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Research Paper

Probing the structure-holding interactions in cheeses by dissociating agents – A review and an experimental evaluation with emmental cheese

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ABSTRACT

Interactions holding protein structure in cheese has been a subject of considerable investigation, with conclusions varying among studies. We present a review on this topic, covering fresh curds, ripened cheeses, and processed cheeses. We discuss the usual chemicals and conditions used to probe different types of interactions. Furthermore, we did our own study with solutions of urea, SDS, EDTA, NaCl, and NaOH, at different concentrations and combinations, for Emmental cheese. To quantify solubilized protein, we developed a modification of a spectrometric-based method that can be conveniently employed to quantify total protein in cheese, with statistically similar results to those obtained by the Kjeldahl method.

Our results point out that caseins in the Emmental cheese are held together by a set of hydrophobic interactions, hydrogen bonds, and other electrostatic ones, including ionic bonds. Hydrogen bonds seem to have an important role, comparable to hydrophobic interactions, a conclusion not commonly reported for cheese structures.

1. Introduction

1.1. Probes for studying interactions in gel systems

Urea, sodium dodecyl sulphate (SDS), and ethylenediaminetetraacetic acid (EDTA) solutions are among the most commonly used protein denaturants. Urea, which is usually used at concentrations up to 9 M, disrupts both intra- and intermolecular hydrogen bonds and weakens hydrophobic interactions, leading to protein denaturation and solubilization (von Hagen, 2008). A few studies (Medronho and Lindman, 2014a, 2014b; Schmid et al., 2015) have also used substituted ureas, such as thiourea, concluding that some of them could be more efficient in breaking hydrophobic interactions. However, in the same studies, urea was shown to be more efficient than the substituted compounds in breaking hydrogen bonds, demonstrating that its overall solubilization power was greater (Rabilloud, 2002).

Several studies conducted in different protein gel matrices, such as surimi gels (Shiku et al., 2004; Zhang et al., 2018), lamb myofibrillar protein gels (Ni et al., 2014), sardine with added proteins (egg white, soy, casein and gluten) gels (Gómez-Guillén et al., 1997), alkali-induced ovalbumin gels (Zhao et al., 2016), and egg yolk gels (Yang et al., 2019), stated that urea, at a concentration of 1.5 M, disrupts hydrogen bonds; at a concentration of 8 M, urea also disrupts hydrophobic interactions. For example, the study conducted with a sardine gel with added sodium caseinate, concluded that, for those gels prepared at 50–60 °C, the solubilities in 1.5 M urea were low, suggesting a small participation of hydrogen bonds in gel formation; but high values of solubility were obtained in 8 M urea, taken as a measure of hydrophobic interactions (Gómez-Guillén et al., 1997).

It is worth mentioning that, in spite of the vast research on the subject, there are still different views about how urea acts as a protein unfolding agent. Some authors propose that it interacts directly with protein side chains to start the unfolding process via hydrophobic interactions (direct interactions between urea and the protein) (Steinke et al., 2017). Others propose that urea acts indirectly, by changing the bulk water-water interactions, leading to a decrease in protein hydration, which destabilizes the protein, causing it to unfold and expose the hydrophobic parts (Steinke et al., 2017). A combined mechanism between these two theories is also proposed by some authors (Steinke et al., 2017; Stumpe and Grubmu, 2007).

SDS is a surfactant detergent that gives a negative charge to all proteins (Hamada et al., 2009), and acts as a denaturant by preventing

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interactions among hydrophobic domains, within and among proteins (von Hagen, 2008). This surfactant molecule is supposed to interact with both charged and nonpolar groups of the side chains of proteins (Lefebvre-Cases et al., 1998).

Ionic bonds are traditionally disrupted by increasing the ionic strength of the media, e.g., with addition of NaCl (Gómez-Guillén et al., 1997; Ni et al., 2014; Strange et al., 1994; Zhang et al., 2018). EDTA is a chelation agent with high affinity for calcium ions. Hence, the addition of this substance to e.g. casein containing samples, such as cheese, is expected to contribute for the destruction of the micelles by the disruption of micellar calcium phosphate nanoclusters (Gaucheron, 2005).

The above agents are used individually or combined, in order to evaluate their cumulative effect in, presumably, different types of interactions.

