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A-type Procyanidins and Their Effects on the Aggregation of Tau Prevalent in Alzheimer's Disease

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by

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Abstract

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Alzheimer's disease (AD) affects over 40 million people worldwide: a number that is expected to grow to over 100 million by 2050.[1] Despite this, there is a shortage of AD drugs on the market, with only five having been FDA approved, and no new ones since 2003.[2] A major problem is that these drugs are merely symptomatic treatments. No drugs exist that stop or prevent AD. One of the hallmarks of AD are intracellular deposits of insoluble neurofibrillary tangles (NFTs) in neurons of the brain.[3] NFTs are composed of aggregates of the microtubule-associated protein, tau. This study focuses on discovering novel treatments that prevent the aggregation of tau.

Peanut skins contain large amounts of procyanidins[4]: molecules proposed to have various health benefits.[5] Peanut skin extract was run

through high performance liquid chromatography (HPLC) to separate the many compounds present, in order to isolate procyanidin molecules. One particular procyanidin was separated and used for inhibition assays with tau and appeared to inhibit its aggregation into NFTs. It was then identified using electrospray ionization mass spectrometry (ESI-MS) and proton nuclear magnetic resonance (^{1}H-NMR) to be the A1 procyanidin dimer isomer.

While running HPLC to isolate more procyanidin A1 for further experimentation, it became apparent that one of the pumps had a leak. This caused a lower percentage of that pump's solvent to go through the column than what it was set to. This resulted in the presumably pure procyanidin A1 eluting later than usual, and separating from another, currently unidentified, molecule. Further experimentation led to the conclusion that the A1 procyanidin has no effect on the aggregation of tau, which was backed up by tests that were then done with a commercially available procyanidin A1 that was purchased. It is assumed that an unidentified contaminant is responsible for the inhibitory activity. Further studies are required to find which compound is responsible for the inhibition seen on tau aggregation.

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Introduction

An ever-growing global population, paired with constant improvements in medicine that raise the average worldwide life expectancy, mean the number of cases of Alzheimer's disease (AD) is always on the rise. Right now there is an estimated 44 million people with the disease worldwide, and the 2017 Alzheimer's disease facts and figures report projects that for the first time, over a quarter of a trillion dollars would be spent on AD related healthcare in the United States.[6] Despite this, no new drugs have been developed for AD since 2003.[2] Only five drugs have been FDA approved, and they all simply slow down AD progression[2]; none exist that cure or prevent it.[7] Three of these, donepezil, galantamine, and rivastigmine, are cholinesterase inhibitors that work by inhibiting enzymes that degrade the neurotransmitter acetylcholine. The cholinergic hypothesis concludes that during the early stages of AD, there is a loss of acetylcholine neurons and a reduction of enzymatic function for acetylcholine synthesis. Cholinesterase inhibiters therefore delay the degradation of acetylcholine and increase cholinergic transmission. A fourth drug, named memantine, works as an antagonist for the N-methyl-D-aspartate receptor that binds to glutamate. It is believed that this protects neurons from excitotoxicity: a process that's associated with AD

as well as a variety of other neurological disorders.[8] The fifth medication is a combination of donepezil and memantine. The problem with these drugs is that they are merely symptomatic treatments. Many studies are focusing on finding novel treatments that can prevent AD from progressing in the first place by targeting a possible root cause.[7]

The two main neuropathological hallmarks of AD are extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs), both of which plague neurons in the brains of patients.[3] Amyloid plaques are buildups of the protein ß-amyloid in-between neurons. It is believed that as ß-amyloid begins to cluster together, it binds to a receptor on the neuron surface. This biochemical signal results in a cascade within the cell that ultimately alters the cell's synapses, and therefore its ability to communicate with other cells.[9] NFTs consist of insoluble fibers of paired helical filaments (PHFs) that arise from misfolded aggregates of the highly soluble microtubule-associated protein tau.[10] This study is centered around the search for small molecules that inhibit the aggregation of tau, and therefore can potentially be new therapeutics for AD.

In humans there are six isoforms of tau, all of which are expressed via alternative splicing of a single gene *MAPT* (microtubule associated protein tau) that is located on chromosome 17 (figure 1).[11] They range from 352 to 441 amino acids, and all are observed in NFTs.[12] The normal

role of tau, which is abundant in neurons of the central nervous system[13], is to interact with tubulin to stabilize microtubule assembly.[11] In AD the tau is hyperphosphorylated (three or four times more phosphorylated than in healthy brains[14]) which results in the disassembly of microtubules and the formation of PHFs.[15] PHFs interfere with cytoplasmic functions and axonal transport which can ultimately lead to cell death.[16]

It's possible to test the effects of molecules on the aggregation of tau by using light scattering. As tau aggregates together, it increases the turbidity of the solution, which can be detected and recorded. With heparin present, tau[187] reaches its maximum level of aggregation in 5-6 hours, as opposed to a minimum of several days without it (full length tau also takes days to aggregate, as opposed to hours with isoforms of tau that only include the binding repeats such as the one used in this study).[17]

Two important factors to consider regarding the effectiveness of a new drug are toxicity and solubility. Regarding toxicity, a functional drug is of no use if its toxicity hurts the patient more than it helps them. As for solubility, a drug must be present in the form of solution in order to be absorbed.[18] During the search for our novel drug, to get around the toxicity obstacle edible plants were used, and to get around the solubility obstacle, only the water soluble fraction was extracted and analyzed.

Previous work by Peterson et al. at UCSB found that a molecule present in cinnamon extract inhibited tau aggregation.[19] Upon further study, it was discovered that the bioactive molecule was a trimer of catechin/epicatechin molecules, named a procyanidin. Procyanidins are members of the proanthocyanidin class of flavonoids, and are naturally occurring polyphenolic compounds that are present in a wide variety of foods such as nuts, fruits, and beans. They're regarded as healthy supplements due to their supposed antioxidant, antimicrobial, and anticancer properties.[20]

At around 867 daltons, trimeric procyanidins are above the rough 500Da upper limit outlined in Lipinski's rule of five, and are therefore likely too large to cross the blood-brain barrier (BBB), an important factor when determining the efficacy of new drugs.[21] Because of this, a change in strategy was formulated. Peanut skins are known to be abundant with the smaller procyanidin dimers[22] (576-578 Da) seen in figure 2, so the focus was shifted to concentrate on isolating these from unroasted peanuts. Their smaller size, while still over the threshold, would give them a higher chance of being successfully delivered to the brain. Studies by Serra et al. involved orally administering procyanidin-rich grape seed extract to rats and measuring the procyanidin concentrations in the gut and blood.[23] The results showed that procyanidin dimers persisted during

gastric transport and were present in significant concentrations in the small and large intestine several hours after ingestion. They were also present in the blood, albeit at lower concentrations, at 1hr post ingestion. This shows that procyanidin dimers survive the harsh conditions of the gastrointestinal system, and are successfully absorbed into the bloodstream, giving them transport to the brain.

B-type procyanidin dimers consist of two catechin/epicatechin monomers linked together with a 4ß-8 or 4ß-6 C-C bond. A-type procyanidins have an additional 2ß-7 C-O-C bond. A-type and B-type procyanidins are very similar in atomic composition, differing by only two extra hydrogens in the B-type. Due to the four stereoisomers of catechin/epicatechin, and the variation in bonds possible, there are a number of possible procyanidin structures.

Electrospray ionization mass spectrometry (ESI-MS) can be utilized to analyze molecules separated by high performance liquid chromatography (HPLC). Once a procyanidin is found, the molecular weight will determine whether it is an A-type or B-type procyanidin (576 vs 578 Da), and proton nuclear magnetic resonance (^{1}H-NMR) can identify the particular isomer.

Preliminary data from Dylan Peterson at UCSB (personal communication, unpublished) showed that procyanidin dimer-rich peanut skin extract (PSE) possibly reduces the amount of PHFs in neurons of the

brains of mice. Transgenic 'AD mice' that overexpressed human tau and ultimately developed AD (strain B6.Cg-*Mapt*$^{tm1(EGFP)Klt}$*Tg(MAPT)8cPdav/J* (005491; Jackson Labs)) were gavage-fed 100µl PSE at 30mg/mL twice a week starting at eight weeks of age for two months. Brain sections were immuno-histochemically stained with the tau NFT binding antibody MC1, and seemed to bind less in the PSE+ brains than in the PSE- control mice. Although not very convincing data in its current capacity, this suggests a possibility that a compound present in PSE is reducing the aggregation of tau within the brain.

Materials and Methods

Tau Expression & Purification

Methods of tau expression and purification were based on methods formulated by Peterson et al.[24] A portion (residues 255 to 441) of the largest tau isoform '2N4R' was amplified by PCR and cloned into pET28 vector. A hexahistidine tag (MGSSHHHHHHSSGLVPRGSH) was fused to the N-terminus to allow purification with nickel resin. This was expressed in *E. coli* strain BL21 DE3 and named 'tau^{187}'.

