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# THE NANO-CASEIN-PHENOLIC PROPERTIES USING DIFFERENT REASSEMBLING TEMPERATURE

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## ABSTRACT

Casein is one of the milk proteins which have the unique characteristic, it is called assembly. Assembly is casein can interact with casein or others to form micelle casein nanoparticle. The nano size of casein can accelerate the process of absorption into the body, making it suitable as a delivery system of nutraceutical food. It can happen with the appropriate temperature. According to the statement, this research aims to determine the best temperature for the reassembling process of casein with phenolic at different temperature (40°C, 50°C, and 60°C) with four replications. The parameter analysis is the interaction between casein and phenolic in the different temperature using spectrophotometer UV Vis, particle size, profile protein using electrophoresis, microstructure and functional group. Temperature treatment gave an effect on casein stability. All treatments produced maximum absorbance at relatively the same wavelength. All treatments still produce nano-sized particles according to the expected range. The exact temperature is required to determine the expected particle size. The smallest size at this stage of the study was obtained at 50˚C. The molecular weights detected in all treatments included $\alpha_{S1}$ casein, β casein, and $\alpha_{S2}$ casein. Good structure formation is determined by the right temperature and plays a role in forming stable casein. The temperature treatment at 50˚C, produced the best microstructure compared to other treatments. The temperature treatment at 50˚C, still contains complete functional groups referring to bioactive compounds when compared to other treatments. It could be concluded that the temperature of reassembling at 50˚C is the best treatment. This research can be used as a base for delivery systems for bioactive compounds using casein applied to nutraceutical products.

**Keywords:** Functional group; microstructure; milk protein; particle size; and protein profile.

## INTRODUCTION

Casein is 80% of the protein in milk with three types, including α-casein, β-casein, and kappa casein. Casein has many advantages including as delivery system material, encapsulated material to protect sensitive material as a bioactive compound, lactic acid, essential oil, etc. Casein can interact with others to form the nanoparticle, the unique characteristic of casein is assembly, they can interact with other casein in material. Casein micelle can be reassembled in vitro in 50˚C and 600 MPa and it increases interaction with vitamin D2. This process delivers nutraceutical hydrophobic vitamin D2 as a nanodelivery system. Casein micelle was reassembled swelling at high pH, however casein particle attraction between casein micelle can maintain micellar integrity until pH 12.6. according to the characteristics casein is an ideal material to deliver bioactive compound (Menendez-Aguirre et al., 2014; Saiz-Abajo et al., 2013; Madadlou, et al., 2009).

Casein is a highly hydrated colloidal particle and the presence of hydrophobic and hydrophilic amino acids makes casein a suitable copolymer for the encapsulation of hydrophobic and hydrophilic compounds. In a study, casein was used as an encapsulant material for keeping curcumin. It gave the result high dispersibility and bioavailability (Liu et al., 2016). Casein is also used as nano encapsulant material for β-carotene for protection from degradation and to increase the stability. The reassembling process could form the nano particle of casein. Nanostructure could protect β-carotene from the high temperature (Saiz-Abajo, 2013). According to the statements, it needs to analyze the different temperatures for the reassembling process on the physicochemical properties of nano-casein-phenolic.

## MATERIALS AND METHODS

### Materials

The materials used in this research include casein, phenolic, 0,38M trisodium citrate, $Na_2HPO_4$, NaOH, HCl, aquades, serbuk KBr, SDS 10 %, 30% acrylamide/Bis solution, 0,5M buffer Tris/HCl, ammonium peroxide (AMPER), tetramethylethylenediamine (TEMED), coomassie blue R – 250, methanol, glacial acetic acid.

