

1 **Quantification of the electrostatic and covalent interaction between whey proteins and**  
2 **low methoxyl pectin using pfg-NMR diffusometry**

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12 **1. Introduction**

13 Nowadays, complexes of proteins and polysaccharides receive a lot of attention due to their  
14 high functionality. This type of complexes can be applied in a wide range of products, such as  
15 in beverages [1, 2], in gels [3], or in microencapsulation [4, 5]. These complexes can be formed  
16 either through electrostatic interaction or covalent bonds. Electrostatic interaction involves  
17 modification of the charge properties of the biopolymers to facilitate the formation of the  
18 complexes and is influenced by different factors such as pH, ionic strength, as well as charge  
19 density of the protein and polysaccharides [6]. There are several studies on electrostatic  
20 interaction between proteins and polysaccharides and its application in emulsions [7-9]. On the  
21 other hand, covalently bound protein-polysaccharide conjugates are normally obtained via  
22 Maillard type reactions [2, 10, 11]. This type of conjugates is obtained by exposing a mixture of  
23 proteins and polysaccharides to elevated temperatures at certain relative humidity for a certain  
24 period of time [12]. Previous studies have shown that protein-polysaccharide conjugates have a  
25 better functionality than protein/polysaccharides alone [2, 13, 14]. It has been reported that  
26 conjugation of WPI and LMP increases the heat stability of WPI as well as its emulsifying  
27 activity [13, 15, 16].

28 pfg-NMR has been used frequently to measure the diffusion coefficient of different materials  
29 and chemicals [17]. Nowadays, NMR gains more popularity in food applications due to its non-  
30 destructive nature [18]. NMR is very useful to study the physical and chemical properties of

31 foods <sup>[19]</sup>. Despite the fact that NMR is less sensitive towards components present at a very low  
32 concentration, such as colorants and flavors, compared to other spectroscopic methods, it is  
33 still a very suitable method for food systems <sup>[19]</sup>. In food applications, NMR has been utilized  
34 in a wide range of studies, for example a study on the adsorption of caseinate in triacylglycerol-  
35 in-water emulsions <sup>[20]</sup>, droplet size measurement in emulsions <sup>[21, 22]</sup>, and electrostatic  
36 interaction between lysolecithin and chitosan in emulsions <sup>[23]</sup>. In the field of milk proteins,  
37 pfg-NMR has been used to study the diffusion of WPI and the gel structure of WPI <sup>[24, 25]</sup>, the  
38 formation of protein and gum arabic coacervates <sup>[26]</sup>, the interaction of casein and maltodextrin  
39 <sup>[27]</sup>, the conjugation between corn fiber gum and milk proteins <sup>[28]</sup>, and the ligand binding to  $\beta$ -  
40 lactoglobulin <sup>[29]</sup>.

41 Nevertheless, to our knowledge, there is limited research that utilizes NMR as a means to  
42 quantify protein-polysaccharide conjugates. By determining the diffusion coefficient of  
43 protein-polysaccharide mixtures, native protein, and native polysaccharides using NMR, it is  
44 possible to confirm the formation of protein-polysaccharide conjugates as well as to quantify  
45 the degree of interaction between proteins and polysaccharides. This is based on the fact that  
46 compounds with a different molecular weight have different diffusion behavior properties and  
47 these properties are altered upon interaction with other compounds. As interaction between  
48 proteins and polysaccharides results in new compounds with a higher molecular weight <sup>[30]</sup>, a  
49 change in the average diffusion coefficient as well as in the distribution width is expected.

50 In previous studies, chemical analyses using reagents such as Trinitrobenzenesulfonic acid  
51 (TNBS) <sup>[11, 13]</sup> and o-phthaldialdehyde (OPA) <sup>[10]</sup> were performed to determine the degree of  
52 covalent interaction between proteins and polysaccharides by determining the residual amount  
53 of available amino groups in the conjugates. Whereas these methods are sensitive, they are also  
54 highly labor intensive and time consuming. NMR diffusometry, on the other hand, hardly  
55 requires any sample pretreatment and hence is less labor intensive.

56 In this study, pfg-NMR diffusometry was used to investigate the interaction of WPI and LMP  
57 as influenced by pH, duration of the dry heat treatment (incubation time), and the concentration  
58 of LMP present in the conjugates. The diffusion coefficient and the distribution width of the  
59 WPI and LMP as influenced by these factors were reported. Moreover, the amount of free WPI  
60 and LMP-bound WPI in the conjugates could be determined. The outcome of this study was  
61 evaluated by comparing the results to those obtained through SDS-PAGE and TNBS analysis  
62 in a previous study [13].

## 63 2. Materials and Methods

### 64 2.1 Materials

65 The WPI was purchased from Davisco Foods International Inc. (Le Sueur, MN, USA). Protein  
66 analysis revealed that the WPI contained approximately 92.6% protein, whereby 85% of the  
67 protein is  $\beta$ -Lactoglobulin [31]. Low methoxyl pectin (LMP) (UnipectinOB700) was obtained  
68 from Cargill (Ghent, Belgium) and contained 89.6% of dry matter. Deuterium oxide ( $D_2O >$   
69 99.8%) was purchased from Armar Chemicals (Switzerland).

### 70 2.2 Methods

#### 71 2.2.1 Conjugate preparation

72 Conjugates were prepared from a 5% (w/v) protein solution and 1% (w/v) LM Pectin solution.  
73 A correction for the protein content and the dry matter was taken into account during the  
74 calculation of the WPI and LMP needed. The WPI and LMP were dissolved in distilled water  
75 and the pH of the solutions was adjusted to 7.0 with 1 N HCl to avoid formation of ionic  
76 complexes that might form at lower pH during mixing. Both solutions were kept overnight in a  
77 refrigerator before mixing. The solutions were then mixed at four different ratios i.e. 1:0, 4:1,  
78 2:1, and 1:1 (on weight basis) and frozen prior to freeze-drying.

79 The frozen samples were lyophilized (Alpha 1-2 LD plus, Christ) to remove all the water and  
80 obtain dry products. The freeze dried products were then incubated at a temperature of 60°C for  
81 16 days in a desiccator containing saturated NaCl solution to keep the relative humidity at 74%  
82 [32]. During incubation, sampling was done at day 1, 2, 8, and 16.