1.2. Chemical interactions in milk gels

In cheese making, milk fat and caseins are concentrated, leading to a complex and heterogeneous system (Gagnaire et al., 2002). Cheese can be described as a bi-continuous gel structure consisting of a porous protein matrix (casein) interspaced with localized domains of fat (Vogt et al., 2015). The way this matrix is formed is a key variable, since it plays a role in the final microstructure of cheese and, consequently, its texture, flavour, and overall quality (Gagnaire et al., 2002; El-Bakry and Sheehan, 2014).

Emmental cheese, the one used in this work, is a Swiss-type, semi-hard cheese. In these cheeses, the matrix acidification occurs after pressing of the curd, i.e., when most of the whey has already been expelled. Hence, the concentration of colloidal calcium increases proportionally with the concentration of casein at drainage, leading to the formation of a highly mineralized, cohesive para-κ-casein matrix, with a calcium-casein ratio close to that of the casein micelles in milk (Gagnaire et al., 2002). Previous studies suggest that, within the casein micelles, several interactions take place among the individual casein chains, such as: (1) weak hydrophobic interactions; (2) salt bridges; and (3) calcium binding to caseins that result in the formation of colloidal calcium phosphate (CCP) (Hinrichs and Keim, 2007; Lucey and Horne, 2018; Stankey et al., 2011). In gel-like systems based on casein, such as cheese, literature proposes that self-association of casein micelles is also driven by different interactions, such as hydrophobic interactions and electrostatic ones (Horne, 1998). However, as far as Emmental cheese is concerned, and to the best of our knowledge, there are no studies on the relationship between such possible interactions and the cheese structure.

A study that looked at different types of fermented milk structures, determined that, for fresh rennet casein gels, specifically Gouda curd grains and Mozzarella cheese, calcium bonds were the dominant interaction in stabilization of the protein structure; for ripened cheeses, such as Camembert or Gouda, hydrogen and other electrostatic bonds were the main interactions (Hinrichs and Keim, 2007). Another study on rennet-induced gels concluded that hydrophobic interactions and calcium bonds were the main forces acting, whereas, for acid-induced gels, hydrophobic, hydrogen bonds, and electrostatic interactions were homogeneously distributed (Liu et al., 2014).

The studies conducted by Lefebvre-Cases et al. (1998) identified interactions among casein gels using urea, SDS, and EDTA. It was determined that hydrophobic interactions and calcium bonds were the main ones in rennet milk gels; while hydrophobic, electrostatic, and hydrogen bonds were the major ones in acid milk gels. The roles of these interactions were reiterated in the study by Zamora et al. (2012), where calcium and hydrogen bonds, as well as hydrophobic interactions, were found to be involved in the protein matrix of drained rennet curds.

In a study by Gagnaire et al. (2002), 2 M urea and 100 mM EDTA were used to disrupt the molecular interactions in fresh Emmental pressed curd. It was determined that the disruption of hydrogen bonds, and possibly some hydrophobic interactions, did not completely disintegrate the paracasein matrix, being the calcium-crosslinking interactions of utmost importance. Based on their findings, the authors hypothesized a joint interaction of those different forces in the maintenance of the curd matrix.

Regarding processed cheese, some authors suggest that hydrophobic interactions play a crucial role in the protein structure (Lucey and Horne, 2018), with some studies indicating that they are the main interaction in this type of cheese (Fu and Nakamura, 2017). However, other studies suggest that different types of interactions must act in cooperation with each other. Namely, hydrophobic, hydrogen bonding and other electrostatic interactions, and disulphide bonds, are all appointed as responsible for stabilizing the protein network in processed cheese (Lucey and Horne, 2018; Marchesseau et al., 1997; Schmid et al., 2015).

Studies on whey protein gel matrices also concluded that different chemical interactions acted together in order to provide structure. For instance, in heat-induced gels of whey protein isolate, the hydrophobic interactions and the intermolecular disulphide bonds were found both responsible for the firmer gel structure with increasing protein concentration (Shimada and Cheftel, 1988). Disulphide bonds were also stated to be important in the early stages of aggregation of whey protein mixtures that formed hydrogels; yet, these hydrogels were found to be mostly stabilized by noncovalent interactions (Mercadé-Prieto et al., 2018).

