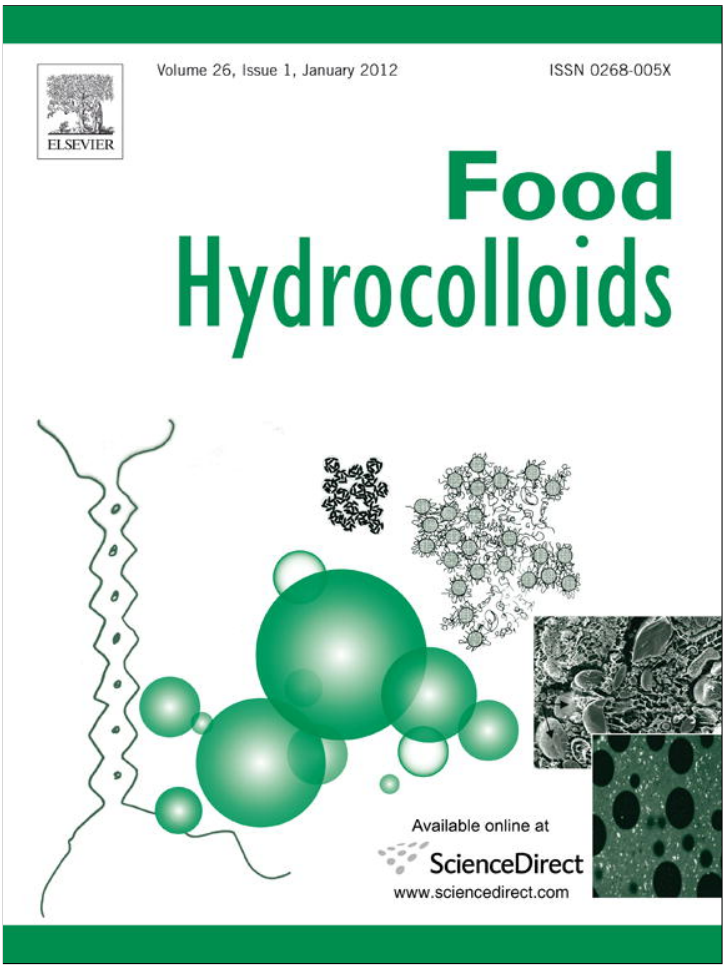

(This is a sample cover image for this issue. The actual cover is not yet available at this time.)

Food Hydrocolloids 29 (2012) 211–218

Contents lists available at SciVerse ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd

Purification of pectin from apple pomace juice by using sodium caseinate and characterisation of their binding by isothermal titration calorimetry

Thomas Happi Emaga [a,b,*], Haikel Garna [c,1], Michel Paquot [a], Magali Deleu [a]

[a] Gembloux Agro-Bio Tech, Department of Industrial Biological Chemistry, Passage des Déportés, 2, B-5030 Gembloux, Belgium
[b] African Research Centre on Bananas and Plantains (CARBAP), PO. Box 832 Douala, Cameroon
[c] Laboratoire de Biotechnologie et Valorisation des Bio- Géo Ressources, Institut Supérieur de Biotechnologie de Sidi Thabet, Pole Technologique de Sidi Thabet, 2020 Sidi Thabet, Tunisia

ARTICLE INFO

Article history:
Received 26 April 2011
Accepted 23 February 2012

Keywords:
Purification
Alcohol
Apple pomace pectins
Sodium caseinate
Binding
ITC

ABSTRACT

The binding of sodium caseinate to pectin using Isothermal Titration Calorimetry (ITC) under different pH values (2, 3, 3.5), and comparison of two purification processes (sodium caseinate or ethanol), based on the physicochemical characteristics of purified pectin was evaluated. The results indicated that ITC titration confirmed the existence of interactions between caseinates and pectin at pH 3 and 3.5. The interaction depicts two interdependent steps, one attributed to an electrostatic interaction and another related to a co-acervation mechanism. The chemical characteristics of pectins are strongly dependent on the purification process. Under some extraction conditions, ethanol is not specific to the recovery of pectin since it causes the precipitation of other compounds together with this polysaccharide.

However, compared to the caseinate, it allows total precipitation of pectins extracted, but caseinates have the advantage of being more specific for the charged polymers.

1. Introduction

In literature, several methods for recovery and purification of pectins from juice extracts were reported: alcohol precipitation (Mañas, & Saura-Calixto, 1993; Phatak, Chang, & Brown, 1988) purification by dialysis (Fishman, Pfeffer, Barford, & Doner, 1984; Pfeffer, Doner, Hoagland, & McDonald, 1981; Shrimanker, 1989), metal precipitation (Chou, 1989; Christensen, 1986; Kaussar & Nomura, 1980, 1981, 1982; Pintauro, 1967; Stevens & Selvendran, 1984), and ion exchange chromatography (De Vries, Voragen, Rombouts 1986).

The pectin extraction conditions have a deep impact on their biochemical characteristics and on their suitability for applications. This technique is based on the elimination of small compounds. However, this method of purification may lead to co-precipitation of non-fibrous compounds (salts, organic acids, etc…) or incomplete precipitation of pectins (Garna et al., 2007; Happi Emaga, Ronkart, Robert, Wathelet, & Paquot, 2008; Mañas & Saura-Calixto, 1993). This phenomenon may be influenced by factors such as pH, ionic strength, temperature and structure of the polysaccharide (Mañas & Saura-Calixto, 1993).

The pectin extraction conditions have a great impact on their biochemical characteristics and on their suitability for applications. Temperature, pH, time, and solvent:solid ratio were considered to be key factors (Kim, Soluski & Lee (1978); Miyamoto & Chang, 1992; Shi, Chang, Schwarz & Wiesenborn, 1995). Generally, the extraction conditions are in the range of pH 1.5–3, temperature 60–100 °C and time 0.5–6 h (Happi Emaga et al., 2008; Levigne, Ralet, & Thibault, 2002). Solid:liquid ratios of 1:17 for apple and 1:35 for citrus are commonly used (Ralet, Bonnin, & Thibault, 2002).