#### 83 2.2.2 High Resolution NMR

84 High-resolution pulsed field gradient (pfg) NMR diffusion analysis was performed with a  
85 Bruker Avance III spectrometer operating at a  $^1H$  frequency of 500.13 MHz and equipped with  
86 a 5 mm DIFF30 gradient probe with a maximum gradient strength of 18 T/m. Pfg-NMR  
87 experiments were performed at 25 °C using a (single) stimulated echo pulse sequence with  
88 bipolar gradient pairs. The samples (565  $\mu$ L) were filled in 5 mm diameter glass NMR tubes  
89 (Armar Chemicals, Switzerland) and were measured upon varying the gradient strength up to  
90 12 T/m, while keeping the gradient duration ( $\delta$ ) and the diffusion delay ( $\Delta$ ) fixed at respectively  
91 1.2 ms and 100 ms. The samples were prepared by dissolving the powder in 10 mM Na-

92 phosphate buffer (pH 6.5 and 7.2) and 5 mM Na-acetate buffer (pH 5.5 and 5.0) in D<sub>2</sub>O to  
 93 obtain a protein and pectin concentration of 20 mg/mL and 10 mg/mL, respectively.

94 The obtained experimental echo intensity attenuation ( $E$ ) as a function of gradient strength  $G$ ,  
 95 which was typically sampled up to 95% decay, was then fitted to Eq. 1a using Matlab 7.5.0.342  
 96 (R2007b) software (Mathworks, Inc.). Hereby, a mass-weighted lognormal distribution  $P_v$  of  
 97 diffusion coefficients was assumed, whereas the echo decay was described by Eq. 1b assuming  
 98 free diffusion<sup>[33]</sup>. The corrected diffusion delay  $\Delta'$  in Eq. 1b corresponds to  $(\Delta - 5\delta/16 - \tau/2)$ ,  
 99 whereby  $\tau$  stands for the delay time between the gradient pulses, which is equal to the sum of  
 100 the gradient recovery delay and the 180° pulse duration. The factor  $s_G$  is a factor correcting for  
 101 the non-rectangular shape of the applied pulsed field gradients (here equal to  $2/\pi$ ,  
 102 corresponding to Sine bell shapes).

$$E_{expt} = \int_0^{\infty} P_v(D_i) \cdot E(D_i) \cdot dD_i \quad (1a)$$

$$E(D_i) = \exp(-D_i \cdot \gamma^2 \cdot (s_G \cdot G)^2 \cdot \delta^2 \cdot \Delta') \quad (1b)$$

$$P_v(D_i) = \frac{1}{\sqrt{2\pi} \cdot D_i \cdot \ln \sigma_g} \cdot \exp\left(-\frac{(\ln(D_i) - \ln(D_g))^2}{2 \cdot (\ln \sigma_g)^2}\right) \quad (1c)$$

$$D_a = D_g \cdot \exp\left(\frac{(\ln \sigma_g)^2}{2}\right) \quad (1d)$$

$$\sigma_a = \sqrt{D_a^2 \cdot (\exp((\ln \sigma_g)^2) - 1)} \quad (1e)$$

103

104 A polydisperse population of diffusion coefficients with probability distribution  $P_v$  was  
 105 assumed because protein and pectin samples display a range of molar masses. For  $P_v$ , we  
 106 assumed a lognormal mass-weighted diffusion coefficient distribution (Eq. 1c). The geometric  
 107 mean diffusion coefficient ( $D_g$ ) and geometric standard deviation ( $\sigma_g$ ) of this distribution were  
 108 converted to the arithmetic mean diffusion coefficient ( $D_a$ ) and arithmetic standard deviation  
 109 ( $\sigma_a$ ) of the lognormal mass-weighted diffusion coefficient distribution using Eq. 1d and Eq. 1e.  
 110 The degree of molecular interaction can be evaluated upon decomposing the WPI diffusion  
 111 signal obtained from the WPI-LMP conjugate sample  $E_{WPI,conjug.}$  into a free WPI fraction  
 112 (whose decay corresponds to the experimentally obtained non-conjugated WPI  
 113 signal  $E_{WPI,free}$ ) and a bound WPI fraction. The bound fraction can be determined upon

114 estimating the diffusion signal of the reacted WPI  $E_{WPI,bound}$  using Eq. 2 and Matlab 7.5.0.342  
115 (R2007b software, The Mathworks). As the molar mass of pectin is much larger as compared  
116 to WPI, the molar mass of the conjugate is mostly determined by pectin. Hence, the bound  
117 fraction can be determined assuming the experimentally obtained LMP diffusion signal to be a  
118 good approximation of the LMP-bound WPI diffusion signal as written in Eq. 3 using the  
119 Solver add-in (Microsoft Excel 2010). For WPI-LMP conjugates, the diffusion signal of dry  
120 heated WPI, which had the same incubation time as the WPI-LMP conjugates, was used as the  
121 diffusion signal of the free WPI. The WPI and LMP signal recorded at 0.6-1 ppm and 3.5-4.0  
122 ppm were used in this calculation, respectively.

$$E_{WPI,conj.} = \varphi_{free} \cdot E_{WPI,free} + \varphi_{bound} \cdot E_{WPI,bound} \quad (2)$$

$$E_{WPI,conj.} = (1 - \varphi_{bound}) \cdot E_{WPI,free} + \varphi_{bound} \cdot E_{LMP,conj.} \quad (3)$$

123

### 124 **2.2.3 Emulsion preparation**

125 10% (w/w) oil in water emulsions stabilized by either 0.5% (w/w) WPI or 0.5% (w/w) WPI-  
126 LMP mixtures (with or without dry heat treatment) were prepared. Emulsions were initially  
127 prepared by dissolving 0.5% of WPI or mixture of WPI-LMP. The aqueous phase was kept  
128 overnight in the fridge prior to the emulsion preparation to fully hydrate the hydrocolloid.  
129 Emulsions containing 10% (w/w) of oil were prepared by adding 10 g of sunflower oil to 90 g  
130 of WPI solution. The mixture was then premixed using an IKA Ultra-turrax TV45 (Janke &  
131 Kunkel, Staufen, Germany) at the highest speed (24000 rpm) for 1 minute. This was followed  
132 by homogenization using a Microfluidizer M110S for 2 minutes at 4 bar of compressed air  
133 pressure, corresponding to 560 bar of liquid pressure. The coil of the Microfluidizer was  
134 immersed in a water bath set at 55°C.

