ORANGE JUICE CLOUD STABILITY AND THE INFLUENCE OF CALCIUM AND HESPERIDIN

by

LINDSEY MICHELLE ELLERBEE

(Under the Direction of Louise Wicker)

ABSTRACT

The clarification mechanisms of citrus juice cloud were evaluated in fresh juice and orange juice serum model systems. Ammonium oxalate (AO) was added to fresh juice at pH 4.0 and 5.5. Only juice at pH 4.0 without AO clarified over the three week study. In a model orange juice serum (OJS), the addition of hesperidin and protein (heated and unheated citrus protein and soy protein) showed that hesperidin interacted with protein. The % transmittance of OJS remained the same, and the protein controls were similar to results at lower hesperidin concentrations (<0.05 mg/ml). Higher concentrations of hesperidin (1.0-0.1 mg/ml) increased in turbidity in the presence of protein. Particle size tended to increase for heated and unheated citrus protein with hesperidin, but hesperidin or protein had little effect on particle size. Changes in particle size and cloud characteristics suggest non-electrostatic, non-covalent interactions are involved in cloud interactions and stability.

INDEX WORDS: Hesperidin, Cloud, Orange juice, Calcium chelation, Clarification, Citrus juice cloud stability

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DEDICATION

I would like to dedicate this thesis to my amazing husband, Phil, for all of his patience, love, and support; to my mom for her constant encouragement and shoulder to cry on; and to the memory of my father who believed that I could do anything I set my mind to.

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CHAPTER 1

INTRODUCTION

Much work has been done to understand the clarification mechanisms of citrus juice cloud; however, it is still not fully understood. Orange juice cloud loss is typically attributed to pectin methylesterase (PME), an enzyme endogenous to oranges. PME de-esterifies high methoxyl pectin, and the resulting negatively charged sites on the pectic acid react with calcium cations forming insoluble calcium pectate. The calcium pectate becomes too large to remain suspended and pulls the juice cloud down with it (Stevens and others, 1950).

The objective of the experiments was to further investigate the role of calcium in fresh juice and flavonoids in citrus juice model systems using techniques that detect association and dissociation interactions of cloud constituents. The first, the Turbiscan®, is an excellent way of detecting clarification and sedimentation in a sample without the need to dilute or disturb the sample. It measures percent transmission and percent backscattering in a graph format. The Malvern measures particle size by volume distribution. The Brookhaven measures both particle size and zeta potential. Both the Brookhaven and Malvern measure by light scattering, but the Brookhaven measures particles in the nanometer range, whereas the Malvern measures particles in the micrometer range.

In the absence of free calcium, fresh orange juice with active pectinmethylesterase will remain cloud stable (Krop and Pilnik, 1974). In the first experiment, the addition of AO to fresh juice at pH 4.0 and 5.5 was tested with the three light scattering techniques. In the second experiment, a model system of orange juice serum (OJS) was used to evaluate the effects of a flavonoid on protein interaction and haze development. The effects of hesperidin and pectin on a

model cloud system have previously been reported by Kanner and others (1982); however, hesperidin-protein interactions have not been characterized.

Different model systems were chosen for each experiment to better fit the objectives of each study. In the first model system, fresh juice was used to examine the effects on particle size growth when the system was under different conditions. The normal pH of juice (pH 4.0) promotes interactions and a higher pH (pH 5.5) inhibits interactions that affect particle size. Serum separation and sedimentation of fresh juice under different conditions were also tested and all were compared to % transmission, which is the juice industry's standard quality control test.

In the second model system, OJS was used because it contains all the components of cloud and has been successfully used as a predictive model for juice cloud interactions (Baker and Bruemmer, 1969; Ackerley and Wicker, 2003). OJS is a clear serum that provided the visibility to see potential hesperidin, protein, and/or hesperidin-protein interactions. Kanner and others (1982) used a model cloud system made up of citric acid, tri-potassium citrate, sucrose, and sodium benzoate at pH 3.8 and were able to show hesperidin-pectin interactions. Though this synthetic model system worked for the stated authors, it was thought that a model cloud system derived from orange juice would potentially give a better indication of the interactions occurring and therefore was used for the current experiment.

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CHAPTER 2

LITERATURE REVIEW

Introduction

Orange juice is the most widely produced fruit juice, and there is a large cost associated with its production, as well as its import and export (USDA, 2004). With so much money spent in the orange juice market, it is important to find ways to best increase its shelf life. Not only is orange juice popular, but it also has many health benefits including vitamins and antioxidants. Fresh squeezed orange juice is typically preferred over processed juice in sensory studies in terms of flavor and color. For this reason, and because processing can decrease the nutritional benefits, minimal processing for orange juice is desired. To better discover ways of minimal processing, it is important to know the components of orange juice and their effect on juice stability.

Orange Juice Cloud

Orange juice cloud is responsible for much of the color, flavor, turbidity, and aroma of orange juice (Baker and Cameron, 1999). The cloud is in fact a separate component of the juice and not merely smaller pieces of pulp (Scott and others, 1965). If the cloud is removed from orange juice, the result is a mostly clear serum that is unacceptable to consumers in taste and appearance.

The composition of juice cloud was estimated by Sinclair (1984) and discussed in reviews (Braddock, 1999; Crandall and others, 1983). Protein is the most abundant material in citrus juice cloud; it contributes approximately 52.4% of the cloud of commercial orange juice

(Klavons and others, 1991). In the protein constituent, 53% is insoluble protein, 30% is complexed with low molecular weight cloud components, and 17% is covalently bonded with components such as hemicellulose. Commercial orange juice cloud contains approximately 4.5% pectin, and in the pectin constituent, 60% is associated with insoluble protein, 25% is calcium pectate, and 15% is protopectin (Klavons and others, 1994).

The color of the juice cloud is due to many factors. The orange – yellow color of orange juice is due to carotenoids found in the flavedo, or peel, and juice vesicles which get into the juice during processing (Gross, 1977). The range of colors seen in orange juice are due to cultivar, season of production, processing methods, and maturity of the fruit (Huggart and others, 1975; Barron and others, 1967). Of the three most common orange cultivars, Valencia has the highest rated color, followed by Pineapple, and then Hamlin (Huggart and others, 1975). The USDA has a set of color standards that orange juice in the United States has to meet (USDA, 1982). In order to meet the USDA orange juice color standards, orange juice from late season Valencia, mid season Pineapple, and early season Hamlin oranges is mixed together; this also helps avoid variation in appearance from season to season of orange juice. Valencia can also be found unblended as a premium product.

Pectin and PME

Pectin is a polysaccharide made up of α1-4-linked D-galacturonic acid units and rhamnose inserts in the backbone, with side chains of primarily galactose, arabinose, and xylose (Voragen and others, 1995). Pectin is found in the flavedo, albedo, membrane, juice vesicles, and core of an orange (Chen and others, 1993). PME is an endogenous enzyme to oranges, and it cleaves the methyl esters on pectin molecules, which gives methanol and free pectic acid. The

cleavage of the methyl esters turns high methoxyl (HM) pectin into calcium sensitive low methoxyl (LM) pectin. If a pectin is more than 50% methylated, it is considered a high methoxyl pectin, and if it is less than 50% methylated, it is considered a low methoxyl pectin. If a pectin is less than 10% methylated, it is then considered pectic acid.

At a specific DE, calcium cations crosslink the free pectic acid units to free units on adjacent pectin molecules, forming insoluble calcium pectate gels that become too large and fall out of suspension, pulling the cloud with it. However, it is not clear how the cloud constituents interact with the calcium – pectate gels. The critical DE at which the calcium cations crosslink with pectic acid is difficult to pinpoint because of the heterogenous nature of pectin. The molecular weight, presence of neutral sugar side chains, and DE distribution varies within each pectin molecule. Pectin contains smooth regions of galacturonic acid backbone and hairy regions of sugars. Because of the hairy side chains, the action of the PME is disrupted, and the DE of the pectin is affected. Several groups have attempted to determine the critical DE value associated with cloud loss, and the numbers range from 14 – 21% (Baker, 1979), 27% (Krop, 1974), and 36% (Ben-Shalom and others, 1985). A study by Ackerley and others (2002) found that pectins from clarified juice had a DE of 13% and pectins from juice that did not clarify had a DE of 19%.

A study was done on the amount of pectin and PME found in several different components of oranges (Rouse, 1953). The flavedo (the outer layer or peel), albedo (the white inner lining of the peel), rag (segment membranes), juice sacs, seeds, and centrifuged juice were the components tested, and Hamlin, Pineapple, and Valencia were the orange cultivars tested. The rag had the highest concentration of pectin in Pineapple and Valencia, and the albedo had the highest concentration in Hamlin. Of the three, Valencia had the highest concentration of

pectin in both the albedo and the rag. The juice sacs had the highest concentration of PME for all three cultivars, and Pineapple orange had the highest PME concentration of the three.

Apple Juice Model

The apple juice cloud model proposed by Yamasaki and others (1967) is well accepted as the mechanism by which apple juice clarification occurs. Yamasaki and others (1967) proposed that in apple juice there is a positively charged protein that is coated with negatively charged pectin. The pectic enzymes present in apple juice break down the pectin coating, exposing the positively charged protein. Neighboring pectin that has not yet been broken down can then electrostatically bind to the exposed protein. This interaction is progressive, eventually leading to sedimentation. Yamasaki and others (1964) also found that no clarification occurs in apple juice with pectic enzymes at pH 6, but upon dropping the pH to 3.5, clarification occurs. The pI of the protein in apple juice is between pH 4 and 5, and at pH 6, the protein is negatively charged and does not electrostatically bind to the negative pectin even though the enzymes are still working. However, dropping the pH below that of the pI causes the cloud protein to return to its normal positive charge and flocculation occurs.

Hesperidin in Orange Juice

Hesperidin is a flavonoid, a class of polyphenols, that is unique to citrus fruits. It has been said to have many health-benefitting properties, such as mild anti-inflammatory, analgesic, and mild antipyresis properties (Emim and others, 1994). Baker and Cameron (1999) have said that flavonoid crystallization is affected by the cultivar of orange, juice extraction pressure, holding time before pasteurization, and pasteurization itself. The authors also found that

Shamouti and Satsuma mandarin cultivars both contain high levels of hesperidin, but that most other orange cultivars do not contain much hesperidin. Dhuique-Mayer and others (2005) measured the hesperidin content in several orange cultivars and found that Shamouti contains about 552 mg/L, Hamlin 317 mg/L, and Valencia 257 mg/L hesperidin in the hand-squeezed juice.

Mizrahi and Berk (1970) did a study on Shamouti orange juice, and found that the clear serum of fresh juice from which the cloud had been removed became turbid upon sitting within two hours. The serum continued to increase in turbidity over the first forty-eight hours. The authors studied the serum by microscope and found needle-like crystals that were determined to be hesperidin. Shamouti oranges were determined to be saturated with hesperidin, and this study showed that hesperidin could be a significant factor in juice cloudiness. Another group saw a large increase in cloudiness in fresh Shamouti orange juice that was also attributed to the crystallization of hesperidin (Rothschild and Karsenty, 1974).

Bennett and Albach (1981) proved that the formation of white spots on freeze damaged Valencia oranges is due to hesperidin crystallization. Hesperidin is unevenly distributed in the cell vacuole of citrus fruit, and crystallization of the hesperidin occurs when the cell membrane is broken. When freezing occurs, the cell membranes are broken, releasing hesperidin, and the uneven distribution of hesperidin in the cells causes the non-uniform white spots on the orange.

Kanner and others (1982) studied the effects of hesperidin in a model cloud system. The authors found that a stable cloud formed with high methoxyl pectin (HMP) and hesperidin in the model cloud system. An increase in cloudiness was observed as the concentration of hesperidin increased, but not when the pectin was increased. The authors found that the most stable cloud formed with a ratio of 13.3 to 1 of HMP to hesperidin. The authors also tested low methoxyl

pectin (LMP), sucrose, polygalacturonic acid, alginates, carrageenans, and carboxymethyl-cellulose in place of the HMP. An increase in turbidity was seen in all cases, but it was less than that of HMP and was not stable. However, another study tested pectins with a DE range of 10% to 73% with hesperidin, and the turbidity of the suspensions did not change over that range (Ben-Shalom and others, 1984). That same group found by microscopic study that the solutions containing pectin had a smaller crystal size than the solutions that did not contain pectin. For crystallization, faster nucleation means large numbers of nuclei are formed before relief of the supersaturation occurs, which in turn leads to a smaller final crystal size; this is based on von Weimarn's law (Adamson, 1976). From von Weimarn's law, the authors concluded that the nucleation site for hesperidin is pectin (Ben-Shalom and others, 1984).

The same group reduced the viscosity of a pectin solution in a model cloud system by polygalacturonase and correlated the viscosity to the molecular weight of the pectin (Ben-Shalom and others, 1984). They found that below a certain molecular weight the turbidity would decrease. From this, the group hypothesized that a critical molecular weight of pectin is needed to stabilize a specific amount of hesperidin. The authors found that pectin with a molecular weight between 76,000 and 84,000 Daltons could stabilize hesperidin without flocculation.

Pectin and hesperidin bind tightly together (Ben-Shalom and others, 1985). This was discovered by separation on a sucrose gradient. The authors took the bands of pectin-hesperidin and were able to resuspend them to form a stable cloud. A ratio of 1:2 of hesperidin to pectin was found in the bands. It was also found that only fifteen percent of the pectin in the total solution was responsible for stable cloud formation (Ben-Shalom and others, 1985), as was also seen by Baker and Bruemmer (1969).

Ben-Shalom and others (1998) discovered that by removing the neutral sugars from hesperidin, no cloud would form in a pectin solution. The neutral sugars on hesperidin are rhamnose and glucose, and the compound without the sugars is known as hesperetin. The authors concluded that it was the neutral sugars on hesperidin that acted as the recognition site for pectin. The authors also tested two different commercial pectins with almost identical molecular weights and DE but with different quantities of neutral sugars; one had 80% neutral sugars and the other 40%. The pectin with 80% neutral sugars reacted with hesperidin to form a stable cloud, but the pectin with 40% neutral sugars reacted with hesperidin and fell out of solution. Therefore the authors concluded that pectin with a higher neutral sugar content reacts with and better stabilizes hesperidin than one with a low neutral sugar content. The authors found that there are three different types of pectins in a given commercial pectin and that the same may be true for the pectin in orange juice. The first type of pectin is found in the clear serum of orange juice and has no interaction with hesperidin. The second type interacts with hesperidin to form stable colloidal particles. The third type interacts with hesperidin but does not form stable colloidal particles; it instead precipitates out of suspension with hesperidin.

Oil in Orange Juice

Scott and others (1965) found that oil in the cloud of orange juice could be involved in the cloud formation. In a study by Mizrahi and Berk (1970), the authors found that oil droplets attached to the surface of the cloud particles in orange juice could help stabilize the cloud by decreasing the density of the particles so that the cloud density becomes closer to the density of the serum. To test this hypothesis, the authors added essential oil to orange juice and found that as the amount of oil was increased, the cloudiness of the orange juice also increased. However,

the addition of too much oil caused the cloud to fall apart and float to the top with the attached oil droplets, which the authors conclude assists the theory on oil stability.

Particle Size and Cloud Stability

Citrus juice cloud particles typically range in size from 0.4 to 5.0 μm (Klavons and others, 1991), while the most stable clouds have particle sizes of 2 μm and smaller (Mizrahi and Berk, 1970). Of the three main orange juice cultivars, Valencia had the highest percentage of particles in the 1-2 μm range (Buslig and Carter, 1974). Pineapple followed as second, and Hamlin had the least percentage of cloud particles in the stable cloud range. Buslig and Carter (1974) obtained particle size using a Coulter counter.

Corredig and others (2001) compared the effect of PME-sensitized pectins and alkali-sensitized pectins on the particle size of orange juice cloud. They found that PME-sensitized pectins showed greater particle size increase than the alkali-sensitized pectins. With increasing amounts of pectin, the particle size of the cloud particles also increased; therefore, the authors concluded that particle size is dependent on the amount of pectin present in the orange juice. Cloud size changes were monitored using integrated light scattering analysis, and the authors found that there were definite interactions between cloud particles and charged pectin. Aggregation of the cloud particles, as shown by an increase in the particle size, is necessary for cloud loss to occur. Furthermore, the authors concluded that the aggregation is caused by bridging of cloud particles to charged pectin. Wicker and others (2002) reported an increase in particle size of cloud particles in reconstituted orange juice samples with added PME and/or cations. This occurred prior to the onset of gross clarification and increase in transmittance of the juice.

Protein and Cloud Stability

Shomer (1988) did a study on the effects of heat treatment on the protein portion of cloud. He found that heat coagulated proteins will encapsulate droplets of oil, colloidal constituents (1988), as well as pectins and neutral sugars (1991). In a subsequent study, Shomer and others (1999) found that clumps develop in the insoluble cloud matter at temperatures above 70° C and at pH 3-4, which are conditions where proteins coagulate and flocculate. Cloud floc was more prominent at pH 3.5, where pectin is more soluble and PME is less active, than at any other pH between the tested range of 1.5 to 6.5. Cloud floc at pH 3.5 was also enhanced by enzymatic pectin degradation plus heating, The authors suggested that protein coagulation/flocculation results in clarification. They concluded that the association between pectin and protein is enhanced by PME activity, which causes cloud protein-pectin flocculation.

Pectin and Cloud Stability

To test the effects of serum pectin on cloud stability, one group used ultracentrifugation to separate the cloud from the serum in fresh juice and found that the resuspended cloud in water was not only stable, but remained so through the addition of pectin, calcium, sugar, and citric acid (Baker and Bruemmer, 1969). The authors also tested heated serum with heated cloud, which was stable; heated serum with unheated cloud, which clarified slower than unheated serum with heated cloud; and both unheated, which clarified rapidly. This suggests that serum pectin does not stabilize cloud, but may in fact play a role in cloud instability.

The same group also took the ultracentrifuged serum and studied the floc that developed after about six days, which is about the same amount of time it takes for clarification of fresh orange juice when both are stored at 60° F (Baker and Bruemmer, 1972a). The authors found that

the floc contained pectates and hesperidin. The floc clarified cloud that had been suspended in serum, but soluble pectin added to the solution inhibited the clarification.

PME Isozymes and Cloud Stability

Several PME isozymes have been confirmed. Versteeg and others (1980) isolated three PME isozymes, two of which were thermolabile and were inactivated by temperatures over 70° C, and one that was thermostable and required a temperature of 90° C for inactivation. The authors found that the thermolabile isozymes, when added to orange juice, clarified the juice very slowly, if at all; the thermostable isozyme however, rapidly clarified the juice.

Six PME isozymes from Valencia juice sac were isolated, two of which remained active at 90°C and a third that retained activity after incubation at 95°C for 30 seconds (Cameron and others, 1994). The most heat stable of these six PME isozymes had a molecular weight between 37.5 kDa and 40.5 kDa, with none of the isozymes having molecular weights above 50 kDa. In a subsequent study, Cameron and Grohmann (1996) isolated a thermostable PME isozyme from Valencia fresh frozen orange juice with a molecular weight of 40.1 kDa that retained 49.2% activity after sixty seconds in a 95°C water bath.

Cameron and others (1998) isolated four PME isozymes, three of which were thermolabile. Two of the thermolabile isozymes showed juice clarification. Ackerley and others (2002) showed that a thermolabile PME with 36 and 27 kDa protein bands would rapidly clarify juice, while one with 36 and 13 kDa protein bands would not rapidly clarify juice.

Han and others (2000) isolated seven PME isozymes from Valencia orange peel. Three of the seven were isozymes with molecular weights of 70, 60, and 27 kDa; the other four were

thermolabile isozymes all with a molecular weight of 35 kDa. PME inactivation of 83.4% was seen after five minutes at 70°C and 98.3% after one minute at 90°C.

Cameron and others (1997) studied the PME extracts from the peel, rag, and juice of oranges. They found that thermostable PME destabilizes cloud faster than a mixture of thermostable and thermolabile PMEs. The authors separated the PME by dialysis supernatant (DS) and dialysis precipitate (DP), and also had a heated dialysis supernatant sample (HDS) (2 minutes at 80°C). The juice DP clarified juice quicker than the rag HDS at room temperature, but the two clarified at the same rate in refrigerated temperatures. Peel HDS clarified juice more quickly than juice HDS, and overall, peel extracts clarified quicker than extracts from the juice or rag. The authors concluded that in processing orange juice, care should be taken to avoid getting particles of peel into the orange juice. To further illustrate this point, another study showed that juice extraction under harsh conditions resulted in more peel content in the juice than did juice extraction under mild conditions; the milder conditions produced more stable orange juice than did the harsher conditions due to less peel content in the juice (Cameron and others, 1999).

Cations and Cloud Stability

MacDonnell and others (1945) stated that monovalent and divalent cations increase the activity of PME. Since then much work has been done on cations and their effect on PME and juice cloud stability. Ben-Shalom and others (1985) studied the effect of calcium on a pectin-hesperidin solution with varying degrees of pectin esterification. It was found that at a DE higher than 36%, a pectin-hesperidin solution with as much as 1000 ppm calcium did not flocculate or lose cloud. Since the solution did not contain active PME, it was concluded that PME and

calcium were not the causes of flocculation in orange juice where the pectin has a DE higher than 36%.

PME can form an inactive enzyme-substrate complex and cations can act as competitive inhibitors by displacing the PME; this is confirmed by Lineweaver Burk plots (Nari and others, 1991). The catalytic rate increases as the cations interact with the substrate instead of the enzyme. The cations can accelerate clarification in orange juice (Wicker and others 2003). Wicker and others (2002) added thermolabile PME to stable reconstituted orange juice in the presence and absence of cations. They found that the higher levels of cations caused clarification more quickly than the lower levels. If PME was left out of the system, the cations did not clarify the juice. If both PME and cations are absent from pasteurized juice, the juice remained stable at 4° C.

Leiting and Wicker (1997) studied the effects of cations and polyamines on the activity of citrus PME extracts and solubilization of PME from the cell wall. Inorganic cations (calcium chloride, ferric chloride, and lead acetate) stimulated PME activity at varying levels and to different degrees of intensity. The polyamines studied (putrescine, spermidine, and spermine) did not stimulate PME activity; however, inhibition was seen at higher levels of concentration. The authors concluded that the activation and solubilization of PME is not due solely to competitive displacement.

pH and Cloud Stability

Commercial orange juice has a pH range of 2.6 – 4.4 (Chen and others, 1993). Stevens and others (1950) observed little change in the rate of flocculation over the pH range of 2.8-3.8. However, they discovered that the rate of flocculation drastically decreases at pH levels of lemon

juice (pH 2.2-2.4). Also observed was that once active PME concentrations were decreased by pasteurization, the pH sensitivity of the juice cloud stability increased.