Garna, Happi Emaga, Robert, and Paquot (2011) have developed a new technique for the purification of electrically charged polysaccharides using protein (sodium caseinate). Commercial pectins from apple pomace were used as a model to verify the feasibility of the process and to define some important parameters. The results indicated that these proteins are very effective for the recovery of charged polymers as pectin. This purification technique is based on the electrostatic interactions between these two polymers and is thus strongly dependent on pH and salt concentration. Two steps are required. The first consists to precipitate the charged pectins using proteins at pH 3.5, whereas, the second step is to separate these polymers by the dissociation of complex formed (pectin and caseinate) and by precipitating protein at pH close to its isoelectric

* Corresponding author. Gembloux Agro-Bio Tech, Department of Industrial Biological Chemistry, Passage des Déportés, 2, B-5030 Gembloux, Belgium. Tel.: +32 81 622232; fax: +32 81 622231.
E-mail addresses: guythappi@yahoo.fr, T.happiEmaga@ulg.ac.be (T. Happi Emaga).
[1] First co-author.

0268-005X/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodhyd.2012.02.019

point (pH = 4.6). The transfer of this purification technique on juice extraction was necessary to check and to prove its efficiency in pectin purification.

It is also important to consider other interactions that might exist in the formation of caseinate–pectin complex. The Isothermal Titration Calorimetry (ITC) based on the measurement of the heat evolved during molecular association (Perrozzo, Folkers, & Scapozza, 2004), is a method of choice to characterize qualitatively and quantitatively (the stoichiometry, the standard Gibbs free energy change, the enthalpy change, the entropy change, equilibrium binding constants) the interaction between two molecules. This technique has already been applied to study the binding process between pectin and calcium ion (Fang, Al-Assaf, Phillips, Nishinari, Funami & Williams Fang, 2008), ionic surfactants (McClements) or beta-lactoglobulin (Girard, Turgeon, & Gauthier, 2003). Available literature does not provide studies carried out on the interactions between sodium caseinate and pectin using this technique.

For these reasons, the aims of the present work are, first, to investigate the binding of sodium caseinate to pectin using ITC under different pH conditions (2, 3, 3.5) in order to better apprehend phenomena involved in the interaction mechanism and secondly, to compare the sodium caseinate and ethanol purification processes, based on the physicochemical characteristics of the purified pectin (yield, Galacturonic content, sugars composition and proteins content) extracted under varied pH, temperature and time.

2. Materials and methods

Chemicals: Glucuronic acid, D-galacturonic acid, 2-deoxy-D-glucose, sodium acetate trihydrate, m-hydroxydiphenyl, sodium tetraborate and apple pectin (76282) were purchased from Fluka chemical Company (Buchs, Germany); sodium hydroxide 50% from JT Baker (Devenier, Holland); sodium caseinate (C-8654) was from Sigma (Steinheim, Germany) and sodium hydroxide pellets from Merck (Darmstadt, Germany).

Standard solutions: The standard solutions of galacturonic acid (1 mM), 2-deoxy-D-glucose (1 mM) and glucuronic acid (1 mM) were prepared from a 10 mM stock solution and were used to determine the relative response factors.

Enzymes: The multi-enzyme complex VL9 was a commercial liquid form produced by a selected strain of Aspergillus (Viscozyme L9) and obtained from Novo Nordisk (Copenhagen, Denmark). It was composed of a wide range of carbohydrases including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase. One millilitre sample of this solution was purified by ultrafiltration (1 h at 2000 g) using Centricon tubes (VivaSoin 20 ml concentrator: cutoff, 30 kDa) before been used to eliminate carbohydrates and glycerol. The residue obtained was washed twice with 15 ml of acetate buffer (20 mM, pH 5) and finally diluted in 100 ml in this buffer.

2.1. Isothermal titration calorimetry (ITC)

Thermodynamic parameters characterizing the binding between two entities and determined by ITC technique are conventionally expressed per mole of the titrant. Determination of an average molecular weight of pectin and caseinate is then required.

2.1.1. Molecular weight of sodium caseinate and pectin

Average Molecular Weight (MW) of sodium caseinate and pectin was determined by High Performance Size Exclusion Chromatography (HPSEC) on a Waters 2690-HPLC system (Waters Inc., Milford, MA), equipped with a TSKgel GMPWxl column (300 mm × 7.8 mm; TosoHaas Co. Ltd., Tokyo, Japan) and coupled on-line with a three detector system: a Waters 2410 differential Refractometer Index (RI), a Right Angle Laser Light Scattering (RALLS) and a differential viscometer detector (Model T-50A, viscotek, Houston, TX). Pectin (2 mg/ml) and caseinate (2 mg/ml) solutions were solubilised under magnetic stirring, then filtered through a 0.45 μm membrane filter (Millipore Co., Milford, MA). A constant volume of pectin or caseinate solution was dried to a constant weight in an air-circulated oven at 106 °C to calculate the exact pectin or caseinate concentration. One hundred μl of the sample was injected in the chromatographic column. Elution was done at a flow rate of 0.7 ml/min with 50 mM sodium nitrate (NaNO3) solution containing 0.05% sodium azide (NaN3) as preservative at 25 °C. The Molecular Weight (MW) was calculated using OMNISEC software (version 4.0.0, provided by Viscotek). The average MW of pectin and caseinate is 198 kDa and 3750 kDa, respectively, which is in accordance with literature (Lucey, Srinivasan, Singh, & Munro, 2000; Renard & Thibault, 1993).