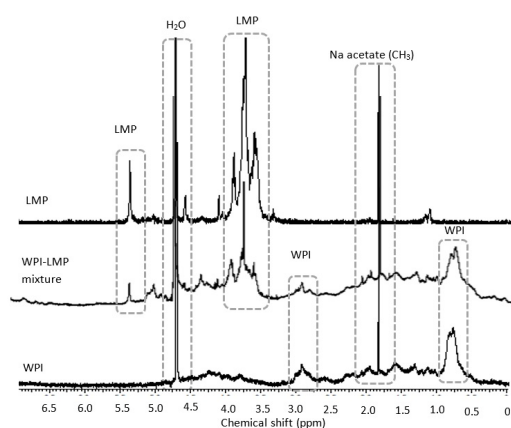
### 135 **2.2.4 Electrophoretic mobility measurement**

136 The electrophoretic mobility of the emulsions stabilized by WPI and WPI-LMP mixtures was  
137 measured in 20 mM of Na-acetate buffer at different pH values using a Zetasizer 2c (Malvern  
138 Ltd, UK). The emulsions were diluted 1000x in the buffers and let to stand overnight prior to  
139 the measurement.

### 140 3. Results and Discussions

#### 141 3.1 Diffusion coefficient of native WPI and LMP

142 In this study, a high gradient probe was needed due to the high molecular weight of WPI and  
143 LMP. From the NMR measurements, 1D spectra of native WPI and LMP were obtained (Fig.  
144 1). By comparing the spectra of WPI and LMP, it was found that the signal observed at 0.6-1  
145 ppm and 2.7-3.1 belonged to WPI, while the signal at 3.3-4.2 ppm and 5.2-5.5 ppm belonged  
146 to LMP. Thus, in a system containing both WPI and LMP, it was possible to distinguish the  
147 contribution of each biopolymer. The arithmetic mean diffusion coefficient ( $D_a$ ) and arithmetic  
148 standard deviation ( $\sigma_a$ ) of WPI and LMP are reported in Table 1.



149

150 Figure 1. 1D  $^1\text{H}$  spectrum of native WPI (20 mg/ml) and LMP (10 mg/ml) and WPI-LMP  
151 mixture (at 2:1 WPI:LMP ratio) in sodium phosphate buffer (pH 7.2) containing 5 mM  
152 sodium acetate, recorded at 25°C.

153

154 The results presented in Table 1 and Figure 2 indicate that WPI ( $D_a = 7.13 \cdot 10^{-11} \text{ m}^2/\text{s}$ ) diffuses  
155 much faster than LMP ( $D_a = 5.49 \cdot 10^{-11} \text{ m}^2/\text{s}$ ). Furthermore, it was obvious that the arithmetic  
156 standard deviation, which represents the distribution width, of LMP was almost ten times  
157 bigger than the arithmetic standard deviation of WPI. The WPI sample used mainly consisted  
158 of  $\beta$ -lactoglobulin which has a molecular weight of 18 kDa (monomer) with a narrow range of  
159 molecular weights. On the other hand, LMP has a bigger molecular weight of several hundred  
160 kDa <sup>[34]</sup> and a broader range of molecular weights. While the former explains the slow diffusion  
161 of LMP compared to WPI, the latter explains the broad diffusion coefficient distribution  
162 exhibited by LMP (Fig. 4).

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Table 1 Arithmetic mean diffusion coefficient ( $D_a$ ) and arithmetic standard deviation ( $\sigma_a$ ) value of WPI, LMP, and WPI-LMP conjugates, dry heat treated for 0 to 16 days obtained upon fitting Eq. 1 to the diffusion signal of the WPI and LMP contributions in the non-conjugated and conjugated samples.

WPI:LMP ratio (w/w)	Dry heat treatment time (days)	pH	WPI (0.6-1.0 ppm)		LMP (3.5-4.0 ppm)		Free WPI (%)
			$D_a$ ( $\times 10^{-12} \text{ m}^2/\text{s}$ )	$\sigma_a$ ( $\times 10^{-12} \text{ m}^2/\text{s}$ )	$D_a$ ( $\times 10^{-12} \text{ m}^2/\text{s}$ )	$\sigma_a$ ( $\times 10^{-12} \text{ m}^2/\text{s}$ )	
1:0	0	7.2	71.3	14.2			
0:1	0	7.2			54.9	161.0	
2:1	0	7.2	70.5	35.2	54.5	59.2	87
	1	7.2	65.1	31.4	58.0	75.3	73
	2	7.2	64.2	35.4	49.4	57.3	66
	8	7.2	62.7	41.4	47.0	57.2	64
	16	7.2	59.7	35.4	47.5	52.8	63
2:1	0	5.5	59.5	33.7	51.9	81.2	75
	0	5.0	56.7	37.0	65.5	137.0	52
4:1	8	7.2	71.5	36.3	64.1	59.6	61
1:1	16	6.5-6.8	88.0	141.0	54.0	150.0	56

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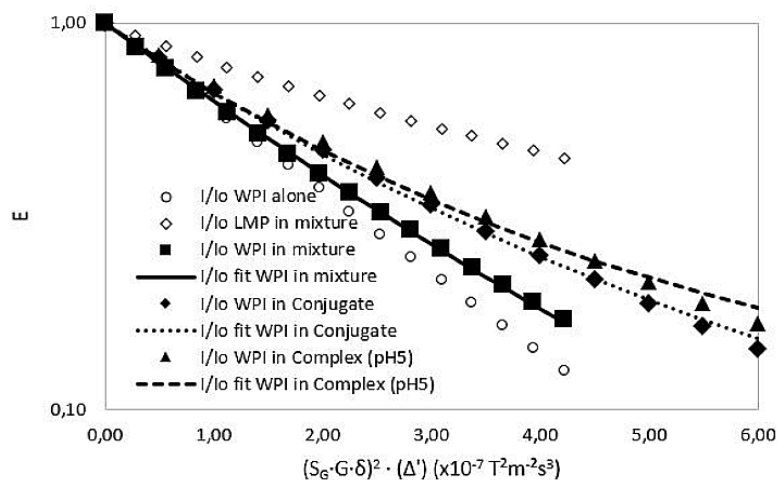
Table 2. Arithmetic mean diffusion coefficient ( $D_a$ ) and arithmetic standard deviation ( $\sigma_a$ ) value of WPI, which has been dry heat-treated for 0-16 days obtained upon fitting Eq. 1 to the diffusion signal of the WPI contributions in the NMR spectrum (at 0.6-1 ppm and 2.7-3.1 ppm, resp.) of the samples.