Rouse and Atkins (1952) found that as the pH of orange juice is decreased, the temperature required for complete inactivation of PME also decreases. Ben-Shalom and others (1984) found that a pectin-hesperidin suspension had a maximum turbidity between the pH values of two and seven. Reducing the pH below two decreased the turbidity and increased precipitation. It was concluded that the amount of hesperidin that can be stabilized by pectin is pH dependent, but cloud formation is independent of pH.

Owusu-Yaw and others (1988) tested the effects of pH on both fresh and reconstituted orange juice. Both were treated with a cation exchange resin that reduced the pH from 3.75 to 2.0 and with concentrated HCl that also reduced the pH to 2.0. Both methods fully inactivated PME, and cloud remained stable over the twelve week study. Microbial death was observed in the cation exchange resin treated samples, but not in the HCl treated samples. The authors suspect this to be due not only to the low pH, but also to the nutrient loss due to resin treatment. Adversely, more than 95% of the vitamin C in the samples was lost. Also, both of these methods of pH reduction cause a loss of sensory qualities that leaves the juice unacceptable (Arreola and others, 1991).

Enzymes and Cloud Stability

Baker and Bruemmer (1972b) found that adding certain commercial pectinases to orange juice that had not been heat treated would stabilize the cloud. The authors suggested that the method by which this occurs is through the degradation of insoluble pectin to soluble pectates instead of insoluble pectates. Baker and Bruemmer (1971) had previously tested the effects of

treating orange juice with a commercial pectinase along with a commercial protease, followed by pasteurization. The pasteurization temperature used in this study was the necessary temperature to make orange juice safe from a microbiological standpoint. The addition of the pectinase and protease for 50 minutes at 27°C, followed by pasteurization at 74°C produced a much denser and more stable cloud than orange juice pasteurized at 74°C, both initially and throughout the study. They also found that overnight treatment with both enzymes at 15.5°C, followed by pasteurization at 74°C produced a much denser and more stable cloud than orange juice pasteurized at 93°C, which is the typical temperature needed for cloud stabilization. The authors stated that the orange juice had to be treated with the enzymes prior to pasteurization; enzyme treatment after pasteurization produced cloud loss.

Enzymes can also be used to treat finisher pulp left after juice extraction (Braddock and Kesterson, 1975). Enzyme-treated pulp results in lower viscosity concentrates, allowing the producers to achieve 70° Brix. Enzyme treatment also resulted in the recovery of more sugar-containing soluble solids. Total sugars can see an increase of 33%, glucose 14%, fructose 28%, and sucrose 37%. This pulp wash concentrate can be used as a beverage base or clouding agent, and the manufacturers can list it as concentrated orange juice or solids on the ingredients list (Braddock, 1999).

Heat Treatment and Cloud Stability

Orange juice cloud is commercially stabilized by pasteurization at 90 – 95° C for 15 – 60 seconds (Chen and others, 1993). The high temperatures are not necessary for microbial destruction but because there is more than one form of PME in cloud that is inactivated only by

these high temperatures (Baker and Cameron, 1999). The problem with the high temperatures of pasteurization is browning and flavor deterioration in the orange juice (Chen and others, 1993).

Stevens and others (1950) looked at the pasteurization of orange juice from 50°C to 80°C. What they saw was the first indication of multiple isozymes of PME in oranges. The authors observed a decrease in flocculation at a temperature of 50°C, and the amount of flocculation continually decreased as the temperature was increased.

One study used a mild heat treatment on fresh juice and saw an increase in cloud stability (Mizrahi and Berk, 1970). The authors concluded that the increase in cloud stability could not be due to the inactivation of PME since the juice was fresh, the cloud stability was measured soon after processing, and the heat treatment was too mild to fully inactivate PME. After the heat treatment, an increase in the serum viscosity was seen. The mild heat treatment on a diluted portion of the orange juice showed greater cloud stability and a lower serum viscosity than that of the undiluted sample. From this, the authors concluded that the stabilizing effects of the heat treatment cannot solely be explained by the increase in serum viscosity.

Another group looked at the effect of holding time before pasteurization on fresh Shamouti and Valencia orange juice (Rothschild and Karsenty, 1974). The authors reported an increase in cloudiness of both Shamouti and Valencia orange juice after just thirty minutes of holding time, and after twenty-four hours, the cloudiness of both juices was still higher than the initial cloudiness. Pasteurization of the juices also caused an increase in cloudiness that was determined to be due more to heat effects than to mechanical effects. It was found that pasteurized, reconstituted Shamouti orange juice had the same turbidity as pasteurized Valencia orange juice, even though Shamouti orange juice has a very low initial turbidity.

Ben-Shalom and others (1984) tested the effects of heat treated pectin and the addition of hesperidin on the stability of the cloud. The pectin solution was heated to a specific temperature and held for a given time. Once the pectin solution was cooled to room temperature, hesperidin was added, and the turbidity was monitored. From 22° to 70° C, no real change in turbidity or viscosity was seen; however, from 70° to 100° C, the turbidity decreased drastically and the viscosity decreased by about 15%. Enzyme degradation of the pectin solution to the same viscosity showed only a 49% decrease in turbidity. The authors concluded that heat treatment causes chemical changes that affect the stability of the cloud more so than enzyme treatment.

Heat treatment is the cheapest, easiest, and most widely used method to stabilize orange juice. It effectively eliminates microorganisms and inactivates PME, allowing safe, cloud stable orange juice. However, heat treatment has some significant sensory disadvantages, which is why other processing techniques without the negative effects are sought. Consumers desire nutritious foods that are minimally processed and retain a fresh flavor and color.

PME Inhibition/Inactivation and Non-thermal Processing Methods

A glycoprotein in kiwi inhibits PME (Balestrieri and others, 1990). It has a sugar portion made up of galactose, arabinose, and rhamnose. The glycoprotein was proven effective as a PME inhibitor in the pH range of 3.5-7.5, and it does not appear to be limited by the source of the PME. Castaldo and others (1991) continued work with the PME inhibitor found in kiwi. Their study used “cutback” concentrates, which are samples of fresh unpasteurized juice added to pasteurized concentrated orange juice. To these “cutback” concentrates, the PME inhibitor was added, and the samples were stored at 5°C for eight months. The inhibitor prevented juice clarification.

High pressure has been used by several people to inactivate PME. Loeffler (1941) was the first to use high pressure (homogenization) and saw that homogenization before pasteurization increases the cloudiness of orange juice. Seyderhelm and others (1996) saw an inactivation of PME in orange juice concentrates at 600 MPa, 45°C for ten minutes. Goodner and others (1999) used high pressure processing on fresh Valencia orange juice and were able to stabilize the juice for 90 days in refrigerated temperatures by using 700 MPa for one minute. The high pressure processing also destroyed microbes present in the juice, making heat pasteurization unnecessary. Basak and Ramaswamy (1996) found that the inactivation of PME was dependent on the pressure level, pressure-hold time, pH, and total soluble solids. A lower pH provided higher levels of PME inactivation. Pressure-hold time inactivation followed first-order rate kinetics. Higher Brix values decreased in PME inactivation. Betoret and others (2008) used homogenization pressures from 0 – 30 MPa and saw that as the pressure was increased, the cloudiness increased, the particle size decreased, and the color improved.

Broeck and others (2000) tested the effects of pressure and temperature on the inactivation of PME. They found that at 70°C and at pressures up to 600 MPa, thermostable PME inactivation was slower than when atmospheric pressure was used. At pressures above 600 MPA, PME inactivation was accelerated; however, the acceleration did not surpass the inactivation rate of atmospheric pressure until the pressure reached 900 MPa. Higher temperatures showed slower inactivation even at 900 MPa.

Dynamic high pressure homogenization (DHP) is defined by Lacroix and others (2005) as a homogenization process where a liquid under high pressure and velocity is pushed through a narrow opening. This process causes physical changes in the product. The authors tested the effects of DHP, using a pressure of 170 MPa, on the inactivation of PME and the stability of

orange juice. DHP alone decreased the activity of PME by 20%, but DHP plus a minimal heat treatment of 50°C for 10 minutes decreased the activity of PME by 50-75%. DHP also decreased the particle size of orange juice, and in combination with the decrease in PME, this resulted in a shelf life comparable to that of pasteurized juice. DHP plus the minimal heat treatment had good sensory qualities, and all of these factors led the authors to conclude that DHP could be an alternative to pasteurization for orange juice processing.

If carbon dioxide is added to the headspace of fresh orange juice, an increase in shelf life is seen (Shomer and others, 1994). The authors tested 10% and 20% carbon dioxide in the headspace of fresh, unpasteurized orange juice. Shelf life at 4°C increased from 15-16 days for fresh juice without carbon dioxide to 25 days with carbon dioxide, and shelf life at 10°C increased from 4-6 days without carbon dioxide to 10-11 days with carbon dioxide. The samples with 20% carbon dioxide in the headspace had very low sensory scores, but the samples with 10% carbon dioxide in the headspace showed no sensory difference from juice without carbon dioxide.

Shomer and others (1994) also compared minimally heat treated juice (60°C for 15 seconds) with minimally heat treated juice plus carbon dioxide. The heat treated juice extended the shelf life to 35 days, while fresh juice with 10% carbon dioxide in the headspace only increased the shelf life to 17 days. Heat treatment plus carbon dioxide only extended the shelf life an additional day or two onto the 35 days from just the heat treatment. Since the authors found that the carbon dioxide treated juice samples were significantly reduced in natural microflora, they inferred that the flora of heat treated juice differs from fresh juice and was not affected by the addition of carbon dioxide.

Balaban and others (1991) used supercritical carbon dioxide on fresh orange juice to inactivate PME. A pH reduction was seen when the orange juice was subjected to pressure; however, the pH returned to normal when the pressure was released and the carbon dioxide evaporated. The cause of PME inactivation was suspected to be from the combined effects of pressure, temperature, pH reduction, and time. A second study by Arreola and others (1991) showed that with supercritical carbon dioxide treatment, an enhanced and stabilized cloud was seen, along with better color. Vitamin C values were higher in the carbon dioxide treated samples than in the controls that were held at the same temperature for the same time period as the carbon dioxide treated samples. Sensory testing showed that the color and cloud of the supercritical carbon dioxide treated samples were preferred over the control samples, and no differences in the flavor, aroma, or overall acceptability were noticed between the control and carbon dioxide samples.

Manothermosonication is defined by Vercet and others (1999) as the simultaneous application of heat and ultrasound under moderate pressure. The ultrasound waves cause cavitating bubbles, and their implosion causes hot spots and localized high pressures. This causes the decomposition (sonolysis) of water and free radical production. The bubble implosions cause high shear forces, which break covalent bonds and split polymeric molecules. This method of PME inactivation was proven quite effective, and would allow for lower heat application in the processing of orange juice. However, the authors concluded that more research would be needed to determine the effect of manothermosonication on the nutrients and sensory qualities of orange juice before it can be effectively applied to industry.

Walking-Ribeiro and others (2008) compared the effects of thermosonication and pulsed electric fields with pasteurization on the sensory, microbial, and color of orange juice. The

authors defined pulsed electric fields (PEF) to be based on electroporation of the cell membrane causing pore formation and possible death of microorganisms. The sensory study showed no significant difference between the pasteurized orange juice and the TS/PEF juice in terms of color, odor, acidity, sweetness, flavor, and overall acceptability. A few of the sensory panelists were able to detect a metallic flavor in the TS/PEF juice, but a few panelists also claimed that the pasteurized juice was more bland than the TS/PEF juice. Color was initially better for the TS/PEF juice; however, over the long term storage study, the color of the pasteurized juice was more consistent. In terms of microbial growth over the almost six month study, both processes were within the acceptable limit. However, the TS/PEF juice had a slightly higher number of microorganisms than did the pasteurized juice. Still, the authors believe that this technique of TS/PEF could be a promising alternative to pasteurization for orange juice.

A different study using high intensity pulsed electric field technology (HIPEF), which is another nonthermal minimal processing technique, found that color differences in the pasteurized juice were greater than in the HIPEF treated juice over a refrigerated storage study (Cortes and others, 2008). HIPEF treated juice could, according to their study, improve the quality of orange juice; however, more research is needed in terms of physical and chemical properties of orange juice during storage.

Power ultrasound is effective because of cavitation bubbles that result in localized high temperatures and pressures, which cause a localized pasteurization effect (Tiwari and others, 2008). Tiwari and others (2008) showed that fresh squeezed orange juice treated with ultrasound did not have changes in pH or °Brix. The authors did see that the cloud increased with sonication at 40% amplitude level, but decreased with an increase in amplitude level above that; however,

the cloud value was still higher at 100% amplitude level than the cloud of the control orange juice.

Conclusion

Orange juice has a large niche in the juice industry and is highly valued due to the nutritional value and fresh juice flavor. Technology to improve quality, fresh juice flavor, and cloud stability with minimal processing remains elusive but vital to continued success. This literature review has given an overview of the components of orange juice and their effects on cloud stability, as well as on the processing techniques evaluated to provide high quality orange juice.

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CHAPTER 3

CALCIUM AND pH INFLUENCE ON ORANGE JUICE CLOUD STABILITY[1]

[1]Ellerbee, L. and Wicker, L. To be submitted to the Journal of Food Science. 2009

Abstract

The cloud stability and physical properties of pulp free, fresh juice with and without ammonium oxalate (AO) at pH 4.0 and pH 5.5 was evaluated. The only juice to clarify in the three week study was the sample without AO at pH 4.0. Particle size analysis showed that the samples at pH 4.0 were larger than those at pH 5.5, and samples at pH 5.5 had a more negative zeta potential than samples at pH 4.0. In addition to electrostatic attraction and calcium binding, cloud particles associate and dissociate via non-covalent, non-electrostatic interactions.

INDEX WORDS: Calcium chelation, Ammonium oxalate, Citrus juice cloud, Cloud stability

Introduction

Cloud in orange juice is desired, and too little or no cloud is considered a quality defect. The cloud contributes much of the color, flavor, turbidity, and aroma of orange juice (Baker and Cameron 1999). Scott and others (1965) suggested that the fraction of orange juice with the finest particles, the cloud, had a composition quite different from the rest of the juice and that the cloud should be treated as a separate component and not as tiny fragments of pulp. Orange juice cloud is made up of protein, pectin, lipids, cellulose, and hemicellulose. Many authors have studied the amounts of each of the components present in the cloud (Crandall and others 1983; Klavons and others 1991, 1994; Baker and Cameron 1999). Citrus juice cloud particles typically range in size from 0.4 to 5.0 μm (Klavons and others 1991), while the most stable clouds have particle sizes of 2 μm and smaller measured by electron microscopy (Mizrahi and Berk 1970).

The most adhered to theory of cloud destabilization says that pectin methylesterase (PME) cleaves the methyl esters of pectin molecules, giving methanol and free pectic acid. At a specific DE, calcium cations crosslink the free pectic acid units to free units on adjacent pectin molecules, forming insoluble calcium pectate gels that become too large and fall out of suspension, pulling the cloud with it. Baker and Bruemmer (1972a) added pectin to an aged floc containing serum of Valencia or Pineapple orange juice and then resuspended the cloud. The added pectin caused the turbidity of the system to increase, and the authors concluded that soluble pectin inhibits clarification when floc is present.

It has been shown that clarification is not directly proportional to the total PME activity of the orange juice. Wicker and others (2002) reported a shift to larger particles and an increase in % T (from approximately 5 to 15 %) of cloud particles in reconstituted orange juice samples with added PME of 1.2 U/mL, but clarification, indicated by % T, was not seen in the 13 day

study at 4° C. Added cations with no PME present did not change % T or increase PS, but samples with both PME and added cations increased in particle size and clarified by day 2 according to % T. The particle size change was evident before the % T showed clarification.

Versteeg and others (1980) isolated three PME isozymes from Navel orange, two of which were thermolabile and were inactivated at 70° C, and one that was thermostable and required a temperature of 90° C for inactivation. The authors found that the thermolabile isozymes, when added to orange juice, clarified the juice very slowly, if at all. The thermostable isozyme however, rapidly clarified the juice. Cameron and others (1998) isolated four PME isozymes from Valencia orange, three of which were thermolabile. Two of the thermolabile isozymes showed juice clarification. Ackerley and others (2002) showed that thermolabile PME extracts from Valencia orange pulp with 36 and 27 kDa protein bands would rapidly clarify juice, while extracts with 36 and 13 kDa protein bands did not rapidly clarify juice.

Shomer and others (1999) found that clumps develop in the insoluble cloud matter at temperatures above 70° C and at pH values of 3-4, which are conditions where proteins coagulate and flocculate. Cloud floc was more prominent at pH 3.5 and floc was enhanced by enzymatic pectin degradation and heating, where pectin is more soluble and PME is less active. The authors suggested that protein coagulation/flocculation results in clarification. They concluded that the association between pectin and protein is enhanced by PME activity, and cloud loss results from cloud protein-pectin flocculation.

Krop and Pilnik (1974) added ammonium oxalate to reconstituted orange juice with added pectinesterase at normal pH to determine the effect of calcium ions on clarification. They gathered turbidity measurements and followed the release of methanol in the juice over a two week period. The results showed that juice cloud was stable with the addition of ammonium

oxalate in the presence of active PME over the two week period. As expected, control juices clarified. Croak and Corredig (2006) also used a calcium chelator and compared the addition of increasing levels of PME at pH 3.8 in juice with 20 mM EDTA, a calcium chelating agent, and in juice without EDTA. They concluded that cloud particles are destabilized even in a calcium depleted environment with the addition of PME (15 and 24 units/mL).

It has been shown that at pH 7, juice clarification does not occur (Ackerley and Wicker 2003) even though this is an optimal pH for PME. It is likely that even though PME is active at this pH, charge repulsion between pectins with a net negative charge prevents association. Reducing the pH back to the natural pH causes flocculation to occur rapidly. Croak and Corredig (2006) showed that juice is more stable against the addition of PME at pH 6 than at pH 3.8.

The nature of interactions in orange juice cloud is not fully understood. In this study we evaluated the colloidal constituent interactions of citrus juice cloud. The objective of this research was to determine cloud particle interactions prior to onset of clarification. The conditions of pH and calcium chelation prevented cloud loss and allowed evaluation of non-electrostatic interactions in cloud stability.

Materials and Methods

Fresh Juice Preparation

Fifteen Valencia oranges were juiced using a laboratory model reamer type citrus juicer (Waring Pro Citrus Juicer, PCJ201, Torrington, CT) and vacuum filtered through a Buchner funnel with Miracloth (Calbiochem, Gibbstown, NJ). Sodium azide (0.4 mM final concentration) was added to the filtered juice. The juice was then separated into two batches and ammonium

oxalate (0.025 M final concentration) was added to one batch from a stock of 0.4 M ammonium oxalate; an equivalent amount of deionized water was added to the second batch (control) to keep the volumes the same. The ammonium oxalate batch was separated into two equal batches, and the pH of one was measured and used without adjustment and set as the "natural" juice pH (pH 4.0); the second ammonium oxalate batch was adjusted to pH 5.5. The control batch was also separated into two equal batches; one was adjusted to pH 4.0, since the ammonium oxalate slightly increased the pH of the juice, and the other was adjusted to pH 5.5. Volumes of NaOH used to adjust the pH were recorded, and the volume of each individual batch was adjusted with deionized water to keep the volumes equal. The four samples were pH 4.0 C (control), pH 4.0 AO (ammonium oxalate), pH 5.5 C, and pH 5.5 AO. The juice was covered and stored at room temperature in beakers.

Analytical Methods

Flat-bottomed screw cap test tubes were used for the turbiscan (Turbiscan Classic MA2000, Sci-Tec Inc., Sandy Hook, CT) measurements, and 7 mL of each sample was placed into a test tube. The tubes were tested with the turbiscan at three hours from the start of the juicing on the first day and once per day thereafter until clarification. The test tubes were stored at room temperature and were disturbed as little as possible. Each turbiscan graph was saved after a scan, and the same graph was opened at the next sampling time and the same sample was scanned again on the same graph. This gave the change in one sample over time on one graph.

Calculations on the turbiscan graphs were done in reference mode, meaning that the first scan was subtracted from each additional scan. From the turbiscan graphs, the mean value kinetics were calculated for both transmission and backscattering for each sample. Backscattering data on the turbiscan can only be used if the transmission is at 0%; otherwise, the

backscattering picks up reflections of the transmission data. Because of this, only the portion at the bottom of each tube that had sediment, causing 0% T, was used for calculations of mean value kinetics on backscattering. Mean value kinetics are calculated by the Turbiscan software when the user indicates the range in the tube to be used in the calculations.

The juice stored in beakers was stirred for 1 minute each day prior to testing. The % T was measured daily until clarification. A 2 mL aliquot from each was placed into centrifuge tubes that were centrifuged for 10 minutes at 500xg (Fisher Scientific Marathon 3200). The supernatant was immediately pipetted into a cuvet, and % T was measured at 650 nm (Milton Roy Spectronic 20+, Thermo Scientific, Waltham, MA). Clarification was determined according to the Quality Control Manual for Citrus Processing Plants (Redd and others, 1986) which considers definite clarification to be from 36-60 % T at 650 nm.

For particle size and zeta potential, the samples were diluted 1:50 with deionized water. Diluted samples were filtered through 5 μm filters (Millex-SV PVDF, Millipore, Billerica, MA) into cuvets. The samples were analyzed using dynamic light scattering and laser Doppler electrophoresis (90-Plus, Brookhaven Inst., NY). For zeta potential, a parallel plate electrode was inserted into the test tube. Changes in particle size and zeta potential were analyzed over time.

Particle size was also measured with a Malvern Mastersizer (Model MSS, Malvern Instruments Limited, Worcestershire, U.K.), using 1.73 and 1.33 as the refractive indices of cloud and dispersed phase, respectively, and 0.1 as the absorption index for cloud particles (Corredig and others, 2001b). Size distributions were calculated and the weight-average sizes were expressed as $D_{3,2} = \Sigma_i n_i d_i^3 / \Sigma_i n_i d_i^2$ and $D_{4,3} = \Sigma_i n_i d_i^4 / \Sigma_i n_i d_i^3$, where n_i is the number of particles of diameter d_i.