### Research equipment

Beakerglass, measuring cup, pH meter, UV Vis spectrophotometer (Hitachi 2900), analytical balance, stirrer, pH meter, SDS PAGE brand Biorad Minirad Protean 3: electrophoresis device (sample cassette arrangement, Electrophoresis Power Supply (EPS)), pipette and suction cup, automatic micro pipette, bottles, water bath, refrigerator, centrifugator, infrared spectrophotometer and PSA (Particle Size Analyzer), Zetasizer Nano Series Software Version 7.01, Malvern Instrument. The interaction between casein and phenolic using spectrophotometer UV Vis, particle size, profile protein using electrophoresis, microstructure and functional group.

### Research method

This study used a laboratory experiment with a completely randomized design and four replications. 5 gr casein was added with 100 ml buffer phosphate pH 6,8. It was dissolved at 30-35˚C for ten minutes using a magnetic stirrer. After that, it was

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added 60 μg/ml of phenolic extract was added and homogenized using a magnetic stirrer. It was heated for the reassembling process with three different treatments at (40˚C, 50˚C and 60˚C). added with 2 ml citric acid 0,38M and homogenized for 5 minutes. added with 12 ml Na2HPO4 0,08 M and homogenized for 5 minutes. The pH was adjusted between 6.7-7 with the addition of 0.1N HCl or 1N NaOH.

**The interaction between casein and phenolic using spectrophotometer UV Vis**

Spectrophotometry determination to know the interaction of protein with a slight modification [9]. 0,1 ml emulsion of casein with catechin (different concentration as treatment) was prepared. After 10 min the solid with adsorbed casein was separated by centrifugation of a suspension at 12,000 rpm (twice, 10 min).

The supernatant absorbance was measured at 280 nm by UV–Vis spectrophotometry. Simultaneously, a reagent blank without catechin was performed according to the same procedure. The absorption spectra of the suspension were measured between 210 and 350 nm by spectrophotometry (Farhadian et al., 2012).

**Particle size**

The particle size of casein was measured by a static laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments, Montreal, QC, Canada), using deionized water as the dispersion medium (refractive index is 1.465) (Shapira, Assaraf, dan Livney, 2010).

**Profile protein using electrophoresis**

Inter-protein crosslinking was evaluated by polyacrylamide gel electrophoresis in sodium dedocyl sulfate (SDS–PAGE) using precast gradient gels (4–15%). Gels were calibrated by the broad range (BRM) SDS calibration standard that contains nine proteins ranging in size from 6.5 to 200 kDa. Samples (approximately 0.5 mg) of casein dissolved in sample buffer (10 mMTris–HCl at pH 8.0 containing 1 mM EDTA, 25 mg/ml SDS, 50 ll/ml b-mercaptoethanol and 0.1 ll/ml bromphenol blue) (Merck) were heated at 40˚C for 4 h. Coomassie blue was used to stain the gels (Aulanni'am, 2005).

**Microstructure**

A scanning electron microscope (SEM-model SU8010, Hitachi High-Technologies Canada, Inc. Toronto, ON, Canada) was used to characterize the surface morphology of casein control and casein with catechin treatment. The sample was placed on object glass and then coated with a layer of gold powder with a coating time of ± 30 seconds. The sample was observed using SEM with a voltage of 15 kV and magnification up to 5000 x (Rahayu et al., 2021).

**Functional group**

Infrared spectra were recorded on a FTIR spectrometer (Impact 420 model, Digilab, Mississauga, ON, Canada). One milligram of casein and casein with catechin treatment dried in a vacuum desiccator was ground and mixed thoroughly with 200 mg of oven-dried KBr powder (Merck, DAC, USP). The powder was placed in a die and compressed into a transparent disk. The sample was observed with the spectral range of 4000–600 cm-1 with a nominal resolution of 2 cm-1 and 100 scans (Maoela et al., 2009).

## RESULTS AND DISCUSSION

**Analysis of interaction casein between phenolic using spectrophotometer UV-Vis**

The characteristic of interaction casein with phenolic was shown using spectrophotometer UV-Vis at λ 210-350 nm. Each treatment gave the same $\lambda_{maximum}$ at 277 nm (Figure 1). The maximum result was consistent with past work of Farhadian et al. (2012), with a maximum of 280 nm and observations ranging from 210 to 350 nm.