Dry heat treatment time (days)	0.6-10 ppm		2.7-3.1 ppm	
	$D_a$ ( $\times 10^{-12} \text{ m}^2/\text{s}$ )	$\sigma_a$ ( $\times 10^{-12} \text{ m}^2/\text{s}$ )	$D_a$ ( $\times 10^{-12} \text{ m}^2/\text{s}$ )	$\sigma_a$ ( $\times 10^{-12} \text{ m}^2/\text{s}$ )
0	71.3	14.2	68.5	5.0
1	71.1	13.6	74.8	20.7
2	71.9	12.8	73.9	21.0
8	73.9	25.6	80.5	35.3

16	71.6	25.2	69.5	20.1
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176 In order to evaluate the influence of dry heat treatment on WPI, freeze dried WPI was dry heat  
 177 treated up to 16 days. Previously, SDS-PAGE analysis revealed that a limited protein  
 178 polymerization occurred in dry heat treated WPI [13]. Polymerization of protein leads to the  
 179 formation of molecules with higher molecular weight [35] that diffuse slower. Table 2 revealed  
 180 that upon dry heat treatment of WPI, the arithmetic mean diffusion coefficient of WPI did not  
 181 decrease significantly. On the other hand, it can be seen in Table 2 that the distribution width  
 182 of WPI became two times broader after 8 days of dry heat treatment, which might be due to  
 183 polymerization of some of the protein molecules in WPI.

184 Hereinafter, the discussion of the results obtained on dry heat-treated mixtures of WPI and  
 185 LMP is divided into 3 parts, which describe the influence of pH, incubation time, and WPI to  
 186 LMP ratio.

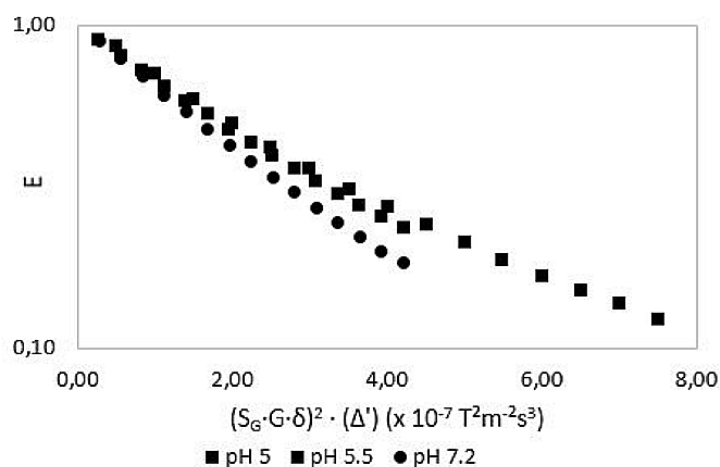


187  
 188 Figure 2. Decomposition of the WPI diffusion signal in the presence of LMP into the  
 189 calculated protein diffusion signal without pectin (lyophilized/dry heat treated WPI only  
 190 (1:0); o ) and the calculated pectin signal (  $\diamond$  ) in WPI-LMP conjugates of ratio 2:1 after 0  
 191 (mixture) and 16 days (conjugates) of dry heat treatment (measured at pH 7.2), as well as in a  
 192 WPI-LMP mixture of ratio 2:1 at pH 5.0 (complexes).

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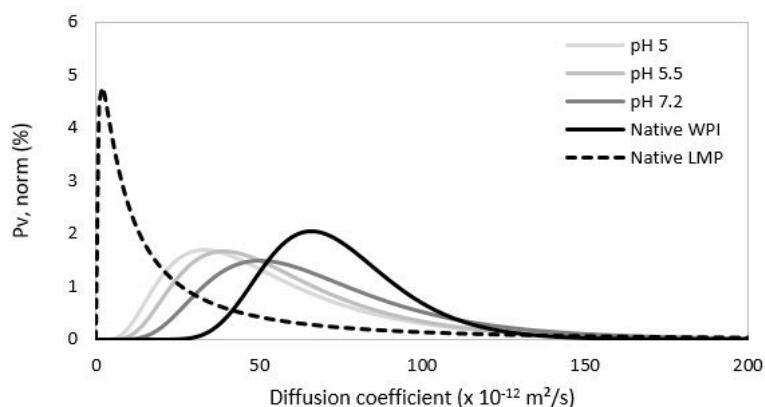


194 **3.2 Influence of pH**



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196 Figure 3. Diffusion echo decay of the WPI signal (0.6-1.0 ppm) in WPI-LMP mixtures  
 197 (without dry heat treatment, ratio 2:1, Day 0) as a function of pH.



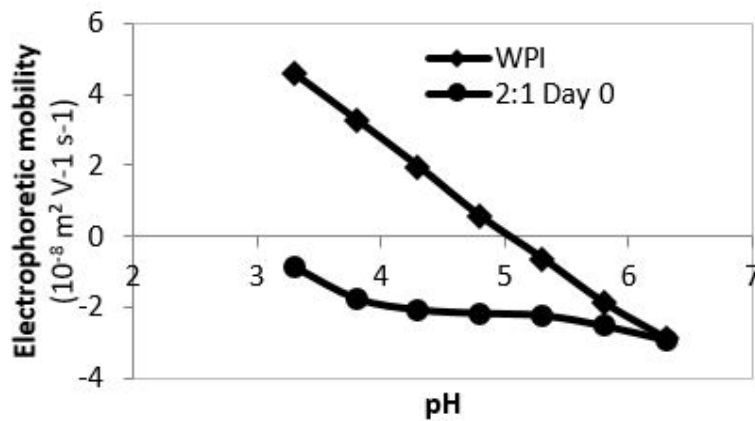
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199 Figure 4. The normalized lognormal mass-weighted probability density function of the  
 200 diffusion coefficient of the native WPI (0.6-1.0 ppm) and LMP (3.5-4.0 ppm) as well as of  
 201 the WPI and LMP in a 2:1 (w/w) mixture of WPI-LMP prepared at pH 7.2, 5.5, and 5.0.