PME activity was quantified using a pH stat titrator (Brinkmann, Westbury, NY) at pH 7.5, 30°C in 1% pectin and 0.1 M NaCl. PME activity was also quantified titrimetrically at pH 4 and pH 5.5 in 1% pectin and 0.1 M NaCl. Titratable acidity was also measured titrimetrically using 0.31 N NaOH. Brix was measured using a refractometer (Milton Roy, Ivyland, PA).

Results and Discussion

The PME activity in pulp free juice, measured at the conventional assay pH of 7.5 was low. The activity at pH 7.5 was estimated to be 0.24 U/mL. When PME activity was measured at juice pH or at adjusted juice pH of 4.0 or 5.5, respectively, the PME units per mL was lower and near 0.03 U/mL and 0.10 U/mL, respectively. Much of the PME activity detected in juice is associated with pulp particles. Low pulp juices typically have lower PME activity levels, but PME is highly variable (Snir and others 1996). The average Brix and titratable acidity values for 4.0 C samples was 10.6° Bx and 0.66% citric acid respectively.

Figure 3.1 shows a Turbiscan graph of 4.0 C juices as presented directly from the Turbiscan software. The top graph is % transmission and the bottom graph is % backscatter. The x-axis refers to the tube length (mm), with 0 mm being the bottom of the tube. The numbers to the right correspond to the time at which each sample was read, and the units are hours: minutes. Each sample was tested daily, and the scans were taken on the same graph for each sample.

The mean value kinetics on backscattering tells the rate of settling for each sample and is obtained from analysis of the peak area of the sedimentation at the bottom of the tube. Figure 3.2 shows the mean value kinetics on backscattering. The rate of settling is as follows: 4.0 AO > 4.0 C > 5.5 AO~ 5.5 C. The rate of settling observed at pH 5.5 in the presence or absence of AO was not markedly different and variation was observed between replications at pH 5.5 in the

presence or absence of AO. However, the rate of settling of juices at pH 5.5 was at a slower rate than either of the pH 4.0 juices.

The mean value kinetics on transmission indicates the speed of clarification in the sample and is obtained from the transmission data at the middle of the tube. Figure 3.3 shows the speed of clarification to be as follows: 4.0 C > 4.0 AO > 5.5 AO > 5.5 C. The addition of AO to pH 4.0 juice slows but does not prevent an increase in % T in 20 days. It is surprising that the 5.5 AO sample clarifies faster than the 5.5 C sample; however, both clarify much slower than either of the pH 4.0 juices.

The data in Figure 3.4 shows the changes in % T over the 21 day study for a replication. Similar trends were seen in other replications. At pH 4.0, both AO and C begin at approximately 7% T and increase until day 4 to approximately 20% T. From there, they remain between ~15-23% T until day 17 when 4.0 C begins to clarify. By day 18, 4.0 C reached definite clarification at 38% T. The 4.0 AO sample remained stable through 21 days. On Day 1, 5.5 C was also at approximately 7% T, but 5.5 AO was at 0% T. The 5.5 C sample remained under 10% T through day 4, where it began to decrease down to 0% T by day 6. Interestingly, 5.5 AO increased to 11% T on day 2, and then began decreasing to 0% T by day 10. Both 5.5 C and 5.5 AO remained at 0% T throughout the remainder of the study. Krop and Pilnik (1974) reported a much more turbid AO sample over their two week experiment, but a similar effect of AO was observed.

From the Brookhaven particle size data (Figure 3.5), it is evident that particle size is larger at pH 4.0 than at pH 5.5 for both C and AO samples. This suggests that at pH 5.5 there is charge repulsion occurring that keeps the cloud particles from aggregating. Zeta potential (Figure 3.6) shows that the samples at pH 5.5 are more negative than the samples at pH 4.0, validating

potentially greater charge repulsion and agreeing with earlier work in other orange juice systems (Croak and Corredig 2006).

The Malvern particle size data indicated particle association and dissociation of cloud particles prior to onset of clarification. In this study, the D[3,2] values increased (from ~7 – 9 μm to ~ 11 – 15 μm), decreased (back to ~ 7 – 10 μm), then increased again (to ~11 – 13 μm) with time before the onset of clarification for 4.0 C. The increase for 4.0 AO was only for one day before the decrease occurred again. The pH 5.5 juice samples showed a similar trend of particle size change, but the first increase in D[3,2] values were higher and more variable than D[3,2] values observed for 4.0 C juices. In agreement with the Brookhaven data, it is apparent that the final particle size for pH 5.5 samples is smaller than that for pH 4.0 samples. Previous research on changes in particle size with either fresh juice (Ackerley and Wicker 2003) or reconstituted juice with added PME (Ackerley and others 2002) report only a progressive increase in particle size. The disassociation and reassociation of particles seen in 4.0 C, 4.0 AO, 5.5 C, and 5.5 AO juices suggests that there are cloud particle interactions occurring other than calcium pectate formation. Since interactions occur at pH 4.0 and 5.5, this suggests non-electrostatic association and dissociation reactions are occurring. Figure 3.7 shows the Malvern data for sample 4.0 C. The range of the first peak occurs from about 2 to 50 μm. The % T, however, does not indicate any clarification. The data in Figures 3.8, 3.9 and 3.10 shows the Malvern data for juices 4.0 AO, 5.5 C, and 5.5 AO, respectively. Particle size of 4.0 AO juices present a similar profile to 4.0 C juices with the exception of higher D[4,3] values at later storage times. Particle size of 5.5 C juices have relatively lower D[3,2] values than 4.0 C juices but with larger D[4,3] values. Particle size of 5.5 AO juices show similar trends to 5.5 C juices.

Ackerley and Wicker (2003), who also used fresh Valencia orange juice, reported a D[3,2] peak at a smaller size near 3 μm. The differences in particle sizes observed for cloud stable juice in this study may be related to the method of particle size determination and/or preparation of pulp free juice. The size of stable cloud particles was determined by Klavons and others (1994) to be in the range of 0.4 to 5.0 μm, with the most stable cloud having particle sizes of 2 μm and smaller (Mizrahi and Berk, 1970). Ackerley and Wicker (2003) and Ackerley and others (2002) centrifuged juice to remove settling pulp. In this study, vacuum filtration was used. Preliminary data showed that with centrifugation, particle size data corresponded better to what was seen by Ackerley and others (2002); however, the % T data showed juice that had already reached definite clarification. The method used in this experiment more closely resembles the procedure industry uses in the preparation of fresh orange juice, allows the rapid separation of juice from settling pulp, and ensures cloudy juice at initial sampling time.

Conclusions

Addition of AO to juices at either pH maintained cloud stability, and juices at pH 5.5 also maintained cloud stability throughout the three week study. Particle sizes of the juices were larger than the upper limit reported for stability, but juices remained cloud stable except for 4.0 C juice. pH independent cloud particle association and disassociation was observed, suggesting that non-covalent, non-electrostatic interactions are involved in cloud particle aggregation.

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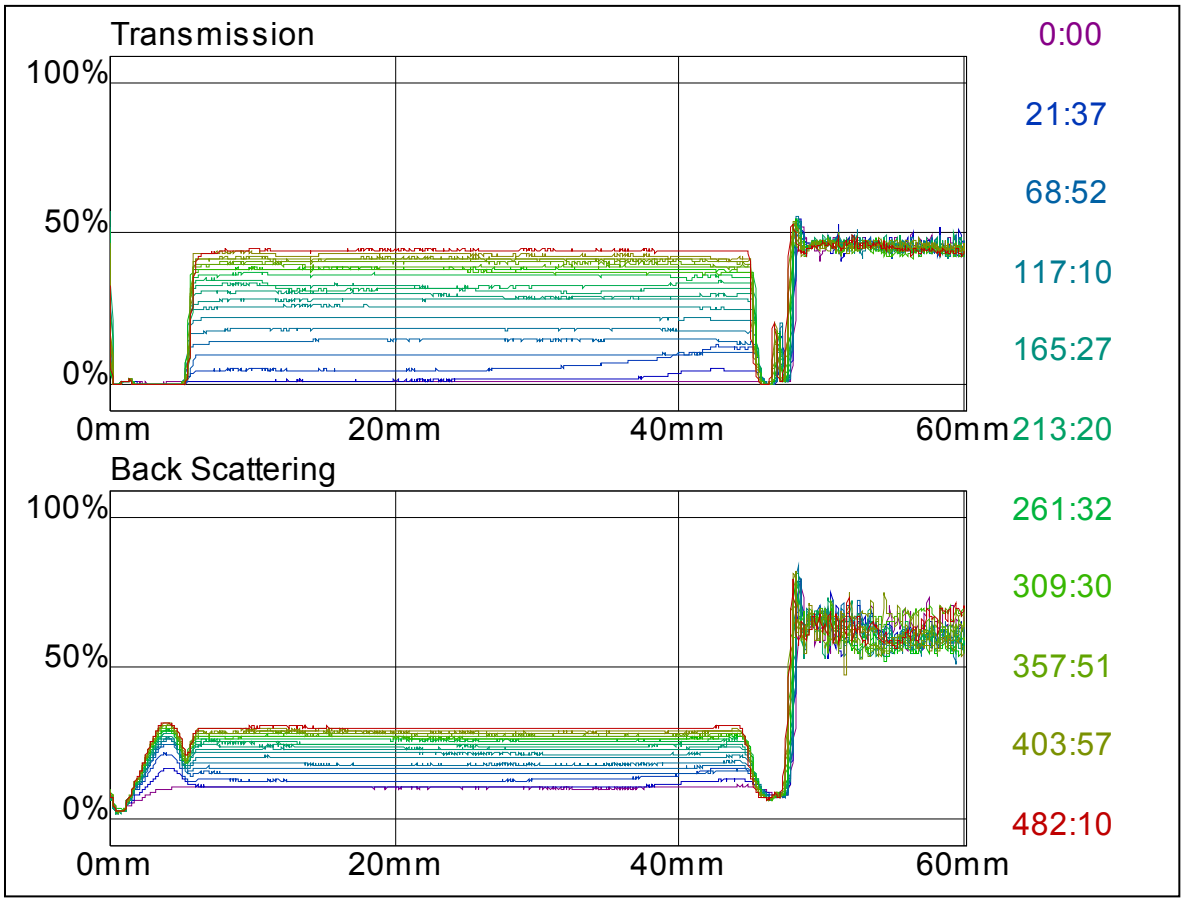

Figure 3.1. Example of Turbiscan graph presented directly from the Turbiscan software. Sample 4.0 Control. X-axis indicates tube length, with 0 mm being the bottom of the tube. The numbers to the right indicate the time (in hours) at which each scan was made.

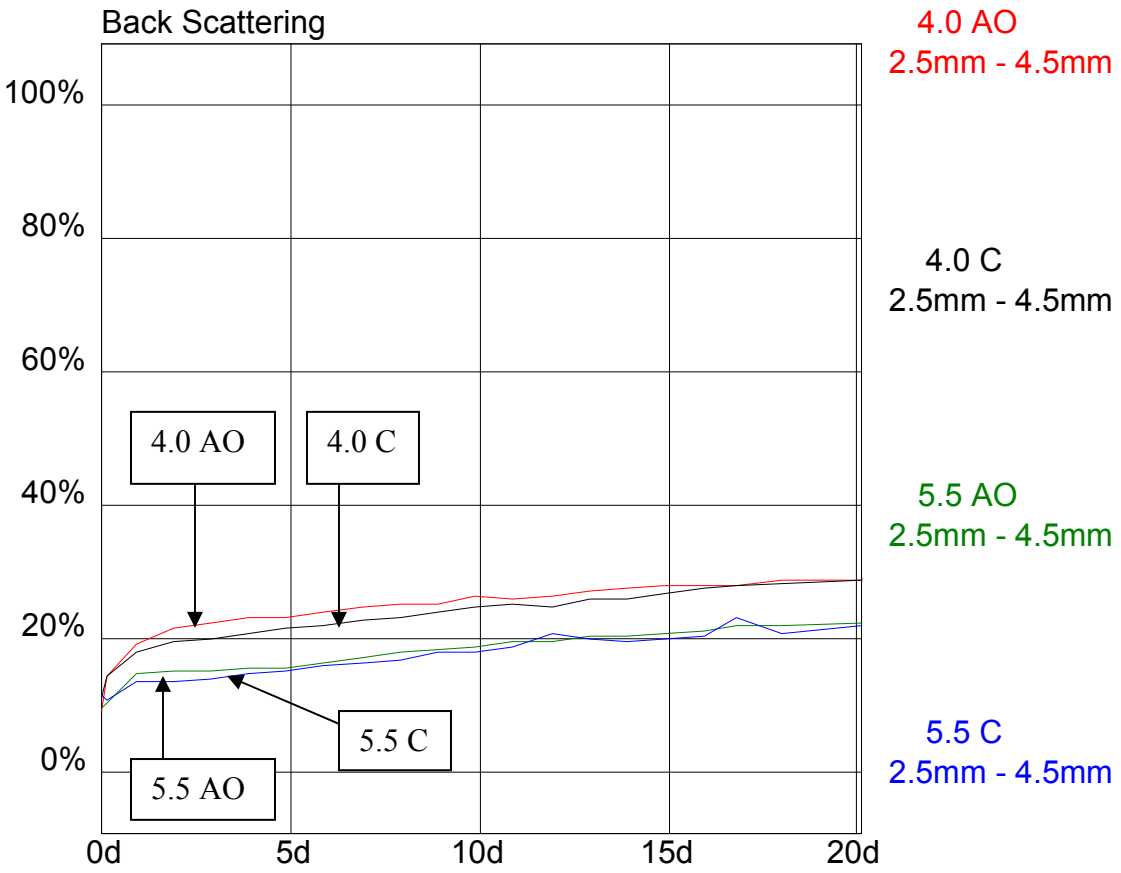

Figure 3.2. Mean Value Kinetics for Backscattering data from Turbiscan for pH 4.0 control, pH 4.0 ammonium oxalate, pH 5.5 control, and pH 5.5 ammonium oxalate

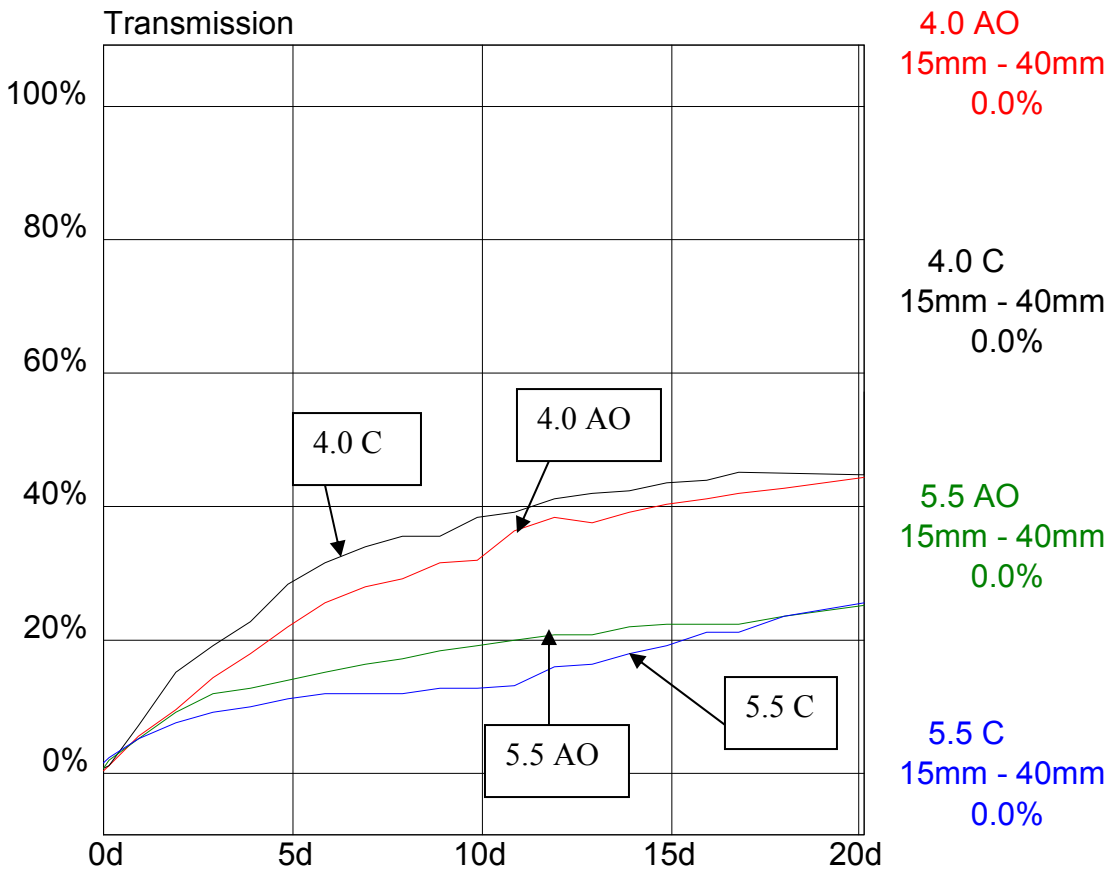

Figure 3.3. Mean Value Kinetics for Transmission data from Turbiscan for pH 4.0 control, pH 4.0 ammonium oxalate, pH 5.5 control, and pH 5.5 ammonium oxalate

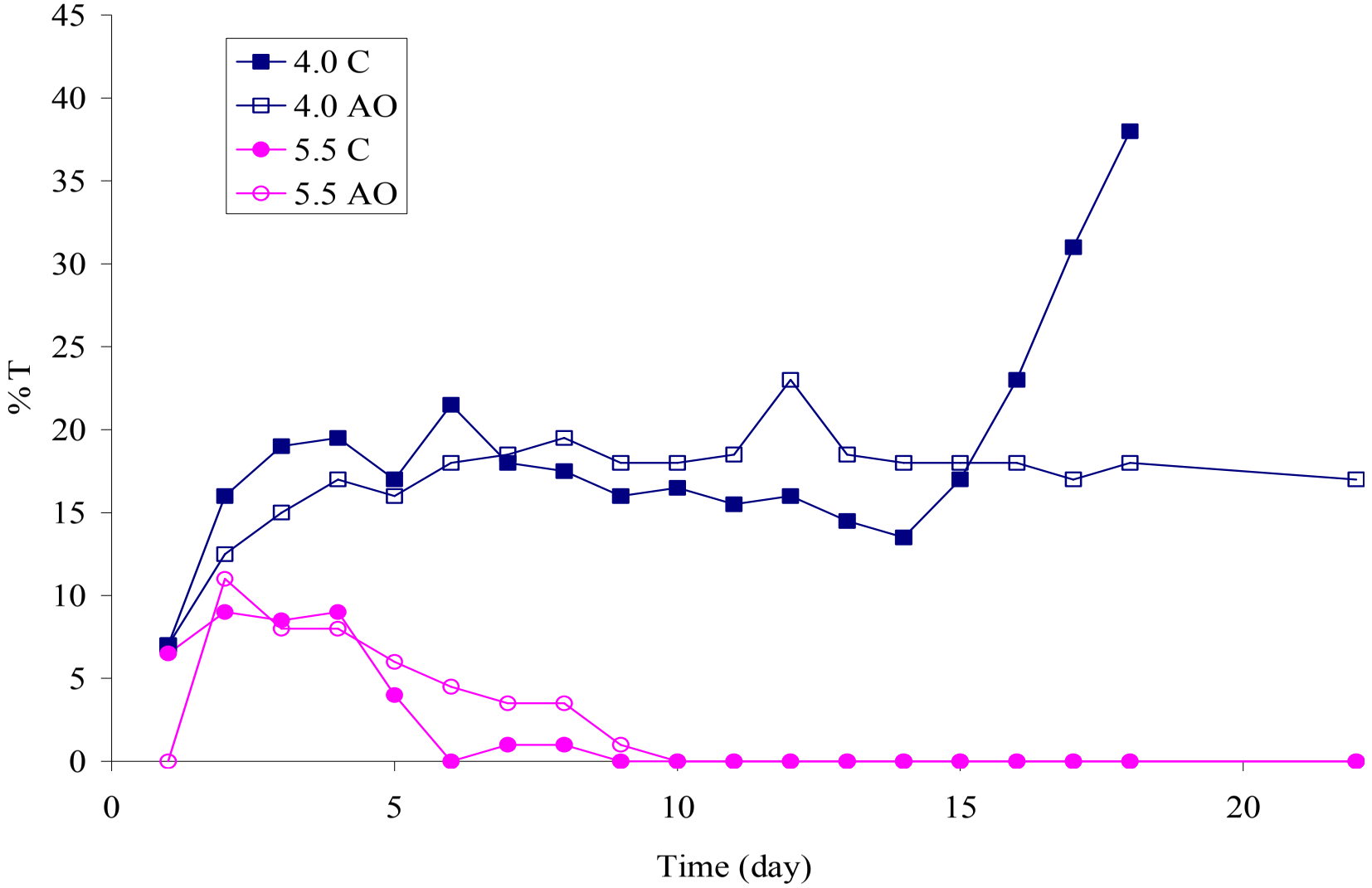

Figure 3.4 % Transmittance data for all samples: pH 4.0 control (filled square), pH 4.0 ammonium oxalate (open square), pH 5.5 control (filled triangle), and pH 5.5 ammonium oxalate (open triangle).