2.1.2. ITC measurement

ITC measurements were performed on a Microcal VP-ITC instrument (Microcal, Northampton, MA, USA) at 25 °C. Pectin and caseinate solutions were prepared by dispersion of the powders into milliQ water and the pH was adjusted by adding HCl 1 M. The solutions were both dialyzed against milliQ water for 12 h at 4 °C. The dialyzed water was used for the blank solution. Prior to the experiments, all the samples were degassed through a vacuum stirring device.

After a first injection of 2 μl, twenty-four 10 μl aliquots of 0.64–0.99 μM caseinate solutions were stepwise injected into a 1.4565 ml reaction cell containing 0.71–1.41 μM pectin, with time intervals of 400 s between two successive injections. Throughout the experiments, the reaction cell was stirred constantly at 480 rpm. The heat of dilution from the blank titration of the caseinate into water was substracted from the raw data. Titration was performed at pH values of 2, 3, and 3.5. Measurements were carried out at least in duplicate.

The results were reported as the change of enthalpy per mole of caseinate injected into the reaction cell as a function of the caseinate/pectin molar ratio. The thermodynamic parameters were determined by using the "one set of sites" model from the software Microcal Origin. The Gibbs free energy (ΔG) was calculated from the equation: $\Delta G = \Delta H - T\Delta S$. Where ΔH is the enthalpy change per mole of caseinate, ΔS is the reaction entropy and T the temperature in Kelvin.

2.2. Extraction of pectin

Apple pomace was obtained from the commercial fruit juice prepared using mixed apple varieties. This was stored frozen at −20 °C before use.

Different extractions were carried out by heating 50 g of apple marc in 1 L of H_2SO_4 solution (pH 1.5 and 2) stirred at 250 rpm in a jacketed stainless steel reactor flask with a thermostatic bath in a discontinuous process at 80 and 90 °C. For each constant pH and temperature, samples were extracted after 1, 2, and 3 h. All experiments were duplicated.

After extraction, the liquid dispersion of apple pomace from the reactor flask was centrifuged at 17500×*g* for 30 min. The supernatant was filtered to remove the impurities. The pH of these juice-extracts was adjusted to 3.5 using 1 M NaOH.

2.3. Purification of pectin by ethanol

The recovery of pectin by alcohol was done as was described previously by Happi Emaga et al., 2008. In brief, the juice extracts

were dispensed in 4 volumes of ethanol (96%). The alcohol precipitate was separated from the supernatants by centrifugation at 17500 *g* for 30 min. This was dried in a vacuum oven at 40 °C to a constant weight and then finely ground using a centrifugal mill.

2.4. Purification of pectin by sodium caseinate and separation

The purification of pectin by sodium caseinate and their separation were carried out as described previously by Garna et al. (2011) (Fig. 1).

2.4.1. Optimisation

Depending on the extraction conditions and the amount of galacturonic acid (GalA) in extract juices, various mass ratios (caseinate to pectin) 1:1, 3:1, 6:1, 12:1, 24:1; 36:1 and 48:1 were chosen. In all the experiments, 2 ml of raw juice at pH 3.5 was used. Once precipitation has taken place after addition of the different amounts of caseinate (pH 3.5; 10 g/l), the precipitate was separated from supernatant by centrifugation at 5250 g for 1 h. The pectin content was then determined by assessing the galacturonic acid in the supernatants based on the above different ratios.

2.4.2. Precipitation of pectin using protein

After determining the ratio for which the maximum of pectin precipitation was observed, we applied it in the purification of one 100 ml of juice extract (pH 3.5). The supernatant was then collected by centrifugation at 17,500 g (10,000 rpm) for 1 h, and then analyzed for their pectin and proteins content.

2.4.3. Separation of pectins from sodium caseinate

The precipitate obtained after treatment with sodium caseinate was transferred into 30 ml of distilled water. The pH was gradually increased to pH 6.5 by addition of 1 M NaOH. After the dissolution of the pellet, an amount of NaCl was added at a concentration of 100 mM. The solution was then brought to pH 4.6 using HCl (0.1 M) and the precipitation of caseinate was observed. The tubes containing the precipitated caseinates were centrifuged at 17,500 g for 30 min and pectins in the supernatant were recovered for further analysis.

Moisture: The moisture content was determined after drying at 105 °C for 24 h. All values were calculated on a dry-weight basis.

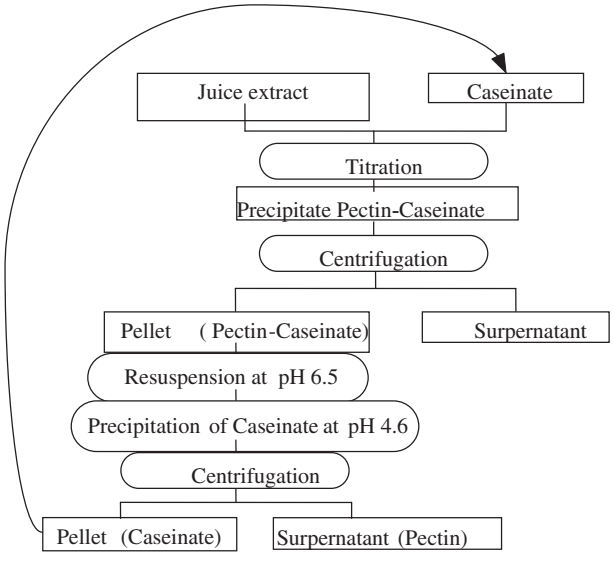

Fig. 1. Principle of pectin recovery by caseinates precipitation.

2.5. Characterisation of the different fraction of pectins

For each extraction condition, the galacturonic acid and neutral sugars content in the juice-extract, in the alcohol precipitate and in the supernatant obtained by the purification of pectin by caseinate during the two steps of the process were determined respectively by HPAEC-PAD and GLC using combined chemicals and enzymatic hydrolysis (Garna, Mabon, Nott, Wathelet, & Paquot, 2004).

