereas citral, also an aldehyde, showed no significant drop in flavor intensity as WPC increased from 0 to 0.5% (Hansen and others 1996). Although both are aldehydes, benzaldehyde seems to be more reactive than other aldehydes with proteins. Using the hydrophobic interaction theory above, Citral (C10) should technically be more reactive with the B-lactoglobulin since it has a higher molecular weight than benzaldehyde (C7), however the opposite is true. It seems that other factors such as the presence of a benzene ring on benzaldehyde and the conformational structure of benzaldehyde could render it more reactive than other aldehydes.

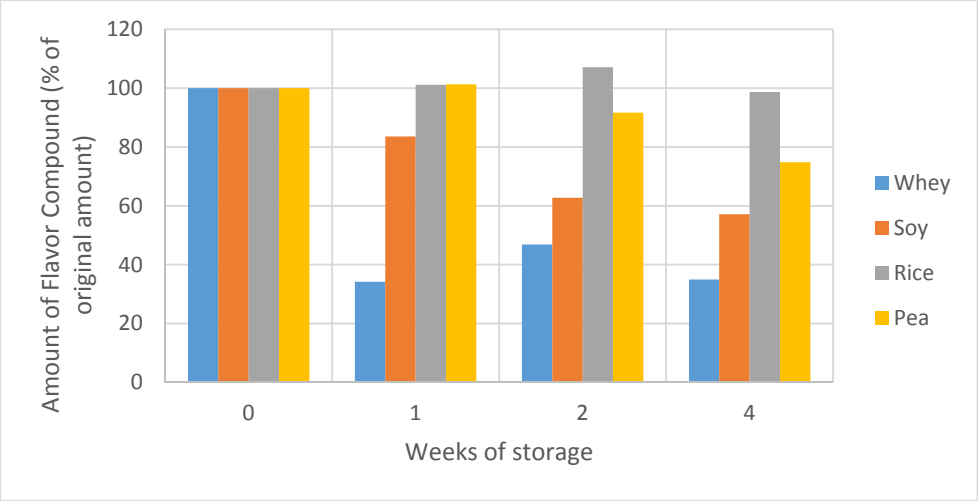


Fig. 10. Loss of eucalyptol over 4 weeks of storage at 45 C

Eucalyptol was a compound that was not added to the protein bar, but appeared in GC-FID peaks and was identified through GC-MS. It could be due to impurities in the flavor compounds used. It is structurally similar to D-carvone, and could have present in the D-carvone since the D-carvone bought was not chemical grade. Eucalyptol showed the highest flavor retention in rice and pea protein, less in soy protein and the least in whey protein.