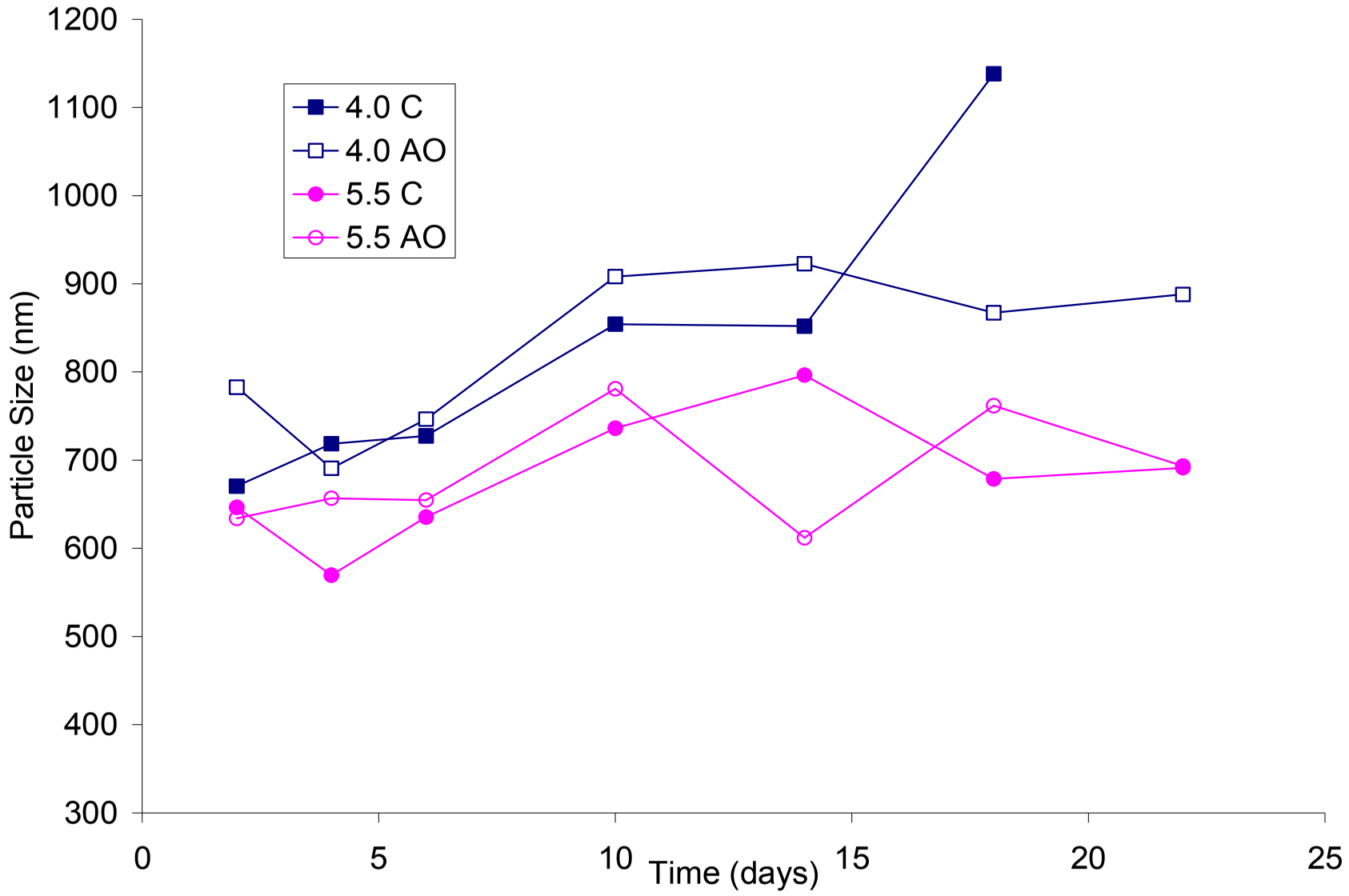

Figure 3.5 Brookhaven Particle Size data for all samples: pH 4.0 control (filled square), pH 4.0 Ammonium oxalate (open square), pH 5.5 control (filled triangle), and pH 5.5 ammonium oxalate (open triangle).

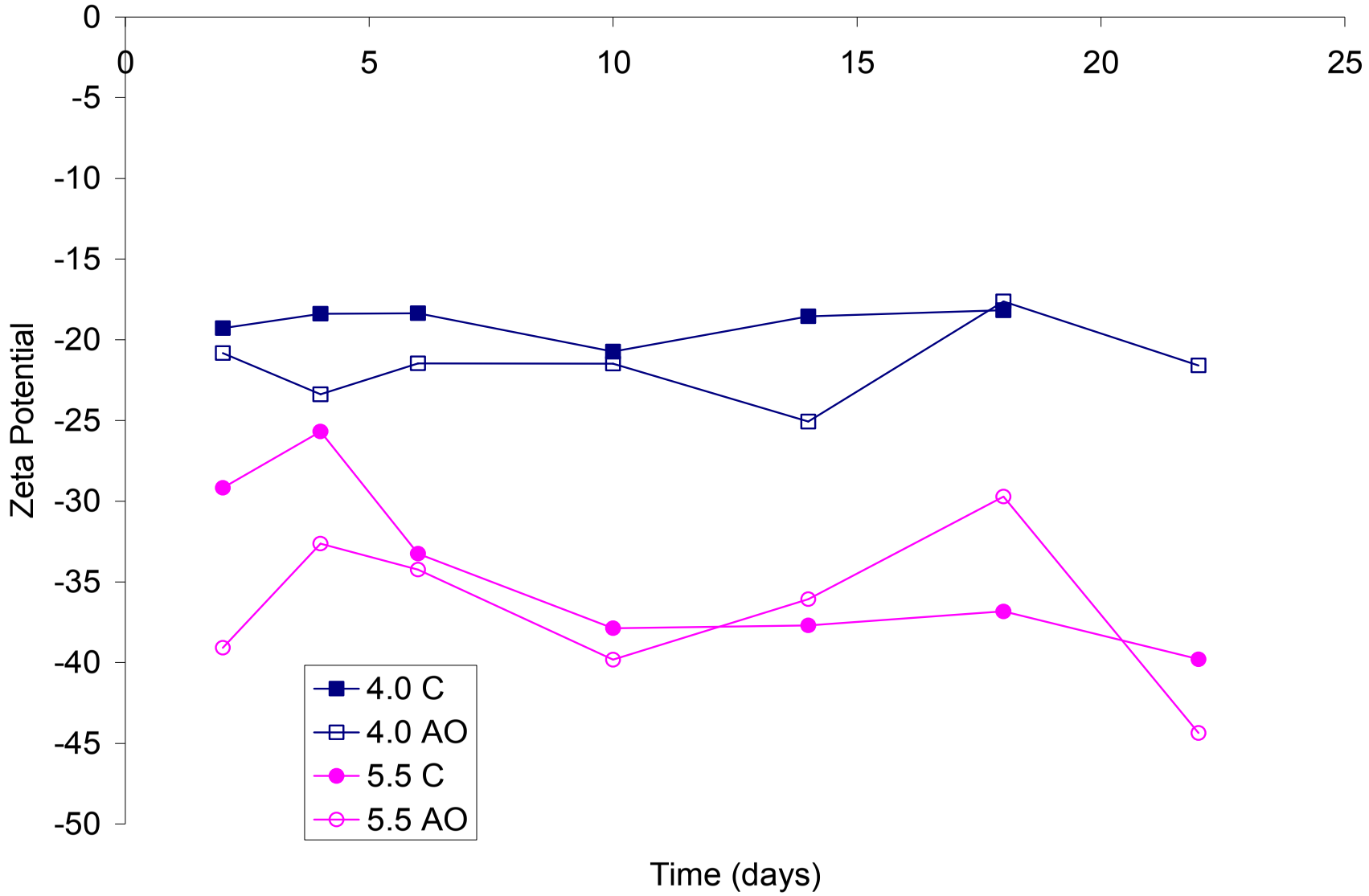

Figure 3.6 Brookhaven Zeta Potential data for all samples: pH 4.0 control (filled square), pH 4.0 ammonium oxalate (open square), pH 5.5 control (filled triangle), and pH 5.5 ammonium oxalate (open triangle).

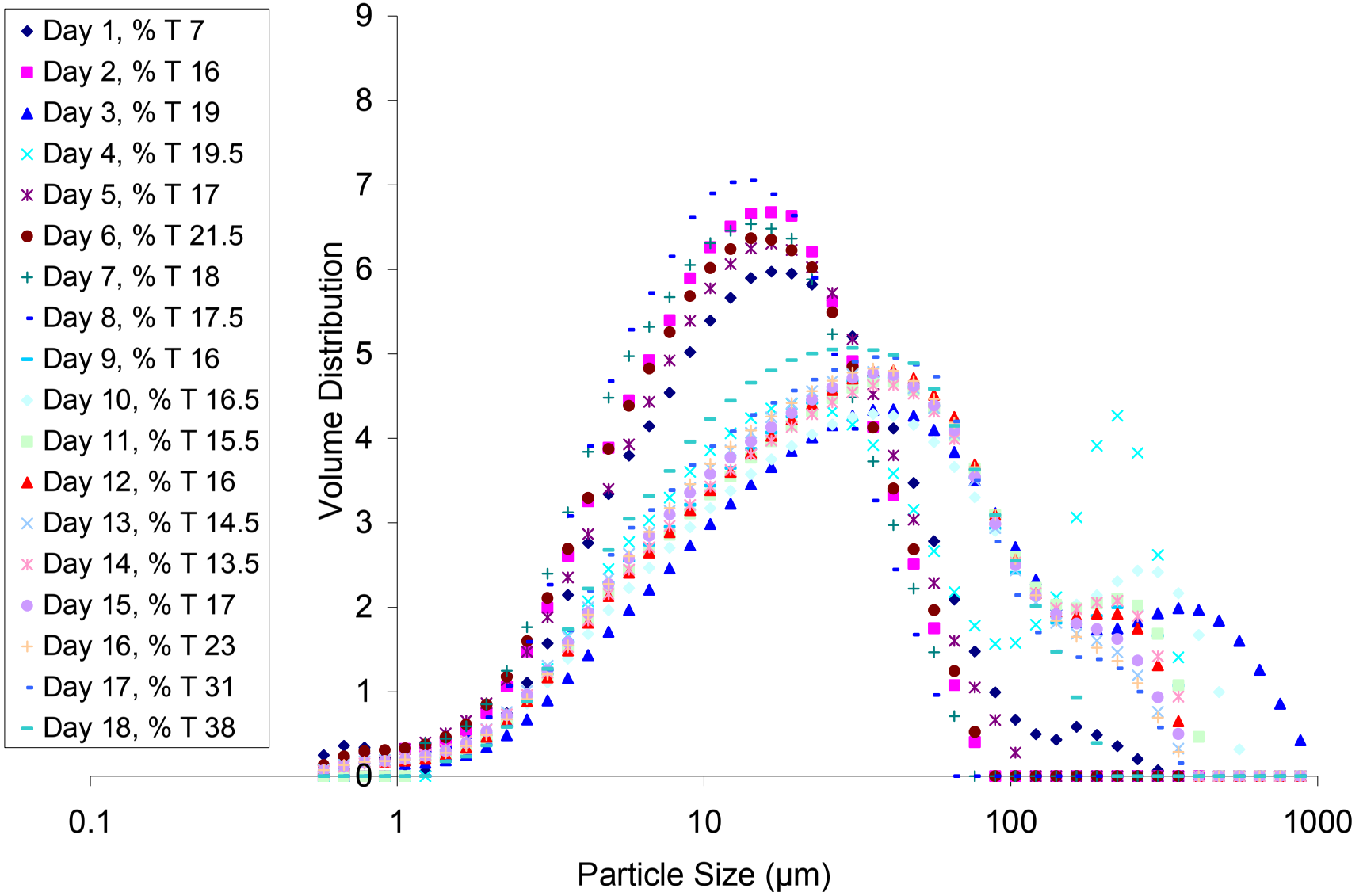

Figure 3.7 Malvern particle size data from sample pH 4.0 control

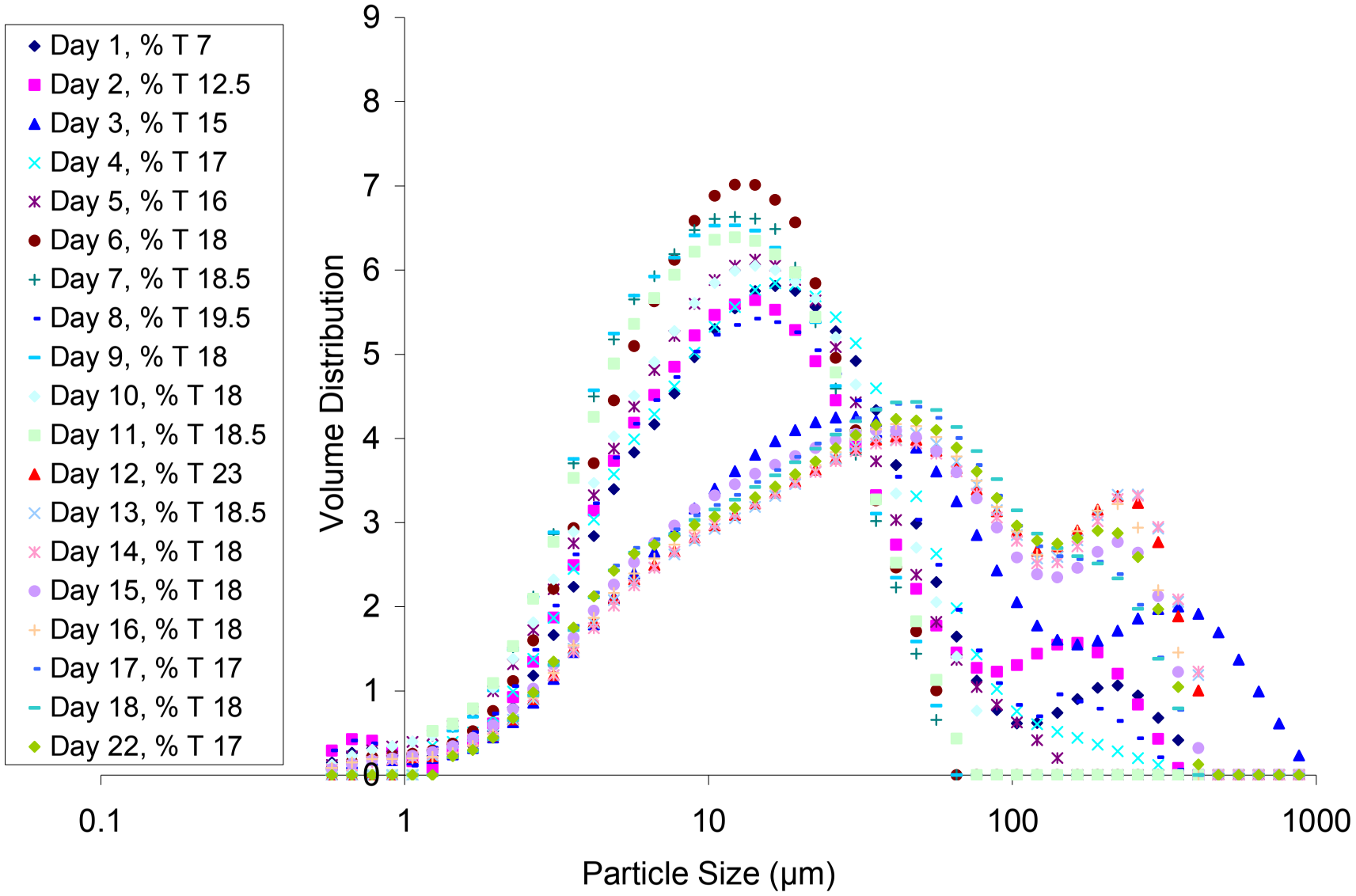

Figure 3.8. Malvern particle size data from sample pH 4.0 ammonium oxalate

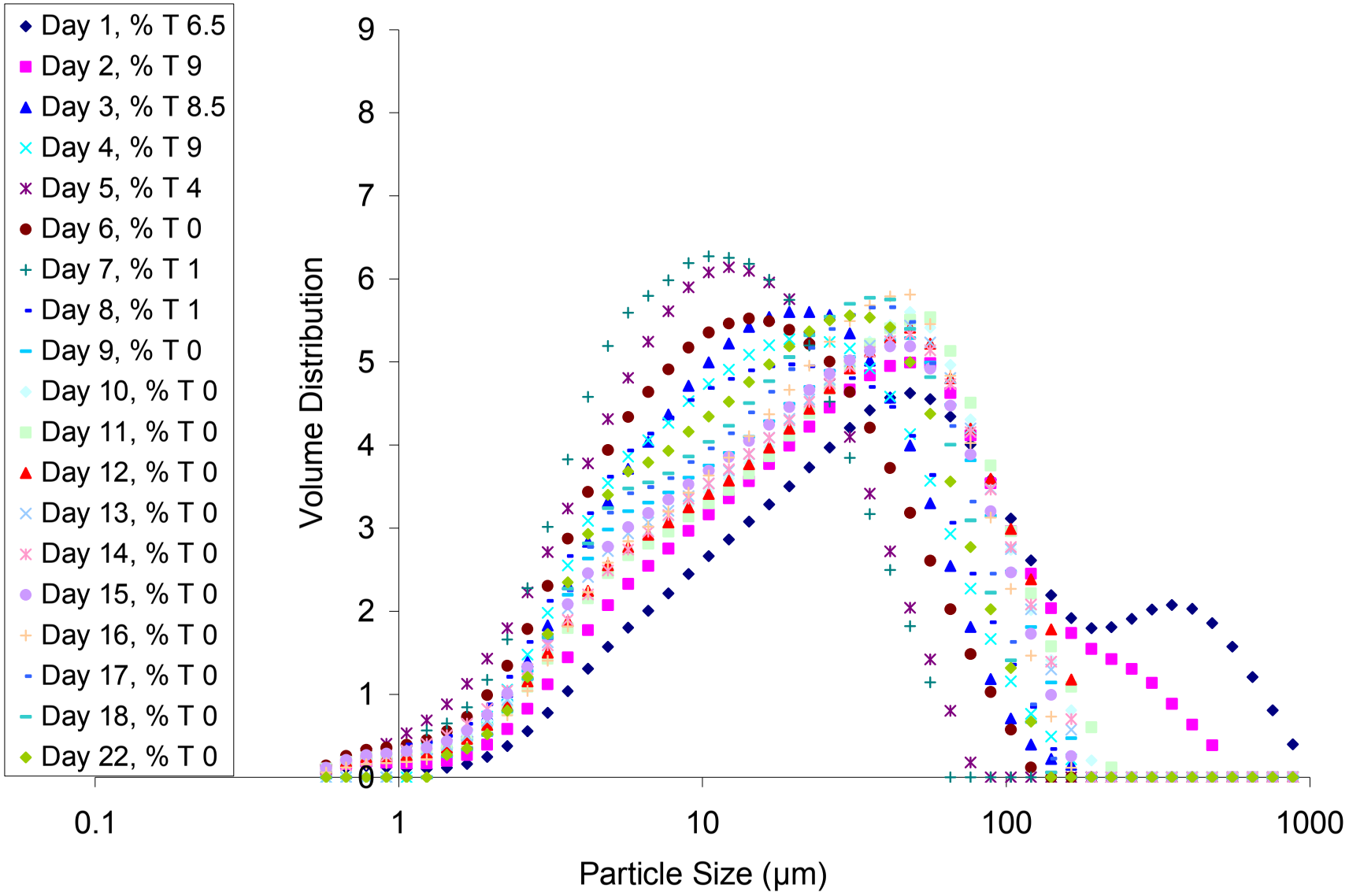

Figure 3.9. Malvern particle size data from sample pH 5.5 control

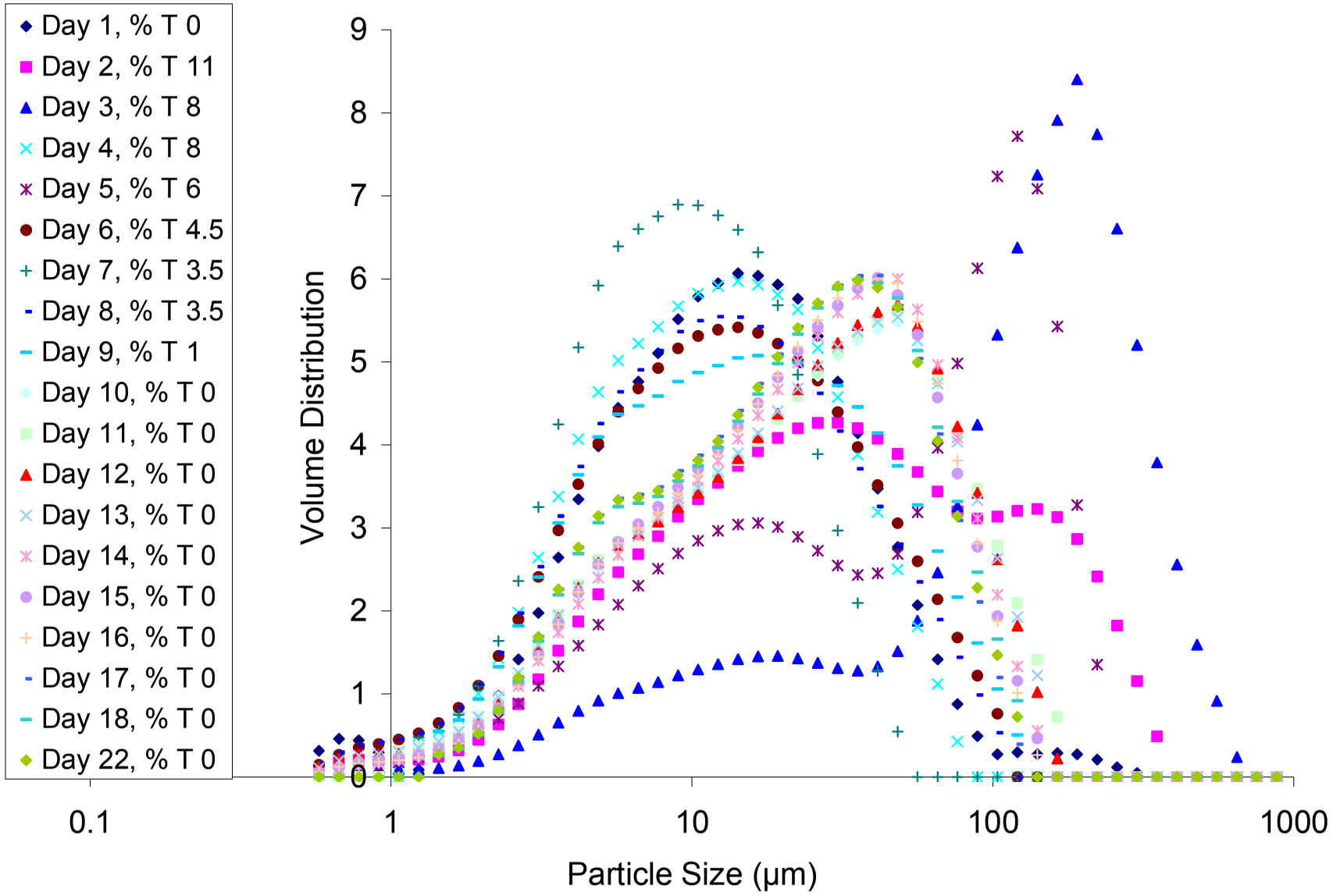

Figure 3.10. Malvern particle size data from sample pH 5.5 ammonium oxalate

CHAPTER 4

FLOC DEVELOPMENT AND STABILITY IN MODEL ORANGE JUICE AND THE INFLUENCE OF HESPERIDIN AND PROTEIN[2]

[2] Ellerbee, L. and Wicker, L. To be submitted to Food Hydrocolloids.