2.5.1. Combined chemical and enzymatic hydrolysis

Two millilitres of juice-extract, alcohol precipitate (1 g/l) and pectin supernatants obtained by caseinate purification were hydrolyzed with 0.4 M TFA (2 ml) at 80 °C for 72 h. The hydrolysate pH was adjusted to pH 4.6 using NH_4OH (14 M) and further diluted to obtain 25 ml. Two millilitres of this solution was mixed with 2 ml of VL9 diluted 250-fold in sodium acetate buffer (20 mM pH 5) and glucuronic acid (2 mM) and 2-deoxy-D-glucose as the internal standard. The mixture was incubated at 50 °C for 2 h then heated at 100 °C for 3 min to inactivate the enzymes and filtered through a 0.45 μm filter membrane.

2.5.2. Analytical methods

HPAEC-PAD: The separation of galacturonic acid was done using a Dionex DX-500 Bio-LC system, with a CarboPAc PA100 column (250*4 mm) in combination with a CarboPac guard column (25*4 mm), Dionex corp., Sunyvale, CA. All the analysis were carried out at 30 °C and at a flow rate of 1 ml/min. The neutral monosaccharides were co-eluted isocratically using 100 mM NaOH for 5min. Uronic acids were eluted using a CH_3COONa gradient reaching (170 mM) and NaOH (100 mM) for 13min. The column was washed with 100 mM NaOH for 10min before the next injection. 25 μl of sample was injected. The detection was realized using a pulsed amperometric detector with a post-injection of 200 μl/min NaOH 900 mM. Potentials of $E_1 = 0.1$ V, $E_2 = 0.1$ V, $E_3 = 0.1$ V, $E_4 = -2$ V, $E_5 = -2$ V, $E_6 = 0.6$ V, $E_7 = -0.1$ V and $E_8 = -0.1$ V were applied for duration time points of $T_1 = 0$ s, $T_2 = 0.20$ s, $T_3 = 0.4$ s, $T_4 = 0.41$ s, $T_5 = 0.42$ s, $T_6 = 0.43$ s, $T_7 = 0.44$ s and $T_8 = 0.50$ s, respectively, at a sensitivity of 1 μC.

GLC : Neutral sugars in the juice-extract, in the alcohol precipitate and in the supernatant were determined by GLC after combined chemical and enzymatic hydrolysis of these products, and after their conversion to alditol acetates following Balkeney, Harris, Henry and Stone method (1983) method modified by Garna et al. (2004). These derivatives were analysed by gas chromatography using a FID detector and fitted with an HP-1 capillary column (30 m × 0.5 mm id) with a film thickness of 0.25 μm. The injector and detector temperatures were 250 °C and 300 °C, respectively. The oven temperature was initiallized at 120 °C, then programmed to rise linearly, 4 °C/min until 220 °C, after the separation of sugar and at 290 °C at a rate of 35 °C/min to condition the column. The carrier gas was helium. The amount of neutral sugars was calculated against a 2-desoxyglucose internal standard.

2.6. Crude protein

Total nitrogen(N) content was determined using the standard Kjeldahl procedure (AOAC, 1990), using a 1000 Kjeltabs MQ tablet and a digestion System 20, 1015 Digester, Tecator, AB, Höganäs, Sweden and the distillation by a Kjeltec Auto 1030 Analyser, Tecator, AB, Höganäs, Sweden. Crude protein was expressed as 6.25 N.

2.7. Statistical analysis

All experiments were done in duplicates. To maintain quality control during chemical analyses, the variation between duplicate

samples was determined. If the variation between duplicate samples was greater than 5%, the analysis was repeated.

3. Results and discussion

3.1. Isothermal titration calorimetry results

Fig. 2 shows typical enthalpy changes ($\delta h_i/\delta n_{cas}$) resulting from the injection of caseinate into pectin solution at three different pH.

The molar heat of reaction is dependent on the pH. At pH 2, $\delta h_i/\delta n_{cas}$ is relatively small and does not significantly vary over the range of caseinate/pectin molar ratios studied. It suggests that the interaction between the polysaccharide and the protein is very low at this pH. Using muteck titration Garna et al. (2011) also showed, that there was no formation of insoluble complex at pH = 2. At pH 3 and pH 3.5, the profile of the enthalpy change is complex. At low caseinate/pectin molar ratio, $\delta h_i/\delta n_{cas}$, it was negative. It progressively levelled off to zero and became positive. After passing by a plateau, $\delta h_i/\delta n_{cas}$ decreasedand tends to zero. This kind of multi-step profile was also observed for the injection of β-lactoglobulin into sodium alginate (Harnsilawat, Pongsawatmanit, & McClements, 2006) and was indicative of the binding of the protein with the polysaccharide.

The fact that exothermic and endothermic $\delta h_i/\delta n_{cas}$ were observed suggested that at least two processes occured in the binding mechanism. The existence of two types of independent binding sites on the polysaccharide has been reported by Fang et al. (2008) in the case of the binding of calcium to alginate. Since the only potential interacting sites on the pectin are the carboxylic groups (Girard et al., 2003), the two different processes were rather suggested to be two different physicochemical phenomena in the caseinate–pectin interaction.

The "one set of sites" model, the ITC Microcal Origin software was successfully applied for the fitting of the negative values of $\delta h_i/\delta n_{cas}$ in the case of pH 3.0 and pH 3.5 and made possible to obtain the thermodynamic parameters for the first step of caseinate-pectin binding (Table 1).

The Gibbs free energy (ΔG) was negative, indicating that the interaction between caseinate and pectin is spontaneous. ΔH was negative and much greater than TΔS. This first step was thus enthalpically driven as it is the case when electrostatic interactions are involved in the mechanism (Girard et al., 2003).

The fitting of the second part of curve failed whatever the model used. However, from the $\delta h_i/\delta n_{cas}$ curve, it can be observed that the enthalpy change of this second phase was positive. Assuming that the process was spontaneous ($\Delta G < 0$), implies that TΔS was positive and greater than ΔH. This indicates that the second part of the process was entropy driven.