202 In this section, the influence of pH on the diffusion coefficient of WPI and LMP in a mixture  
 203 containing both biopolymers is discussed. For this purpose, mixtures of WPI and LMP with  
 204 ratio of 2:1 were prepared at a pH close to the IEP of WPI (5.0 and 5.5) and at relatively neutral  
 205 pH (7.2). It is indeed well known that the electrostatic interaction between WPI and LMP is  
 206 governed by pH. A typical 1D <sup>1</sup>H-NMR spectrum of a system containing both WPI and LMP  
 207 can be seen in Fig. 1. Fig.3 shows that when the pH was decreased to around the IEP of WPI,  
 208 the diffusional attenuation of b WPI in the mixture decreased less rapidly which indicated

209 slower diffusion due to molecular interaction between WPI and LMP. At pH 5.0, this effect  
210 was more pronounced than at pH 5.5, which indicates that there was more WPI bound to LMP  
211 at pH 5.0 than at pH 5.5. This was supported by the value of the arithmetic mean diffusion  
212 coefficient of WPI in the mixtures (2:1 Day 0) prepared at pH 7.2, 5.5 and 5.0, which was  
213  $7.05 \cdot 10^{-11} \text{ m}^2/\text{s}$ ,  $5.95 \cdot 10^{-11} \text{ m}^2/\text{s}$ , and  $5.67 \cdot 10^{-11} \text{ m}^2/\text{s}$ , respectively (Table 1). These values were  
214 smaller compared to that of native WPI ( $7.13 \cdot 10^{-11} \text{ m}^2/\text{s}$ ). Hence, for the WPI in the presence  
215 of LMP, the arithmetic mean diffusion coefficient decreased as the pH was lowered towards  
216 the protein's IEP. The distribution width of WPI increased from  $1.42 \cdot 10^{-11} \text{ m}^2/\text{s}$  (native WPI)  
217 to about  $3.5 \cdot 10^{-11} \text{ m}^2/\text{s}$  in the presence of LMP. On the other hand, the diffusion coefficient of  
218 the LMP (3.5-4.0 ppm) at pH 7.2, 5.5, and 5.0 was  $5.45 \cdot 10^{-11} \text{ m}^2/\text{s}$ ,  $5.19 \cdot 10^{-11} \text{ m}^2/\text{s}$ , and  $6.55 \cdot 10^{-11}$   
219  $\text{m}^2/\text{s}$ , respectively. Strong electrostatic interaction between biopolymers reduces their  
220 diffusion coefficient [26]. Hence, the diffusion coefficient of LMP at pH 5.0 was expected to be  
221 the smallest. However, it was found that at pH 5.0 the arithmetic mean diffusion coefficient of  
222 LMP was higher than that at pH 7.2 and 5.5. However, it has to be noted that the LMP peak at  
223 pH 5.0 exhibited a broader diffusion coefficient distribution ( $\sigma_a = 1.37 \cdot 10^{-10} \text{ m}^2/\text{s}$ ) than that at  
224 pH 7.2 ( $\sigma_a = 5.92 \cdot 10^{-11} \text{ m}^2/\text{s}$ ) and 5.5 ( $\sigma_a = 8.12 \cdot 10^{-11} \text{ m}^2/\text{s}$ ) (Table 1).

225 From the decomposition of the WPI signal in the mixture into the calculated WPI diffusion  
226 signal without LMP (lyophilized/dry heat treated WPI only) and the calculated pectin signal  
227 with LMP (WPI-LMP conjugates/ WPI-LMP complex) (Figure 2), the fraction of unreacted  
228 WPI or free WPI in the mixture was calculated. At pH 7.2, there was approximately 13% of  
229 WPI bound to LMP and 87% of free WPI. At pH 5.5 and 5.0, the amount of LMP-bound WPI  
230 increased, as expected, to 39% at pH 5.5 and 44% as the pH was further decreased to 5.0. This  
231 result explains the trend observed in the diffusion coefficient of WPI previously mentioned. In  
232 addition, it can be noticed in Figure 2 that the diffusion signal of WPI shifted further towards  
233 the diffusion signal of LMP as more WPI was bound to LMP.

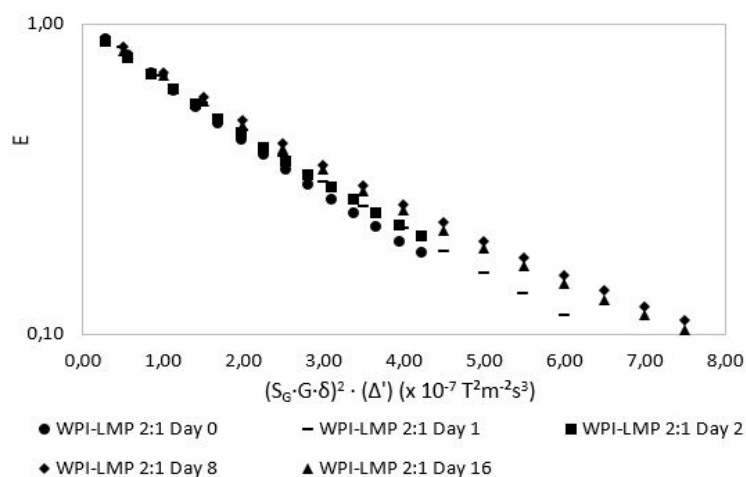


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235 Figure 5. Electrophoretic mobility profile of emulsions stabilized by native WPI (  $\blacklozenge$  ) and by  
 236 a 2:1 (w/w) WPI-LMP mixture (without dry heat treatment) (  $\bullet$  ).

237 These results were further supported by electrophoretic mobility measurement of emulsions  
 238 stabilized by either WPI only or a WPI-LMP mixture at different pH values. Figure 5 indicates  
 239 that at pH 5.0-5.5, the EM of emulsions stabilized by a WPI-LMP mixture was more negative  
 240 than that stabilized by WPI only. This was due to the presence of the negatively charged LMP  
 241 at the surface of the oil droplets via electrostatic interaction with the positive patches on WPI.  
 242 At a higher pH, electrostatic attraction between WPI and LMP is not favorable due to charge  
 243 repulsion since both biopolymers carry a net negative charge <sup>[7]</sup>. . Hence, it might be expected  
 244 that there would be no WPI bound to LMP at pH 7.2. However, the NMR results clearly  
 245 indicated some interaction between WPI and LMP, even at pH 7.2. This is thought to be due  
 246 to a weak electrostatic attraction between the anionic pectin and positively charged regions of  
 247 the WPI <sup>[36, 37]</sup>. The same authors also mention that hydrogen bonds are also involved in the  
 248 interaction between WPI and pectin at a pH slightly above the IEP of the protein, albeit to a  
 249 lesser extent.