Abstract

A model system of orange juice serum was used to determine the influence of hesperidin and protein addition on floc development. Soy protein, unheated and heated citrus protein at 0.4 mg/ml in the presence or absence of hesperidin (1.0-0.01 mg/ml) were added to orange juice serum, and turbidity, particle size, and zeta potential were measured. OJS control did not change in %T over the 48 hours. Hesperidin addition increased transmittance initially and sedimentation was observed after 48 h. Soy protein was less reactive and formed less floc than unheated or heated citrus protein. Hesperidin and unheated or heated citrus protein decreased transmittance initially and sedimentation was observed after 48 h. Protein controls stayed at the same % T as the lower concentrations of hesperidin. At higher hesperidin concentrations of 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, and 0.1 mg/mL, increased turbidity was observed. Particle size was in the range of 400-600 nm and tended to increase for heated and unheated citrus protein, but hesperidin addition had little effect on particle size.

INDEX WORDS: Hesperidin, Heated citrus protein, Citrus juice cloud, Cloud stability

Introduction

Orange juice cloud is responsible for much of the color, flavor, turbidity, and aroma of orange juice. It is composed of protein, pectin, lipid, hemicelluloses, cellulose, and other minor constituents (Baker & Cameron, 1999). Cloud is a distinct component of juice and not merely smaller pieces of pulp (Scott, Kew, & Veldhuis, 1965). If the cloud is removed from orange juice, the result is a mostly clear serum that is unacceptable to consumers in taste and appearance. Citrus juice cloud particle sizes typically range from 0.4 to 5.0 μm (Klavons, Bennet, & Vannier, 1991), with 2 μm and smaller, estimated by electron microscopy, forming stable cloud (Mizrahi & Berk, 1970). There is a distinct increase in particle size, estimated by laser light scattering, with onset of cloud loss from about 3 to 4 μm (Ackerley & Wicker, 2003).

Cloud loss is typically attributed to pectin de-esterification by pectin methylesterase (PME) and chelation of de-esterified blocks of pectic acid with calcium. The large, insoluble calcium pectate falls out of suspension, pulling the citrus cloud with it (Stevens, Pritchett, & Baier, 1950). Clarification in citrus juice is most likely a complex set of reactions and not readily explained by a single mechanism. Evidence for multiple mechanisms is provided by the lack of strong correlation between PME activity, PME isozyme activity, %DE of pectin, and cloud loss. The isozyme profile (Cameron, Baker, & Grohmann, 1997), presence of cations (Wicker, Ackerley, & Corredig, 2002), formation of inactive PME- pectin complexes (Leiting & Wicker, 1997), juice pH (Versteeg et al. 1980; Ackerley et al., 2003), and juice temperature (Versteeg, Rombouts, Spaansen, & Pilnik, 1980; Cameron, Baker, & Grohmann, 1998) all influence the rate or existence of cloud loss.

The calcium pectate theory was challenged after a study on the effects of heat treatment on the protein portion of cloud showed that heat coagulated proteins encapsulate droplets of oil

and colloidal constituents (Shomer, 1988), as well as pectins and neutral sugars (Shomer, 1991). In a subsequent study, Shomer, Yefremov, and Merin (1999) found that clumps develop in the insoluble cloud matter at temperatures above 70° C and at pH 3-4, which are conditions where proteins coagulate and flocculate. At pH 3.5, where pectin is more soluble and PME is less active, cloud floc was more prominent and improved by enzymatic pectin degradation and heat (Shomer et al., 1999). The authors suggested that protein coagulation/flocculation results in clarification. They concluded that the association between pectin and protein is initiated by PME activity, which causes cloud protein-pectin flocculation. Further evidence for protein-pectin interactions and structure formation was provided by gelation of pectin, sodium chloride, and inactivated PME (Schmelter, Vreeker, & Klaffke, 2001). The gelation was seen over a pH range of 3-7, with those below pH 5 forming the stronger gels. Gelation, in the absence of active PME, indicates specific interaction of protein with pectin, unrelated to catalytic PME activity.

Formation of cloud during juicing or shortly thereafter is less well defined and the mechanism may be related to haze formation in beverages. Mizrahi et al. (1970) observed that the clear serum of fresh, cloud-free Shamouti juice became turbid within two hours. The serum continued to increase in turbidity over the first 48 hours, which was attributed to needle-like crystals of hesperidin. Shamouti oranges are saturated with hesperidin, and this study showed that hesperidin could be a significant factor in juice cloudiness. Another group saw a large increase in cloudiness in fresh Shamouti orange juice that was also attributed to the crystallization of hesperidin (Rothschild & Karsenty, 1974).

Hesperidin is a flavonoid that is unique to citrus fruits. It has been said to have many health-benefitting properties, such as mild anti-inflammatory, analgesic, and mild antipyretic properties (Emim, Oliveira, & Lapa, 1994). Hesperidin is unevenly distributed in the cell

vacuole of citrus fruit, and crystallization of the hesperidin occurs when the cell membrane is broken (Bennett & Albach, 1981). The neutral sugars on hesperidin are rhamnose and glucose, and the compound without the sugars is known as hesperetin. The amount of hesperidin found in the juice of some common orange cultivars is as follows: Hamlin 313±1 mg/L, Shamouti 552±3 mg/L, and Valencia 257±3 mg/L (Dhuique-Mayer, Caris-Veyrat, Ollitrault, Curk, & Amiot, 2005).

Kanner, Ben-Shalom, and Shomer (1982) studied the effects of hesperidin and pectin in a model cloud system of 1% citric acid, 0.67% tri-potassium citrate, 10% sucrose, and 0.1% sodium benzoate at pH 3.8. Stable cloud formed with high methoxyl pectin (HMP) with a degree of esterification of 77% and hesperidin in the model cloud system at a specific HMP to hesperidin ratio of 13.3 to 1. An increase in cloudiness was observed as the concentration of hesperidin increased, but not when the pectin was increased.

Pectin and hesperidin form tight complexes that elute at 60% on a sucrose gradient (Ben-Shalom, Pinto, Kanner, and Berman (1985). Resuspension of the fraction in 10% sucrose formed a stable cloud that could not be separated by gravity. Removal of the neutral sugars from hesperidin resulted in no cloud formation in a pectin solution (Ben-Shalom & Pinto, 1998), indicating that the recognition site for pectin was the neutral sugar portion and not the phenolic ring structure.

Clear serum of fresh, unpasteurized Valencia and Pineapple orange juice likely contains the components that are essential for cloud loss. Baker and Bruemmer (1972a) ultracentrifuged fresh juice serum without cloud and observed that floc, which contained pectates and hesperidin, developed after about six days. Fresh, cloud containing juice clarified in about the same timeframe. Ackerley et al. (2003) also saw floc formation in ultracentrifuged serum that

occurred at the same time as clarification in fresh, cloudy juice. They reported proteins in the floc at 13, 27, and 36 kDa that were thought to be PME, indicating that PME influences floc formation as well as clarification.

Klavons, Bennett, and Vannier (1992) formed a stable cloud using 0.337 mg/mL soy protein isolate and citrus pectins in a model orange juice serum at pH 3.7. They found that pectin with free carboxyl groups helped prevent aggregation of the cloud suspension by association of the pectin with the soy protein. Siebert, Troukhanova, and Lynn (1996) stated that haze formation is seen between polyphenols and proteins when the proteins contain the amino acid proline and little to no haze forms when there is no proline in the protein. In a subsequent study, Siebert, Carrasco, and Lynn (1996) added free proline to a protein-polyphenol model system to test the effects on haze formation. The authors thought the proline would compete with protein to bind with the polyphenol and the proline-polyphenol complex would be smaller and more soluble than a protein-polyphenol complex. However, the free proline did not cause haze formation above what was seen in the control, and there was no indication that proline competes with protein to bind with polyphenols. In this study, the authors found that their highest level of haze formation in the protein-polyphenol model system formed at ~ 0.08 mg/mL polyphenol and 0.4 mg/mL protein. Kanner et al. (1982) found a stable cloud formation with 150 ppm hesperidin and 0.2 % high methoxyl pectin in a model cloud system.

Corredig, Kerr, and Wicker (2001) compared the effect of PME-sensitized pectins and alkali-sensitized pectins on the particle size of orange juice cloud. Aggregation of the cloud particles, as shown by an increase in the particle size, is necessary for cloud loss to occur. Furthermore, the authors concluded that the aggregation is caused by bridging of cloud particles to charged pectin. Wicker et al. (2002) reported an increase in particle size of cloud particles in

reconstituted orange juice samples with added PME and/or cations. This occurred prior to the onset of gross clarification and increase in transmittance of the juice. Croak and Corredig (2006) saw an increase in particle size in reconstituted orange juice with the addition of PME at low pH values (2.5 and 3.8), but at a higher pH (6.0), the same amount of PME did not cause an increase in particle size.

In addition to PME activity and calcium pectate formation, there are other interactions between cloud components that may or may not impact on cloud stability of citrus juice. In the case of weakly interacting aggregates, sample preparation and measurement may actually disrupt the nascent flocs. The Turbiscan® utilized in this study, provides information on stability, particle size variation, and particle migration without further sample preparation. The instrument collects transmission and backscatter data at 135º from two detectors following a near infrared light pulse. Data is collected without sample dilution or other sample disruption. Potentially, it would provide information on the nature of the interaction of cloud constituents. Haze active proteins in apple juice, beer, and wine react readily with phenolic compounds to produce haze (Beveridge & Tait, 1993; Eastmond & Gardner, 1974; Hsu, Heatherbell, Flores, & Watson, 1987). An analogous situation may exist that may relate to cloud formation and stability in citrus juices. While pectin-hesperidin interactions have been characterized, the role of protein in a citrus juice model system has not. The objectives of this study are to determine the effect on floc formation of hesperidin and protein (heated citrus protein, unheated citrus protein, and soy protein isolate) in orange juice serum, and to determine the effect of hesperidin and protein (heated protein, unheated protein, and soy protein isolate) on the particle size and surface charge of the orange juice serum.

Materials and methods

Orange Juice Serum

Tropicana Pure Premium Orange Juice (no pulp) was centrifuged (Sorvall RC6 Plus, Thermo Electron Corporation, MA) at 1500 x g for 10 minutes at 4°C to remove the settling pulp. The pulp-free, cloudy juice supernatant was centrifuged at 27,000 x g for 90 minutes at 4°C. The resulting clear supernatant was adjusted to pH 4.0 with HCl and designated as orange juice serum (OJS). Sodium azide (0.1% total volume) was added to OJS.

Protein and hesperidin preparation

A citrus protein extract was prepared from frozen Valencia orange pulp as described previously to extract citrus pectinmethylesterase (Wicker et al. 1988). Briefly, pulp was homogenized (Pro 300A, Proscientific Inc., Monroe, Conn., U.S.A.) in four parts of 0.1 M NaCl and 0.25 M Tris at pH 8, filtered, and centrifuged at 8,000 g, 4°C, 20 minutes (Sorvall RC 6 Plus, Thermo Electron Corporation, Waltham, MA). Protein was concentrated by making a 75% ammonium sulfate cut. The precipitate was collected by centrifugation, resuspended, and dialyzed overnight against 50 mM sodium phosphate buffer at pH 7. A micro Bradford protein assay was conducted using IgG as standard to determine the concentration of the protein in the extract. Citrus pulp was donated by Citrus World (Lake Wales, FL). A portion of the citrus protein was heated in a 100°C water bath for 15 minutes to inactivate endogenous enzymes, including pectinmethylesterase. Another portion of the citrus protein was used without heating and retained enzyme activity.

Soy protein isolate was also tested because of previous reports of soy protein as a natural clouding agent (Klavons et al., 1992). Soy protein isolate was prepared by adding 0.4 mg/mL soy protein to the orange juice serum, stirring for one hour at ambient temperature, followed by

overnight hydration at 4°C. The proteins were added at a constant final concentration of 0.4 mg/mL based on studies by Siebert et al. (1996) on the formation of protein-phenol haze in apple juice. A 20 mg/mL, the limit of hesperidin solubility (Windholz, Budavari, Blumetti, & Otterbein, 1983), stock concentration of hesperidin was made with 0.2 M NaOH.

Sample preparation

To 7 mL OJS, 0.4 mg/mL (final concentration) heated or unheated citrus protein was added and stirred on a magnetic stir plate. Hesperidin from the stock solution and/or 0.2 M NaOH were added to the beakers to form final hesperidin concentrations of 1mg/mL, 0.5mg/mL, 0.25mg/mL, 0.1mg/mL, 0.05mg/mL, 0.025mg/mL, and 0.01mg/mL (with the exception of soy protein which did not have the 1mg/mL hesperidin concentration). Protein controls for each of the three proteins consisted of 7mL of OJS, respective protein solution, and appropriate volume of 0.2M NaOH. An OJS control with no protein and no hesperidin was used as another control. The beakers were mixed on a magnetic stir plate for 5 minutes before transferring 7 mL to a flat-bottomed test tube. The Turbiscan (Turbiscan Classic MA2000, Sci-Tec Inc., Sandy Hook, CT), was used to record backscatter and percent transmission at 850nm, at times of 0, 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours, and 48 hours. Data was collected in groups of like proteins, going from highest to lowest hesperidin concentration, followed by the protein control and OJS control. The total time for measurements was less than seven minutes and there were no measureable changes in this time frame. Samples were stored at 21°C and care was taken not to disturb the samples when running the measurements. Hesperidin and OJS only controls were also run. Duplicate sample preparation and analysis was conducted. Results are shown from one replicate.

Particle size and zeta potential

Particle size and zeta potential measurements were made at times of 0, 1, and 2 hours at a hesperidin concentration of 1mg/mL. Samples were diluted 1:50 with deionized water, and filtered through 5 μm filters (Millex-SV PVDF, Millipore, Billerica, MA) into cuvets. The samples were analyzed using dynamic light scattering and laser Doppler electrophoresis (90-Plus, Brookhaven Inst., NY, USA). For zeta potential, a parallel plate electrode was inserted into the test tube. Sample preparation and analysis was done in duplicate. Average of duplicates is reported and statistical analysis was performed using the general linear model procedure (SAS Institute). The change in size over time for each sample was analyzed, differences were considered to be significant for $p < 0.05$ according to Tukey's test.

Results and Discussion

% T and backscatter

OJS controls were translucent initially and did not develop haze or particulates during the study. The %T values ranged from ~ 45 to 50 %. None of the three protein controls produced a visible haze in the absence of hesperidin. The 1mg/mL hesperidin control, in the absence of protein, produced a visible haze around one hour. The samples with either unheated or heated citrus protein plus 1 mg/mL hesperidin developed a visible haze within 30 minutes.

The data in Figure 4.1 depicting the hesperidin controls indicates that the addition of hesperidin to OJS at any concentration slightly increases the % T from about 40% in the control OJS to about 45% in the OJS with hesperidin. This suggests that there is only a slight interaction of hesperidin with constituents in the OJS. After 48 hours (Figure 4.2), a progressive increase in

sedimentation is observed as evidenced by the movement of sedimentation front at the bottom of the tube in OJS with hesperidin added at 0.1 to 1 mg/mL.

When soy protein (SP) was added to the OJS, the % T decreased from about 38% to about 28% (Figure 4.3). The decrease in % T and increased cloudiness of the OJS supports earlier work by Klavons et al. (1992), who reported that soy protein could be used as a natural clouding agent. After the addition of hesperidin to the OJS/SP dispersion, a decrease in %T was observed initially followed by a gradual increase to about 50% during storage for 48 hours (Figure 4.4). Furthermore, sedimentation was observed at 0.1 -0.5 mg/ml hesperidin. At intermediate times, similar trends were observed but to lesser extent than observed at 48 hours. At time 0 (Figure 4.3), the % T of all concentrations hesperidin with SP are above the % T of SP only, and range from ~30-40% T. OJS is at ~38% T. A progressive increase in sedimentation is seen in the presence of hesperidin from 0.1 to 0.5 mg/mL in SP dispersions after 48 hours (Figure 4.4). At positions in the tube higher than ~ 12 mm, the % T is about the same as the % T of OJS. At lower concentrations of hesperidin (0.01 to 0.05 mg/ml) and SP, the % T, ~ 45 % T, is higher than the % T of OJS.

In the presence of unheated citrus protein (UCP) or with the subsequent addition of hesperidin, there is a decrease in % T at time 0 (Figure 4.5) from above ~45%T in OJS to about ~35-38 % T. UCP and HCP (heated citrus protein) changed the most within the first two hours of the 48 hour testing period. For UCP, by 30 minutes (Figure 4.6), sedimentation was already seen in the bottom 10 mm of the 1 mg/mL sample, while the rest of the tube had a dense cloud around 10% T. The rest of the samples, excluding the OJS, also decreased in % T in the bottom 10 mm of the tube; this trend continued throughout the 48 hours. By 1 hour (Figure 4.7), the 0.5 mg/mL sample had separated from the group and decreased in % T, with the bottom 10 mm

around 0-10% T and the rest of the tube around 28% T. The 1 mg/mL sample did not gain anymore sedimentation by 1 hour, but there appears to be two more distinct sections within the tube from about 10 mm to 30 mm and about 30 mm to the top of the sample. These two sections are increasing in % T as you go up the tube. By two hours (Figure 4.8), the % T of the 1 mg/mL sample has decreased more in the middle of the tube and has increased more at the top of the tube; this trend continues throughout the 48 hours. By 5 hours (Figure 4.9), the 0.25 mg/mL sample began to separate out from the group. After 48 hours (Figure 4.10), there is a definite decrease in %T in the 0.5 and 0.25 mg/mL concentrations. At 1 mg/mL hesperidin concentration, the %T is between 0% -20%, but there is also sedimentation in the bottom of the tube to ~30 mm.

In the presence of HCP and subsequent addition of hesperidin, there is a decrease in % T relative to the OJS. Compared to UCP, there was considerably more noise and a broader range of % T in these scans. Scans for HCP at time 0 (Figure 4.11) are similar to scans for UCP. The HCP with hesperidin and HCP all range from ~ 31-45% T, and OJS is 48%T. The similarity of the scans at the initial time suggests that the interactions of citrus cloud components are unrelated to catalytic PME activity. Through 4 hours, HCP is unique from the other proteins in that it increases in turbidity at the top of the tube instead of at the bottom. Figure 4.12 shows the HCP plus indicated concentrations of hesperidin at time 30 minutes. By 1 hour (Figure 4.13) this trend is emerging by way of the 1 mg/mL sample. By 2 hours (Figure 4.14), the 1 mg/mL sample has separated completely from the group, and the % T at the top of the tube is approaching 0%. The other samples, with the exception of OJS, are increasing in % T at the bottom of the tube and decreasing in % T at the top of the tube. The 1 mg/mL sample has obtained a very dense cloud of <5% T across the entire tube length by 4 hours (Figure 4.15). By 48 hours, all of the

samples including the HCP control have dropped drastically in % T at the bottom of the tube and have clarified above the % T of OJS by midway up the tube. After 48 hours (Figure 4.16), at higher hesperidin concentrations between 0.25 -1.0 mg/mL, there is a progressive increase in sedimentation between 23-27 mm of the tube. The % T is near 0% at 0.5-1.0 mg/mL hesperidin and about 10% for 0.25 mg/mL hesperidin. At the top of the tube, there is a higher % T than observed in OJS at all hesperidin concentrations and for the heated citrus protein control. However, there is no evidence of serum separation at the top of the tube. At intermediate and lower hesperidin concentrations, while there is sedimentation at the bottom of the tube and higher % T at the top of tube, it is not correlated with hesperidin concentration.

While the scans were very similar for unheated and heated citrus protein with or without hesperidin initially, the profile at intermediate times and at 48 hour was vastly different. The OJS and heated citrus protein interacted more strongly with hesperidin than unheated citrus protein. These results parallel those of Shomer et al. (1999) who observed that heated citrus extracts were more unstable than unheated citrus extracts.

Throughout the 48 hours, the OJS controls did not change. This implies that any change seen in the samples cannot be ascribed to the OJS. The lower concentrations of hesperidin (0.05 – 0.01mg/mL) have approximately the same % transmittance as the protein controls throughout the 48 hours, meaning that low concentrations of hesperidin do not have much effect, if any, on cloud.

The hesperidin controls do not readily react with OJS, since at time 0 the controls have a higher initial % T than the OJS (Figure 4.1); however, for all three protein samples at time 0, almost all concentrations of hesperidin are below the % T of OJS (Figure 4.3, 4.5, and 4.11), indicating a reaction of the protein that increases turbidity. Hesperidin clearly begins affecting

the cloud at 30 minutes for the UCP (Figure 4.6), 1 hour for SP (data not shown), and 2 hours for the HCP (Figure 4.14); hesperidin controls begin to show haze formation at 30 minutes (data not shown). Though the highest concentration of hesperidin with SP has clouding at 1 hour, a comparison of its graph to the graph of hesperidin controls at 1 hour shows that they appear the same.

The scans of UCP at 30 minutes show that hesperidin plus active PME does have an effect on cloud that cannot be attributed solely to PME activity since the 1mg/mL hesperidin sample has a much lower % T than any other sample. The effect of hesperidin is much slower when there is not active PME, since the 1mg/mL hesperidin sample in the HCP scans does not separate out with a lower % T until 2 hours. The HCP scans show that there is a non-enzymatic reaction occurring even in the protein control, which agrees with the study done by Schmelter et al. (2001) saying that the structure of PME has a non-catalytic reaction with pectin. From the HCP scans, it is evident that if enough hesperidin is present in orange juice, destabilization of the cloud will occur.

Both the HCP and UCP plus hesperidin graphs show a more distinct effect than the SP plus hesperidin or hesperidin controls. The UCP plus hesperidin has the greatest effect on % T, followed by the HCP plus hesperidin. SP plus hesperidin has the least effect on % T and most closely resembles the hesperidin controls. Since Klavons et al. (1992) did their work on soy protein at a similar pH (3.7) and soy concentration (0.337 mg/mL), it is interesting that the soy did not form a better cloud.

The turbiscan was used to test hesperetin and pectin in a model cloud system as described by Ben-Shalom et al. (1998). As the authors reported, no cloud was formed indicating that the recognition site of hesperidin is in fact in the neutral sugars.