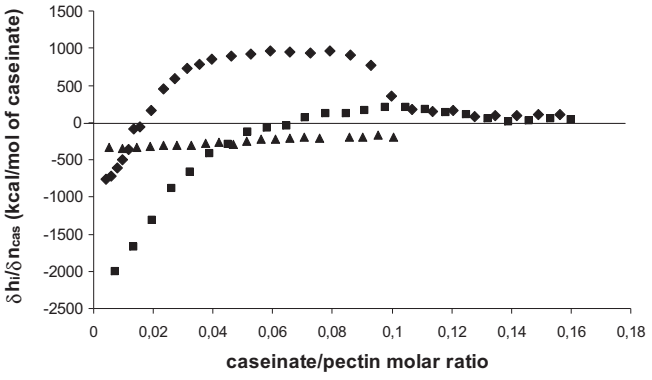

Fig. 2. Typical enthalpy changes ($\delta h_i/\delta n_{cas}$) resulting from the injection of caseinate into pectin solution at three different pH: pH 2 (▲), pH 3 (■) and pH 3.5 (◆).

Table 1
Thermodynamic parameters of the first step of caseinate-pectin interaction at pH 3.0 and pH 3.5 and at 25 °C determined with the "one set of sites" of the Microcal origin software.

Thermodynamic parameters	pH 3.0	pH 3.5
K (M − 1)	$(2.36 \pm 0.22) \times 10^8$	$(3.03 \pm 2.08) \times 10^9$
ΔH (kcal mol^{-1})	$(-2.60 \pm 0.05) \times 10^3$	$(-1.04 \pm 0.13) \times 10^3$
TΔS (kcal mol^{-1})	-216.6 ± 4.4	-86.5 ± 6.4
ΔG (kcal mol^{-1})	$(-2.38 \pm 0.04) \times 10^3$	$(-9.58 \pm 0.8) \times 10^2$

K: binding constant.
ΔH : enthalpy change per mole of caseinate.
ΔS : reaction entropy and T the temperature.
ΔG : Gibbs free energy.

The existence of two steps in the interaction mechanism between caseinate and pectin can be explained by the Veis-Aranyi theory as adapted by Tainaka. According to this theory (Burgess, 1990), a spontaneous aggregation of the oppositely charged polyelectrolytes takes place in a first step by electrostatic interaction, forming aggregates of low configurational entropy. These aggregates then rearrange to form coacervate phase. Rearrangement is driven by the gain in configurational entropy. The Tainaka theory was also used to explain the two-step complexation process observed by ITC for β-lactoglobulin and pectin (Girard et al., 2003).

In the case of this studt, it appears that, at the first step, enthalpically driven, is mainly due to the direct interaction between the carboxylic groups of the pectin and positively charged groups of the sodium caseinate. The second step, entropically driven, could be mainly assigned to the association of the caseinate-pectin aggregates into coacervates or precipitates involving a molecular rearrangement of one or both components and/or a release of hydration water molecules or ions (Harnsilawat et al., 2006). This second step seems to occur at a caseinate/pectin molar ratio higher than 0.06 for pH 3 and 0.015 for pH 3.5. This is in agreement with Rediguieri, de Freitas, Lettinga, and Tuinier (2007) study which showed of the influence of the polyectrolyte ratio in the mixture on the complex formation. However, we cannot say that the two steps are completely distinct as ITC measures the sum of all events occurring in the reaction cell.

The end of the titration curve indicates the total saturation of the pectin sites by caseinate. It occured at ~ 0.14 mol of caseinate per mole of pectin for both pH. Then, only a part of the protein molecules is complexed to the pectin ones. The similarity between both pH is not in accordance with the higher charge density of the pectin at pH 3.5 comparatively to pH 3.0 (Garna et al., 2011) which would suggest a greater number of electrostatic reaction sites at pH 3.5 than pH 3.0. However, the binding constant (K) calculated for the step associated with the electrostatic interaction (Table 1) was one order of magnitude higher at pH 3.5 than at pH 3.0, showing that the electrostatic affinity of caseinate for pectin was higher at pH 3.5 than at pH 3.0.

3.2. Optimisation of the pectin precipitation by caseinate

The optimization of the precipitation of pectin by sodium caseinate was performed using different ratios of caseinates:Gal A to determine the amount of protein needed for optimal precipitation. The results of this optimization are presented in Table 2.

Whatever the extraction conditions, the recovery rate increased with caseinate to pectin ratio and remained constant beyond a determined value. A ratio Caseinate:GalA of 48:1 and 36:1 was necessary at pH 1.5 depending on the extraction temperature.

Table 2
Recovery rate of pectins extracted at pH 1.5 and pH 2 at 80 °C or 90 °C after precipitation by sodium caseinate at pH 3.5 according to different mass ratios caseinate/GalA.

Ratio caseinate/GalA	pH 1.5; 80 °C			pH 1.5; 90 °C			pH 2; 80 °C			pH 2; 90 °C		
	% recovery			% recovery			% recovery			% recovery		
	1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h
1:1	19.0	14.1	17.5	18.8	19.0	16.5	28.0	22.3	24.9	27.0	23.9	26.2
3:1	23.3	28.5	22.1	22.9	23.5	22.5	32.1	28.3	33.8	32.3	28.6	30.3
6:1	33.6	32.1	32.6	26.8	26.1	32.1	40.0	37.5	41.2	39.2	33.7	39.9
12:1	39.0	36.7	38.3	33.1	38.3	61.0	59.9	68.7	71.4	68.5	71.0	75.3
24:1	47.4	60.7	72.9	65.2	74.7	87.7	90.7	89.0	90.4	85.7	87.1	87.3
36:1	62.9	81.6	89.5	87.5	91.9	93.1	90.4	90.1	90.3	86.4	87.9	87.5
48:1	83.0	89.1	91.4	87.6	92.0	93.5	90.2	90.8	90.8	85.2	87.3	87.2

Conversely,, it was 24:1 at pH 2. Under these conditions amount of pectin recovered ranged from 87% to 91%. This variation in the amount of caseinate based on the extraction conditions could be explained by the fact that the juice-extracts at pH 2 were purer in pectin than at pH 1.5. At this pH, the possibility of the presence of others substances coextracted with pectin that may hinder their precipitation can be proposed.