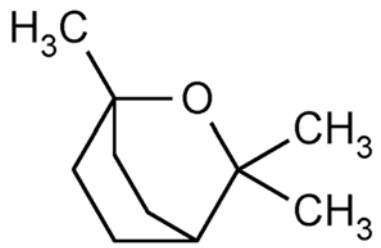


Fig. 11. Molecular structure of eucalyptol

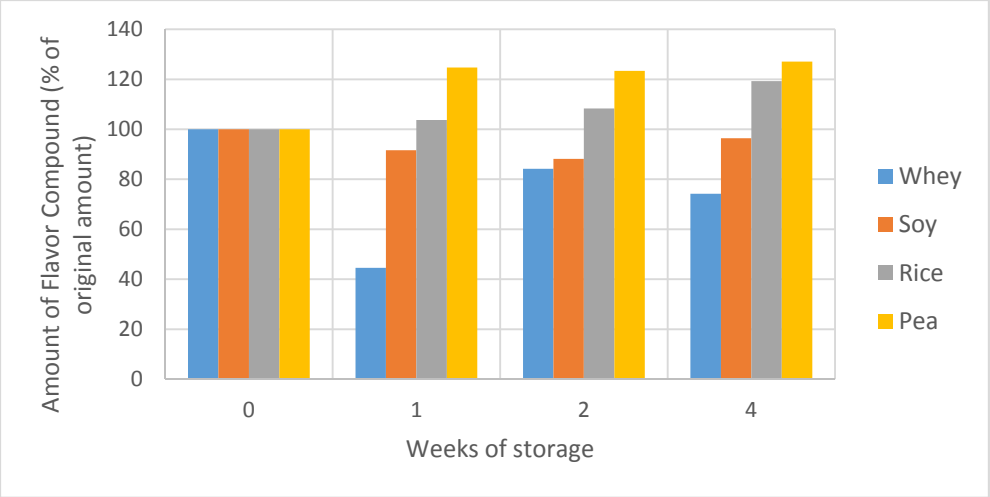


Fig. 12. Loss of menthol over 4 weeks of storage at 45°C

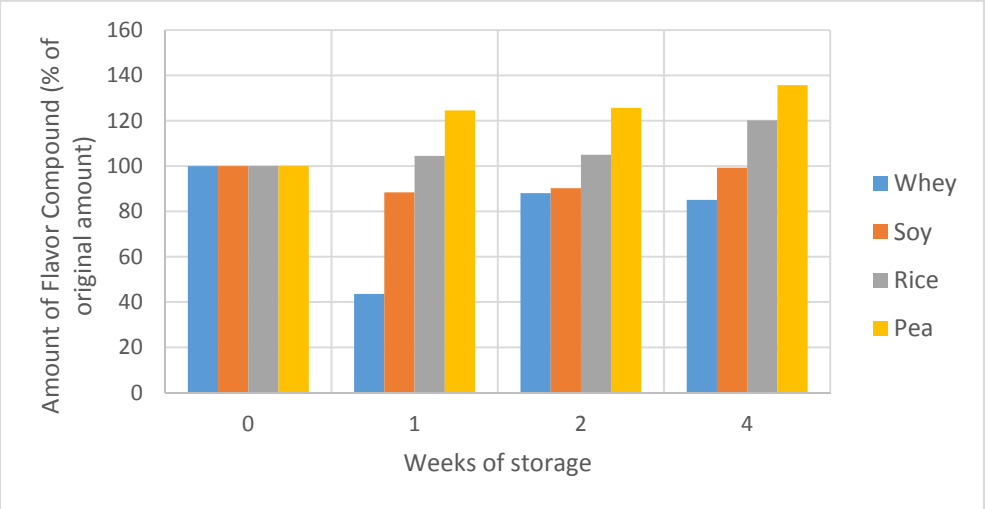


Fig. 13. Loss of methyl salicylate over 4 weeks of storage at 45 C

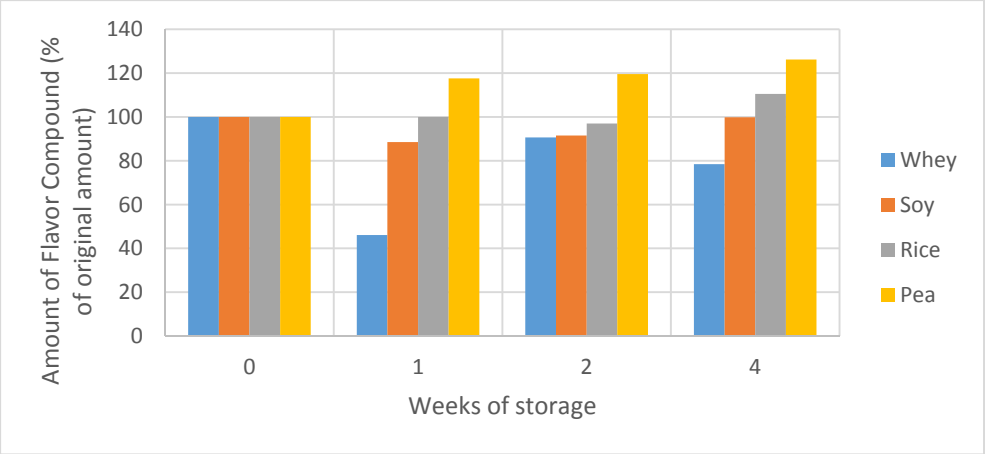


Figure 14. Loss of D-carvone over 4 weeks of storage at 45 C

Menthol had a similar loss profile to methyl salicylate and D-carvone (Figure 8, 9, 10). For these three flavor compounds, rice, soy, pea and possibly whey protein showed similar rates of losses. The initial amounts of the three flavor compounds was highest in soy protein, with comparable amounts in rice and pea protein.

Despite the alcohol group on menthol, it did not seem particularly more reactive than methyl salicylate or D-carvone with all four proteins. Methyl salicylate and D-carvone could be resonance-stabilized (Figure 11), making it less prone to covalent interactions with amino acids.

A previous study done by O'Neill (1996) showed that the binding constants for aliphatic ketone were almost 2.5 times higher for B-lactoglobulin compared to soy protein (O'Neill, 1996). Although D-carvone is a cyclic ketone, it might exhibit similar binding patterns and have higher binding affinity for B-lactoglobulin since the loss of D-carvone in whey protein is greater than the loss in soy protein (Figure 10).

Despite the alcohol group on menthol, it does not seem to be very reactive with any of the proteins with the exception of whey protein. This is unsurprising, as previous studies done by Gremli (1974) showed that in a 5% aqueous soy protein solution with 40 ppm flavor compounds, none of the alcohols reacted with the proteins whereas aldehydes and ketones showed significant interactions with the protein.

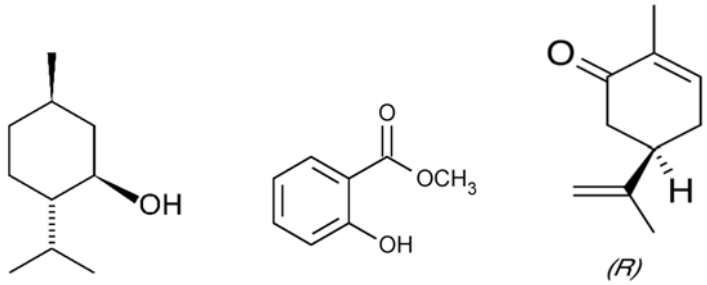


Fig. 15. Molecular structure of menthol, methyl salicylate and D-carvone, respectively.