Particle size and zeta potential

Particle size and zeta potential data are presented in Table 4.1. There was no significant ($p<0.05$) difference in particle size over two hours for OJS or the hesperidin controls. The particle size for OJS was near 510 nm and the particle size for hesperidin in OJS ranged from 548 to 519 nm. When SP was added to OJS, there was no significant change in particle size in 2 hours. Likewise, when hesperidin was added to the SP and OJS dispersion, there was no change in particle size in 2 hours (Table 4.1). Particle size significantly ($p<0.05$) increased from 498 nm at 1 hour to 592 nm at 2 hours for UCP. Upon addition of hesperidin, the particle size of the dispersions increased ($p<0.05$) from 482 to 563 nm at 1 and 2 hr, respectively. Particle size for HCP increased significantly ($p<0.05$) between time 0 and 1 hour from 515 to 583 nm. When hesperidin was added, the particle size ranged between 533 and 561 nm but was not significantly different.

The particle size for UCP and HCP controls are about the same in the presence or absence of hesperidin. The increase in particle size with time in the UCP is likely influenced by catalytic PME activity and increase in carboxylic acid groups. Previously, Croak et al. (2006) reported an increase in particle size from ~400 to 800 nm in 0.5 hours in reconstituted orange juice with added PME (15 units/mL). This increase was attributed to cleavage of methyl esters by PME and the formation of calcium pectate. Notably, the particle size of HCP is larger than UCP, suggesting a conformational change in citrus protein on heating.

The particle size of the OJS control remains the same and agrees with %T data from the Turbiscan, where OJS remained constant throughout the 48 hours (Table 4.1). With the addition of hesperidin to OJS, the particle size does not increase in 2 hours. However, within 1 to 2 hours, the % T decreased and formed peak, uniform cloud density in samples tested by the

Turbiscan. Hence, turbidity increases in OJS with hesperidin in the absence of a measurable particle size increase.

For zeta potential (Table 4.1), there was no significant difference over the two hours tested for OJS, OJS with hesperidin control, HCP, or HCP with hesperidin. For both the UCP and UCP with hesperidin, there was a significant increase in negative charge between time 0 and 1 hour. Dispersions which contained UCP had the lowest negative charge, followed by dispersions with HCP. SP also had no significant difference in surface charge in two hours, but SP with hesperidin had a significant increase in negative charge between 1 and 2 hr.

The net charge on pectin is negative at pH values greater than about pH 3.5. It is likely that the net charge on the SP, UCP, and HCP are positive. The pI values are ~4.5 for soy proteins (Barbosa et al., 2006) and > 7 for citrus PME protein. Cloud particles may be covered in a protective coat of pectin, accounting for an overall negative surface charge for the cloud particles. All the samples were negative, and hesperidin had a minimal effect on the surface charge.

Conclusions

The difference in % transmittance, particle size, and zeta potential is much more pronounced for OJS in the presence of hesperidin with the heated and unheated citrus proteins than with soy proteins. The early changes in particle size, zeta potential, and turbidity of the samples with unheated or heated citrus protein and OJS with hesperidin were unique, suggesting a non-catalytic role for citrus protein in cloud stability. The differences in the unheated and heated citrus protein in OJS with hesperidin indicate a different interaction of cloud particles upon heating.

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Table 4.1. Particle size and Surface Charge of Orange Juice Serum (OJS), Hesperidin, Soy Protein (SP), Unheated Citrus Protein (UCP), and Heated Citrus Protein (HCP)

Sample	Time (hrs)	Particle Size (nm)	Zeta Potential
OJS	0	510[a]	-24[a]
	1	510[a]	-24[a]
	2	516[a]	-21[a]
OJS + Hesperidin	0	548[a]	-24[a]
	1	497[a]	-24[a]
	2	520[a]	-24[a]
OJS + SP	0	503[a]	-21[a]
	1	502[a]	-23[a]
	2	504[a]	-21[a]
OJS + SP + Hesperidin	0	498[a]	-23[ab]
	1	488[a]	-21[a]
	2	484[a]	-24[b]
OJS + UCP	0	460[a]	-26[a]
	1	497[a]	-31[b]
	2	592[b]	-31[b]
OJS + UCP + Hesperidin	0	454[a]	-28[a]
	1	481[a]	-31[b]
	2	563[b]	-32[b]
OJS + HCP	0	515[a]	-27[a]
	1	583[b]	-28[a]
	2	626[b]	-25[a]
OJS + HCP + Hesperidin	0	533[a]	-25[a]
	1	533[a]	-25[a]
	2	561[a]	-24[a]

Different letters within each sample indicate significant difference at p=0.05.

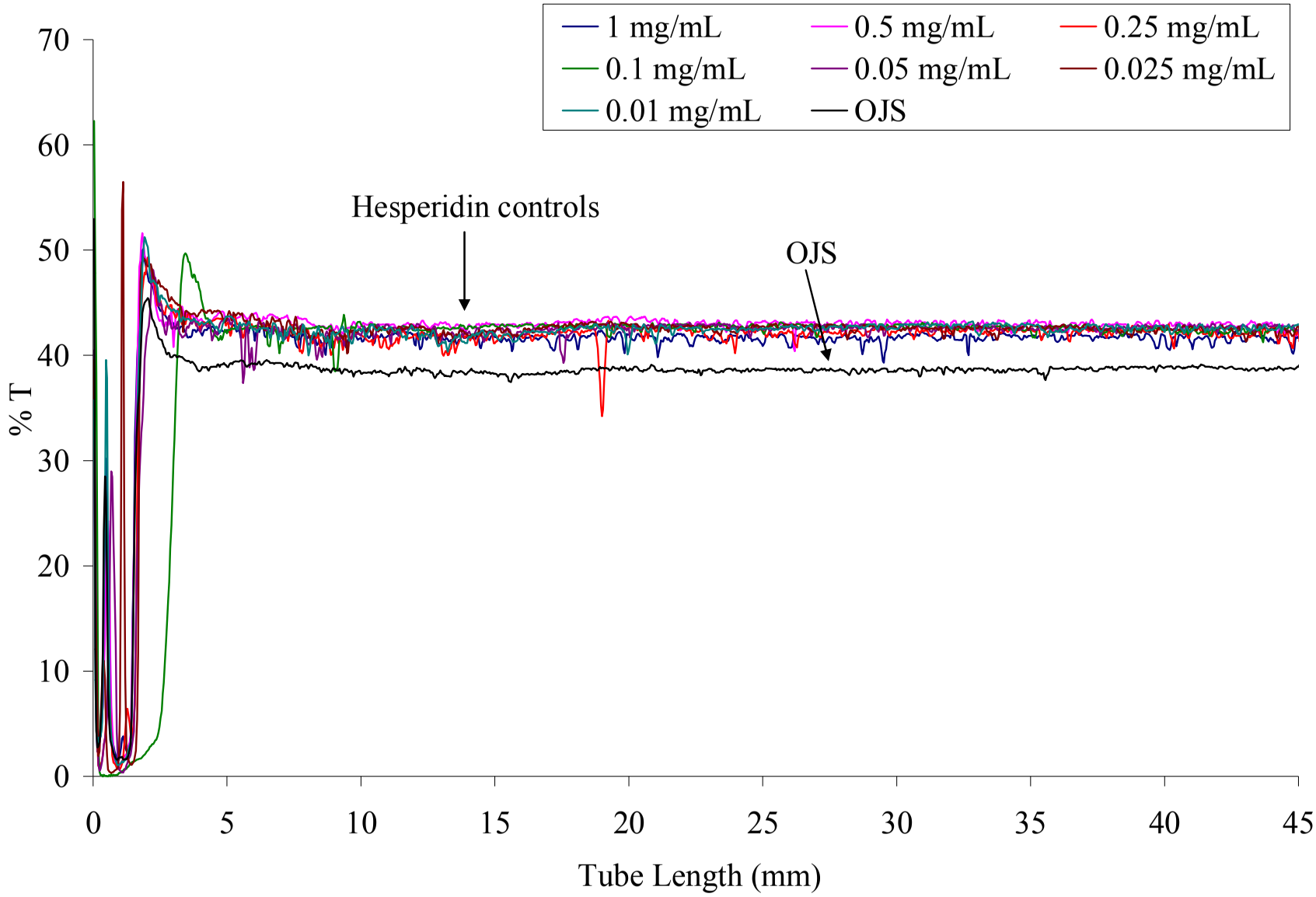

Figure 4.1. Turbiscan graph of Orange Juice Serum (OJS) and hesperidin controls at time 0.

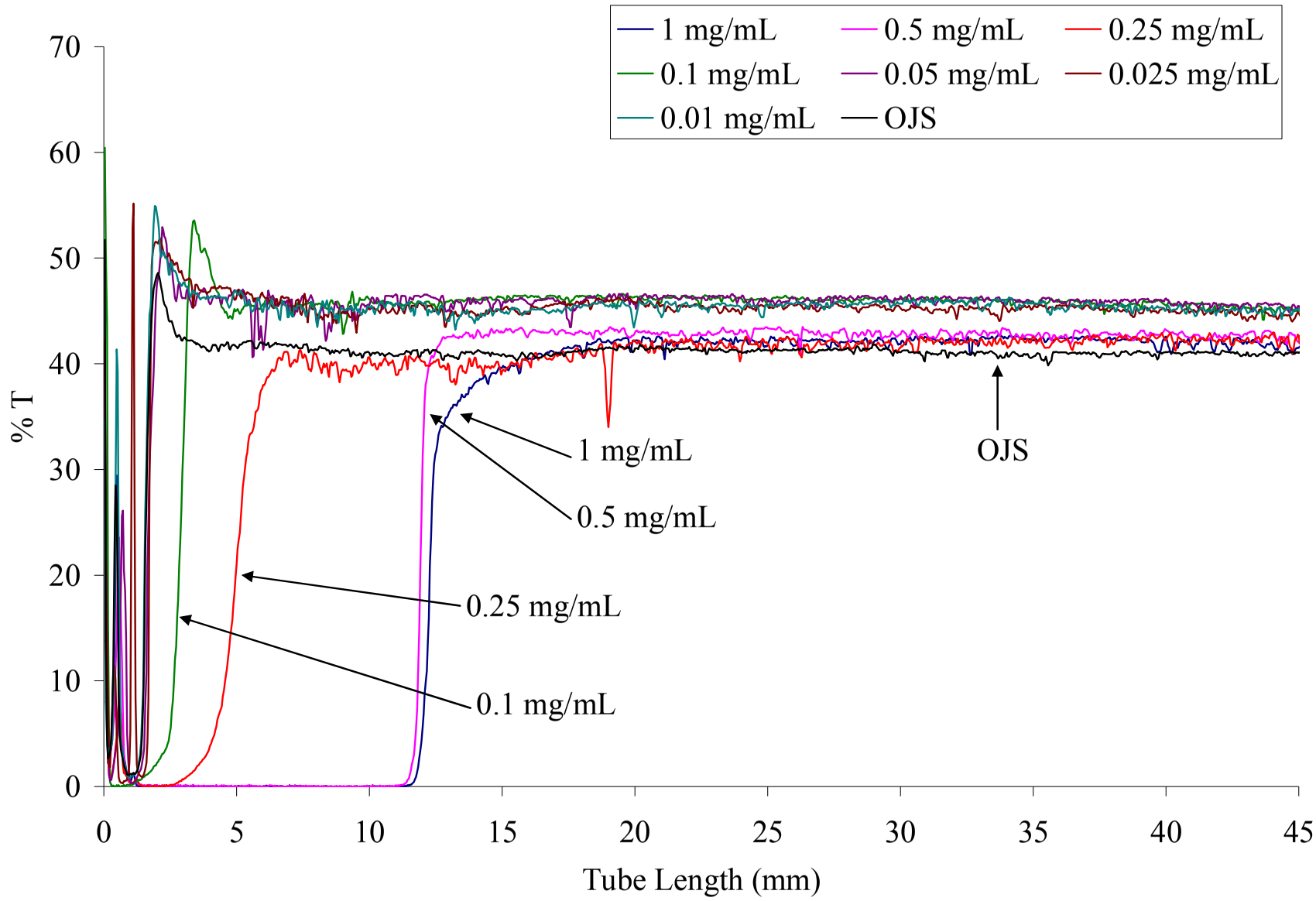

Figure 4.2. Turbiscan graph of OJS and hesperidin controls at 48 hours.

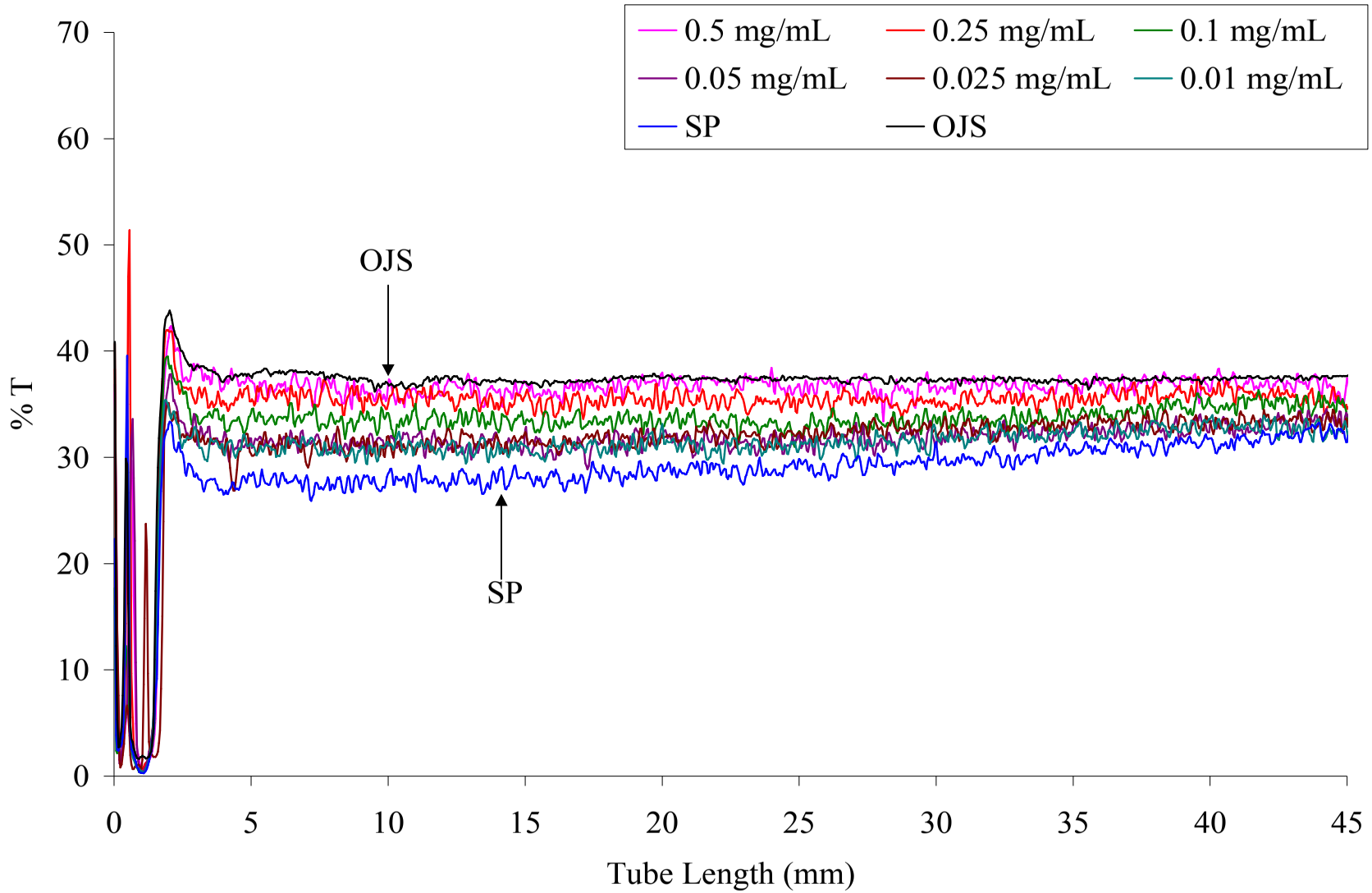

Figure 4.3. Turbiscan graph of OJS with 0.4 mg/ml soy protein and OJS, soy protein and indicated mg/ml hesperidin concentration between 0.01 and 0.5 mg/ml at time 0.

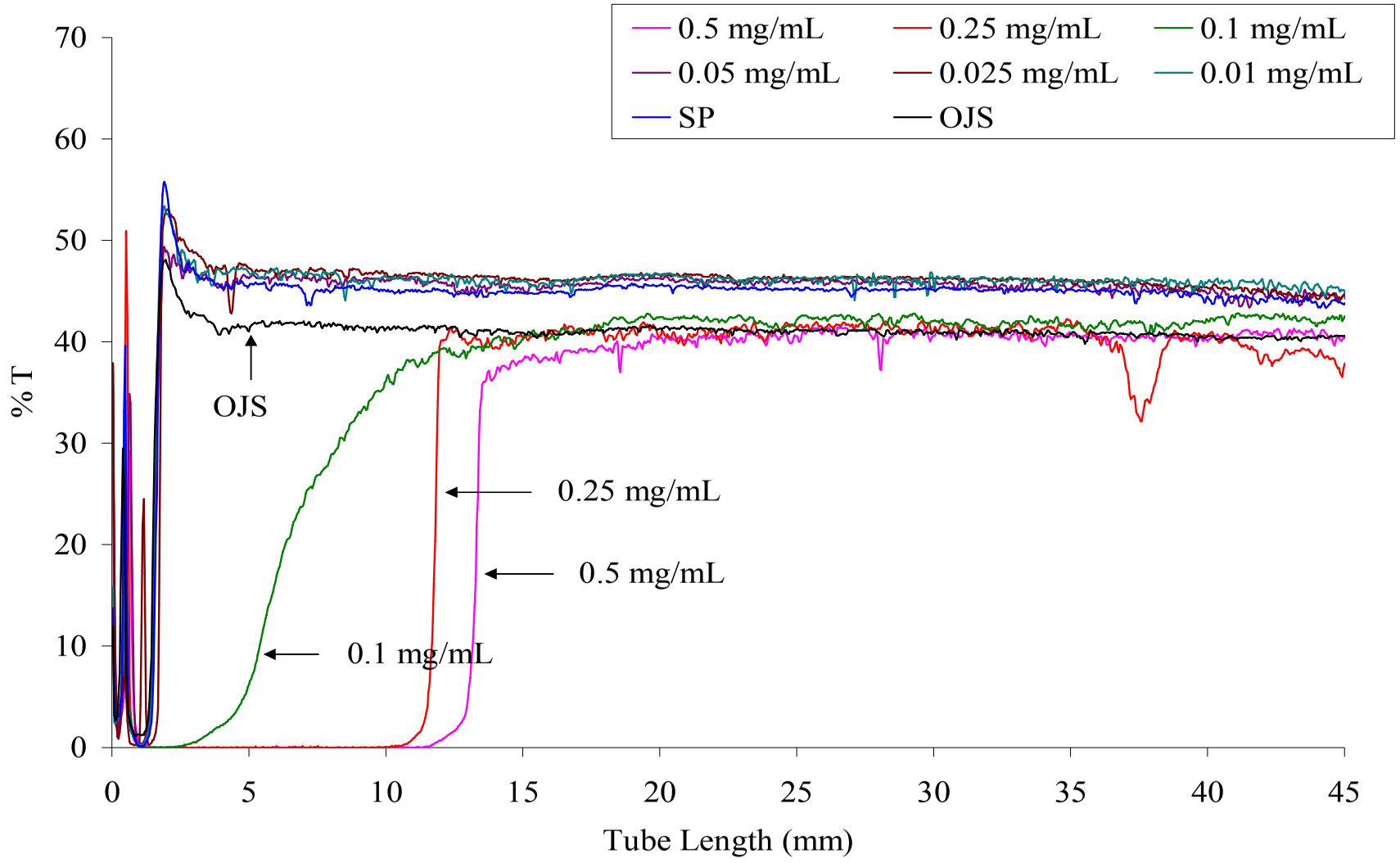

Figure 4.4. Turbiscan graph of OJS with 0.4 mg/ml soy protein and OJS, soy protein and indicated mg/ml hesperidin concentration between 0.01 and 0.5 mg/ml at 48 hours.

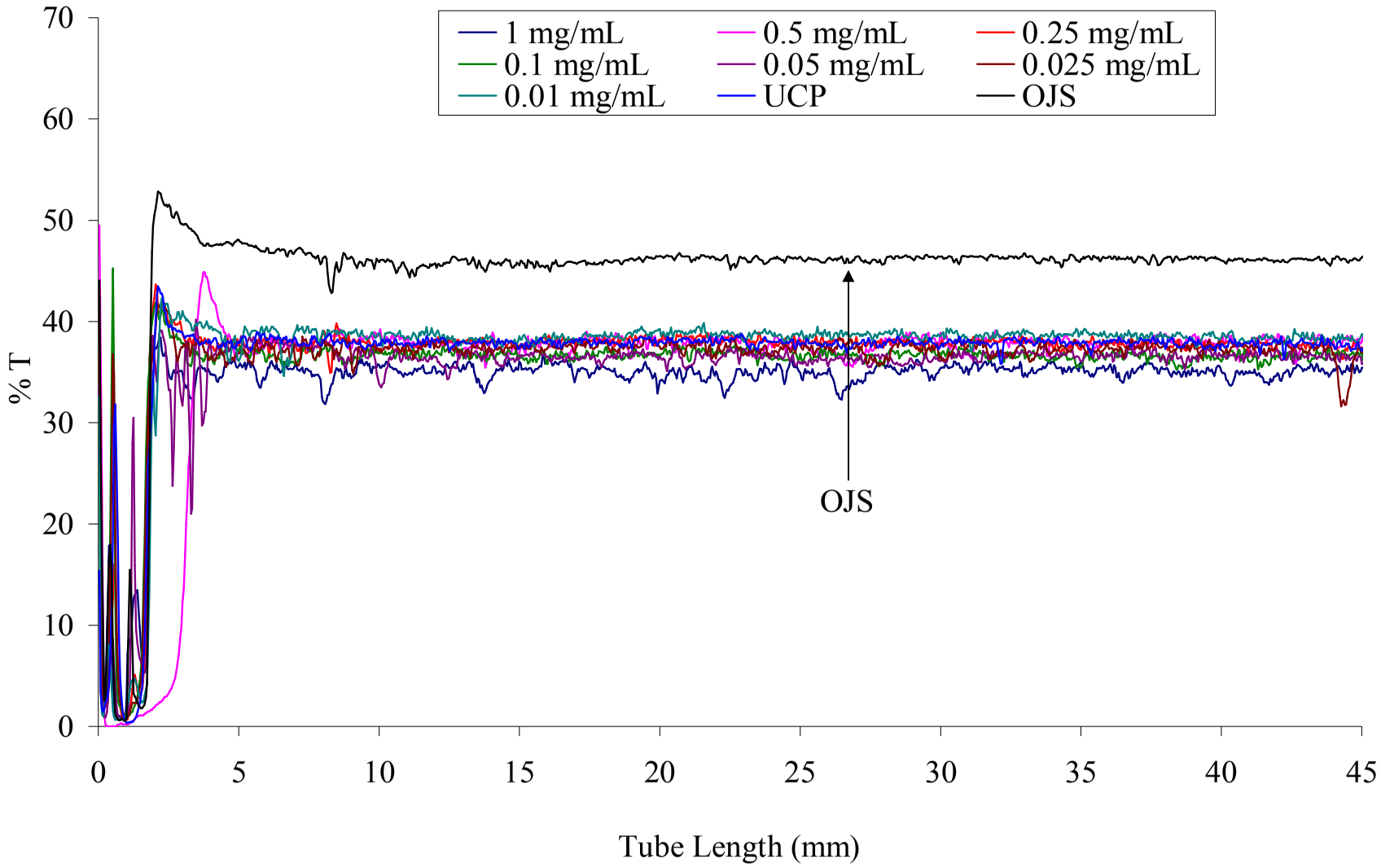

Figure 4.5. Turbiscan graph of OJS, unheated citrus protein and indicated hesperidin concentrations at time 0.