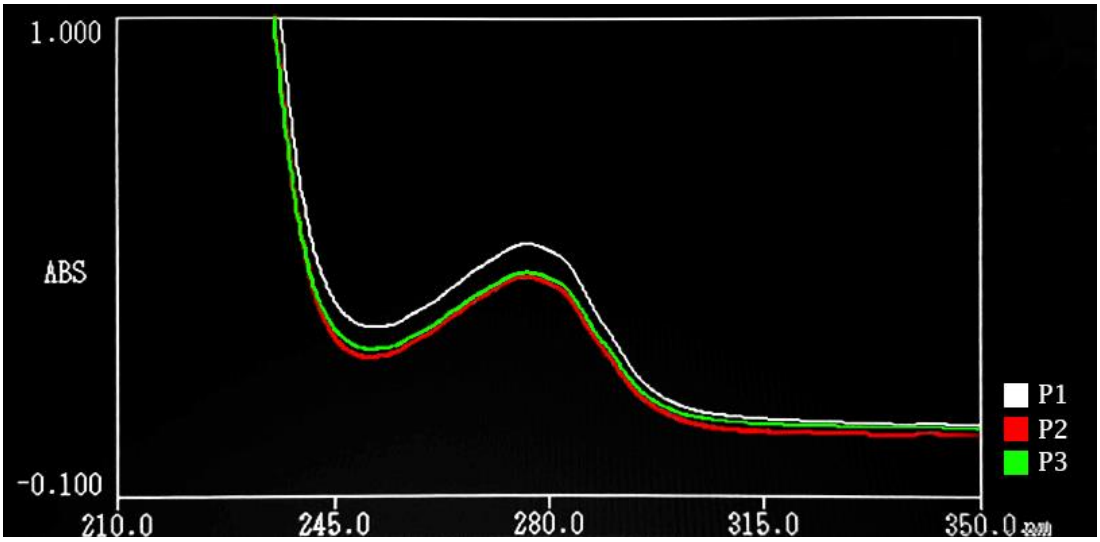



**Figure 1.** Characteristic of interaction casein with phenolic using spectrophotometer UV-Vis
Note: reassembling with temperature P1: $40^0$C; P2: $50^0$C; P3: $60^0$C

Any medium that can absorb UV light is due to the presence of a functional group, namely a chromophore. UV radiation has a wavelength range from 100-400 nm. The interaction of UV or visible light produces electronic transitions of bonding electrons, both sigma sigma (σ), pi (π) and non-bonding (n) electrons present in organic molecules. Chromophores have unsaturated bonds or contain functional groups with double bonds. When a molecule absorbs light, the energy will cause a transfer of electrons due to light energy from the ground state to a higher energy state.

The absorbance value produced in this study tends to decrease along with the increase for the temperature treatment used (Table 1). Treatment P1 produced an absorbance value of 0.515, which was the highest result compared to other treatments. Treatment P1 to P3 temperature decrease the absorbance values. The results of the absorption indicated that there was an interaction between casein and phenolic that formed a complex. This complex can be stabilized by hydrophobic bonds (Hasni et al., 2011) and hydrophilic interaction (Mehanna et al., 2014). This can be seen in different absorbance values with the same $\lambda_{maximum}$.

The high absorbance value caused the existence of chromophore groups. It was found for reassembling at $50^0$C. This indicated that phenolic was trapped in casein micelles. It could be concluded that temperature has a role in casein micellar reassembling process. It could show the interaction of casein micelle and phenolic formed complex compound.

This is due to the interaction formed between the amino side and the phenol ring which can improve the mechanical properties of casein. There are several roles of interaction in the formation of the complex including hydrophobic interactions, hydrogen bonds and van der Waals (Farhadian et al., 2012; Dezhampanah et al., 2016).