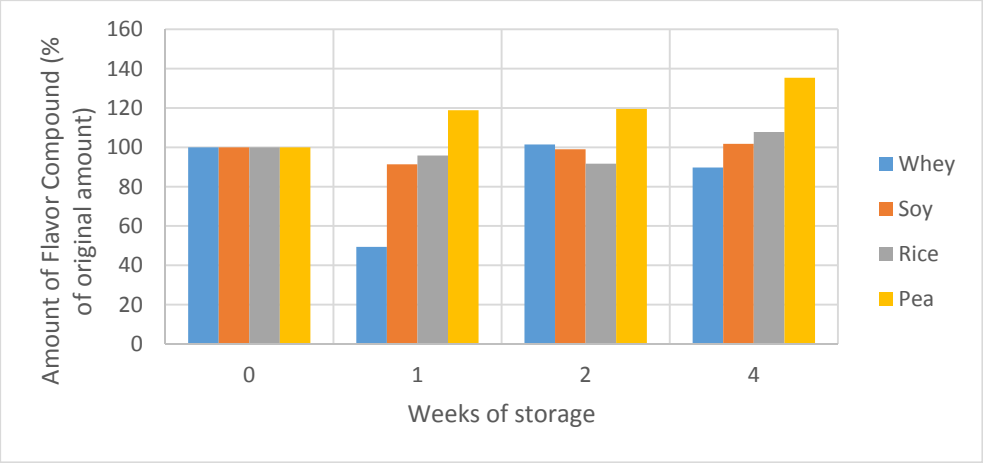


Figure 16. Loss of γ-nonalactone over 4 weeks of storage at 45 C

γ-nonalactone showed high flavor retention in all proteins, and potentially slightly higher in pea protein. Pea protein has significantly less % methionine than rice protein, and a bit lesser than whey and soy (Table 3). Pea protein also has the lowest % cysteine compared to all other proteins (Table 3). Less sulfhydryl nucleophiles on the methionine and cysteine of pea protein can result in less interaction with γ-nonalactone.

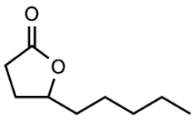


Fig. 17. Molecular structure of γ-nonalactone

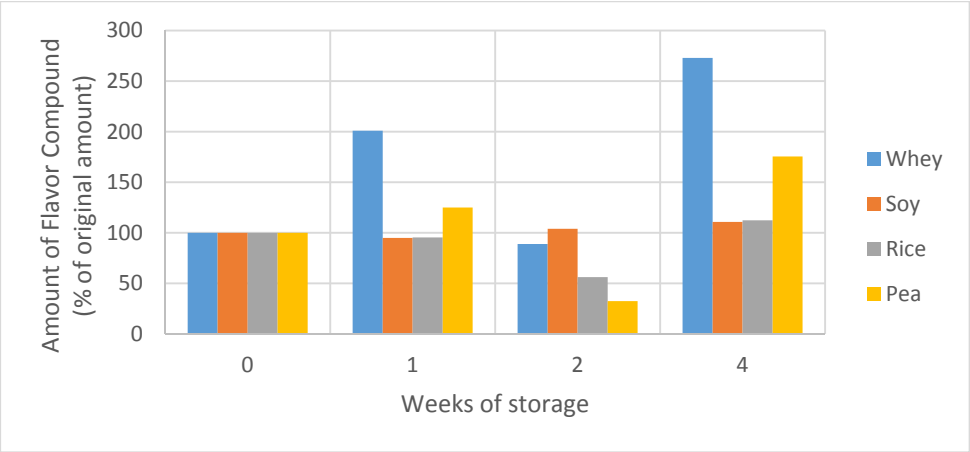
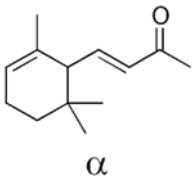


Figure 18. Loss of trans-α-ionone over 4 weeks of storage at 45 C

Soy, rice and pea protein showed minimal losses of trans-α-ionone over the storage period. One reason is due to the extremely low levels of trans-α-ionone detected during GC. Trans-α -ionone

had one of the lowest peak areas for all of the flavor compounds, making it more susceptible to small fluctuations in sensitivity during detection and data points with lower precision.



α

Fig. 19. Molecular structure of trans-α-ionone

Discussion

Effect of Protein Type

Rice protein. Rice protein showed the slowest rate of loss of flavor compounds amongst all proteins, making it a relatively stable flavor compound carrier. Its only drawback is that it is second to soy protein in terms of having the least amount of flavor compound bound initially in Week 0. Rice protein also appears to have significantly less binding affinity for allyl sulfide, isoamyl acetate, benzaldehyde compared to the other proteins, leading to lesser loss and higher flavor retention to these three flavor compounds. The low reactivity of rice protein with allyl sulfide could be due to rice protein's low cysteine content (1.8%) compared to soy (3.0%) and whey (2.8%). What is particularly interesting is rice protein's lack of interaction with benzaldehyde, which was very reactive with pea and whey protein. Rice protein has the slowest rate of protein-flavor compound interactions over storage time, which can make it a suitable flavor carrier for protein products that are going to be stored for long periods of time before consumption.