E.coli were grown from a glycerol stock of tau^{187} in 2mL LB culture to an OD_{600} of 0.6-0.8, which was then used to inoculate 200mL minimal media. This was grown overnight, at which point 40mL was added to 1L of fresh minimal media. This was grown to an OD_{600} of 0.6-0.8, at which point protein expression was induced with 1mM isopropyl ß-D-1-thiogalactopyranoside (IPTG) for 3 hours. The bacteria were then pelleted by centrifugation for 15min at 5000 x g in a Sorval SS-34 rotor at 4°C. The pellet was resuspended in 20mL lysis 'buffer A' (20mM MOPS [pH7.4], 10mM imidazole, 0.1mM EDTA, 50mM NaCl, 1mM DTT), PMSF (1mM), lysozyme (2mg/mL), DNase (20µg/mL) and $MgCl_2$ (1mM). The sample was then placed on ice and kept as cool as possible for the remainder of the

purification. The sample was sonicated (6 x 30sec intervals with 1min cooling in between each sonification) with a Branson Sonifier 250 ultrasonic cell disruptor. The lysate was then centrifuged for 30min at 15,000rpm (Sorval SS-34) and the supernatant was loaded onto 2mL of Ni-NTA agarose. The resin was then washed with buffer A, buffer B (same as buffer A but with 500mM NaCl), and then the tau was eluted with buffer C (same as buffer A but with 300mM imidazole) and collected in fractions. SDS-PAGE gels were then run to determine which fractions contained tau. These fractions were pooled and loaded onto 5mL of SP-sepharose that was equilibrated with buffer SP-A (20mM MOPS pH 7.4, 0.1mM EDTA, 50mM NaCl). SP-A was flowed through, followed by SP-B (same but with 150mM NaCl), and then the tau was eluted with SP-C (same but with 500mM NaCl) and collected in fractions. SDS-PAGE was again utilized to determine which fractions contained protein. These were pooled and concentrated to approximately 0.5-1mL using an Amicon ultra-4 centrifugal filter unit. The sample was then applied to a gel filtration column containing sepharose CL6B and fractions were collected. Fractions were once more analyzed with SDS-PAGE and those containing protein were pooled together. These were then concentrated and stored at -80°C. The concentration of tau was determined by an assumed extinction coefficient at 274nm of $2.8 cm^{-1} mM^{-1}$ that was calculated using the known

extinction coefficient for tyrosine, two of which are present in tau[187] (tryptophan is absent). Tau is natively unfolded so denaturing is unnecessary for obtaining A_{274}.

Tau Aggregation Assay

Tau aggregation assays took place in an 8-well microcell in a Shimadzu 1601 spectrophotometer running UV probe software, measuring A_{350} every minute for up to 600min. Each well contained 50μM tau[187], 100mM NaCl, 20mM sodium phosphate buffer (pH 6.9), 0.11mg/mL heparin (Sigma H3393, average weight of 18kDa, used to induce rapid aggregation), whichever molecule (and at a particular concentration) was being tested for activity (dissolved in water), and water to bring it to a total of 100uL. An absorbance control with only water, and a negative inhibition control with no inhibitory molecule, were included in all assays. Procyanidin concentration was initially chosen to be around 50uM, only so that it would be equal to the tau concentration. This was calculated using an extinction coefficient at 279nm of $7.32 cm^{-1} mM^{-1}$.

Preparation of PSE

Skins were peeled off of unroasted Spanish peanuts and placed in a round bottom flask containing a stir bar and water preheated to 40°C. One gram of skins was added to 50mL of water and then stirred rapidly

for 10min. The mixture was then strained off, the liquid was centrifuged at 4°C in an SS-34 rotor at 10,000rpm to separate solid material, and then the supernatant was passed through a 2µm vacuum filter. This was then lyophilized with a Virtis 4K benchtop freeze dryer overnight.

Isolation of Procyanidin

Solid PSE was dissolved in water/0.1% trifleuroacetic acid (TFA) at a concentration of 10mg/mL. 2mL was injected onto reverse phase HPLC (Waters 2487 dual wavelength detector, Waters 1525 binary pump, Breeze software version 3.30) at flow rate 4.7mL/min. Solvents used were water/0.1% TFA and HPLC grade acetonitrile/0.09% TFA. A Waters Atlantis prep T3 column (5µm 10x250mm) with attached guard column was used for separation, and a Symmetry C18 Column (100Å, 3.5µm, 3x100mm, flow rate 0.5mL/min) was used for analytical purposes. Solvents were initially run on a changing gradient that increased the acetonitrile composition over time. The peak of interest was collected, frozen with dry ice, lyophilized overnight, and dissolved in water/0.1% TFA. This was then run again on HPLC on a 15% acetonitrile isocratic method to further separate and purify the compound. Collected liquid was frozen, lyophilized, and resuspended in water. A_{279} was measured to determine concentration.

Determination of Procyanidin Molar Extinction Coefficient

5mg of procyanidin A2 was purchased from BOC sciences and was dissolved in 1mL of water, diluted 100 fold, and measured on a UV spectrophotometer at a wavelength of 279nm. A-type procyanidins are 576Da so 576mg/mL = 1M. 5mg/mL therefore equals 8.68mM. Diluted 100 fold at 86.8uM it gave an A_{279} of 0.635. Using Beer's law $A = \varepsilon \cdot c \cdot l$, the molar extinction coefficient of procyanidin A2 at 279nm was calculated to be 7.32$cm^{-1}mM^{-1}$. It was assumed that the extinction coefficients for all A-type and B-type procyanidin dimers would be fairly similar, since the four conjugated systems which are responsible for the absorbance in the UV range aren't disrupted between different isomers. This is proven to be correct by Bohr et al.[25] as they measured procyanidin dimers B2-B5 to have an average extinction coefficient of 7.53$cm^{-1}mM^{-1}$ with a range of only 1.7$cm^{-1}mM^{-1}$.

Mass Spectrometry

The samples were dissolved in water/0.1% formic acid and run via autosampler of a Waters Acquity H-class Quarternary Separations Module pump and Xevo G2-XS Tof MS detector. All runs were in positive ion mode. MassLynx software (Micromass Inc.) was used to analyze the data.

^{1}H-NMR Spectroscopy

Approximately 5mg of sample was dissolved in 0.5mL of deuterated methanol and run on a Varian Unity Inova 600 MHz spectrometer at room temperature. VnmrJ software was used to analyze the data.

Results

When PSE was run on HPLC there were many peaks, each corresponding to one of the numerous different molecules present. In preliminary experiments involving PSE and HPLC conducted by Dylan Peterson prior to this study, several of the more prominent, high absorbing peaks were isolated and tested with mass spectrometry in order to find one at 576 or 578Da. It was discovered that the first prominent peak on the chromatogram was 576Da (unpublished data). This was repeated for this study and the peak was collected at approximately 36min as pointed out in figure 3. The collected material was frozen using dry ice and lyophilized overnight. A beige white powder remained, and was dubbed '1X' (one times purified). This was fully soluble in water/0.1% TFA and was reinjected back on to the same HPLC column. The 15% acetonitrile isocratic run separated the material further as seen in figure 4. The largest peak that eluted first was collected, lyophilized, and dubbed '2X'. This dried material was entirely white powder. 2X was run on HPLC through a C18 analytical column and appeared to be very pure (figure 5). This was tested in a tau aggregation assay and showed inhibition in a dose-dependent manner with regard to concentration, as shown in figure 6.

ESI-MS was conducted on the 2X sample which revealed it to be 576 Da, equal to the molecular weight of A-type procyanidins (figure 7). Mass spectrometry from the National Institute of Standards and Technology (NIST)[26] on procyanidin A1 shows the 2nd and 4th most prominent peaks present to be 425Da and 287Da respectively. Experiments by Karchesy et al. showed that fast atom bombardment on procyanidin A1 gave prominent fragments of 425 and 287Da.[27] These masses are the next two most prominent peaks on the 2X data that was obtained, which is further evidence that the isolated molecule is an A-type procyanidin. In the same study, Karchesy et al. also tested procyanidin B7 which gave peaks at 579, 427, and 289Da.

To determine which isomer of procyanidin was in the 2X sample, ^{1}H-NMR spectroscopy was conducted and compared to the findings from a study by Lou et al.[22] in which they conducted ^{1}H-NMR on a variety of A-type procyanidins that they isolated from peanut skins. The results of the 2X sample (figure 8a) most closely resembled their data for the A1 isomer (table 1). The A1 and A2 isomers were commercially available and purchased from Cerilliant Corporation and BOC Sciences, respectively. ^{1}H-NMR was conducted on these samples (figures 8b and 8c) and the results corresponded closely with the results from Lou et al. (table 1) with only small changes in the chemical shifts of hydrogens. It was concluded

from these results that the isolated 2X material was the procyanidin A1 isomer.