250 **3.3 Influence of incubation time**



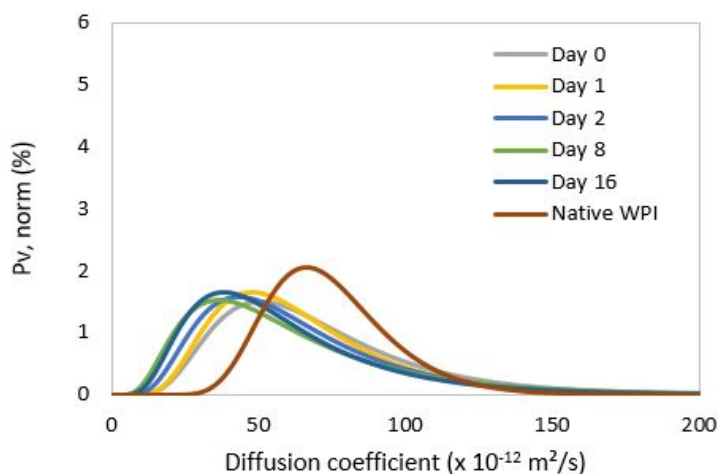
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252 Figure 6. Diffusion echo decay of the WPI signal (0.6-1.0 ppm) in WPI-LMP conjugates  
 253 prepared at pH 7.2 as a function of dry heat incubation time.

254 The interaction between WPI and LMP as influenced by incubation time was determined at pH  
 255 7.2 to minimize the electrostatic interaction which might cause an overestimation of the results.  
 256 Figure 6 shows the diffusion echo decay of the LMP and WPI signal in the conjugates as a  
 257 function of dry heat incubation time. As it can be observed, the longer the dry heat incubation  
 258 time, the more the graphs shifted upward. However, it can be seen that the diffusion echo decay  
 259 of the WPI in WPI-LMP conjugates obtained after 8 or 16 days of dry heat treatment almost  
 260 coincided. The observed upward shift was due to the increase of the molar weight of WPI/LMP  
 261 caused by the conjugation. Upon dry heat treatment, amino groups of WPI become covalently  
 262 linked to the carbonyl groups of LMP through a Maillard type reaction [38]. A longer incubation  
 263 time is expected to result in a higher degree of interaction between WPI and LMP, leading to  
 264 the reduction of the free WPI fraction in the system.

265 Table 1 reveals that dry heat treatment for one day decreased the arithmetic mean diffusion  
 266 coefficient of WPI from  $7.05 \cdot 10^{-11} \text{ m}^2/\text{s}$  (Day 0) to  $6.51 \cdot 10^{-11} \text{ m}^2/\text{s}$ . Further incubation of the  
 267 conjugates resulted in a further reduction of the WPI diffusion coefficient to  $6.42 \cdot 10^{-11} \text{ m}^2/\text{s}$   
 268 after 2 days of dry heat treatment,  $6.27 \cdot 10^{-11} \text{ m}^2/\text{s}$  after 8 days of dry heat treatment, and  $5.97 \cdot 10^{-11}$   
 269  $\text{m}^2/\text{s}$  after 16 days of dry heat treatment. A change in the distribution width was also observed  
 270 (Table 1). Compared to native WPI, the WPI signal in conjugates had a broader distribution  
 271 width (Fig. 7). As for LMP, the trend was similar: the diffusion coefficient of LMP decreased  
 272 as the incubation time was prolonged. The LMP signal had a diffusion coefficient of  $5.45 \cdot 10^{-11}$

273  $11 \text{ m}^2/\text{s}$ ,  $5.80 \cdot 10^{-11} \text{ m}^2/\text{s}$ ,  $4.94 \cdot 10^{-11} \text{ m}^2/\text{s}$ ,  $4.70 \cdot 10^{-11} \text{ m}^2/\text{s}$ , and  $4.75 \cdot 10^{-11} \text{ m}^2/\text{s}$  after dry heat  
274 treatment for 0, 1, 2, 8 and 16 days, respectively.



275

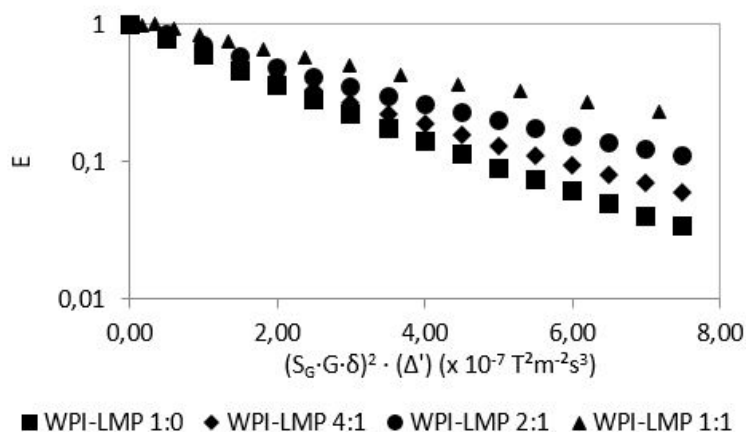
276 Figure 7. The normalized lognormal mass-weighted probability density function of the  
277 diffusion coefficient of WPI (0.6-1.0 ppm) in WPI-LMP conjugates which were incubated for  
278 0, 1, 2, 8, and 16 days.

279 Signal decomposition of the WPI signal in the conjugates obtained after 16 days of dry heat  
280 treatment into the WPI signal without and with LMP can be seen in Fig. 3. From the calculation,  
281 it was found that in the unheated WPI-LMP mixture (Day 0), there was already 13% of WPI  
282 bound to LMP which was most probably formed due to weak electrostatic interaction between  
283 WPI and LMP. It was observed that upon dry heat treatment, the percentage of free WPI  
284 decreased from 87% (Day 0) to 73%, 66%, 64%, and 63% after 1, 2, 8, and 16 days of dry heat  
285 treatment. Table 1 reveals that the WPI-LMP mixtures were characterized by rapid formation  
286 of conjugates in the first 2 days of dry heat treatment. Extension of the dry heat treatment for  
287 more than 2 days did not considerably increase the rate of conjugation.