Overall, rice protein did not interact with other flavor compounds as much as compared to other proteins. With flavor compounds of low molecular weight, rice protein showed lesser interactions compared to other proteins. It performed just as well with flavor compounds of higher molecular weights, but was slightly worse than pea protein. Rice protein has a high proportion of hydrophobic amino acids (Table 4), which might lead to more hydrophobic interactions with flavor compounds, but can also lead to rice protein's tendency to aggregate with itself instead. Traditionally, rice protein's tendency to aggregate has made it unpopular due to the lack of functionality. However, this might also mean that rice protein is suitable for retaining flavor compounds since it prefers binding to itself than to flavor compounds.

Another reason for the low levels of interaction might be because the rice protein used was a concentrate, while all the other proteins used were isolates. Concentrates tend to have a lower protein content than isolates, which may mean that less rice protein was available to bind to flavor compounds. However, given that the flavor compounds were added in such miniscule amounts in comparison to the amount of protein added, a slightly lesser amount of binding sites on the protein should not be a big factor in the extent of binding interactions between rice protein and flavor compounds.

Table 4. Percentage distribution of charged, polar and hydrophobic amino acids

	BiPro™ Whey protein Isolate	**NOW Foods™ Soy Protein Isolate**	**NOW Foods™ Pea Protein Isolate**	**NOW Foods™ Sprouted Brown Rice Concentrate**
Charged	39.8	44.3	45.6	38.9
Polar	21.6	21.3	18.5	22.4
Hydrophobic	38.5	34.5	35.9	38.7

Pea protein. Pea protein seems to have a high degree of interaction with allyl sulfide, furfuryl mercaptan, benzaldehyde and trans-ionone, making it an unsuitable carrier of flavors that depend strongly upon these compounds. Pea protein has the highest percentage of charged amino acids (45.6%) compared to others, such as rice (38.9%) and whey (39.8%). The high degree of interactions between pea protein and allyl sulfide and furfuryl mercaptan was surprising, given the low levels of cysteine and methionine in pea protein. Perhaps the high percentage of charged and polar amino acids have led to a high degree of hydrogen bonding between the amino acid and allyl sulfide and furfuryl mercaptan. Besides these flavor compounds however, pea protein in general showed extremely low levels of interaction with other flavor compounds.

Pea protein seems to have low binding affinity for longer chain molecules, indicating perhaps that pea protein has an amino acid profile that does not promote as much hydrophobic interactions compared to other protein types, possibly due to its low percentage of hydrophobic amino acids (Table 4). Additionally, pea protein has a low percentage of two aromatic hydrophobic amino acids, tryptophan and threonine (Table 3). Pea protein has only 0.9% tryptophan and 3.9% threonine, compared to 2.9% and 4.7% in whey protein. If tryptophan is the main amino acid responsible for the binding of non-polar flavor compounds in B-lactoglobulin, it is possible that tryptophan also takes part extensively in hydrophobic interactions in pea protein. The low percentage of aromatic amino acids indicate that less aromatic stacking can occur in pea protein, which might explain pea protein's extremely low levels of interaction with flavor compounds of high molecular weight, the only exception being trans-α-ionone. Similar to pea protein, soy protein has a tendency to aggregate. Its preference for interacting with itself might be why it shows little interaction with flavor compounds.

Soy Protein. Although soy protein exhibited the least amount of binding with all the flavor compounds initially in Week 0, its rate of interactions with flavor compounds increased much faster than rice protein for these compounds: allyl sulfide, isoamyl acetate, furfuryl mercaptan, benzaldehyde.

Soy protein interacted less with flavor compounds of higher molecular weight, and interacted more with flavor compounds of lower molecular weight. Soy has the lowest percentage of hydrophobic amino acids compared to other proteins (Table 4), which might explain why it interacts less with flavor compounds of higher molecular weight and longer chain length. On the other hand however, most commercial soy protein on the market is denatured, compared to whey protein isolate, in which denaturation is markedly less. Denaturation of soy protein would theoretically expose more hydrophobic amino acid groups and allow for more hydrophobic interactions to happen. The interactions that occurred between soy and other flavor compounds were lower than expected for soy protein, given how denatured it was. This could be due to the soy protein bar's relatively low water activity of 0.66, compared to other protein bars that were between 0.77 - 0.84 in water activity (Table 5). Zhou and others (2006) found that although the binding of non-polar flavor compounds were not affected by different relative humidity levels, the binding of polar flavor compounds with soy protein isolate was reduced when relative humidity increased, suggesting that water compete with the flavor compounds for high-energy

binding sites on the protein. The soy protein bar's relatively lower water activity could have allowed more free binding sites on the protein for flavor compounds.