3.3. Weight balance of the purification

Pectins extracted from apple pulp were precipitated by sodium caseinate and separated from the complex pectin/caseinates by the precipitation of this protein at its isolelectric point. The results of this purification according to the extraction conditions are shown in Tables 3 and 4.

From these tables, the amount of caseinates necessary for the precipitation of pectin was highly dependent on extraction conditions. Indeed, this amount varied from pH 2 to pH 1.5, although quite similar quantities of galacturonic acids were extracted at 90 °C. Compared to the purification of commercial pectin (Garna et al., 2011), larger quantities of caseinates were necessary to precipitate the pectin. Roughly 31 g and 12 g protein were used at pH 1.5 and pH 2. This difference was due first to the variation in the purity of juice-extracted pectin and on the other hand to their low concentration in these polysaccharides (0.7 to 1 g/l).

After precipitation of pectins by caseinate at pH 3.5, there was always a quantity of non-precipitated pectin in the supernatant. The percentage of pectin loss varied from 6.9% to 17% depending on extraction conditions. This percentage was lower at pH 1.5 than at pH 2. Garna et al. (2007) showed that pectins extracted at pH 1.5 were characterized by lower degree of methylation (DM) than those extracted at pH 2. Moreover, the DM decreased significantly with increasing extraction time. For these reasons, pectins extracted at pH 1.5 had a higher negative charge than those extracted at pH 2 precipitation was facilitated by caseinates.

The recovery rate of pectin after caseinate precipitation at pH 4.6 ranged from 71.9% to 83.7%. This was slightly higher at pH 1.5 than at pH 2. This variation is mainly due to a higher precipitation

Table 3
Evolution of the galacturonic acid content of apple pectin extracted at pH 1.5 at 80 °C or at 90 °C; in the pellet and the supernatant after precipitation of pectins using caseinate to pectin ratio 48:1 and 36:1 at pH 3,5 and after the dissolution of the pellet and precipitation of caseinate at pH 4.6.

	pH 1.5 80 °C			pH 1.5 90 °C				pH 1.5 80 °C			pH 1.5 90 °C		
	1 h	2 h	3 h	1 h	2 h	3 h		1 h	2 h	3 h	1 h	2 h	3 h
Quantity GalA in the extraction juice (mg)	700.6	804.6	817.7	907.8	982.4	1023.7							
Amount of protein precipitated with pectins at pH 3.5 (g)	29.8	31.5	31.4	28.9	32.2	30.4							
Quantity GalA purified using caseinate (mg)	557.3	642.5	684.1	757.7	797.3	768.9	% Gal A purified with caseinate	79.5	79.9	83.7	83.5	81.2	75.1
Quantity GalA not precipitated at pH 3.5 (mg)	119.3	87.8	70.6	113.3	79.6	70.9	% Loss GalA at pH 3.5	17.0	10.9	8.6	12.5	8.1	6.9
Quantity GalA precipitated with caseinate at pH 4.6 (mg)	24.0	74.3	63.0	36.8	105.5	183.9	% Loss GalA at pH 4.6	3.4	9.2	7.7	4.1	10.7	18.0

Table 4
Evolution of the galacturonic acid content of apple pectin extracted at pH 2 at 80 °C or at 90 °C; in the pellet and the supernatant after precipitation of pectins using using caseinate to pectin ratio 24:1 at pH 3,5 and after the dissolution of the pellet and precipitation of caseinate at pH 4.6.

	pH 2 80 °C			pH 2 90 °C				pH 2 80 °C			pH 2 90 °C		
	1 h	2 h	3 h	1 h	2 h	3 h		1 h	2 h	3 h	1 h	2 h	3 h
Quantity GalA in the extraction juice (mg)	678.4	753.4	872.1	866.2	946.4	993.8							
Amount of protein precipitated with pectins at pH 3.5 (g)	10.9	10.7	12.5	10.6	13.0	14.0							
Quantity GalA purified using caseinate (mg)	549.3	570.4	659.1	623.7	680.1	725.0	% Gal A purified with caseinate	81.0	75.7	75.6	72.0	71.9	72.9
Quantity GalA not precipitated at pH 3.5 (mg)	63.2	82.7	83.6	124.0	122.2	126.6	% Loss GalA at pH 3.5	9.3	11.0	9.6	14.3	12.9	12.7
Quantity GalA precipitated with caseinate at pH 4.6 (mg)	65.9	100.3	129.4	138.6	144.1	142.2	% Loss GalA at pH 4.6	9.7	13.3	14.8	16.0	15.2	14.3

Table 5
Yield and chemical composition of juice and pectin purified with ethanol and by sodium caseinate at pH 1.5 at 80° C (values in parenthesis are the percentages of recovery).