From the above overview, we conclude that different works point to different interactions as the main ones in maintenance of cheese structure (at the curds stage, cured, or processed type). Our experimental work aimed to clarify the contribution that each type of interaction has in ripened Emmental cheese. For that purpose, we used the dissociating agents referred above, evaluating their ability to solubilize protein (Shukla and Trout, 2010), under a careful experimental protocol. The knowledge of the interactions governing cheese structure is relevant technologically, as it can guide conditions during cheese and cheese-containing products manufacture, in particular processed cheeses and cheese sauces.

1.3. Protein quantification methods

Although several other methods can be used to quantify the protein content in milk and other dairy products, the Kjeldahl method is still the standard one. However, it is very time-consuming, expensive to run, and has some safety issues (Liu and Pan, 2017). Furthermore, this method cannot be used on samples containing urea (since it measures the nitrogen content of the sample). Colorimetric methods commonly used for protein quantification are not as accurate, due to the various interferences associated therewith, including SDS or high concentrations of urea (Redmile-Gordon et al., 2013). Another possible option is through UV absorbance, which is a direct method using a simple calibration curve (Olson and Markwell, 2007; Zheng et al., 2017). However, it can be applied only in quite pure protein solutions.

The alkalinisation of milk systems, e.g. by the addition of NaOH, results in changes in the physical properties of the casein micelles, namely destabilization and ultimately their disruption (Lam et al., 2018; Liu and Guo, 2008). Studies show that NaOH can disrupt various chemical bonds in milk systems, including disulphide bonds (Florence, 1980; Reichardt and Eckert, 1991). This leads to protein solubilization: Post et al. (2012) showed that micellar casein, sodium caseinate, or calcium caseinate solutions at pH 10–11 leads to complete solubilization of both α_s-casein and β–casein. Sinaga et al. (2017), and the previous works therein referred, demonstrate that milk turbidity disappears at pH above 8.5, due to casein micelles dissociation. Here, in order to quantify the protein in cheese, a modification of the method reported by Reichardt and Eckert (1991) was used. Cheese samples were dispersed in 0.1 M NaOH, which leads to protein solubilization, and, afterwards, the soluble protein was evaluated by UV absorbance. The protein present in the different dissociating media was quantified also by the same method.

2. Materials and methods

2.1. Materials

The dissociating agents used were urea (molecular biology grade, Sigma-Aldrich, USA), SDS (ultrapure, ITW, USA), EDTA (Fluka, USA), NaOH (Eka, Sweden) and NaCl (Fluka, USA). The cheese was commercial grated Emmental (Milbona, Germany). Casein was from bovine milk (Sigma-Aldrich).

2.2. Experimental methods

2.2.1. Determination of total protein in cheese

The total protein in the Emmental cheese was determined by the Kjeldahl method (AOAC, 1990) and by a modification of the method by Reichardt and Eckert (1991). In this, approximately 1.3 g of cheese was dispersed in 30 mL 0.1 M NaOH, and the mixture was warmed up to 70 °C, under gentle magnetic stirring, for about 10 min, and without a holding period at that temperature. After cooling down, the samples were centrifuged at 5000 rpm (4410×*g*), 4 °C, for 30 min. After centrifugation, the lipid layer was removed, and the middle clear solution was separated from the pellet, which was discarded. Then, the absorbance of the solution at 280 nm was determined, as detailed below. The absorbance values were converted to protein concentration using a calibration curve of casein in 0.1 M NaOH. Three replicates were made.

2.2.2. Determination of solubilized protein by different dissociation media

Aliquots of 35 mL of 0.17 M NaCl solution were drawn into 100 mL glass beakers and then different dissociating agents were dissolved in the saline. Additionally, solutions of NaCl at 50 mM and 0.6 M were also prepared. The dissociating agents used and respective concentrations are listed in Table 1.

1.33 ± 0.5 g of cheese was added to each beaker, and each solution was slowly heated to 70 °C (in about 10 min), with constant magnetic stirring. This step of heating the cheese suspension, although not common in similar works, was included in order to mimetize the conditions found in the preparation of several cheese-containing foods, such as sauces or processed cheeses, among others. However, in order to study the contribution of heating on solubilization of the cheese proteins, the test with 6 M urea was also carried out at room temperature.