Table 5. Water Activity of Protein Bars

Protein	Water Activity
Whey	0.823
Soy	0.659
Pea	0.773
Rice	0.835

There was little interaction between soy protein and menthol, which is consistent with literature that states soy protein does not really react with alcohols (Gremli 1974). Past studies have showed that aldehydes, especially unsaturated aldehydes, react strongly with soy protein (Gremli 1974). Accordingly, there is a marked decrease of free benzaldehyde over 4 weeks of storage to about half the original amount (Fig. 6) in soy protein. However, soy protein performed much better than whey and pea protein, both of which had no detectable benzaldehyde as early as in Week 1. One possible reason could be that for aldehydes, a certain percentage tends to be irreversibly bound to soy protein but a greater portion engages in reversible binding to soy protein (Gremli 1974). Gremli (1974) noted that flavor compounds that are reversibly bound could be protected against loss and then gradually released as the consumer chews on the food. It is possible that the SBSE method utilized is unable to extract the flavor compounds bound reversibly to the protein, so any flavor compounds that appear to be "lost" might actually not be lost. This quality might make soy protein unsuitable for use in protein beverages but might be a good way to retain flavor in foods that require mastication, such as protein bars, as they can be released gradually during consumption.

Whey protein – Whey protein performed the worst, with overall the fastest rate of flavor compound loss amongst all four proteins. Whey protein showed much higher reactivity compared to most other proteins with compounds of lower molecular weight, such as allyl sulfide, isoamyl acetate, benzaldehyde and eucalyptol. As explained in the methods section, the relative initial amount of flavor compounds unbound to the whey protein cannot be compared with other proteins. Data analysis only gave us whey protein's rate of loss and amount of loss as a percentage of the initial level.

BiPro whey protein isolate from Davisco was used for this experiment. BiPro WPI is very hydrophilic on the surface. In comparison, the three other proteins are rather hydrophobic as the commercial production process renders them quite hydrophobic on the surface. However, if hydrophobic interactions are the main form of interactions between proteins and flavor compounds, whey protein should technically have less interactions with flavor compounds and better flavor compound retention.

During storage, bars made with whey protein were the only bars to turn a much darker shade of brown as early as in Week 1. The whey protein bars also turned perceptibly harder in texture compared to bars made with other protein. It is possible that Maillard reaction has taken place

between the whey protein and oligosaccharides (present in the VitaFiber™ syrup used to bind the bar together). It is unclear why this would happen to a much greater extent compared to other proteins.

Effect of Chain Length

The results of this experiment suggest that the longer the chain length of the flavor molecule, the less it interacts with the protein, and the higher the flavor retention rate. Smaller flavor molecules might interact with proteins more since they can conform to the protein better, especially if these interactions require the flavor molecules to bind to the core of the protein molecule, such as how hydrophobic flavor compounds and fatty acids tend to bind to the central calix of B-lactoglobulin (Brownlow and others 1997, Qin and others 1998, Wu and others 1999).

On the other hand, extensive studies done on the effect of chain length on ketones and aldehydes have shown that the longer the chain length up to a certain level, the higher the degree of interaction between the protein and the ketone/aldehyde (Damodaran and others 2014, Gremli 1974), possibly due to more extensive hydrophobic interactions between the flavor compounds and the protein (Wang and others 2014). This was observed across several types of proteins, including canola, pea and wheat proteins (Wang and others 2014, Heng and others). This effect was observed not just in 5% soy protein aqueous solution but also 50% soy dough, which might make it applicable to a protein bar matrix system too (Gremli 1974).

This past work appears to be in direct conflict with the results of this experiment, in which compounds of higher molecular weight actually showed less interactions with protein. However as mentioned, the increasing in binding interactions as chain length increases is only true up to a certain level. For instance, ketones' retention rate increase up to 2-decanone (C10) and started decreasing with larger compounds (Gremli 1974). The longer chain flavor compounds used in this experiment ranged from C9 (D-carvone) to C13 (trans-α-ionone), which would theoretically show a decrease in binding affinity and experimentally appeared so too.

The chain-length effect has only been observed mostly in aliphatic ketones and aldehydes. The effects of chain length on the binding affinity of cyclic ketones and aldehydes as well as the other types of flavor compounds have not been studied as extensively. However, if the increase in binding affinity is mainly due to more extensive hydrophobic interactions, it would suggest that even for flavor compounds that are not ketones and aldehydes, longer chain length would lead to more extensive hydrophobic interactions and higher binding affinity as well.

Effect of functional groups

Ketones, aldehydes, alcohols. While binding of nonpolar flavor compounds to soy proteins was attributed to mostly to van der Waals forces, more polar flavor compounds including ketones, aldehydes and alcohols, showed both nonspecific van der Waals forces as well as specific forces such as hydrogen bonding and dipole forces (Zhou and others 2006). Although the study pertained to soy protein, it could possibly be extrapolated to whey, rice and pea proteins, but can only be confirmed by more extensive investigation.

Zhou and others (2006) showed by using heat of adsorption that 1-hexanol, hexanal and 2-hexanone showed respectively a decrease in strength of interaction with soy protein. They claimed that that the strongest interaction forces were observed for alcohols compared to aldehydes and ketones due to the possible electron donor and acceptor role of the hydroxyl group and possible hydrogen bonding. However, Gremli (1974) showed that alcohols have low reactivity with soy proteins in 5% aqueous solution. There seems to be conflicting information on the strength and types of interactions observed between alcohols and different protein groups, which is definitely an area that should be more researched. In this experiment, menthol, the only alcohol used, showed very little interaction with all four of the proteins, which was contrary to Zhou and others (2006) but similar to what Gremli (1974) had found.