The purchased A1 dimer was tested in a tau aggregation assay at 50μM and did not inhibit aggregation, as seen in figure 11. The green line shows how tau aggregation is inhibited with the addition of 50μM 2X sample, and the blue line shows that the addition of the purchased A1 dimer at the same concentration has little effect on tau aggregation. The A2 isomer was also tested and had a similar result (data not shown). Since the purchased pure A1 isomer does not inhibit tau aggregation, there must be another unidentified molecule present that is responsible for the 2X sample's inhibitive activity.

During preparation of more 2X material, it became apparent that there was a leak in the pump that fed acetonitrile through the column. This resulted in the actual concentration of acetonitrile to be less than the set proportion of 15%. This caused the sample to elute later than usual. Acetonitrile is the hydrophobic component of the mobile phase responsible for moving the 1X sample molecules through the column, so a reduction in acetonitrile percentage resulted in a longer retention time. It also resulted in an increase in separation of the molecules present, so the single peak that was normally collected was split into two different peaks as seen in figure 12 at ~92min and ~102min. These separated peaks were collected

and analyzed with ESI-MS. This showed that the second of the peaks was the A1 procyanidin, and the most prominent signal for the first molecule was 466Da (figure 13). They were both tested on tau aggregation and the collected A1 procyanidin did not inhibit, while the 466Da molecule did in a dose dependent manner, shown in figure 14. Studies in the Lew laboratory are continuing in order to identify the molecule with intrinsic tau aggregation inhibitory activity.

Discussion

Something present in peanut skins inhibits the aggregation of tau into paired helical filaments, and this study shows that it isn't procyanidin A1 or A2, or likely any A-type procyanidin, but rather another unidentified molecule. This molecule must have hydrophobic properties comparable to procyanidin A1 since with HPLC it elutes at the same time under the conditions used. Changing different aspects of the overall HPLC configuration would likely aid in identifying this mystery molecule.

Since the new molecule of interest is unknown, it is impossible to know exactly which configuration will be the best at isolating it from the many other molecules that are present in PSE. Instead of using acetonitrile as a solvent, other common solvents, such as methanol, could be used which interact with molecules differently. This would change the peak shape, the elution time of individual molecules, and the amount of time between the elution of each molecule (resulting in an increase or decrease in separation). Instead of using a C18 column where the hydrophobic carbon chains packed inside the column are 18 carbons long, C8 or even C4 could be utilized. The shorter carbon chains are less hydrophobic and so would reduce hydrophobic interactions with the samples. This would cause the hydrophobic molecules in the PSE to bind less strongly to the column which would change the elution times of the different molecules in the

sample. Reverse phase HPLC was used in the entirety of this study but normal phase or ion exchange may be a better choice for these particular samples. Since the molecule of interest is unknown, all of these alterations in the configuration would have to be blindly tested, but they may prove to be much more efficient at separation.

Of the six 576Da A-type procyanidins analyzed by Lou et al. shown in figure 15 (the other compounds identified in the study were methylated derivatives), the ^{1}H-NMR data for the A1 isomer most closely matches the data retrieved from the 2X sample. There are small differences in the chemical shifts of some hydrogens (an average deviation of only 0.027ppm per hydrogen), but this is most likely due to differences in equipment and/or technique and procedure. When the purchased A1 dimer was analyzed with ^{1}H-NMR, the spectrum was incredibly similar to that of the 2X sample, which implies that the 2X is the A1 procyanidin isomer. In fact, the two samples were virtually identical with regards to chemical shifts and splitting patterns. The only noteworthy difference is the presence of what appears to be a singlet at approximately 7.95ppm on the purchased A1 spectrum, but the intensity is less than 25% of that which is equivalent to a single proton so it does not represent the A1 that was being tested, but instead most likely a contaminant at a much lower concentration.

Even though the extreme similarity of the ^{1}H-NMR spectrums for the 2X and purchased A1 samples strongly implies that they are the same molecule, it is still wise to rule out isomers A2-A6 by additional means. Some of the equivalent hydrogens across the six isomers do not change much on ^{1}H-NMR spectra due to them being in very similar environments. The minor changes between the isomers is enough, however, to see changes in the chemical shifts of certain hydrogens. To rule out A2, the strongest evidence is that the ^{1}H-NMR data was obtained and it does not resemble that of the 2X sample. Additionally, there is only one doublet present at ~7.13ppm where A2 should have two. The #10 and #10' hydrogens are either side of the #14 doublet of doublets while with A2, both #10 and #10' are downfield from the two doublets of doublets of #14 and #14' that are separated by only 0.08ppm. With A3 and A6, the peaks for #4α and #4β are no longer two separate doublets of doublets like the 2X sample, but instead one multiplet. For A3, as with A2, #10' is alongside #10 at ~7.13 ppm. The 2X sample only has one doublet that far downfield. The integration on the 2X spectra shows that there are three protons present at ~6.8ppm, but A3 only has two in that location. A4 and A5 both have #14' alone at 6.68ppm, while the sample has it at 6.80ppm alongside #13' and #13 at 6.80ppm and 6.79ppm respectively. On top of all of this, if one attempts to line up the data from Lou et al. (table 1) for

the A2-A6 isomers with the ^{1}H-NMR spectra for the 2X sample, the average deviation in chemical shift is much larger than with their data for A1.

The leak in the HPLC pump split the previously presumed pure 2X material into two: the A1 procyanidin and an unknown. Mass spectrometry on the unknown (figure 13) shows a peak with the highest intensity of mass 466.53Da. Francisco et al. isolated molecules from peanut skins[28] and one possible match could be sodiated catechin gallate (442.37Da + 22.99Da). This only equals 465.36Da: a full mass unit below the unknown molecule's mass. Testing this sample again with different combinations of solvent and ion mode would perhaps increase its ionizability and give better results, making it easier to identify.

Experiments were previously performed in the Lew laboratory (unpublished data) on tau[187] cys-mutants with which one of the sole two cysteines, residues cys291 or cys322, was changed to a serine (cysteine and serine are very similar, differing only in the switching of the thiol for a hydroxyl group. This change renders the protein incapable of forming disulfide bonds at this residue). A double mutant with both mutations was also used. All three mutants aggregated normally with heparin which shows that the cysteines aren't required by heparin in order for it to have its effect on tau aggregation. When treated with an isolated sample from

PSE that had inhibitory activity (thought to be an A-type procyanidin at the time), the two single mutants were inhibited but the double mutant aggregated normally. These experiments rule out the possibility that the inhibiting molecule is interacting with heparin instead of tau, and strongly suggest that it interacts with cysteine. Whether cysteine is present or absent, the tau aggregates; but if the inhibitor is added then the double mutant is the only one that isn't inhibited. The mechanism of action for the inhibitor requires at least one of the cysteines, and heparin is not targeted.

The original aim of this study was to identify the isomer of procyanidin that was thought to be inhibiting tau aggregation. Due to malfunctioning equipment, a sample that was believed to be a pure isolate of a particular procyanidin turned out to contain a contaminant which could be responsible for the inhibitory activity. The status of the previously isolated procyanidin trimer also becomes questionable. Further experiments in the Lew laboratory will seek to find the true inhibitor of tau aggregation.

References

1. Prince M, et al. (2015). World Alzheimer Report 2015. The global impact of dementia. An analysis of prevalence, incidence, cost and trends. *World Alzheimer's Disease International.*

2. Cummings JL, Morstorf T, Zhong K. (2014). Alzheimer's disease drug-development pipeline: few candidates, frequent failures. *Alzheimer's Research & Therapy.* 6 (4), 37.

3. Serrano-Pozo A, et al. (2011). Neuropathological alterations in Alzheimer disease. *Cold Spring Harbor Perspectives in Medicine.* 1, 1-23.

4. Yu J, et al. (2006). Peanut skin procyanidins: Composition and antioxidant activities as affected by processing. *Journal of Food Composition and Analysis*. 19, 364-371.

5. Hammerstone JF, Lazarus SA, Schmitz HH. (2000). Procyanidin content and variation in some commonly consumed foods. *The Journal of Nutrition*. 130 (8S suppl), 2086S-2092S.

6. Alzheimer's Association. (2017). 2017 Alzheimer's disease facts and figures. *Alzheimer's & Dementia*. 13 (4), 325-373.

7. Yiannopoulou KG, Papageorgiou SG. (2013). Current and future treatments for Alzheimer's disease. *Therapeutic Advances in Neurological Disorders*. 6 (1), 19-33.

8. Dingledine R, et al. (1999). The glutamate receptor ion channels. *Pharmacological Reviews*. 51 (1), 7-62.

9. Selkoe DJ, Hardy J. (2016). The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Molecular Medicine*. 8 (6), 595-608.