The cheese suspensions were allowed to cool down and the dispersions were centrifuged in 50 mL tubes for 30 min, at 5000 rpm (4410×*g*), and at 4 °C. That supernatant was left at room temperature overnight, in order to account for any possible alterations that might still occur.

The protein concentration of this solution was then evaluated by UV absorbance at 280 nm. For this measurement, 920 μL of 0.1 M NaOH was mixed with 80 μL of the sample solution in a 1 mL quartz cuvette. The corresponding blank was made with the same dissociating solution, and with the same dilution in 0.1 M NaOH.

Preliminary tests of UV spectra of the blanks were carried out, in order to check if the dissociating agents would interfere with the method. There were no significant interferences for any of the agents, at the concentrations employed, and at the wavelength of 280 nm.

Table 1
Dissociating agents and their concentrations used in the study.

Dissociating Agent	Concentrations
NaCl	0.05 M and 0.6 M
Urea	1.5 M and 6 M
SDS	2.5%
EDTA	4 mM and 50 mM
Urea + SDS	6 M + 2.5%
Urea + EDTA	6 M + 4 mM
Urea + SDS + EDTA	6 M + 2.5% + 4 mM

The amount of solubilized protein was evaluated based on the calibration curve of casein, and the result compared with the total protein in cheese.

Each experiment was repeated three times.

2.2.3. Statistical analysis

A t-student test was applied to the results of total protein in cheese evaluated by Kjeldahl method and by the dissolution in 0.1 M NaOH, as reported here. An ANOVA one-way was used for the statistical analysis of the effect of the different dissociating agents.

3. Results and discussion

3.1. Determination of total protein concentration in Emmental cheese

Total protein in the Emmental cheese was determined by two methods: the reference Kjeldahl and by dissolution in 0.1 M NaOH followed by UV measurement. This is a modification of the method by Reichardt and Eckert (1991), that used the biuret method for quantifying solubilized protein. Colorimetric methods for protein determination, and in particular the biuret method, are much less sensitive than UV absorbance (Chutipongtanate et al., 2012; Olson and Markwell, 2007). Furthermore, it is common with colorimetric methods to use calibrations made with BSA, while we used the analyte in question, casein, for that purpose. It should be recalled that colorimetric methods can give considerable deviations in results with different proteins (Olson and Markwell, 2007). The calibration curve for the latter method can be found in Figure A1. The mean value of protein content of the cheese by the two methods was 0.297 ± 0.030 and 0.315 ± 0.010 g protein/g of cheese, respectively, indicating no statistically significant difference ($p > 0.05$). This shows that the NaOH/UV method can be conveniently applied, enabling a quite precise determination of protein in cheese. Based on these observations, we believe that this method is likely to be applicable to several other dairy matrices, justifying a separate work to perform a full validation of the method.

3.2. Determination of solubilized protein by dissociating agents

The solubilized protein by each dissociating medium was evaluated by the NaOH/UV method, and the results compared to the total protein in cheese. The values are reported in Table 2.

The statistical analysis of these results used an ANOVA one-way test, with the application of the Tuckey test for pairwise comparisons between particular samples.

Table 2
Dissociating media and values of respective solubilized cheese protein ($n = 3$).

Dispersing medium	Solubilized Protein (g protein/g cheese)	Solubilized Protein (% total protein)
Deionized Water	0.0319 ± 0.006^{a}	9.97 ± 1.90^{a}
NaCl 50 mM	0.0439 ± 0.003^{a}	13.70 ± 0.95^{a}
NaCl 0.6 M	0.0746 ± 0.011^{a}	23.33 ± 3.52^{a}
Urea 1.5 M	0.167 ± 0.018^{b}	52.10 ± 5.47^{b}
Urea 6 M	0.269 ± 0.025^{c}	85.30 ± 6.13^{c}
Urea 6 M (no heating)	0.261 ± 0.016^{c}	82.57 ± 2.66^{c}
EDTA 4 mM	0.0905 ± 0.0079^{a}	28.67 ± 2.02^{a}
EDTA 50 mM	0.0730 ± 0.0063^{a}	22.83 ± 1.96^{a}
SDS 2.5%	0.213 ± 0.007^{b}	67.37 ± 1.40^{b}
Urea 1.5 M + SDS 2.5%	0.282 ± 0.012^{c}	88.20 ± 3.60^{c}
Urea 6 M + SDS 2.5%	0.270 ± 0.016^{c}	85.40 ± 2.40^{c}
Urea 6 M + EDTA 4 mM	0.275 ± 0.008^{c}	87.27 ± 3.56^{c}
Urea 6 M + SDS 2.5% + EDTA 4 mM	0.279 ± 0.022^{c}	92.13 ± 10.61^{c}

Samples with the same superscript letter do not present statistically significant differences among them, according to the Tuckey test.