		1 h			2 h			3 h		
		Juice	Alcohol	Caseinate	Juice	Alcohol	Caseinate	Juice	Alcohol	Caseinate
	Yield (g)	11,1	2,65 (23,9)	3,2 (28,8)	11,1	3,17 (28,6)	2,7 (24,3)	10,8	3,0 (27,5)	3,2 (29,6)
	Gal. A (mg)	700,6	648,3 (92,5)	557,3 (79,5)	804,6	773,3 (96,1)	642,5 (79,9)	817,7	799,8 (97,8)	684,1 (83,7)
Sugars (mg)	Rhamnose	66,8	19,1 (28,6)	26,1(39,1)	65,5	20,7 (31,6)	32,8 (50,1)	67,5	22,2 (32,9)	32,8 (48,6)
	Arabinose	719,3	82,6 (11,5)	44,9 (6,2)	757,8	47,7 (6,3)	33,2 (4,4)	709,4	35,5 (5,0)	27,1 (3,8)
	Xylose	71,1	16,4 (23,1)	13,0 (18,3)	70,9	20,9 (29,5)	14,8 (20,8)	67,9	24,1 (35,6)	16,8 (24,8)
	Mannose	1499,8	35,6 (2,4)	98,8 (6,6)	1512,4	43,7 (2,9)	128,2 (8,5)	1393,3	42,5 (3,1)	87,5 (6,3)
	Glucose	4071,4	330,9 (8,1)	129,1 (3,2)	3841,8	323 (8,4)	39,8 (1,0)	3630,8	322,7 (8,9)	106,5 (2,9)
	Galactose	109,6	71,1(64,9)	32,4 (29,6)	121,4	80,8 (66,6)	39,8 (32,8)	127,6	92,5 (72,5)	41,5 (32,6)
	Total	6537,9	555,7 (8,5)	344,4 (5,3)	6369,8	536,8 (8,4)	288,7 (4,5)	5996,5	539,6 (9,0)	312,4 (5,2)
	Protein (mg)	77,5	2,3 (3,1)	111,3	52,0	2,6 (5,0)	132,2	70,6	3,6 (5,2)	109,0

Table 6
Yield and chemical composition of juice and pectin purified with ethanol and by sodium caseinate at pH 1.5 at 90 °C (values in parenthesis are the percentages of recovery).

		1 h			2 h			3 h		
		Juice	Alcohol	Caseinate	Juice	Alcohol	Caseinate	Juice	Alcohol	Caseinate
	Yield (g)	11.2	3.2 (28.2)	3.4 (30.4)	11.3	3.0 (26.7)	3.3 (29.2)	11.2	3.4 (30.6)	3.0 (26.8)
	Gal. A (mg)	907.8	874.2 (96.3)	757.7 (83.5)	982.4	916.5 (93.3)	797.3 (81.2)	1023.7	977.6 (95.5)	768.9 (75.1)
Sugars (mg)	Rhamnose	59.0	23.5 (39.9)	46.7 (79.2)	65.3	28.5 (43.7)	51.7 (79.2)	84.6	32.8 (38.8)	49.7 (58.7)
	Arabinose	847.4	34.3 (4.0)	34.1 (4.0)	850.4	23.5 (2.8)	31.7 (3.7)	738.4	16.3 (2.2)	29.4 (4.0)
	Xylose	83.7	29.3 (35.0)	20.4 (24.4)	95.7	32.9 (34.4)	22.9 (23.9)	93.5	37.2 (39.8)	23.8 (25.5)
	Mannose	1582.5	45.1 (2.8)	96.7 (6.1)	1546.4	43.3 (2.8)	97.9 (6.3)	1375.4	40.4 (2.9)	90.0 (6.5)
	Glucose	3715.5	342.6 (9.2)	128.0 (3.4)	3712.8	307.8 (8.3)	48.6 (1.3)	3780.3	289.0 (7.6)	97.6 (2.6)
	Galactose	134.5	101.5 (75.5)	43.2 (32.1)	179.8	109.8 (61.1)	48.6 (27.0)	181.7	107.1 (59.0)	45.4 (25.0)
	Total	6422.7	576.3 (9.0)	369.1 (5.7)	6450.5	545.8 (8.5)	301.4 (4.7)	6253.9	522.8 (8.4)	335.8 (5.4)
	Protein (mg)	109.8	3.0 (2.7)	115.4	79.2	3.6 (4.5)	102.2	76.4	3.6 (4.7)	68.6

of pectin at pH 3.5 in the extraction conditions which was more acidic (pH 1.5). At pH 1.5, an average of 701.3 mg of pectins was purified by caseinate against 634 mg at pH 2.

Pectin loss during caseinate precipitation at pH 4.6 varied from 3.4% to 18%. This was more important at pH 2 than at pH 1.5; and higher than those obtained when the commercial powdered pectin was used (1.4%). Indeed, contrary to the simple model, the complex pectin/caseinate formed at pH 3.5 was characterized by high protein content (12 g–31 g) that favour their interaction with pectin during the complex dissociation at pH 6.5 and lead consequently to the co-precipitation of a high amount of this polysaccharide with caseinate at pH 4.6.

3.4. Comparative study of the purification with ethanol and sodium caseinate

A comparative study between the precipitation of pectin with ethanol and sodium caseinate was performed based onthe weight balance during the extraction and the purification (yield, galacturonic acid, sugars and proteins). The results are presented in Tables 5–8.

3.4.1. Extraction yield

As presented in these tables, an average 28.5% and 22% solids were extracted from apple pulp respectively, at pH1.5 and pH 2. These values represented 11.3 g and 8.3 g of extracts from 50 g of pulp. 28% and 16% of this extract was recovered by alcohol precipitation against 28% and 34% obtained using caseinate at pH 1.5 and pH 2, respectively.

Yields obtained after purification of pectin with ethanol or caseinates were almost similar at pH 1.5 (3.2 g). At pH 2, the ethanol yields were lower (1.1 g–3.4 g). However, these yields obtained by caseinate method were overestimated because the salts were taken into account (100 mM) during the dissolution of the complex pectin/caseinate and the caseinate precipitation at pH 4.6.

3.4.2. Galacturonic acid content

According to the above tables, the juice-extracts have a galacturonic acid content ranging from 6.3% to 11.5% of material

Table 7
Yield and chemical composition of juice and pectin purified with ethanol and by sodium caseinate at pH 2 at 80 °C (values in parenthesis are the percentages of recovery).