10. Brion J. (1998). Neurofibrillary tangles and Alzheimer's disease. *European Neurology*. 40, 130-140.

11. Wang Y, Mandelkow E. (2015). Tau in physiology and pathology. *Nature Reviews Neuroscience*. 17, 22-35.

12. Andreadis A. (2005). Tau gene alternative splicing: expression patters, regulation and modulation of function in normal brain and neurodegenerative diseases. *Biochimica et Biophysica Acta.* 1739 (2-3), 91-103.

13. Bakota L, Brandt R. (2016). Tau biology and tau-directed therapies for Alzheimer's disease. *Drugs*. 76, 301-313.

14. Jouanne M, Rault S, Voisin-Chiret A. (2017). Tau protein aggregation in Alzheimer's disease: An attractive target for the development of novel therapeutic agents. *European Journal of Medicinal Chemistry*. 139, 153-167.

15. Godyn J, et al. (2016). Therapeutic strategies for Alzheimer's disease in clinical trials. *Pharmacological Reports*. 68, 127-138.

16. Mudher M, Lovestone S. (2002). Alzheimer's disease - do tauists and baptists finally shake hands? *Trends Neuroscience*. 25, 22-26.

17. Barghorn S, Mendelkow E. (2002). Toward a unified scheme for the aggregation of tau into Alzheimer paired helical filaments. *Biochemistry*. 41 (50), 14885-14896.

18. Ketan T, et al. (2012). Drug solubility: Importance and enhancement techniques. *ISRN Pharmaceutics*. 2012 (article ID 195727), 1-10.

19. Peterson D, et al. (2009). Cinnamon extract inhibits tau aggregation associated with Alzheimer's disease in vitro. *Journal of Alzheimer's Disease.* 17, 585-597.

20. Prior RL, Gu L. (2005). Occurrence and biological significance of proanthocyanidins in the American diet. *Phytochemistry.* 66 (18), 2264-2280.

21. Lipinski CA, et al. (1997). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews.* 46 (1-3), 3-26.

22. Lou H, et al. (1999). A-Type proanthocyanidins from peanut skins. *Phytochemistry.* 51, 297-308.

23. Serra A, et al. (2010). Bioavailability of procyanidin dimers and trimers and matrix food effects in in vitro and in vivo models. *British Journal of Nutrition*. 103, 944-952.

24. Peterson D, et al. (2008). A soluble oligomer of tau associated with fiber formation analyzed by NMR. *Biochemistry*. 47 (28), 7393-7404.

25. Rohr GE, Meier B, Sticher O. (2000). Analysis of procyanidins. In Atta-ur-Rahman (Ed.), *Bioactive Natural Products*. Volume 21 (B), p546.

26. National Institute of Standards and Technology (NIST) mass spectral library v17.

27. Karchesy JJ, et al. (1986). Sequencing procyanidin oligomers by fast atom bombardment mass spectrometry. *Analytical Chemistry*. 58, 2563-2567.

28. Francisco ML, Resurreccion AV. (2008). Functional components in peanuts. *Critical Reviews in Food Science and Nutrition*. 48, 715-746.

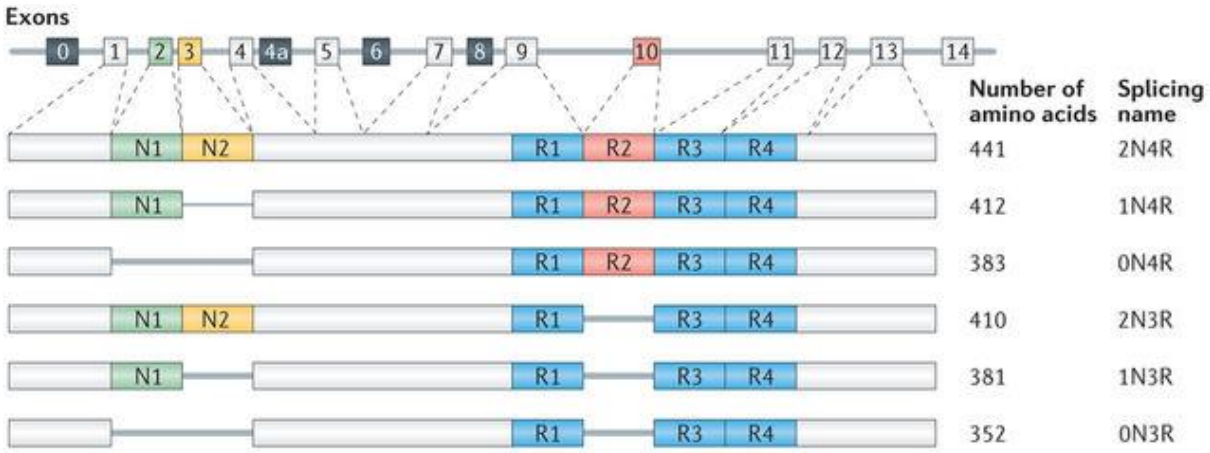

Figure 1. Alternative splicing of *MAPT* gene that encodes for the six isoforms of tau. Exons 2, 3, and 10 are alternatively spliced giving six combinations. 'R' exons are the conserved sequences of microtubule binding repeats. Tau[187] begins 14 amino acids in from the first repeat and aggregates more rapidly than wild type tau. Image obtained from Wang et al.[11]

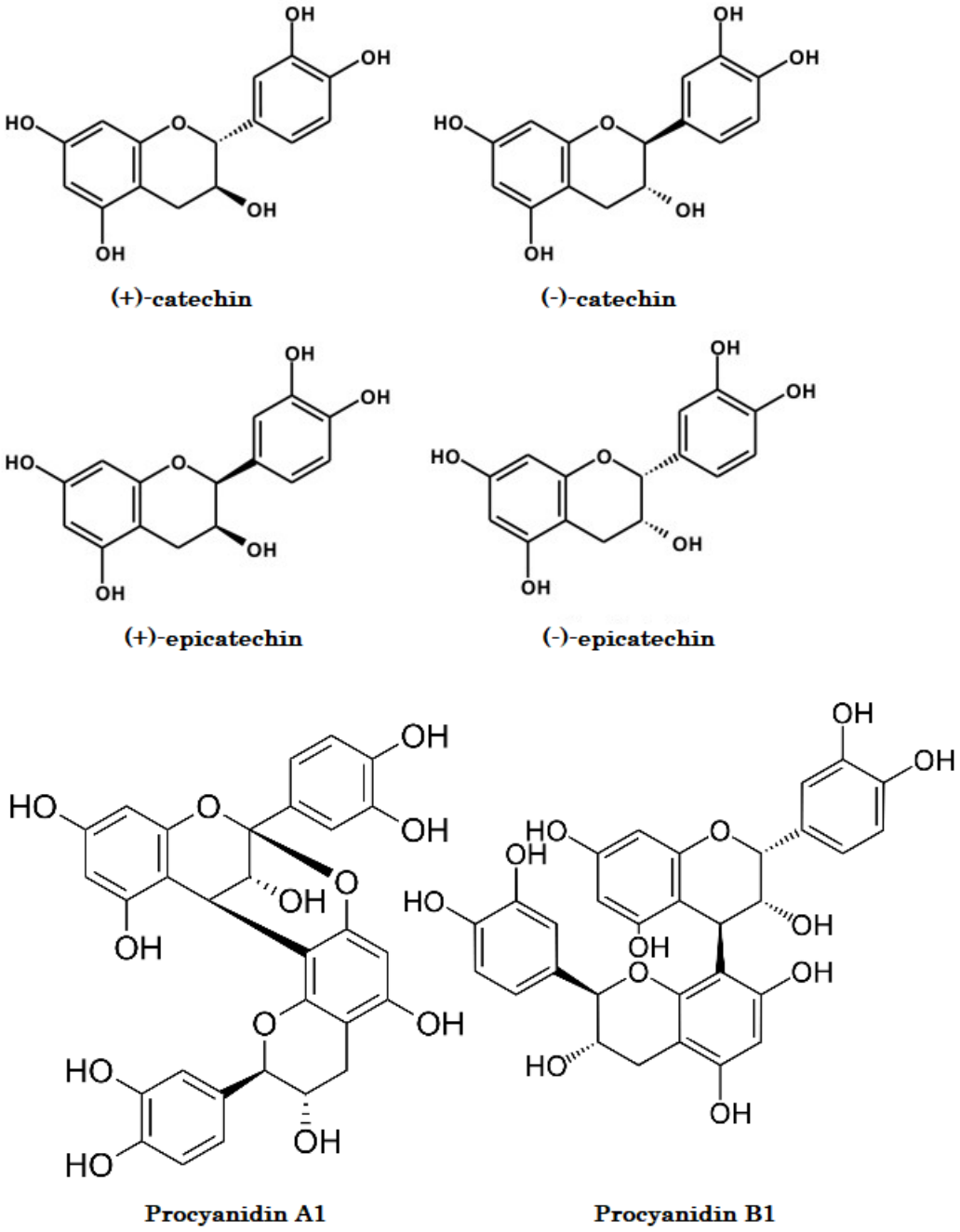

Figure 2. Structures of stereoisomers of catechin (290Da) and procyanidins A1 (576Da) and B1 (578Da). A-type procyanidins are composed of two catechin and/or epicatechin molecules joined with two covalent bonds while B-type procyanidins are joined with only one. Both A1 and B1 consist of (-)-epicatechin and (+)-catechin. Other procyanidins consist of variations of catechin stereoisomers and location of linkage(s).