Although the data on alcohols is not always congruent, aldehydes consistently showed higher binding affinity than ketones in pea proteins, with aldehydes having a binding affinity 2 – 5 times higher than ketone. (Wang and others 2014, Heng and others 2004, Gremli 1974). This was observed in canola, pea and wheat proteins through GC/MS, and might be applicable to all the proteins studied in this project, all of which are globular proteins. Gremli's (1974) study also showed that unsaturated aldehydes had higher flavor retention than unsaturated aldehydes.

Similar to what has been observed in literature, the only aldehyde flavor compound used in this study, benzaldehyde, had one of the highest rate of loss for all four proteins except for rice protein, suggesting there is a high binding affinity between aldehydes and whey, soy and pea protein. The two ketones used, D-carvone and trans-α-ionone, showed significantly lower losses. D-carvone exhibited almost complete flavor retention in all four proteins, and trans-α-ionone showed almost complete retention in soy and rice protein.

Thiols. Previous research has shown that volatile flavor disulfides can partake in in disulfide interchanges with ovalbumin (a type of protein in egg white), and that disulfides containing furfuryl groups were more reactive than saturated alkyl disulfides (Adams and others 2001). Although furfuryl mercaptan is a thiol and not a disulfide, the sulfhydryl group has a similar structure that likely allows it to undergo disulfide interchanges with proteins and be more reactive than other sulfides because of the furfuryl component. This is consistent with this experiment, where furfuryl mercaptan was undetectable past Week 0. That said, thiols also have low stability in aqueous solutions as the hydrogen atom can be easily abstracted, and the furfuryl mercaptan used could easily be oxidized into furfuryl disulfide via Fenton-type reactions (Weerawatanakorn 2015). Thus, the loss of furfuryl mercaptan could be due to Fenton-type reactions occurring when the protein bar was dispersed and stirred in water during SBSE, instead of due to furfuryl mercaptan binding with the proteins.

Esters. Although there has been some investigation on the interactions between carbonyl compounds and proteins, little of that research included esters. However, structural comparisons suggest that esters are more stable than other carbonyls like ketones and aldehydes. Esters are less nucleophilic than ketones as esters have more resonance-stability due to the two oxygen groups that can partake in sharing charges. Results from this experiment were mixed. One of the esters used, isoamyl acetate, showed moderate amount of interaction with all proteins except for rice. The other ester, methyl salicylate, showed little interaction with all proteins. In this

scenario, chain-length and the degree of hydrophobic interactions might have been bigger determinants of flavor stability.

Lactones. Little research has been done on the interaction of protein with flavor compounds that are lactones.

Experimental Design Improvements

For future purposes, areas of improvement that would lead to more reliable results were identified. Improvements included maintaining the same water activity for the protein bars, relative humidity of storage environment, consistent pH across bars, and the addition of other analytical techniques in combination with SBSE. These external conditions can induce protein conformational changes which will affect the binding of flavor compounds, and should be controlled so that the change in flavor compound retention is due to the protein used and not due to external conditions.

Water and moisture. Water activity of the protein bars was found to range from 0.659 for soy protein to 0.835 for rice protein (Table 5), despite the same amount of water used for each formulation. The range of water activity is similar to that of commercial protein bars on the market, which ranges in moisture content from 10% to 30% and water activity from 0.6 to 0.8 (Taoukis & Labuza 1996). However, an a_w of 0.659 and 0.835 are still quite different and would result in different amounts of water available for interaction with the protein and flavor compounds. At different relative humidity, the differences in binding due to functional groups are also much less pronounced between soy protein and polar compounds (Zhou and others 2006). The effect of stereochemistry of flavor compounds and protein molecules on their binding interactions was of lesser impact than the relative humidity in Zhou and other's experiment, which heightens the level of impact water and water activity can have on protein-flavor interaction.

Moisture content is one of the factors that affect the conformation of the protein molecules, which can lead to changes in the binding of flavor compounds. Through the use of X-ray crystallography and fluorescence spectroscopy, O'Neill (1996) monitored the conformation changes in protein structure and found that exposure to aqueous solvent can result in the partial unfolding of the B-lactoglobulin molecule, which can expose the hydrophobic core and reduce the affinity of the protein for nonpolar molecules. This means during the SBSE extraction step which includes dispersing the protein bar in water and stirring with the Twister for 10 minutes, the hydrophobic core of the proteins used would be exposed and nonpolar molecules that are bound to the protein have a chance to unbind. As a highly polar compound, water is also able to displace polar flavor compounds on the polar binding sites on the protein (Zhou and others 2006). This means that during SBSE, water molecules can substitute polar flavor compounds on the binding sites of protein, resulting in the polar compounds that were previously bound to the protein to now become free which can go into the SBSE phase. This results in an over-estimation of "free" polar flavor compounds, when more could have been bound to the protein. This might

not be representative of human perception of the more polar flavor compounds, as we don't necessarily chew our food for as long as 10 minutes to release the polar flavor compounds.