### 288 3.4 Influence of WPI to LMP ratio

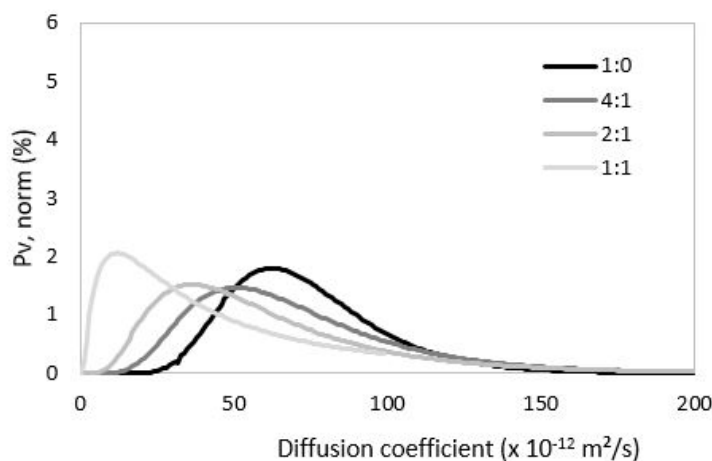
289 The influence of the WPI to LMP ratio was also investigated. In this study, the concentration  
290 of the WPI was kept constant at all ratios, while the concentration of the LMP was varied. WPI-  
291 LMP conjugates with four different WPI to LMP ratios, namely 1:0, 4:1, 2:1, and 1:1 were  
292 prepared. The conjugates with ratio of 1:0, 4:1, and 2:1 were incubated for 8 days, while  
293 conjugates with ratio 1:1 were incubated for 16 days. This comparison was made since an  
294 extension of the dry heat treatment time from 8 days to 16 days did not considerably increase

295 the amount of conjugates. The conjugates were measured at pH 7.2 in a phosphate buffer  
 296 containing 5 mM of sodium acetate, except for the conjugates with ratio 1:1. The latter was  
 297 measured in D<sub>2</sub>O containing 5 mM sodium acetate with a final pH of approximately 6.5-6.8.



298  
 299 Figure 8. Diffusion echo decay of the WPI signal (0.6-1.0 ppm) in WPI-LMP conjugates at  
 300 ratio 1:0, 4:1, 2:1, and 1:1 prepared at pH 7.2 after 8 days (ratio 1:0, 4:1, and 2:1) or 16 days  
 301 (ratio 1:1) of dry heat incubation time.

302 The diffusion echo decay of the LMP and WPI signal as influenced by the WPI to LMP ratio  
 303 can be seen in Figure 8. In the case of LMP, the graphs shifted upward as the concentration of  
 304 LMP was higher. The same trend was observed for WPI. It can be seen in Table 1 that the  
 305 conjugates with ratio 1:0, 4:1, 2:1, and 1:1 had an arithmetic mean diffusion coefficient of 7.45,  
 306 7.15, 6.27, and 8.80·10<sup>-11</sup> m<sup>2</sup>/s, respectively, when considering the WPI peak at 0.6-1.0 ppm.  
 307 On the other hand, the arithmetic mean standard deviation of the distribution of the conjugates  
 308 was reported to be 2.56, 3.63, 4.14, and 14.1·10<sup>-11</sup> m<sup>2</sup>/s for the conjugates with ratio of 1:0, 4:1,  
 309 2:1, and 1:1, respectively.



310

311 Figure 9. The normalized lognormal mass-weighted probability density function of the  
 312 diffusion coefficient of the WPI (0.6-1.0 ppm) signal in WPI-LMP conjugates with ratio of  
 313 1:0, 4:1, 2:1, and 1:1.

314 While it was expected that the diffusion coefficient of the WPI signal at ratio 1:1 would be the  
 315 smallest, it was found that it had the biggest diffusion coefficient. Nevertheless, it has to be  
 316 noted that the WPI signal at this ratio exhibited a much broader distribution width than the  
 317 others (Fig. 9). A similar behavior was previously observed in a mixture of WPI and LMP  
 318 prepared at pH 5 (paragraph 3.2). Besides, Figure 9 clearly indicates that the distribution mode  
 319 (*i.e.*, the diffusion coefficient corresponding to the maximum in the distribution curve)  
 320 gradually decreased as the LMP concentration increased.

321 The signal decomposition and calculation of the free WPI fraction in the conjugates confirmed  
 322 the phenomena explained above (Table 1). After 8 days of dry heat treatment, there was 75%  
 323 of unreacted WPI in the conjugates with ratio 4:1. By Increasing the proportion of LMP in the  
 324 conjugates to a WPI:LMP ratio of 2:1, the fraction of free WPI present in the conjugate  
 325 decreased to 64%. Finally, at a ratio 1:1 almost half of the WPI was bound to LMP (Table 1).  
 326 Therefore, it can be concluded that increasing the concentration of LMP in the WPI-LMP  
 327 conjugates resulted in a higher degree of conjugation between WPI and LMP.

### 328 3.5 Discussion

329 The current study was performed to investigate the capabilities of diffusion NMR as a tool to  
 330 confirm and to quantify the formation of WPI-LMP conjugates or complexes, as an alternative  
 331 of chemical analysis. Based on the aforementioned results, it can be seen that interaction of  
 332 WPI and LMP, through both electrostatic and covalent interaction, resulted in the decrease of

333 the arithmetic mean diffusion coefficient and the increase of the arithmetic mean standard  
334 deviation of WPI. The latter is due to the broad molecular weight range of LMP. Since native  
335 WPI has a very narrow distribution width due to its uniform molecular weight, its interaction  
336 with LMP dramatically influenced the average diffusion coefficient and distribution width of  
337 WPI. On the other hand, the diffusion coefficient properties of the LMP were much less  
338 affected by the presence of bound WPI. The results also indicate that as whey protein interacted  
339 with LMP, it acquired the diffusion properties of LMP, which points to the fact that smaller  
340 WPI molecules become bound to larger LMP molecules according to a pearls on a necklace  
341 geometry. For the sake of completeness, it has to be mentioned that an increase of the diffusion  
342 coefficient was sometimes observed when there was strong interaction between the WPI and  
343 LMP, as was the case in WPI-LMP conjugates with a ratio of 1:1 which was dry heat treated  
344 for 16 days. This phenomenon was due to the fact that the increased distribution width  
345 sometimes overruled the expected effect on the arithmetic mean value: in fact, considering a  
346 lognormal distribution, the arithmetic mean increases with increasing distribution width for a  
347 constant geometric mean.