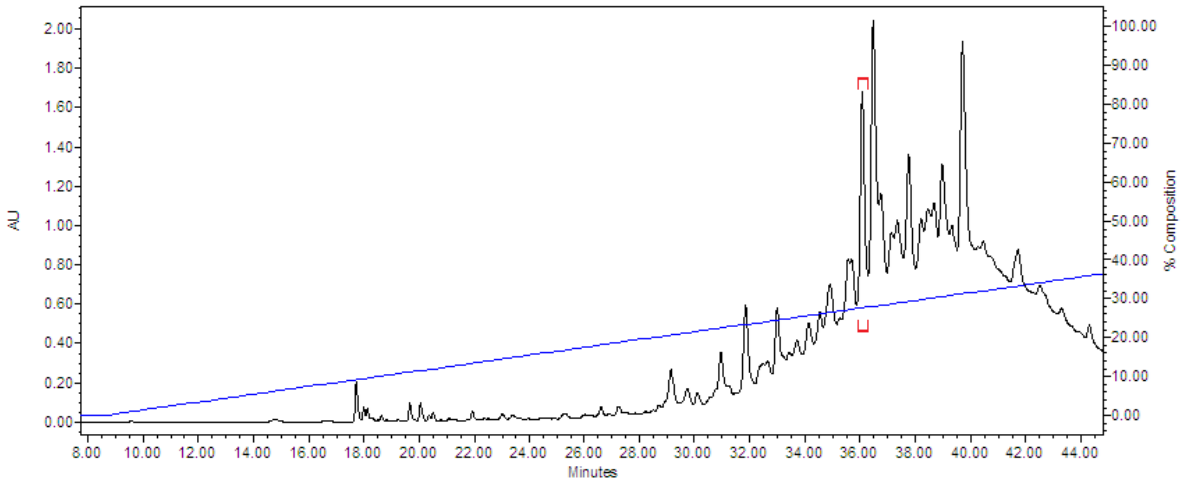

Figure 3. HPLC chromatogram of PSE on C18 semi-prep column. Absorbance wavelength is 214nm. Blue shows % composition acetonitrile. Red markers show peak of interest that was collected and purified further.

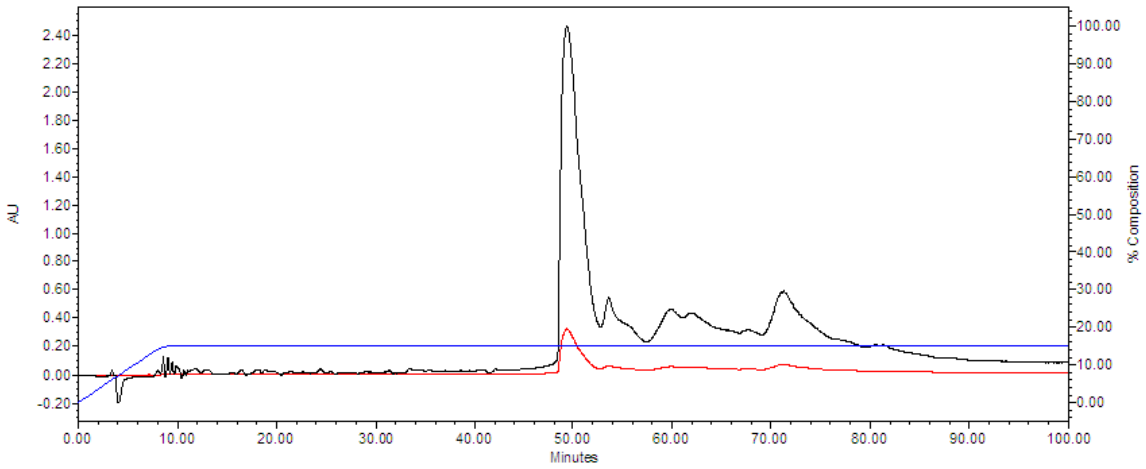

Figure 4. HPLC chromatogram of 1X sample on C18 semi-prep column. Major peak at 48min is peak of interest that was collected. Absorbance wavelengths are black-214nm, red-280nm. Blue shows % composition acetonitrile.

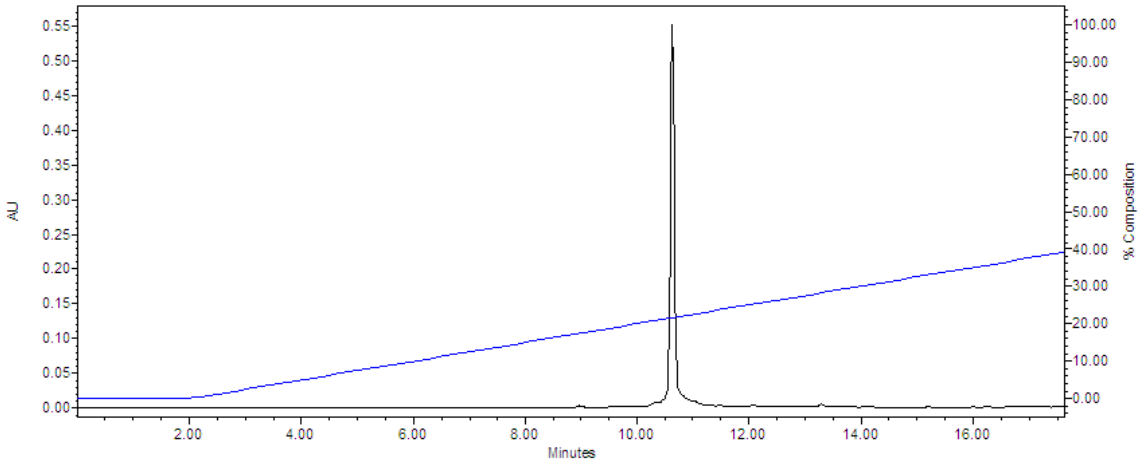

Figure 5. HPLC chromatogram of isolated 2X sample on C18 analytical column. Black is absorbance at 280nm. Blue shows % composition acetonitrile.

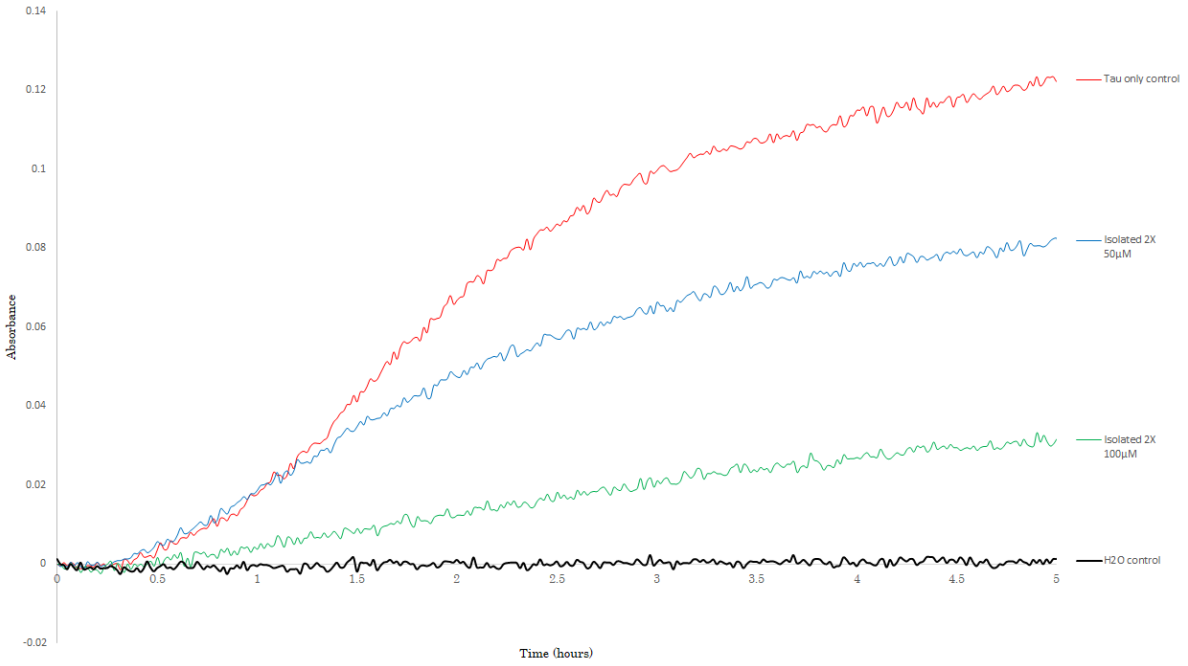

Figure 6. Absorbance spectrum of tau aggregation with isolated 2X sample. Wavelength is 350nm.