From the results, we can conclude that ionic interactions might play some role in this cheese matrix, since 0.6 M NaCl can dissolve up to 23% of cheese proteins. In contrast, deionized water and 50 mM NaCl, which have low ionic strength, dissolve up to 13% protein, which can represent "free" protein in cheese, particularly non-drained whey proteins. Despite these slight variations, the results obtained with deionized water and with NaCl (50 mM and 0.6 M) were statistically similar ($p > 0.05$).

Urea at 6 M, with or without heating to 70 °C, solubilized a great part (up to 85%) of the cheese proteins. This result is in line with a previous study that reports that the proteins and large polypeptides in cheese are completely soluble in 4–6 M urea (McSweeney and Fox, 1997). However, 1.5 M urea dissolves considerably less protein. As stated previously, some published analyses on protein gels and films (Gómez-Guillén et al., 1997; Zhang et al., 2018) used that concentration as a means to probe hydrogen bond interactions, and urea 8 M to measure, additionally, hydrophobic interactions. Our results can be interpreted under the same principles, albeit a clear dependence of urea concentration on each type of bond can still be questioned.

Hydrophobic interactions are known to strengthen with temperature (Alessi et al., 2007). However, some authors (Gómez-Guillén et al., 1997) point out that temperatures beyond 58 °C can, instead, weaken hydrophobic interactions, by destabilizing the hydrogen bonds among water molecules, which would facilitate hydrophobic hydration and make it easier for protein denaturation. Furthermore, temperature increases molecular motions, facilitating separation of aggregated proteins, such as in cheese. Notably, in our experiments with Emmental cheese, there was no statistically significant difference ($p > 0.05$) between the amount of solubilized protein by 6 M urea heated to 70 °C (85%) and at room temperature (82%) (Table 2).

EDTA, as a chelating agent, at both 4 mM and 50 mM, only solubilized up to 28% of the cheese proteins, a value similar to the one obtained with 0.6 M NaCl solution. This suggests that, in this matrix, the calcium bonds do not require a specific chelating agent for their disruption (Lefebvre-Cases et al., 1998). Furthermore, there was no statistically significant difference between 4 mM and 50 mM EDTA ($p > 0.05$).

SDS, as opposed to EDTA, solubilized up to 67% of the cheese proteins, which shows that hydrophobic interactions must play an important role in this matrix (Lefebvre-Cases et al., 1998). This is not unexpected given the significant number of nonpolar regions found along the caseins polypeptide chains. Solutions with combinations of urea (6 M or 1.5 M) with SDS, or with EDTA, dissolved practically the same amount of protein as 6 M urea alone (up to 88%). In fact, there were no statistically significant differences observed between the results of any pair of the solutions 6 M urea, 6 M urea + SDS, 1.5 M urea + SDS, 6 M urea + EDTA, and 6 M urea + SDS + EDTA ($p > 0.05$). The fact that 6 M urea was similar to 1.5 M urea with SDS, supports the view that, at high concentrations, urea disrupts hydrophobic interactions.

The effect of temperature of the dispersion media was also carried out with 1% and 2.5% SDS, and with 4 mM EDTA solutions. Unlike urea, EDTA solutions only solubilized a significant amount of the cheese proteins when heated to 70 °C (up to 28%); at room temperature, no significant amounts (ca. 6%) were solubilized. With 2.5% SDS solution at room temperature, some cheese proteins were solubilized, however, after the centrifugation, a phase separation occurred, and the turbidity of the solution turned quantification inviable. At 1% SDS concentration, no phase separation was noticeable, but the solution remained quite opaque for UV measurement. These problems were not observed when heating to 70 °C was included. Therefore, apart from the case of urea, heating was needed in order to attain significant levels of protein solubilization. An interpretation of such observation is that the thermal agitation of the molecular chains in the protein aggregates in cheese complements the action of the dissociating agents.