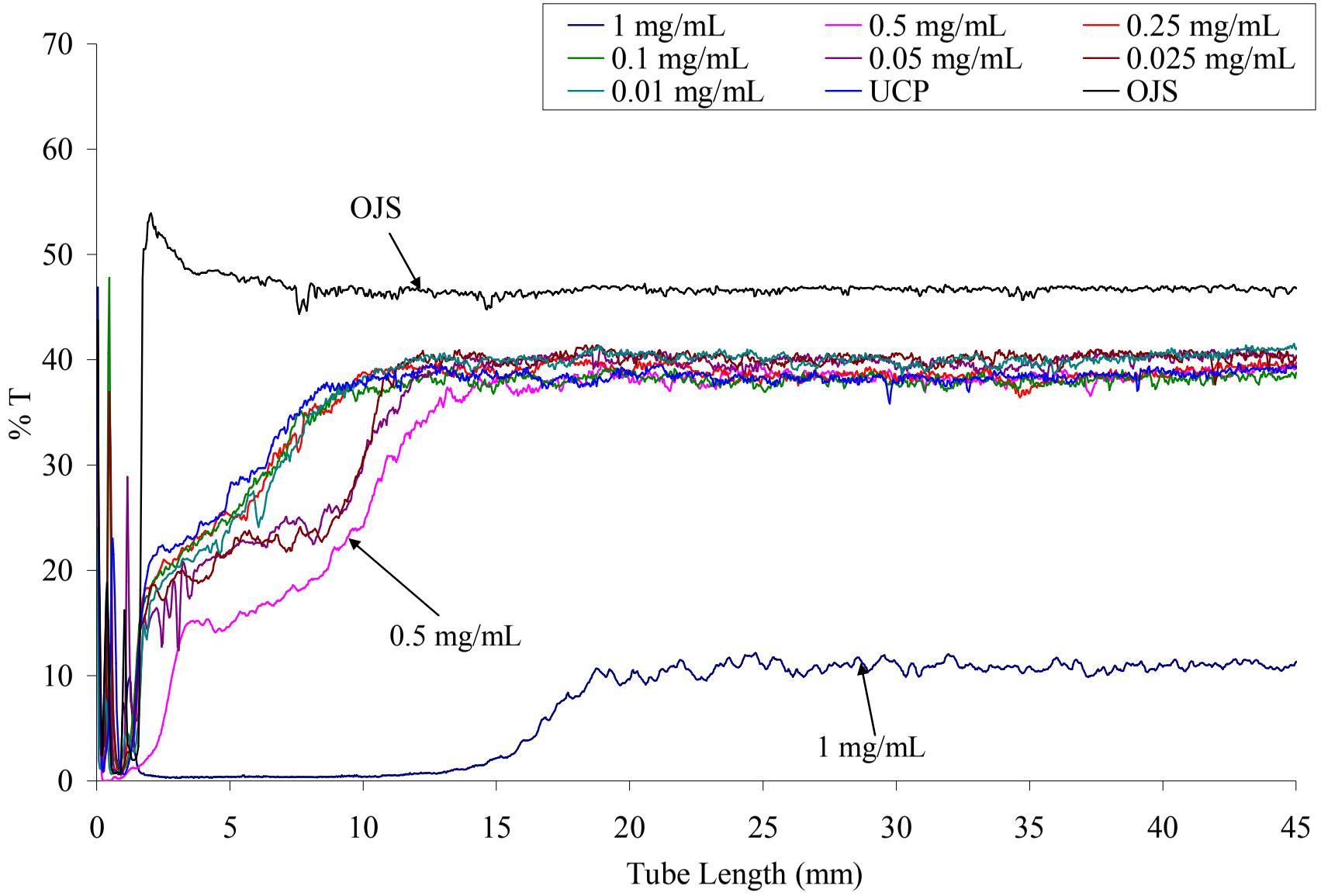

Figure 4.6 Turbiscan graph of OJS, unheated citrus protein and indicated hesperidin concentrations at time 30 minutes.

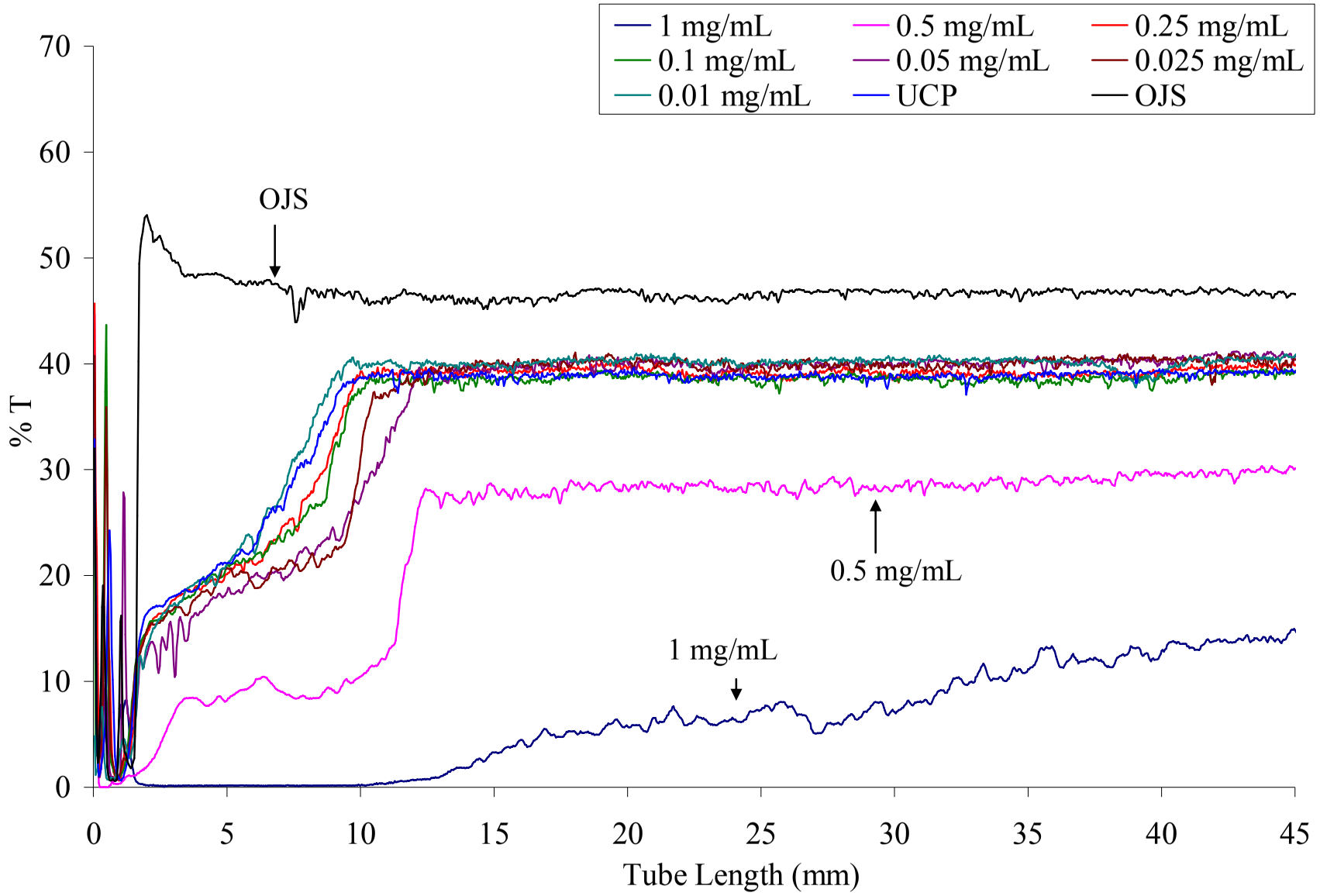

Figure 4.7. Turbiscan graph of OJS, unheated citrus protein and indicated hesperidin concentrations at time 1 hour.

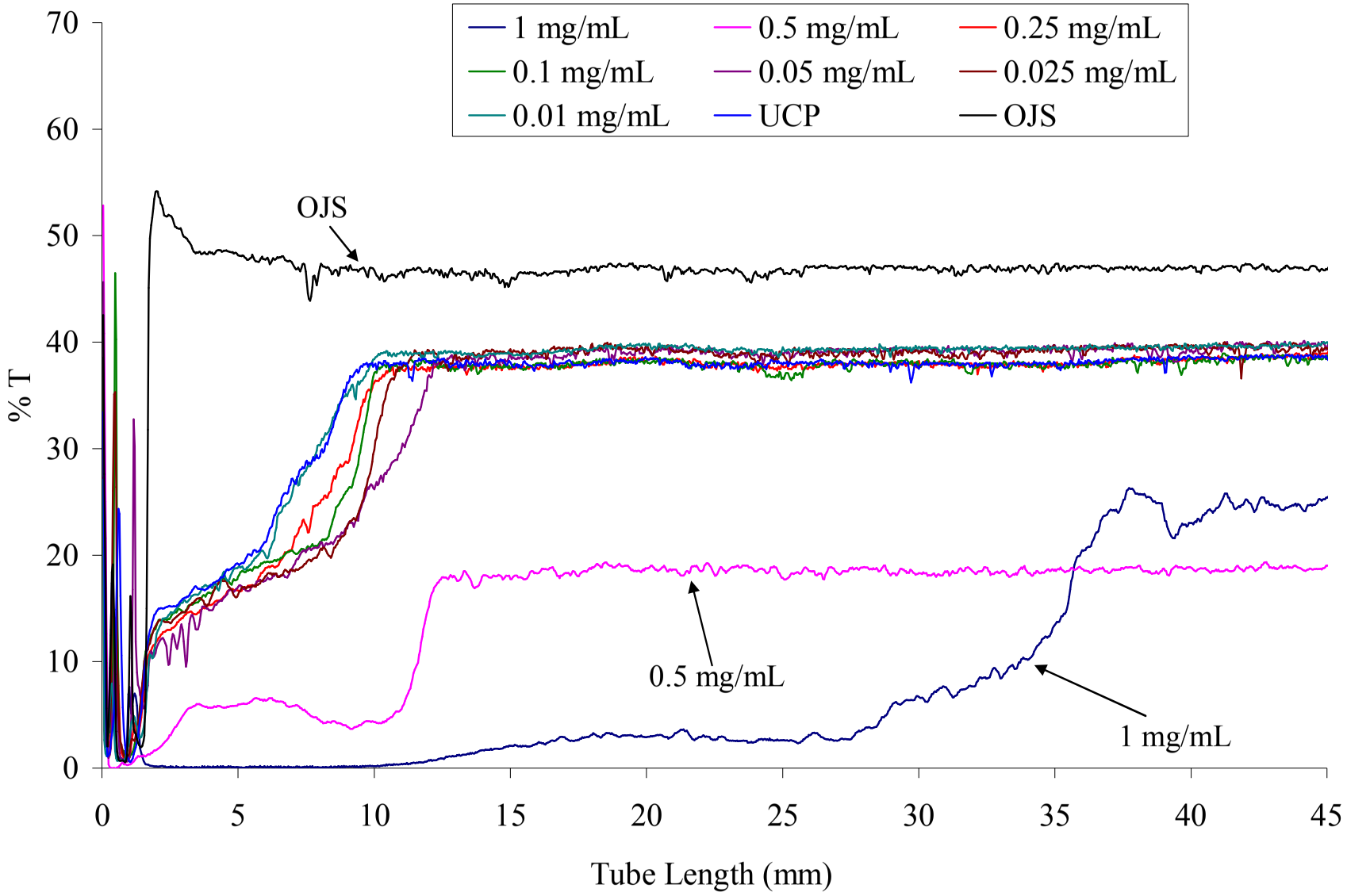

Figure 4.8 Turbiscan graph of OJS, unheated citrus protein and indicated hesperidin concentrations at time 2 hours.

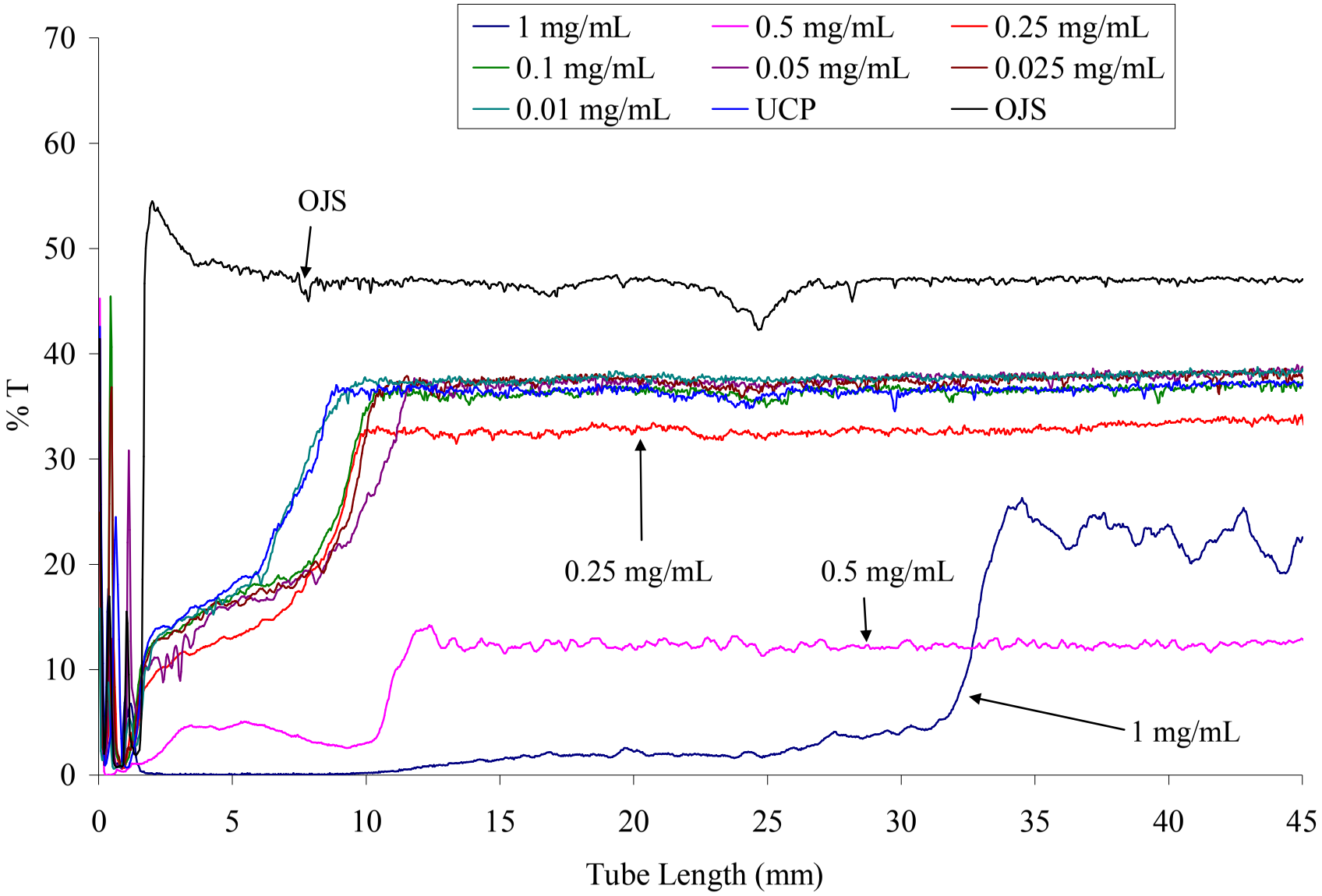

Figure 4.9. Turbiscan graph of OJS, unheated citrus protein and indicated hesperidin concentrations at time 5 hours.

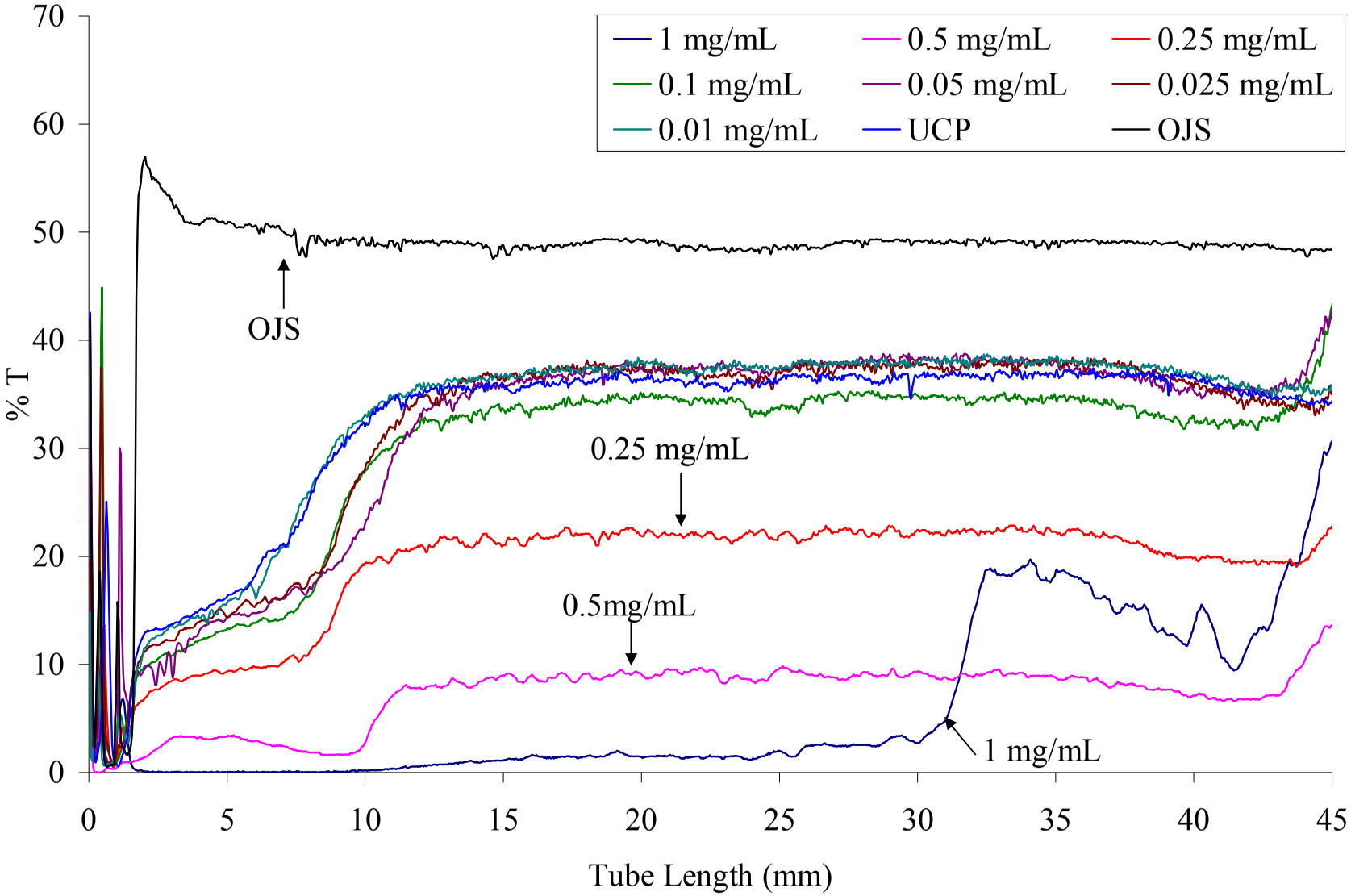

Figure 4.10. Turbiscan graph of OJS, unheated citrus protein and indicated hesperidin concentrations at 48 hrs.