**Table 1.** The absorbance value of nano-casein-phenolic at different reassembling temperature

| Treatment | Average of absorbance value |
|---|---|
| $P_1$ | 0.515 |
| $P_2$ | 0.428 |
| $P_3$ | 0.410 |

Note: reassembling with temperature $P_1$: $40^0$C; $P_2$: $50^0$C; $P_3$: $60^0$C

Based on the discussion above, it can be concluded that heating can affect the interaction of casein with phenolics, but excessive heating can reduce the interactions formed

**Particle size of nano-casein-phenolic**

Particle analysis of nano-casein-phenolic to know the effect of different temperatures for reassembling process. The small particle size of nano-casein-phenolic will be easy to apply when it will be fortified in milk product. According to the result in Table 2, the highest particle was 159 nm ($P_2$). Temperature could change the size particle but the result of particle size is appropriate with Some literatures that nano particle was 50-10000 nm, casein diameter was 150-300 nm (Defrates *et al*., 2018; Nagi *et al*., 2012), according to this statement, the particle size in this study is nano size.

Based on the data in (Table 2) obtained the smallest particle size results in P2 treatment with a temperature treatment of 50˚C. These results explained that to produce the appropriate particle size, it is necessary to decide the temperature that can be used for heating. Insufficient temperatures allow some particles to not dissolve completely, but excessive temperatures can also cause damage to proteins.

**Table 2.** The particle size of nano-casein-phenolic

| Treatment | Average of particle size (nm) |
|---|---|
| $P_1$ | 205.1 |
| $P_2$ | 159.8 |
| $P_3$ | 164 |

Note: reassembling with temperature $P_1$: $40^0$C; $P_2$: $50^0$C; $P_3$: $60^0$C

Temperature has a role in the casein reassembling process. This process occurs because casein has the property to bind other caseins and other proteins. This is due to the presence of hydrophobic interactions in casein. The interaction increases when there is heating because the hydration ability of the water molecules is reduced. This is supported by several studies which explain that the interaction between casein and phenolics and the casein reassembling process is influenced by several factors including temperature, pH and concentration. The formation of nanoparticles in these interactions can be conditioned by determining the right temperature (Faizullin et al., 2013; Pool et al., 2012; Mehranfar et al, 2013).

The interactions that occur between casein and phenolic include hydrophobic interactions, hydrogen bonds and van der Waals interactions. Casein is composed of proline which has an open structure so that it easily interacts with other compounds. Phenolic compounds can be bound by casein because of the high proline in casein. The interactions that occur can cause changes in the characteristics of casein which include changes in its structure, stability, solubility and functional properties (Patel and Velikov, 2014; Mootse, et al., 2014; Yildirim-Elikoglu and Erdem, 2017).

Based on the results and discussion above regarding particle size analysis in the reassembling process with different temperature treatments, it can be concluded that all temperature treatments used, still produce casein with nanoparticle size. The results of particle measurements in the reassembling process at a temperature of 50˚C obtained the smallest particle size compared to other treatments.

**Protein profile using electrophoresis SDS PAGE**

The analysis of protein profile using SDS PAGE electrophoresis aims to determine the molecular weight. The working principle of electrophoresis is to separate charged biomolecules based on the rate of migration of biomolecules in an

electric field. The results of the protein profile analysis are shown in (Fig. 2).

Based on the curve in (Fig. 3). the equation y = -1.518x + 2.302 with $R_2$ = 0.959. The molecular weight of the sample can be obtained from the linear formula. Based on electrograms from electrophoretic analysis using SDS-PAGE, it can be seen that the bands that appear in all treatments $P_1$, $P_2$, and $P_3$ have the same molecular weight. The molecular weights (MW) of samples $P_1$, $P_2$ and $P_3$ were 22.14 kDa, 22.41 kDa, 28.27 kDa, 36.11 kDa, 31.18 kDa, 61.85 kDa, 75.22 kDa, 82.95 kDa, 87.11 kDa. The molecular weights were 22.14 kDa, 22.41 kDa, 28.27 kDa, and 82.95 kDa in the samples suspected of $\alpha_{S1}$ casein, β casein, $\alpha_{S2}$ casein and lactoferrin.