We also monitored the pH of the separated supernatants of the dispersion media. All values ranged between 5.5 for 0.6 M NaCl and 6.64 for 6 M urea +2.5% SDS solution; when dispersing cheese in 0.1 M NaOH, the corresponding pH was 8.6. Except in this last case, the pH should not have a significant impact on the extent of protein solubilization, as backed by previous works (Lam et al., 2018; Marchesseau et al., 1997).

We note that the literature uses the mentioned dissociating solutions as "sensors" of specific interactions, but clearly several of these solutions might disrupt more than one type of interaction, with no absolute specificity. Therefore, the amounts of solubilized protein should be interpreted as indicators of the influence of the different types of interactions in a structure.

4. Conclusions

Even though a consensus has not yet been reached regarding the main molecular interactions acting in the casein matrix of cheese, this work was able to provide some insights, for the case of ripened Emmental cheese.

We suggest that the caseins in this matrix are maintained by a set of hydrophobic interactions, hydrogen bonds, and other electrostatic interactions, including ionic bonds. The results obtained with urea suggest that hydrogen bonds have an important role, comparable to hydrophobic interactions, a conclusion not commonly reported for cheese structures. Rather surprisingly, and different from what is often stated, calcium bonding seems not to have a relevant role in this case.

Solutions without urea required heating for solubilization of proteins to some extent, a fact interpreted as thermal agitation helping separation of the protein chains in aggregates.

It will be interesting to extend the methodology here reported to different cheeses, including hard and soft ones, and cheeses with different maturation times. This will enable to see the influences of these factors on cheese interactions. We can speculate that a softer cheese, due to higher water content, will lead to higher protein solubilization, as there are fewer molecular interactions among casein strands. Also, a longer ripening period is expected to lead to higher protein solubilization, as cheese strands are progressively hydrolysed by the action of proteases originally in milk and, mainly, from the starters (Coker et al., 2005). A study with different cheeses under the same experimental conditions is justified.

Finally, in terms of protein quantification, we point out that 0.1 M NaOH solubilizes the totality of cheese proteins and this fact can be explored for their quantification, as previously reported (Reichardt and Eckert, 1991). However, we introduced analytical improvements in the method that enabled results quite close to the standard Kjeldahl one.

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Declaration of Competing Interest

None.

CRediT authorship contribution statement

Tatiana Paula Vilela: Investigation, Methodology, Formal analysis, Writing - original draft, Visualization. **Ana Maria Gomes:** Conceptualization, Writing - review & editing, Supervision. **João Paulo Ferreira:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Project administration.

Appendices.

Appendix A: calibration curve of casein dissolved in 0.1 M NaOH

The calibration curve of casein in 0.1 M NaOH has shown to be linear up to 1.4 mg/mL concentration, with a value of R^2 of 0.9903, as shown in Figure A1.

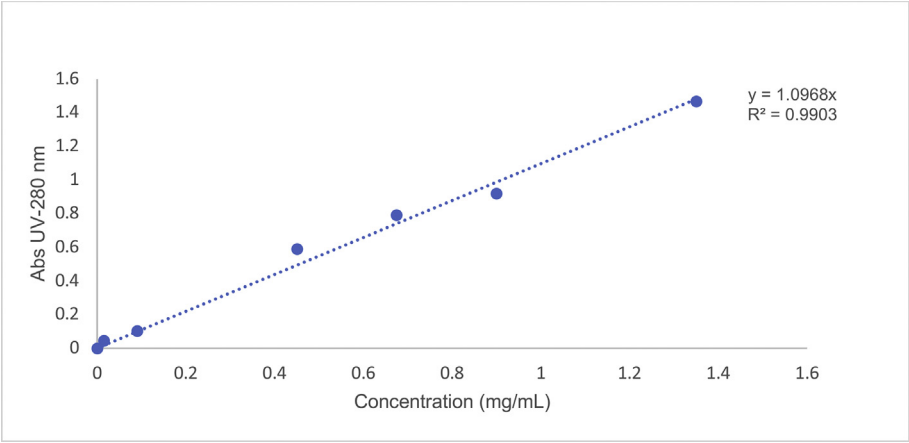

Figure A.1. Calibration curve of casein dissolved in 0.1 M NaOH.

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