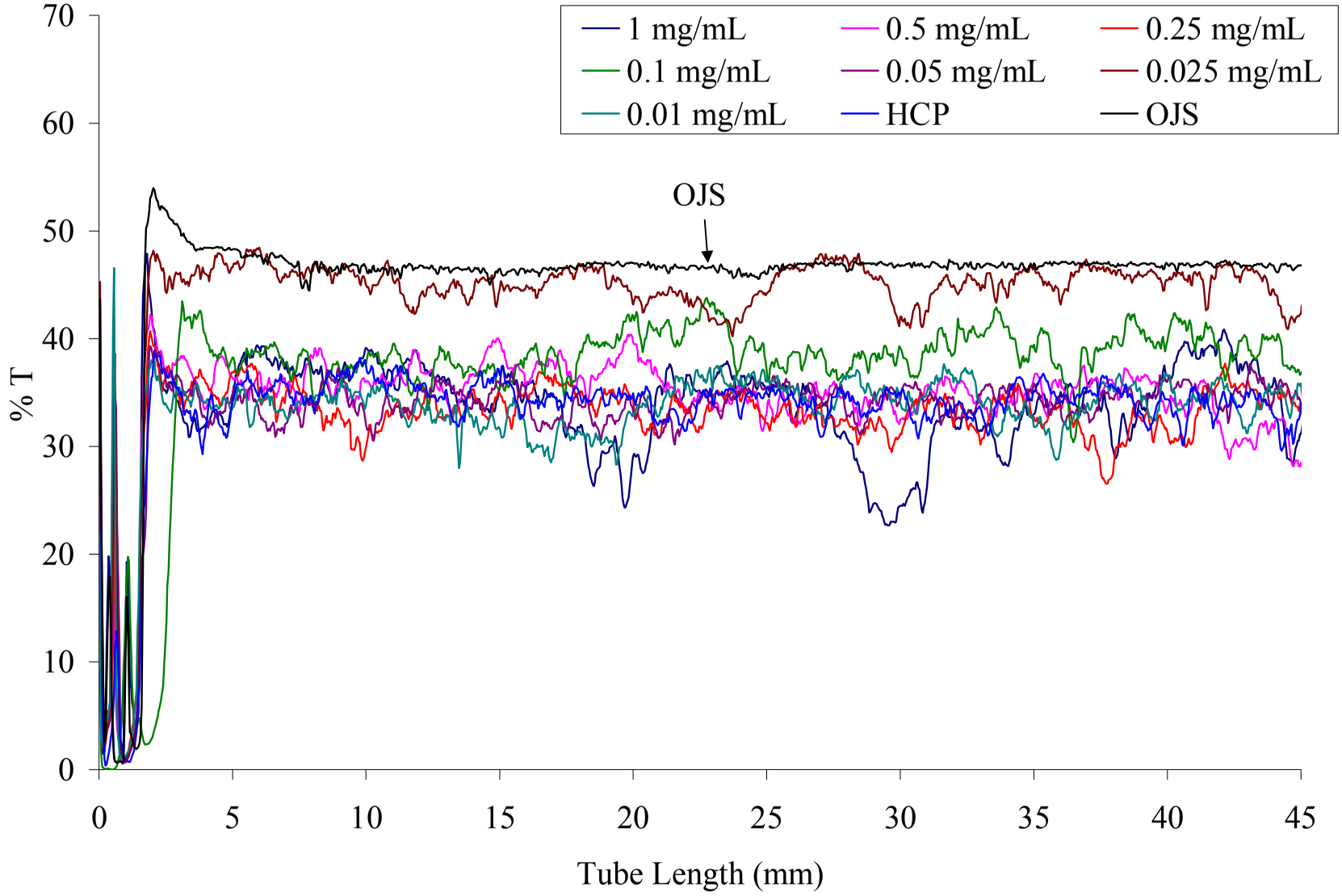

Figure 4.11. Turbiscan graph of OJS, heated citrus protein and indicated hesperidin concentrations at time 0.

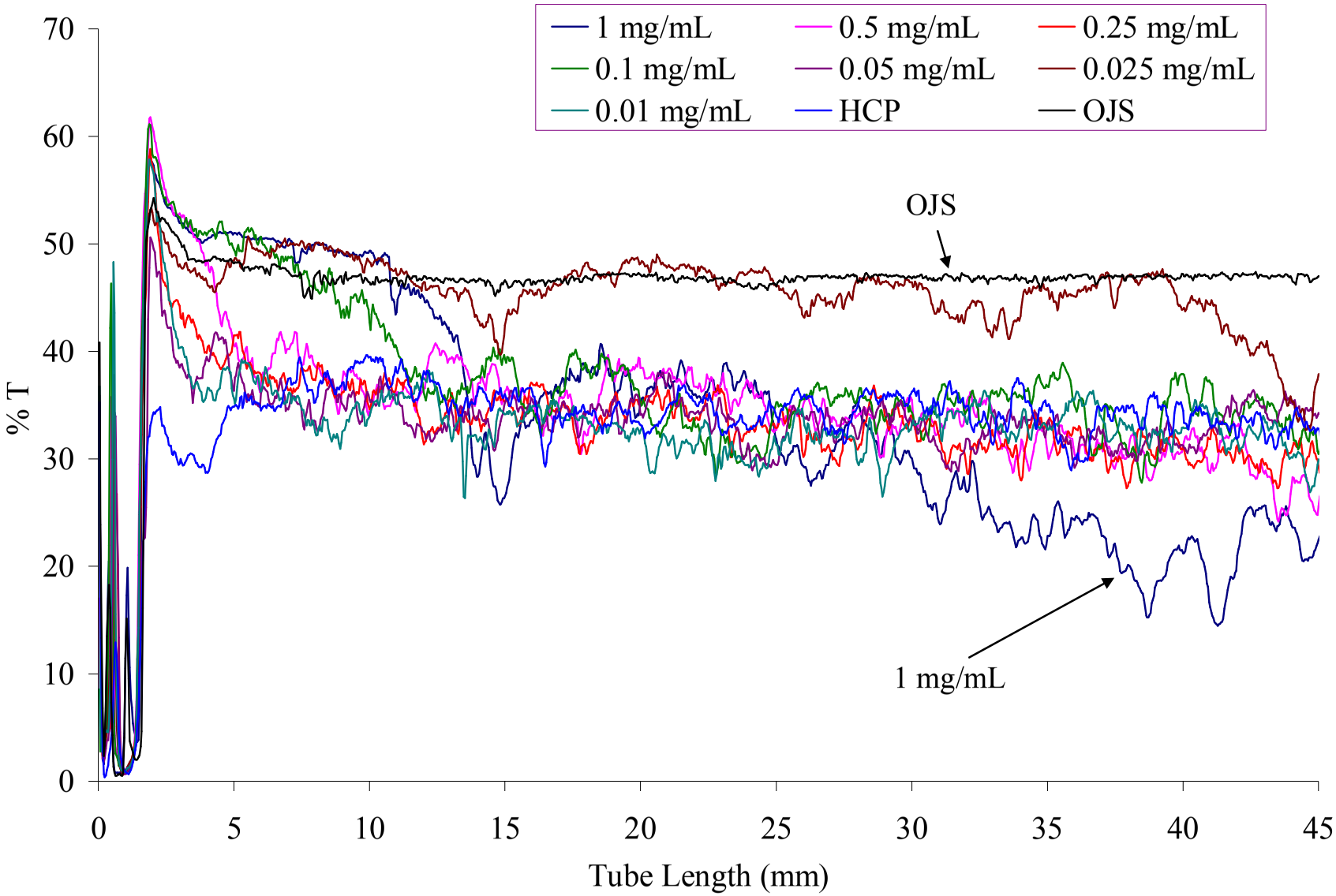

Figure 4.12. Turbiscan graph of OJS, heated citrus protein and indicated hesperidin concentrations at time 30 minutes.

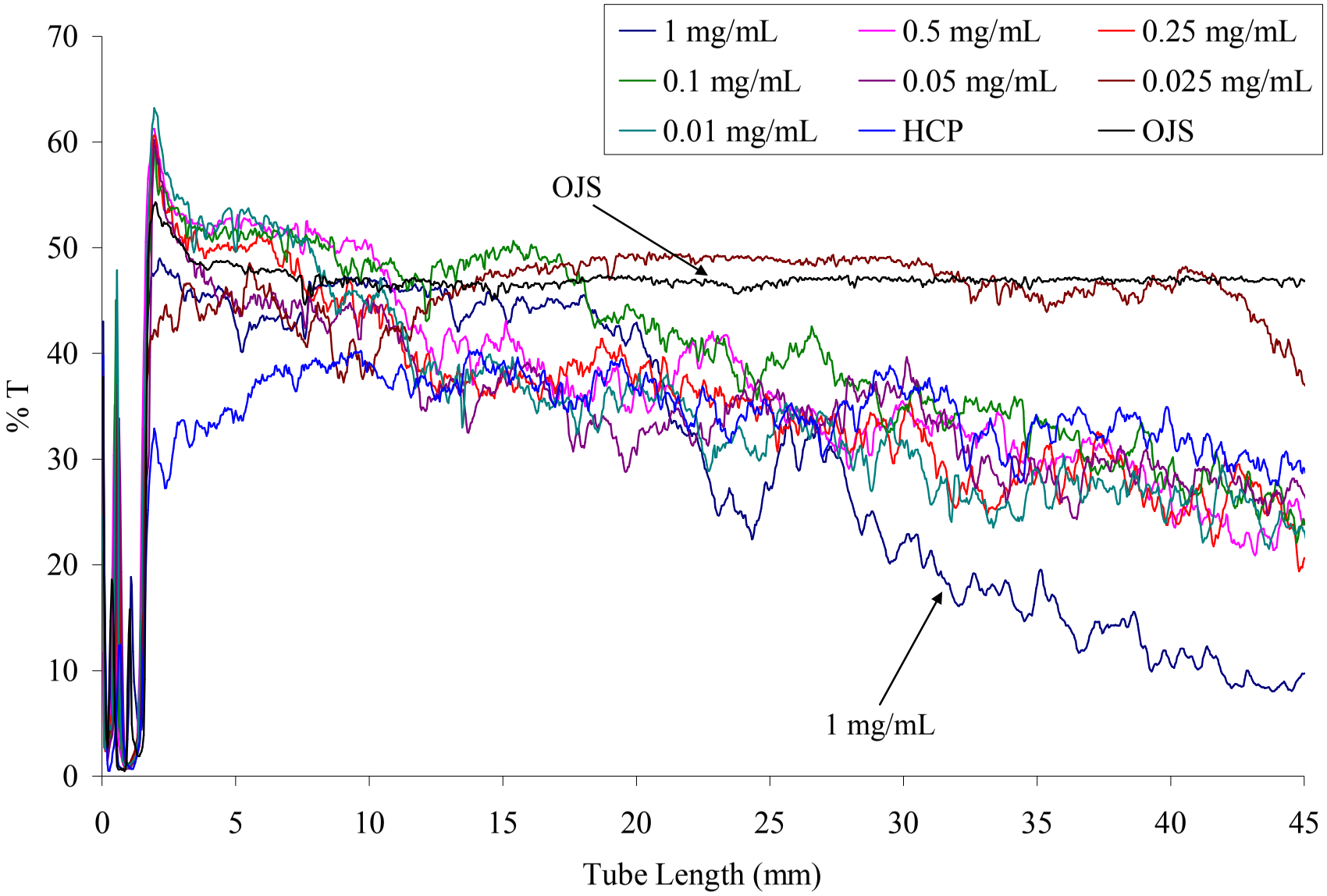

Figure 4.13. Turbiscan graph of OJS, heated citrus protein and indicated hesperidin concentrations at time 1 hour.

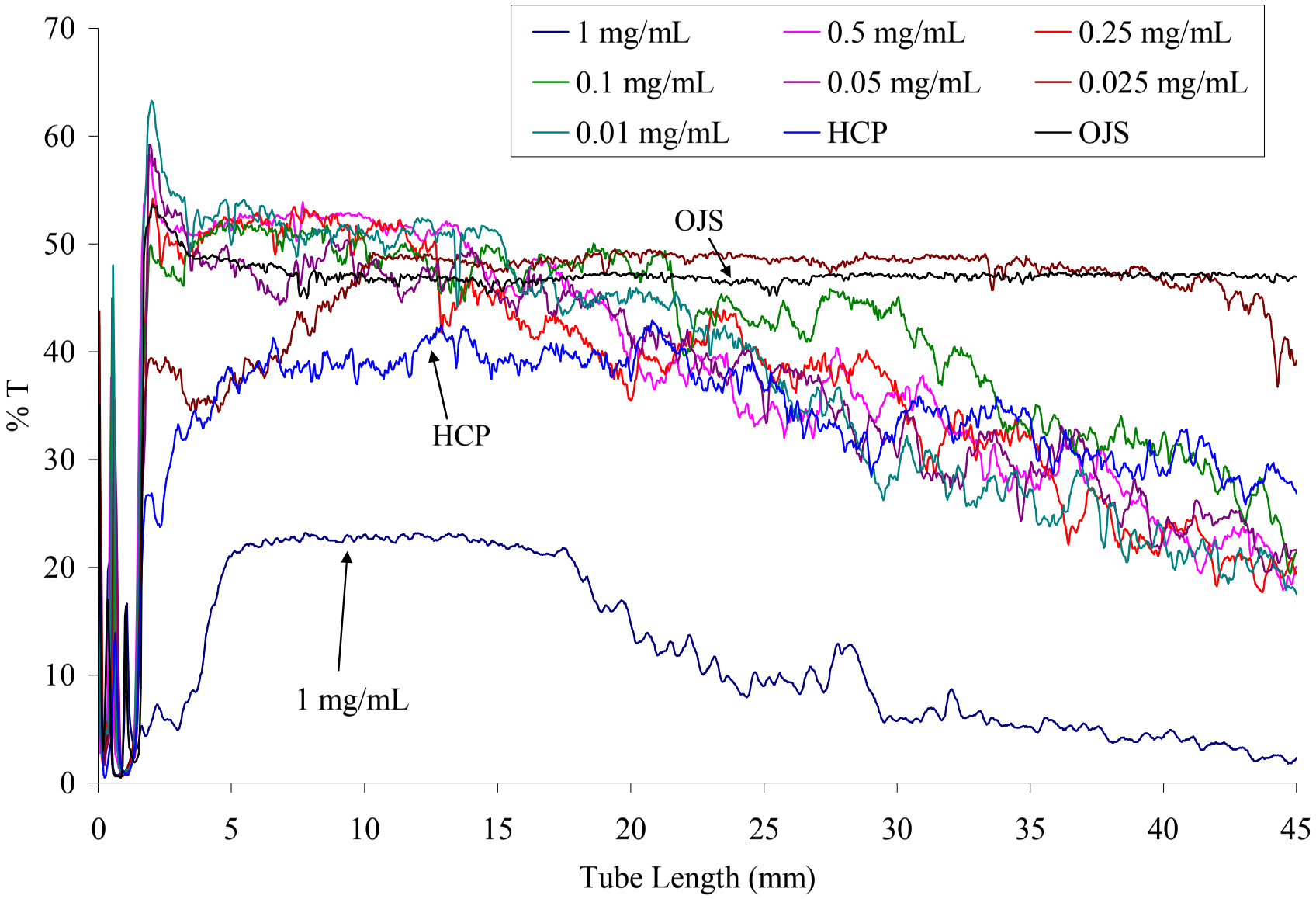

Figure 4.14. Turbiscan graph of OJS, heated citrus protein and indicated hesperidin concentrations at time 2 hours.

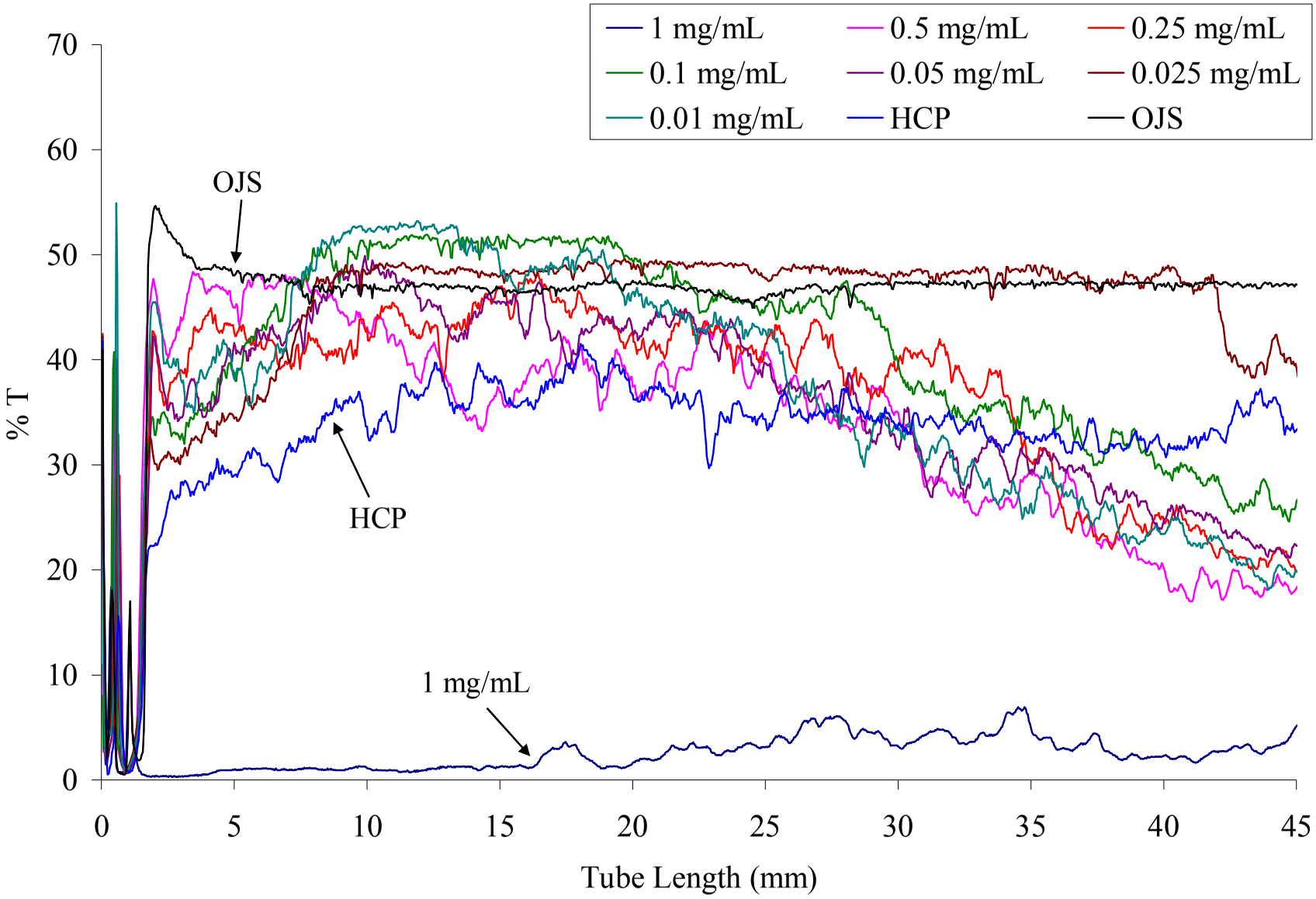

Figure 4.15. Turbiscan graph of OJS, heated citrus protein and indicated hesperidin concentrations at time 4 hours.

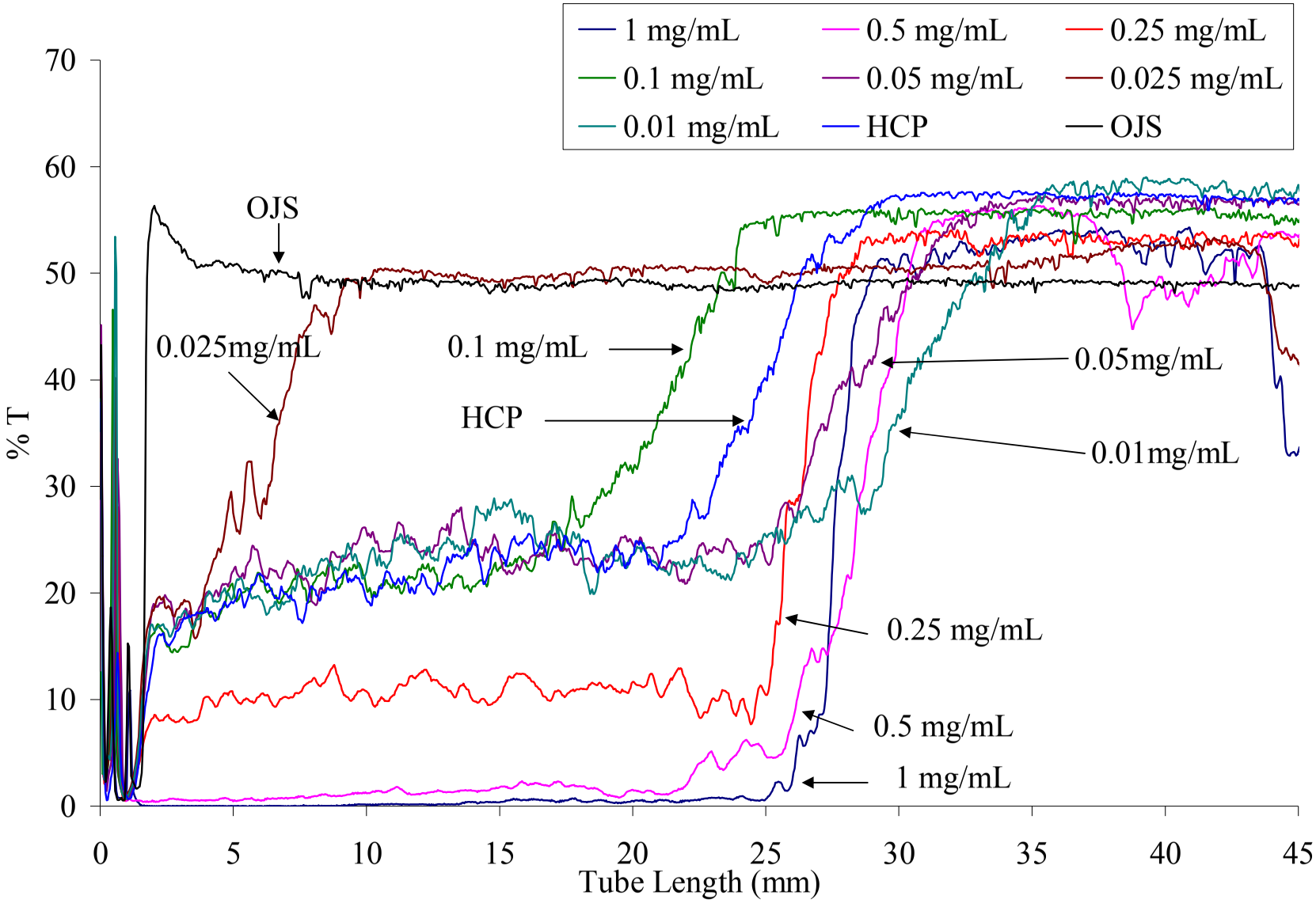

Figure 4.16. Turbiscan graph of OJS, heated citrus protein and indicated hesperidin concentrations at 48 hours.

CHAPTER 5

CONCLUSIONS

There is a need for a better understanding of the mechanisms of citrus cloud destabilization in order for the industry to improve upon consumer products. It is clear that there are many factors involved in the destabilization of citrus cloud. The research presented here has shown that calcium chelation at normal juice pH does improve the stability of orange juice cloud. There are non-covalent and non-electrostatic interactions involved in cloud particle aggregation, as evidenced by the association and disassociation of cloud particles that was independent of pH.

Hesperidin affects the stability of orange juice cloud as well. It was seen that higher levels of hesperidin (0.1 mg/mL – 1 mg/mL) interact with citrus protein, both heated and unheated, causing instability in the juice cloud. Soy protein and hesperidin had very little interaction. A non-catalytic role for citrus protein is indicated by the interaction of heated citrus protein with hesperidin. The difference in heated and unheated citrus protein with hesperidin indicate a different interaction of citrus particles upon heating.