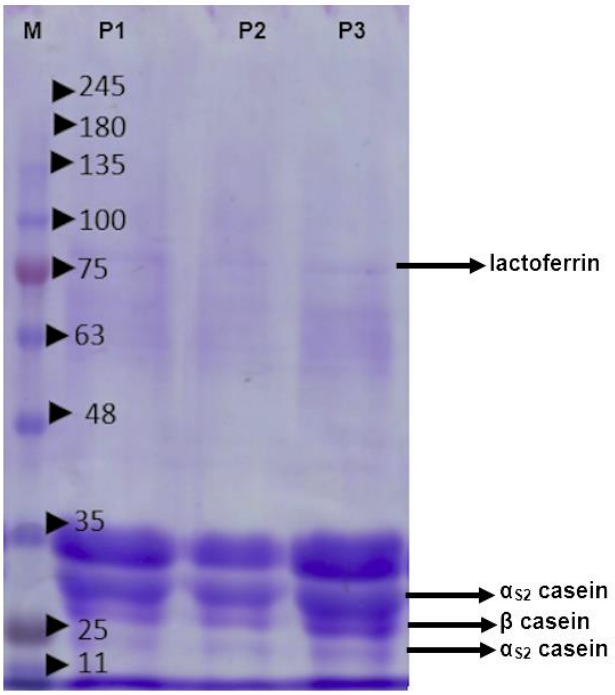



**Figure 2.** Electrophoregram of nano-casein-phenolic
Note: $P_1$: $40^0$C; $P_2$: $50^0$C; $P_3$: $60^0$C

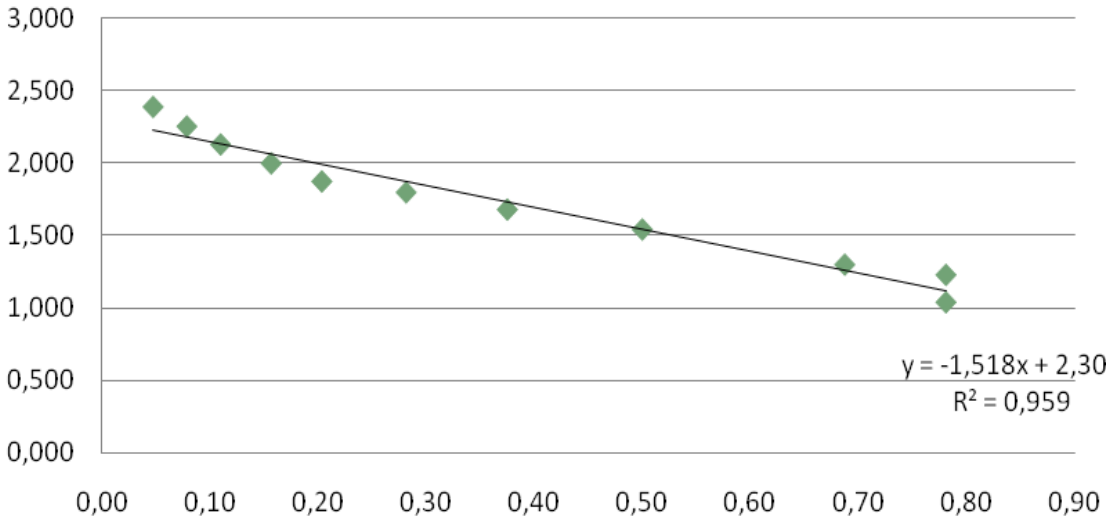


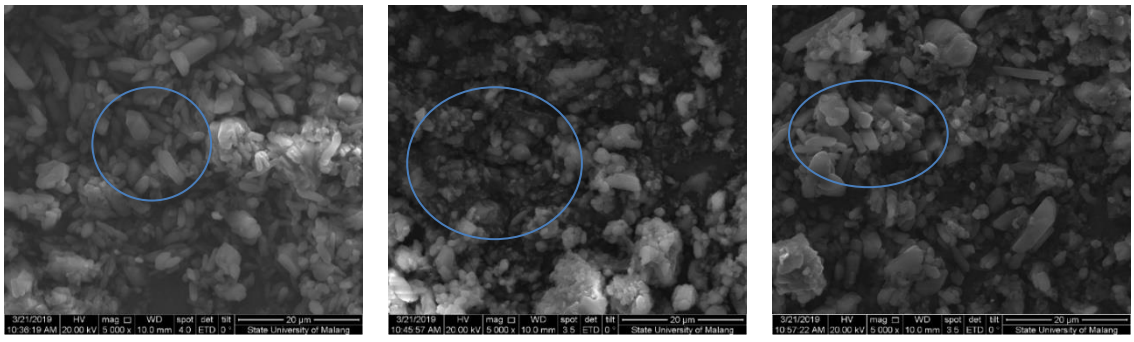

**Figure 3.** curve for the linear formula of molecular weight

It is according to with Yildirim-elikoglu and Erdem (2017) who explained that the MW of $\alpha_{S1}$ casein was 22.1-23.7 kDa, $\alpha_{S2}$ casein was 25.2-25.4 kDa, β casein was 23.9-24.1 kDa and k casein by 19 kDa. Wang et al. (2017) explained that lactoferrin has a BM of 78 kDa). All samples at different reassembling temperatures were thought to contain $\alpha_{S1}$ casein, β casein and $\alpha_{S2}$ casein. This explains that the reassembling process at temperatures between 40˚C and 60˚C does not remove the bands for $\alpha_{S1}$ casein, β casein and $\alpha_{S2}$ casein.

The k-casein band with MW 19 kDa is not visible. This is presumably the smaller the particles that may not be detected. Heating with a temperature of 40-60˚C still produces the same size variations, but if the temperature used is more than 60˚C with a certain time it can increase the size. It can be ascertained that the resulting BM will also increase. Heating treatment at 80-90˚C can cause the hydrophobic bond intensity to decrease. Heating can change the conformation of proteins (Faizullin et al., 2013 and Dissanayake et al., 2013). The reassembling process is supported because of the hydrophobic interaction that occurs in casein and phenolic and is assisted by the right temperature. The formation of a complex between casein and phenolic will stabilize when it is heated. Based on the results of the protein profile and discussion, it was found that the protein bands that appeared at a temperature treatment between 40-60˚C produced the same molecular weight and detected the presence of $\alpha_{S1}$ casein, β casein and $\alpha_{S2}$ casein.

**Microstructure of nano-casein-phenolic using *Scanning Electron Microscopy***

Microstructural analysis was to determine the external appearance of casein phenolics during the reassembling process at different temperatures. The test uses samples in dry form so that initial preparation is needed before observations are carried out. The results of microstructural observations are shown in (Figure 4).



$P_1$ $P_2$ $P_3$

**Figure 4.** Microstructure of nano-casein-phenolic at different temperature
Note: $P_1$: 40˚C; $P_2$: 50˚C; $P_3$: 60˚C

The results of casein with the different reassembling temperature produced different microstructures. $P_2$ with heating at 50˚C still had a rounded casein shape and looked more uniform than the other treatments. Samples $P_1$ and $P_3$ have seen the shape of casein which is not only round but oval. This could be due to the influence of the temperature used. The optimal temperature will produce casein that is in accordance with its characteristics, namely the globular form of casein. The results of microstructure analysis have a size that tends to be larger than the particle size analysis using PSA. This is due to the different shape of the samples. SEM observations use dry samples, so it is necessary to dry the samples first. The dried samples have hygroscopic properties so they tend to cluster when observed. The

hygroscopic nature of the sample indicates the ability of the sample to absorb water molecules well so that the solubility is high.