Use of SBSE vs Headspace. The method of extraction used (SBSE) assumes that whatever flavor compound is bound on the protein stays on the protein and what is not bound then gets adsorbed onto the PDMS. In reality, flavor compounds might bind to the protein bar but when hydrated with water, it can unbind again due to the exposure to water, as the highly polar water molecules are able to displace flavor compounds that might have bound. Headspace analysis could be a better option, but a serious drawback is its lack of sensitivity, thus requiring high concentrations of flavor compounds to be added to the protein bar. In comparison, SBSE has a much lower detection limit which allows for more representative amounts of flavor compounds to be added during formulation.

Another flaw of the experimental design is that during storage, more volatile flavor compounds such as the shorter chain flavor compounds could have equilibrated into the headspace of the container since a glass container was used to hold the protein bar and only half of the container was filled. Upon opening the glass jar, flavor compounds that was unbound to the protein but also highly volatile could be "lost" to the atmosphere, and thus were not extracted during SBSE resulting in low detection during GC. This could have led to an over-estimation of the flavor compounds bound to the protein. A better method might be to store the protein bars in smaller portions in headspace analysis jars, and to analyze these jars directly through headspace GC.

Other methods of analysis. Quantitative analysis of flavor compound loss through the use of headspace-GC-MS can be combined with the monitoring of conformational changes in protein through X-ray crystallography or fluorescence spectroscopy might give more reliable results. Fluorescence spectroscopy has been used in various studies of interactions between flavor compounds and B-lactoglobulin to track the binding of flavor compounds to tryptophan residues, which are the primary source of fluorescence emission (Marin and others 2000, O'Neill 1996). Tryptophan residues reside in the calix of the B-lactoglobulin, and was deemed the most likely site for the binding of nonpolar molecules, so the reduction of fluorescence emission is associated with the binding of nonpolar molecules in the calix of the B-lactoglobulin (O'Neill 1996). Fluorescence quenching was exhibited with the binding of B-ionone, retinol, fatty acid lactones, curcumin, retinol, benzaldehyde, aliphatic aldehydes and methyl ketones (Dufour and others 1990, Muresan and others 2001, Lakowicz 1999). Whereas the use of tryptophan spectrofluorimetry is more relevant to B-lactoglobulin, other spectroscopy and assays can be used to track the binding sites in other proteins.

Lastly, sensory evaluation will perhaps be the most effective and reliable way to test for the loss of flavor compounds in protein bars over time, due to the complex food matrices which is difficult to predict with a protein bar model. The drawback of sensory evaluation is getting quantitative data. Furthermore, protein conformational changes can occur during mastication due to the aqueous environment and presence of electrolytes in the saliva, all of which can affect the amount of flavor compounds that are "set free".

Accelerated Shelf Life. Due to a lack of time, an accelerated shelf life study was performed over the course of one month at 45 C. Given that temperatures as low as 41°C can denature proteins, storage at elevated temperatures over an extended period could induce conformational changes in the proteins, especially globular proteins, which is what predominantly makes up all four of the proteins. At higher temperatures, globular proteins such as B-lactoglobulin can unfold and allow more flavor compounds to bind to the protein and reduce the amount available for consumer perception (Hansen and others 1996). This means that storage at elevated temperatures could cause an overestimation of the amount of flavor compounds that are lost to protein interactions. The conformational properties of the proteins might not be representative of how the proteins behave at actual storage conditions in warehouses at consumers' homes. However, given how processed the proteins used in this study are, the extent of denaturation that can take place during the one month of storage at 45°C is relatively small.

Conclusion

Most of the work done on protein-flavor interactions has been conducted in aqueous protein systems with GC-headspace and equilibrium dialysis techniques. This might be a useful model prediction for products such as protein beverages, but might not be indicative of protein-flavor interactions in solid-state products such as protein bar, where water activity could be a factor that affects flavor retention and release. There has also been little work done on mixed flavor systems.

Milk and soy proteins have been extensively studied, with less emphasis on pulse and seed proteins. Given that different proteins can have different structures and amino acid profile, the amount and types of flavor interactions that can occur would differ too, and it would serve industry well to consider the other types of protein that can be utilized to reduce flavor loss. Prediction of which flavors work well in different proteins can help manufacturers know which flavor compounds to add in excess and develop strategies to prevent flavor loss.

Overall, rice protein showed little binding interactions with the flavor compounds used, especially flavor compounds of lower molecular weight. Pea protein showed little binding interactions with flavor compounds of higher molecular weight. Soy protein showed a fast rate of flavor compound loss despite having the most free flavor compounds at the beginning, and whey protein showed the highest rate of loss with most flavor compounds.

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Appendices

Appendix A – Abundance of flavor compounds used at each week, as a percentage of triacetin used except for whey protein, where abundance was listed as peak area.