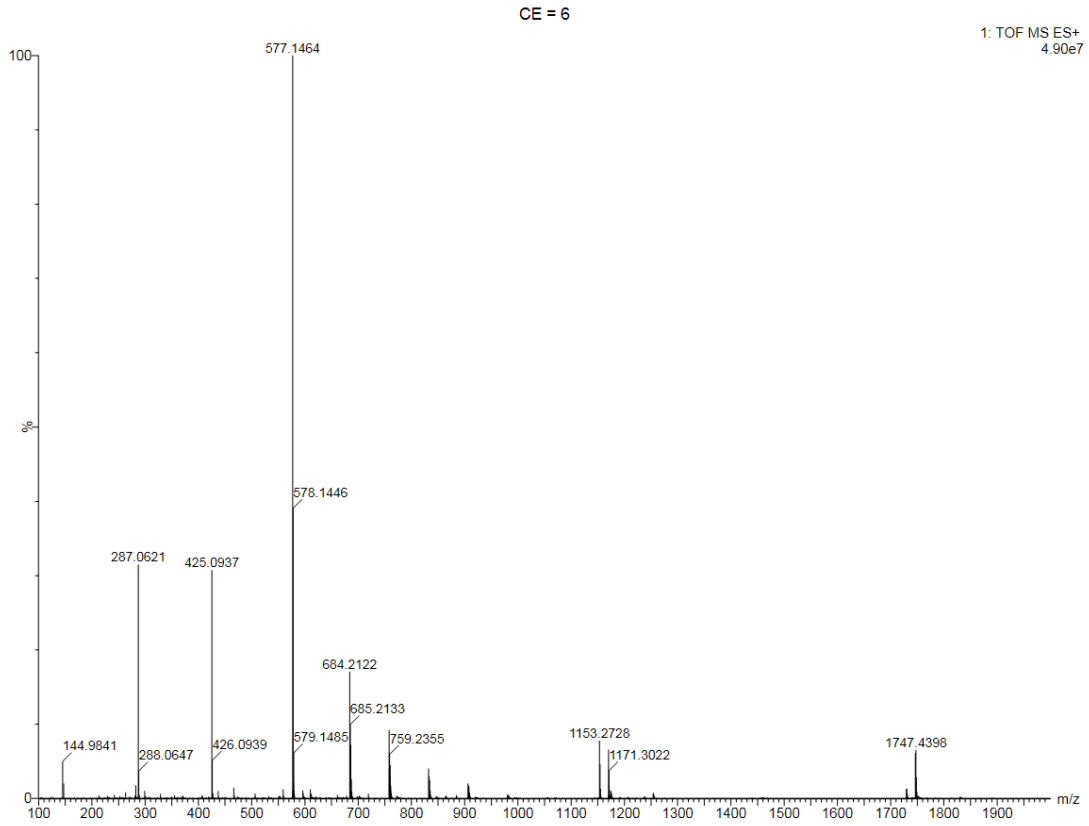

Figure 7. Mass spectrometry of isolated 2X sample. Major peak is 577Da corresponding to protonated A-type procyanidin (576Da). Secondary peaks are present at 425Da and 287Da.

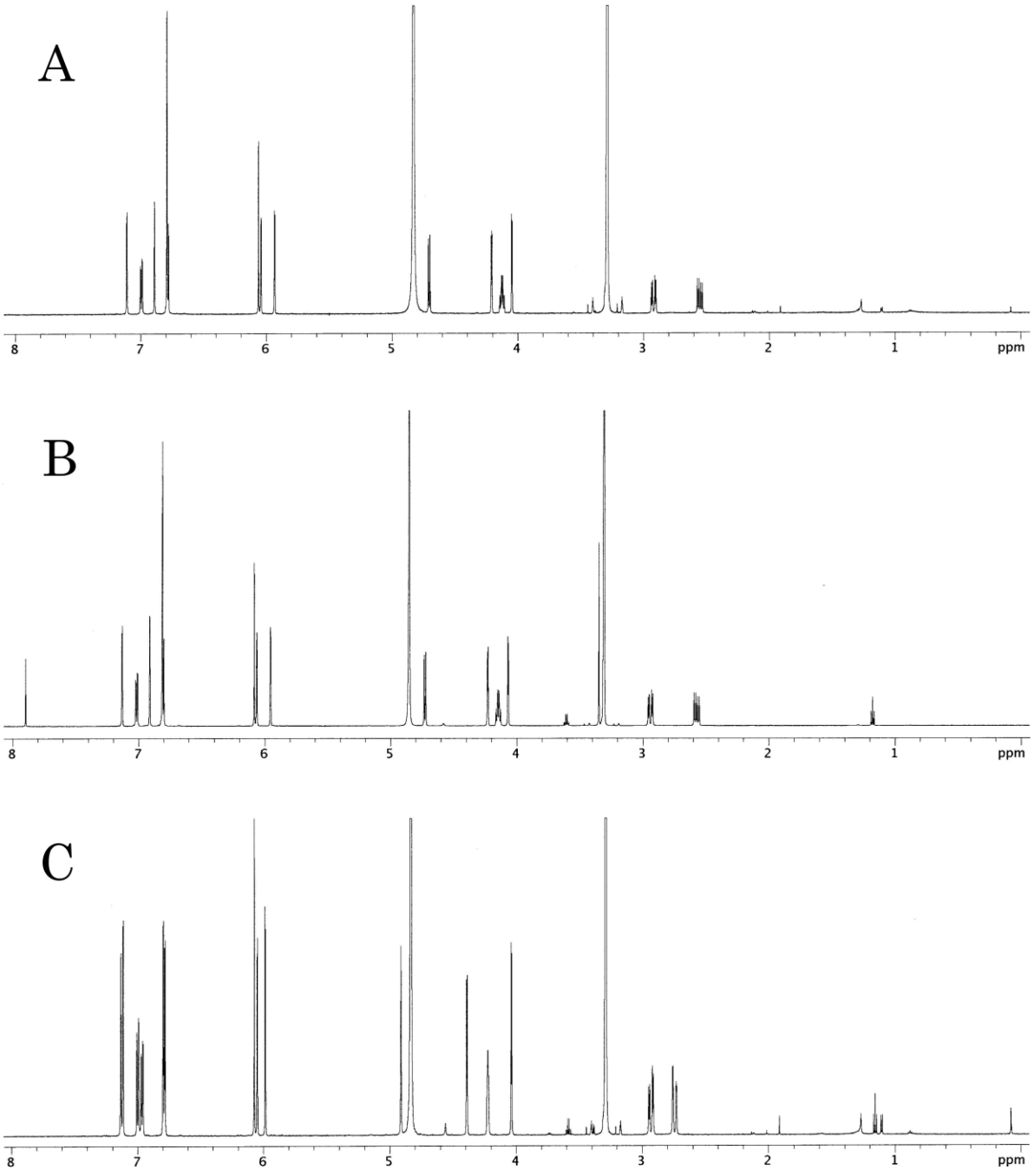

Figure 8. 1H-NMR spectrums of **A)** 2X isolated procyanidin from PSE, **B)** purchased procyanidin A1, and **C)** purchased procyanidin A2. Major peaks at ~3.3 and ~4.85ppm are the solvent (CD_3OD).