It was observed that the $P_2$ treatment with a temperature of 50˚C resulted in a more uniform, compact structure seen from the increasingly intensive bond formation, no visible gaps between particles and a round shape still visible. This is in accordance with Gaucheron et al. (2001) who explained that casein is very stable to heat because it rarely occurs tertiary structure formation, but the modifications made to casein depend on the heating used. Treatment of $P_1$ and $P_3$ produced almost the same structure. This is because the formation of a good structure is determined by the right temperature. Temperature plays a role in stabilizing the complex structure formed between casein and phenolics. These complexes form nanoparticle sizes due to the role of phenolic OH in binding amino acids to casein (Pool et al., 2012; Mehranfar et al, 2013). It was concluded that the best temperature treatment in the reassembling process for the microstructure was at a temperature of 50˚C.

**Functional group analysis using *Fourier Transform InfraRed* (FTIR) of nano-casein-phenolic**

IR spectroscopy aims to determine functional groups for the purpose of identifying compounds, determining molecular structures, analyzing purity and studying reactions that occur and can be used in organic and inorganic analysis (Ramos-Tejada et al., 2002). The results of observing the functional groups in nano casein which were reassembled at different temperatures using FTIR can be seen in (Figure 5) and a summary of the absorption areas of the compounds can be seen in (Table 3).

Observation of functional groups in the process of reassembling nano-casein-phenolics at different temperatures aims to determine the functional groups that refer to phenolic compounds after heating according to treatment. The heating used starts from 40˚C, 50˚C and 60˚C. The results of observations of functional groups can be used to control the presence of phenolic compounds and the changes experienced by casein with that temperature. The resulting functional groups were analyzed by comparing the control and previous studies.

Based on (Table 3) it shown that all the reassembling temperature treatments made a difference to the resulting absorption area. In the $P_1$, $P_2$ and $P_3$ treatments, the spectra appeared in the absorption area containing phenolic groups, although there were some shifts in the absorption area. Bioactive components are found in absorption areas ranging from 500-1900 $cm^{-1}$, it refers to the O-H bond of the phenol number and the C=C Strecth group which is the aromatic number. Changes in number groups can be caused by the process used during reassembling. This is in accordance with several studies which explain that the group number changes by several factors, namely time and temperature during the process (Chen et al., 2006; Senthilkumar et al., 2017). It can be seen that the spectra produced in the three treatments varied. $P_2$ produces the most spectra compared to other treatments. There are several differences in spectra, starting from the results of the second phase of the study which produce the best spectra (Table 3). There are several spectra that begin to disappear and there are groups of numbers that experience a shift in the absorption area.

This is because the temperature used at this stage affects the spectra that appear. The spectra in the $P_1$ and $P_3$ treatments produced a slightly smaller spectrum when compared to the $P_2$ treatment so that it can be concluded that $P_2$ is the optimal temperature in the reassembling process. All treatments at this stage had absorption referring to the O-H bond of the phenol number, but there was a slight shift in absorption. There are several absorption areas that appear and are different from other temperature treatments. One of the important absorption areas refers to the C=C Strecth group which is an aromatic number.

Based on the qualitative analysis of functional group observations, it was concluded that all temperature treatments still contained several functional groups which refer to the important numbers of phenolics.