Allyl sulfide

Protein Used	Rep	Week			
		0	1	2	4
Whey	1	372958	142665	55437	15585.8
	2	618384	340733	196322	113273
Soy	1	37.48928918	20.7666425	1.7437753	1.392845113
	2	29.37568319	14.10915576	6.88007606	2.789366546
Rice	1	6.778974867	3.170456573	4.343899957	3.482583417
	2	14.09413149	11.61556822	5.650481377	3.812257033
Pea	1	8.76099289	0	0	0
	2	10.02662536	0	0	0

Isoamyl acetate

Protein Used	Rep	Week			
		0	1	2	4
Whey		618384	340733	196322	113273
		655042	130990	34752.7	114425
Soy		58.0133897	42.08048217	8.630319453	9.666267164
		47.06916549	23.72204992	30.96750896	19.23808412
Rice		18.05249796	18.11118599	24.31299976	18.4395338
		30.29112359	33.60426166	31.57797124	19.64618763
Pea		22.64626221	16.34255458	7.138003959	2.951589813
		24.38459118	16.49428889	11.64050913	3.36735792

Furfuryl mercaptan

Protein Used	Rep	Week			
		0	1	2	4
Whey		0	0	0	0
		0	0	0	0
Soy		0.367860943	0	0	0
		1.462238095	0	0	0
Rice		0	0	0	0
		0	0	0	0
Pea		0	0	0	0
		0	0	0	0

Benzaldehyde

Protein Used	Rep	Week			
		0	1	2	4
Whey	1	98010	0	0	0
	2	5969	160063	0	0
Soy	1	6.16179697	4.339273576	1.474846174	2.274282703
	2	5.232153734	3.787926445	3.181980524	2.693919119
Rice	1	1.707684389	2.561338679	2.422314942	3.136605129
	2	4.087830836	4.373605181	3.040120947	2.622397539
Pea	1	0	0	0	0
	2	0	0	0	0

Eucalyptol

Protein Used	Rep	Week			
		0	1	2	4
Whey	1	377550	258267	180871	130948.2
	2	410475	1116450	188339	144615
Soy	1	28.232785	24.11202248	11.08599558	11.15893663
	2	23.50007927	19.20742583	20.27530102	17.57978374
Rice	1	15.16276039	15.35579309	17.51595002	16.45300723
	2	18.9702534	19.17301715	18.73820159	16.87076678
Pea	1	17.037638	16.14797785	13.86396426	11.76290264
	2	14.57657614	15.72677902	14.86631914	11.75214963

Menthol

Protein Used	Rep	Week			
		0	1	2	4
Whey	1	429585	382817	368058	306086.4
	2	458128	2097810	378834	353605
Soy	1	23.16381202	21.81923234	15.75241878	18.71026068
	2	19.45194963	17.32645692	21.06076923	21.80512386
Rice	1	11.12397585	11.90335333	12.59188951	14.86694185
	2	12.4903995	12.54710948	12.92521643	13.11069008
Pea	1	10.24207067	12.32068833	11.98978055	12.15602469
	2	10.06886286	13.00517793	13.05290602	13.64037967

Methyl salicylate

Protein Used	Rep	Week			
		0	1	2	4
Whey	1	191496	166924	171346	154371
	2	201462	858532	174479	180252
Soy	1	20.16534684	18.08832193	13.77531482	16.67176377
	2	16.12303597	14.04273452	18.07160548	18.67907504
Rice	1	10.81980661	11.80199097	11.29205126	14.62044819
	2	12.21529877	12.19453382	12.89607339	12.85166424
Pea	1	8.733866772	10.49072153	10.73689011	10.96727009
	2	8.419260622	10.85331398	10.80389301	12.27516331

D-carvone

Protein Used	Rep	Week			
		0	1	2	4
Whey	1	598482	551583	556215	450776
	2	644484	2542240	569124	525493
Soy	1	38.85151741	35.04769565	28.71936723	32.57369275
	2	31.72942934	27.56165491	34.63326227	36.7387043
Rice	1	23.56482663	24.16712256	22.35874703	28.21936733
	2	25.16923445	24.51661855	24.9626845	25.50415439
Pea	1	21.12430946	24.81908345	24.93655961	25.16422014
	2	20.6933485	24.35220914	25.05367135	27.573421

γ-nonalactone

Protein Used	Rep	Week			
		0	1	2	4
Whey	1	818322	807947	843102	687848
	2	837621	3592790	836820	799846
Soy	1	58.60086063	54.43512938	49.39658295	51.1566636
	2	48.83489232	43.86807124	55.53036217	56.77511224
Rice	1	37.18079025	35.98511268	31.42114089	42.11335424
	2	35.51913632	33.69853092	35.11152346	36.3624506
Pea	1	29.71222368	35.03127659	35.30408449	39.38119197
	2	29.81616401	35.71737959	35.84857798	41.20664421

Trans-α-ionone

Protein Used	Rep	Week			
		0	1	2	4
Whey	1	38031	25334	20269	76662.6
	2	48306	161905	60215	166275

Soy	1	1.547207653	1.507752526	1.274019339	1.547924825
	2	1.170389593	1.083202277	1.468577233	1.421052888
Rice	1	1.042326784	1.024551692	0.245544306	1.227270572
	2	0.983532066	0.910352231	0.873080104	1.051158375
Pea	1	0.831937804	1.075009082	0.291197344	1.870579387
	2	1.043758371	1.260380594	0.312643709	1.317025925