348 In the case of protein-polysaccharides conjugates or complexes, the degree of interaction is an  
349 essential parameter as it is highly related to the functionality of these conjugates or complexes  
350 [39]. Furthermore, research on improving the functionality of proteins through interaction with  
351 other biopolymers is currently receiving a lot of attention [40]. It has been reported that the yield  
352 of the conjugation (dry heat treatment) (i.e. the degree of interaction) has an influence on the  
353 emulsifying activity and heat stability of protein-pectin conjugates [40-42]. The better  
354 emulsifying activity of the protein upon dry heat treatment is attributed to the present of  
355 polysaccharides. Upon emulsification the proteins are adsorbed to the surface of oil droplets  
356 and thus polysaccharides are carried by the proteins and become available on the surface of the  
357 oil droplets [12]. Adsorption of polysaccharides to the surface of oil droplet is not possible or  
358 very limited unless it is bound to proteins. The present of polysaccharides on the surface of oil  
359 droplet stabilizes the oil droplets in an oil in water emulsion system by providing additional  
360 viscoelastic layer on the surface of oil droplet and through steric hindrance [12, 43]. Hereby,  
361 knowing the degree of interaction or the minimum amount of polysaccharides required to be  
362 linked to proteins and present on the surface of oil droplet is essential to have a good  
363 understanding on the mechanism of protein-polysaccharides functionality. Since the degree of  
364 interaction is influenced by some factors such as the biopolymer concentration, pH, time,  
365 relative humidity and water activity (for dry heat treatment), and temperature, quantifying the



366 degree of interaction will help finding the optimum conditions for the preparation of conjugates  
367 or complexes with the desired functional properties. In addition, it will be also possible to  
368 determine the minimum degree of interaction required to achieve optimum functional  
369 properties.

370 The results obtained from NMR measurements were generally in agreement with those  
371 obtained by chemical analysis. SDS-PAGE analysis performed in a previous study showed that  
372 dry heat treatment of WPI-LMP mixtures resulted in the formation of high molecular weight  
373 compounds (WPI-LMP conjugates) [13]. In the same study, the degree of interaction between  
374 WPI and LMP was determined by measuring the availability of the primary amino group of  
375 WPI in the conjugates as a function of incubation time using the TNBS method [13]. Compared  
376 to the results obtained from TNBS measurement [13], the degree of interaction between WPI  
377 and LMP obtained using NMR was higher: TNBS analysis indicated that after 16 days of dry  
378 heat treatment, WPI-LMP conjugates with a ratio 2:1 had a degree of conjugation of  
379 approximately 15%. However, it has to be noted that  $\beta$ -lactoglobulin has 15 lysine residues.  
380 Thus, if only one out of the 15 amino groups reacts (*i.e.* 6.67% based on the number of free  
381 amino groups), all WPI may become bound to LMP (*i.e.* 100% based on diffusion NMR).  
382 Despite of showing a different magnitude of the degree of interaction, the general trend  
383 obtained from the NMR measurements was comparable with that obtained from the TNBS  
384 measurements in which the rate of WPI and LMP interaction was intensive in the first 4 days  
385 and decreased upon extension of the dry heat treatment incubation time. Furthermore, the  
386 TNBS analysis results also showed that the degree of interaction increased as the concentration  
387 of LMP in the mixture was higher, which was also in line with the results of NMR  
388 measurements.

389 A lot of useful information can be extracted from NMR measurements, such as the molecular  
390 structure, diffusion coefficient, and distribution width, which are related to the molecular  
391 weight distribution of the targeted compound [26-29, 39, 44]. Some studies related to milk protein  
392 employed NMR to confirm the interaction between the protein and other compounds using  
393 only the NMR spectra [26, 27] or by determining the diffusion coefficient of the protein [28, 29]. In  
394 fact, the results obtained from NMR were useful to quantify the degree of interaction between  
395 proteins and polysaccharides or other biopolymers. This was performed by decomposing the  
396 protein signal of the conjugates or complexes into the WPI signal without pectin (native state)  
397 and the pectin signal of the mixture using Eq. 2 and 3.

398 The results of this study indicate that NMR diffusometry is a promising method to study and  
399 quantify protein and polysaccharide interaction, in which the results are comparable to that  
400 obtained from chemical analysis. Whereas chemical analysis, e.g. using the TNBS and OPA  
401 method, cannot be used for protein-polysaccharide electrostatic interaction, NMR  
402 measurement works well for both electrostatic and covalent interactions. Compared to  
403 chemical analysis, e.g. using the TNBS method, which is time consuming and labor intensive,  
404 this method is more user-friendly and the sample preparation time is relatively short.  
405 Furthermore, the required amount of sample for the NMR analysis is low and no sample  
406 pretreatment is needed.

#### 407 **4. Conclusions**

408 Whereas the diffusion properties of LMP were less affected, the results showed that interaction  
409 between WPI and LMP, either through electrostatic or covalent interaction, decreased the  
410 diffusional speed of WPI. Deconvolution of the experimentally acquired signal of complexes  
411 or conjugates enabled the quantification of the fraction of free and reacted WPI in mixtures and  
412 conjugates of WPI-LMP. The results obtained from the NMR analysis were also found to be  
413 comparable to those obtained by alternative methods, such as SDS-page, spectrophotometric  
414 determination of the concentration of available free amino groups by the TNBS method, as  
415 well as electrophoretic mobility evaluation, as reported by Setiowati et al. [13]. However, NMR  
416 measurements are relatively user friendly and require relatively little sample preparation. Some  
417 work dealing with the application of NMR to study the interaction of protein with other  
418 compounds has been published [26-28, 39]. However, these studies did not perform any signal  
419 decomposition to quantify the degree of interaction, which is an important factor for the  
420 application of conjugates or complexes. Future application of this system to determine the  
421 molecular weight of the complexes or conjugates would be very interesting.

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