**Table 3.** Function group analysis of nano-casein-phenolic using FTIR

| Phenolic | $P_1$ | $P_2$ | $P_3$ | Function groups | Chemical compound |
|---|---|---|---|---|---|
| | 817.82 | 823.6 | 835.18 | N-H | Amina |
| 952.84 | 933.55 | | 925.83 | C-H | Alkane |
| | 993.34 | 999.13 | 997.2 | C-H | Alkane |
| 1037.7 | 1047.35 | 1051.2 | | C-O stretch | Alcohol, ester, carboxylic acids |
| 1228.88 | 1259.52 | 1259.52 | 1236.37 | C-N Strech | Aliphatic amines |
| | | 1311.59 | | N-H | amide III |
| 1456.26 | | 1516.05 | | C-C Strech (in ring) | Aromatic |
| 1712.79 | 2185.35 | 2115.91 | | C=O Strech | Carbonyl |
| 1766.8 | 2250.93 | 2166.06 | 2266.36 | C=C Strecth | Aromatic |
| 2472.74 | 2382.09 | 2380.16 | 2380.16 | N-H | Amida A |
| | 2736.99 | 2762.06 | 2750.49 | C-H | Strain |
| 2883.58 | 2800.64 | 2879.72 | 2814.14 | C-H Strech | Alkane |
| 2931.8 | | 2912.51 | | | |
| | | | | C-H Strech | Alkane |
| 2970.38 | | 3088.03 | | O-H. H-Bonded | Phenol, alcohol |
| | | 3124.68 | 3057.17 | N-H | Amida |
| 3344.57-3352.28 | | 3442.94 | | N-H | Amida |

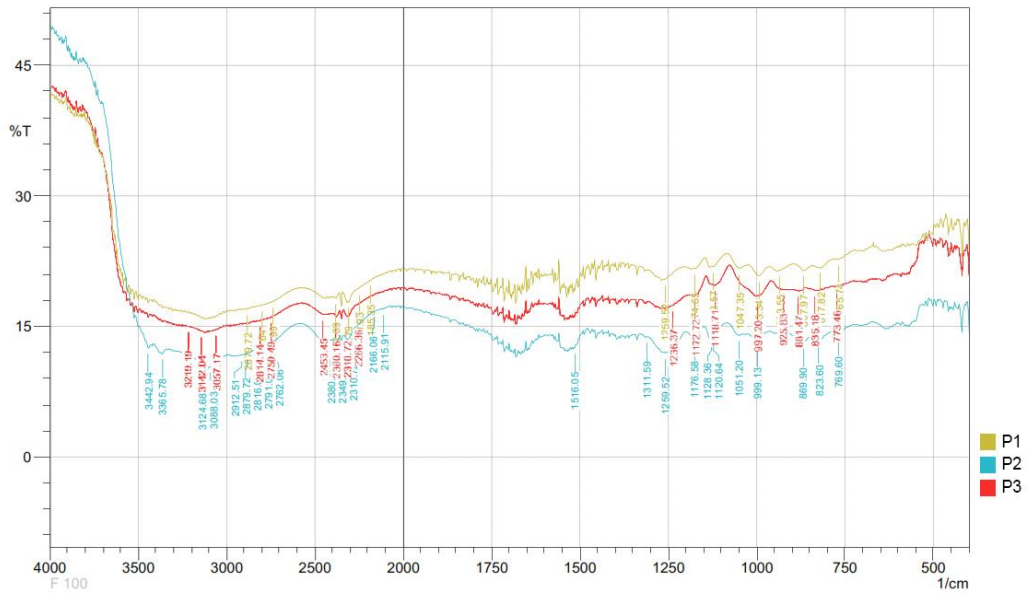



**Figure 5.** IR Spectra of nano-casein-phenolic with different *reassembling* temperature.
Note: reassembling with temperature P1: 40˚C; P2: 50˚C; P3: 60˚C

Treatments $P_1$ and $P_3$ experienced some decrease in the resulting spectra. The $P_3$ treatment produced the fewest spectra and the group numbers started to disappear. The $P_2$ treatment produced the most spectra, there were several shifts in the absorption area of the important group referring to the phenolics, but these compounds did not

disappear. Based on the discussion above, it can be concluded that the best treatment was found in the $P_2$ treatment with a temperature of 50˚C.

## CONCLUSION

It concluded that temperature of reassembling at 50˚C is the best treatment. It can increase the stability of casein, maintain nano size, casein components are still detected in protein profiles, the resulting microstructure looks compact and functional groups of bioactive compounds are still detected in FTIR.

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