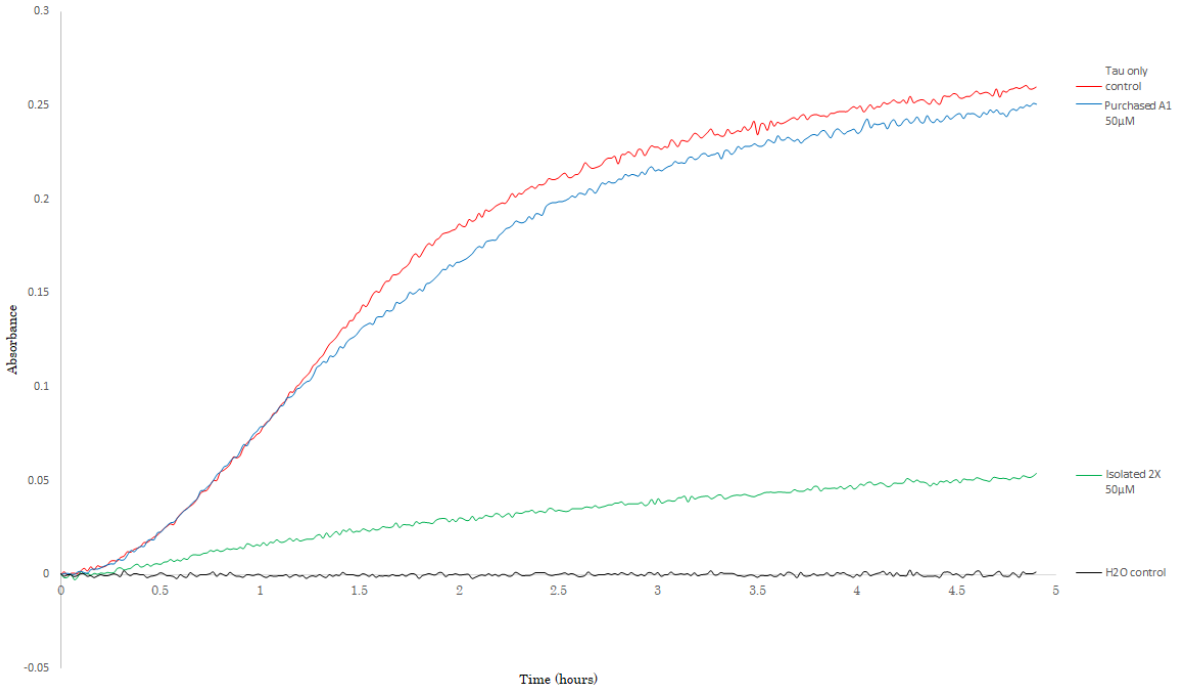

Figure 11. Absorbance spectrum of tau aggregation with purchased procyanidin A1 dimer at 50µM and isolated 2X at 50µM as positive control. Wavelength is 350nm. Differences in absorbances of equivalent samples across different spectra is due to the usage of different tau preparations.

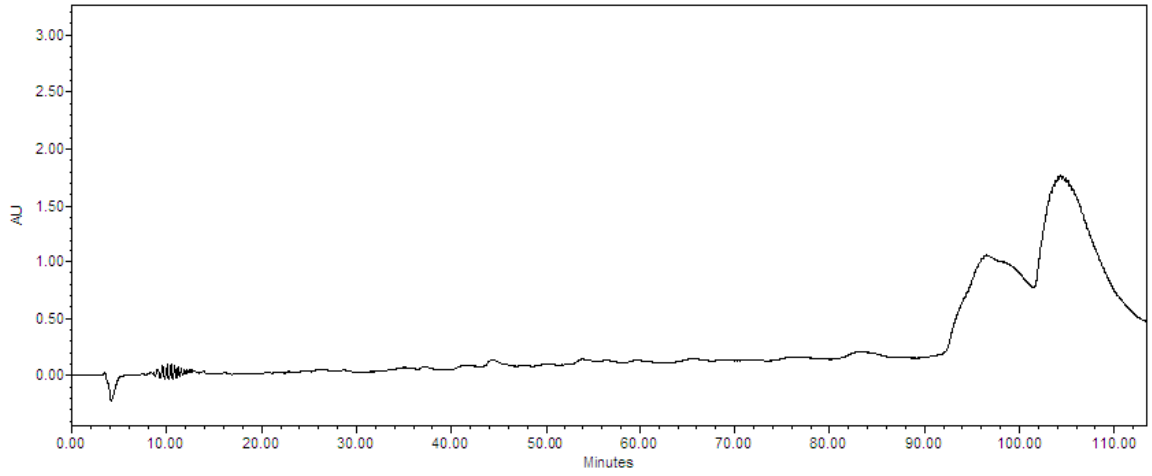

Figure 12. HPLC chromatogram of 1X sample on semi-prep column. Absorbance at 214nm. Acetonitrile concentration set to 15% but is lower due to pump leak. Leak led to single peak from figure 4 (at ~48min) to be split into two peaks. Mass spectrometry determined the second peak to be the procyanidin and the first to be an unknown molecule, possibly 466Da as seen in figure 13.

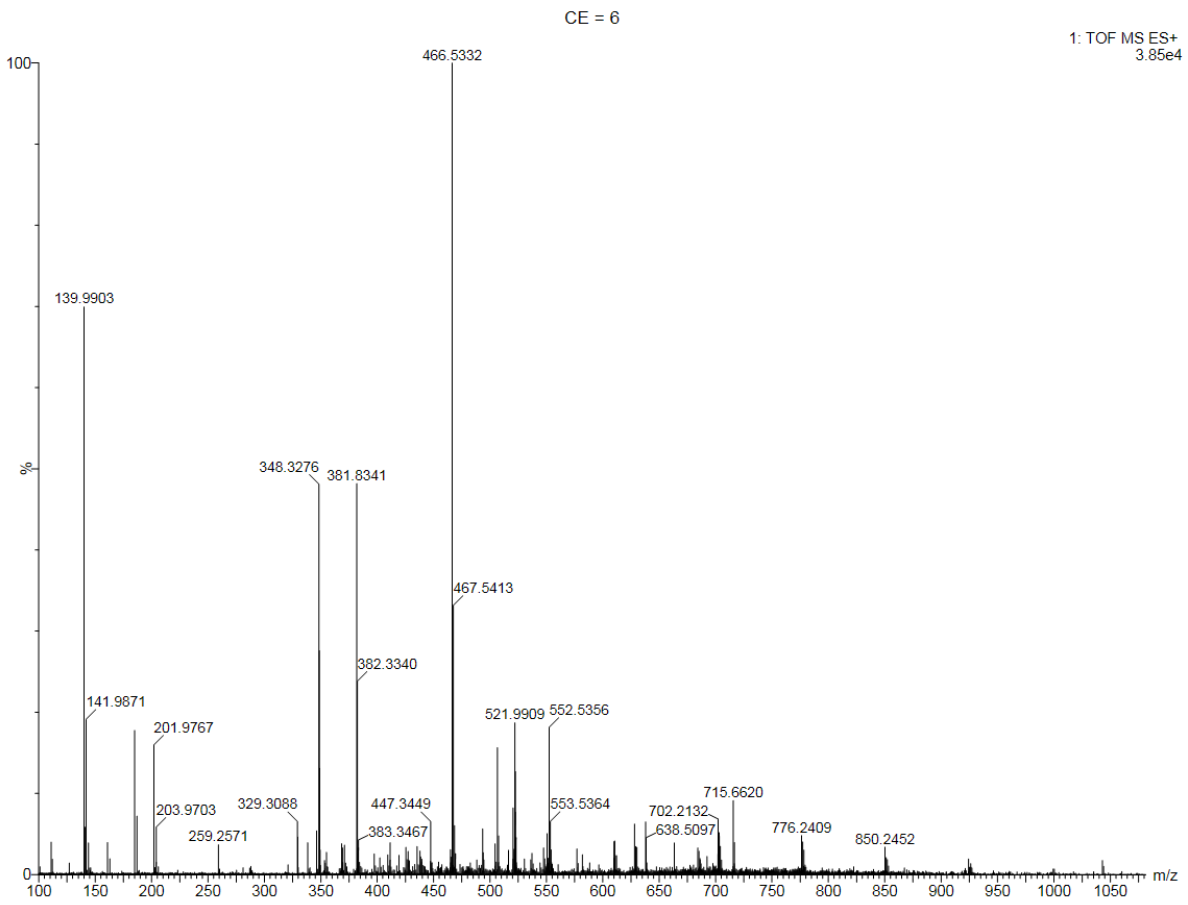

Figure 13. Mass spectrometry of first peak in figure 12. No procyanidin is present. Most abundant molecule is 466Da.

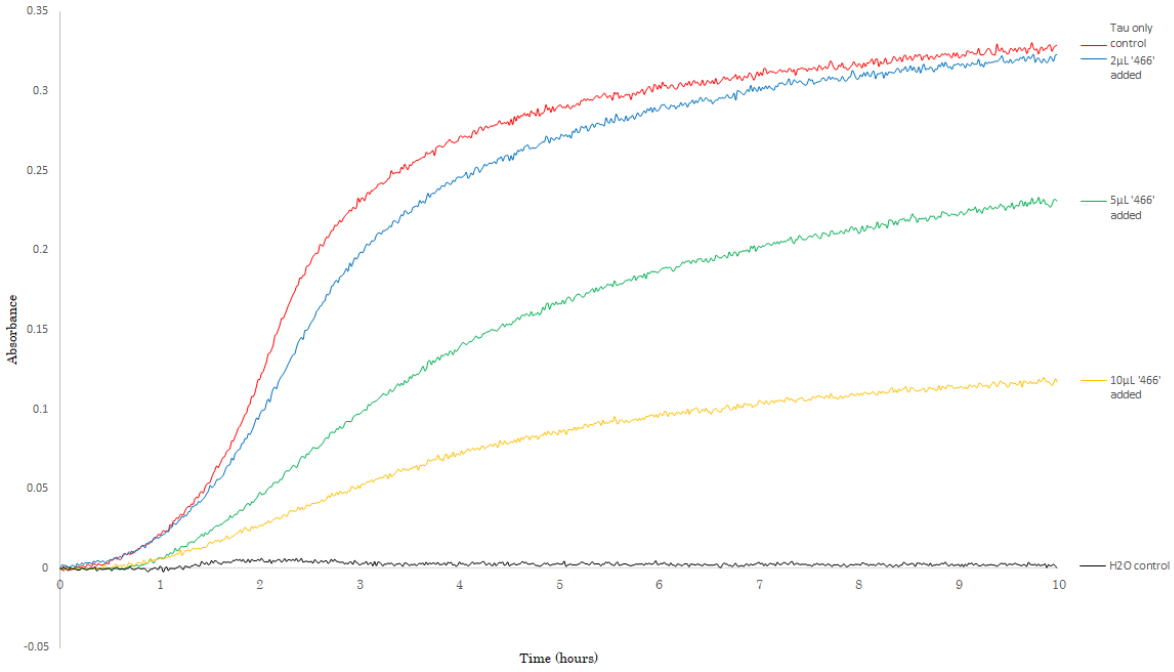

Figure 14. Absorbance spectrum of tau aggregation with sample '466' added at arbitrary amounts of 2, 5 and 10µL of stock solution. Molecule is unknown but using extinction coefficient for procyanidin A2 it would be the equivalent of 33, 84, and 167µM. Wavelength is 350nm.

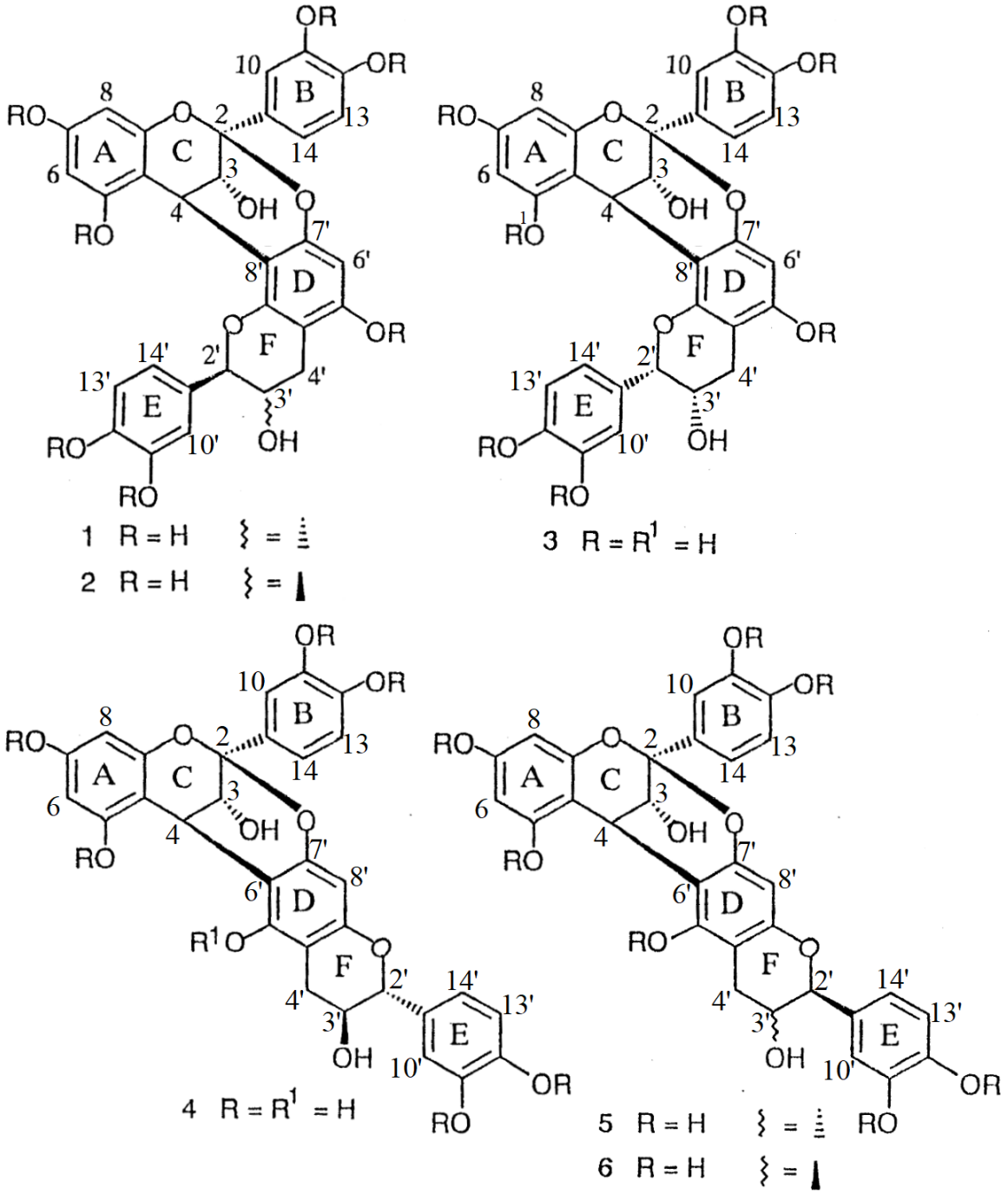

Figure 15. Procyanidins A1 through A6 structures from Lou et al.[22] All have the A-type 2ß-7 linkage. Structures 1-3 have a 4ß-8 linkage and 4-6 have a 4ß-6 linkage. Image was edited to include numbering for all carbons.

Ring	H	1	2	3	4	5	6
C	3	4.07 d (3.5)	4.05 d (3.4)	4.16 d (3.5)	4.12 d (3.5)	4. 10 d (3.5)	4.12 d (3.2)
	4	4.24 d (3.5)	4.39 d (3.4)	4.41 d (3.5)	4.29 d (3.5)	4.28 d (3.5)	4.30 d (3.2)
A	6	5. 90 d (2.4)	6. 00 d (2.3)	5.89 d (2.4)	6.03 d (2.2)	6.03 d (2.2)	6.01 d (2.3)
	8	6.05 d (2.4)	6.07 d (2.3)	6.07 d (2.4)	6.08 d (2.2)	6.09 d (2.2)	6.07 d (2.3)
B	10	7.13 d (2.4)	7.14 d (2.4)	7.15 d (2.4)	7.17 d (2.4)	7.16 d (2.4)	7.16 d (2.2)
	13	6.79 d (8.4)	6.82 d (8.2)	6.84 d (8.4)	6.82 d (8.4)	6.81 d (8.4)	6.82 d (8.1)
	14	7.01 dd (8.4, 2.4)	7.07 dd (8.2, 2.4)	7.04 dd (8.4, 2.4)	7.05 dd (8.4, 2.4)	7.06 dd (8.4, 2.4)	7.05 dd (8.1, 2.4)
F	2′	4.74 d (7.8)	4.91 br.s	5.02 br.s	4.67 d (6.5)	4.63 d (7.3)	4.80 br.s
	3′	4.16 m	4.23 m	4.23 m	4.05 m	3.99 m	4.16 m
	4′α	2.58 dd (16.5,8.1)	2.74 dd (17.3, 3.2)	2.87 m	2.81 dd (16.5, 5.4)	2.91 dd (16.7, 5.4)	2.87 m
	4′β	2.81 dd (16.5,5.4)	2.95 dd (17.3, 4.6)		2.62 dd (16.5, 8.1)	2.58 dd (16.7, 7.6)	
	6′	6.08 s	6.09 s	6.08 s			
	8′				6.09 s	6.07 s	6.09 s
D	10′	6.91 d (2.0)	7.13 d (2.2)	7.14 d (2.0)	6.80 d (2.2)	6.79 d (2.2)	6.93 d (2.2)
E	13′	6.80 d (7.8)	6.81 d (8.2)	6.81 d (8.2)	6.75 d (8.4)	6.70 d (8.4)	6.73 d (8.3)
	14′	6.80 dd (7.8, 2.0)	6.99 dd (8.2, 2.2)	6.95 dd (8.2, 2. 0)	6.68 dd (8.4, 2.2)	6.68 dd (8.4, 2.2)	6.75 dd (8.3, 2.2)

Table 1. ^{1}H-NMR data of A-type procyanidins 1-6 from study by Lou et al.[22] Units are ppm. s = singlet, d = doublet, dd = doublet of doublets, m = multiplet, br.s = broad singlet.