		1 h			2 h			3 h		
		Juice	Alcohol	Caseinate	Juice	Alcohol	Caseinate	Juice	Alcohol	Caseinate
	Yield (g)	8.0	1.1 (14.0)	2.6 (32.5)	8.1	1.3 (16.3)	3.0 (37.1)	8.8	1.4 (15.4)	3.0 (34.2)
	Gal. A (mg)	678.4	485.2 (71.5)	549.3 (81.0)	753.4	589.7 (78.3)	570.4 (75.7)	872.1	708.4 (81.2)	659.1 (75.6)
Sugars (mg)	Rhamnose	43.8	13.7 (31.2)	26.0 (59.3)	46.3	17.3 (37.3)	27.5 (59.4)	52.0	18.2 (35.0)	35.3 (67.9)
	Arabinose	553.5	160.3 (29.0)	69.9 (12.6)	649.3	122.3 (18.8)	56.2 (8.7)	741.8	84.5 (11.4)	50.8 (6.8)
	Xylose	57.3	15.1 (26.4)	11.8 (20.6)	61.1	18.8 (30.7)	12.7 (20.8)	66.3	19.4 (29.3)	15.0 (22.6)
	Mannose	1538.7	29.0 (1.9)	109.4 (7.1)	1541.6	32.1 (2.1)	83.4 (5.4)	1586.2	32.8 (2.1)	75.5 (4.8)
	Glucose	3656.9	240.3 (6.6)	148.8 (4.1)	3612.0	318.8 (8.8)	29.8 (0.8)	3783.3	315.0 (8.3)	76.7 (2.0)
	Galactose	73.0	49.1 (67.3)	26.7 (36.5)	88.4	64.4 (72.9)	29.8 (33.7)	109.2	69.9 (64.0)	38.4 (35.2)
	Total	5923.3	507.6 (8.6)	392.6 (6.6)	5998.6	573.5 (9.6)	239.4 (4.0)	6338.9	539.9 (8.5)	291.8 (4.6)
	Protein (mg)	58.9	0.7 (1.1)	46.9	51.1	1.0 (2.0)	41.3	49.1	0.9 (1.7)	65.5

Table 8
Yield and chemical composition of juice and pectin purified with ethanol and by sodium caseinate at pH 2 at 90 °C (values in parenthesis are the percentages of recovery).

		1 h			2 h			3 h		
		Juice	Alcohol	Caseinate	Juice	Alcohol	Caseinate	Juice	Alcohol	Caseinate
	Yield (g)	8.7	1.4 (16.1)	2.1 (24.0)	8.8	1.6 (18.0)	3.7 (42.0)	8.6	1.6 (18.5)	3.8 (44.1)
	Gal. A (mg)	866.2	779.5 (90.0)	623.7 (72.0)	946.4	926.1 (97.9)	680.1 (71.9)	993.8	884.9 (89.0)	725.0 (73.0)
Sugars (mg)	Rhamnose	51.6	19.5 (37.8)	26.5 (51.3)	59.6	23.8 (39.8)	39.8 (66.8)	56.7	26.0 (45.8)	41.1 (72.6)
	Arabinose	734.7	87.5 (11.9)	46.4 (6.3)	785.2	64.4 (8.2)	37.5 (4.8)	806.5	47.7 (5.9)	30.4 (3.8)
	Xylose	70.1	21.9 (31.3)	11.9 (16.9)	74.9	28.4 (38.0)	16.6 (22.2)	78.8	30.5 (38.7)	18.8 (23.8)
	Mannose	1615.6	32.2 (2.0)	45.2 (2.8)	1595.0	43.7 (2.7)	67.7 (4.2)	1498.6	41.4 (2.8)	60.5 (4.0)
	Glucose	3855.3	337.9 (8.8)	58.6 (1.5)	3699.7	344.0 (9.3)	78.9 (2.1)	3533.6	352.9 (10.0)	75.1 (2.1)
	Galactose	110.7	74.2 (67.0)	34.4 (31.0)	142.6	96.2 (67.4)	46.2 (32.4)	150.0	98.0 (65.4)	49.8 (33.2)
	Total	6438.1	573.2 (8.9)	222.9 (3.5)	6357.0	600.5 (9.4)	286.8 (4.5)	6124.1	596.5 (9.7)	275.7 (4.5)
	Protein (mg)	33.6	3.7 (11.0)	68.0	97.3	3.8 (3.9)	68.6	62.4	5.7 (9.1)	64.5

extracted. The highest content (11.5%) was obtained at pH 2 at 90 °C after 3 h of extraction. The lowest content (6.3%) was obtained at pH 1.5 at 80 °C after 1 h. At both extraction pH, the galacturonic acid content increased with time and temperature.

For caseinate extraction, the purity of extracts (expressed as percentage of galcturonic acid in the final extract) ranged between 17% and 29%, while for ethanol extraction the range was from 24 to 58%. The purity of ethanol extracts seemmed to be more affected by extraction pH than the purity of caseinate extracts.

An average of 94.3% galacturonic acid extracted at pH 1.5 was recovered by alcohol precipitation. This recovery rate was highly variable (71%–97%) at pH 2 depending on temperature and the extraction time. Otherwise, the recovery of galacturonic acid by caseinates varied from 75.1% to 84% respectively for both pH.

Whatever the extraction conditions, the concentrations of galacturonic acid of purified pectin by ethanol precipitation were always higher (except at pH 2, 80° C in 1 h extraction) than those purified by caseinate. This can be explained by the difference in the precipitation mechanism of pectin. Indeed, with ethanol, low and high molecular weight pectin are precipitated by removal of water molecules, contrary to the case with caseinate, where highly charged substances with high molecular weight are targeted.

3.4.3. Sugar composition

Juice-extracts are very rich in neutral sugars, varying from 57% to 75% of crude extract. These sugars can be under free form or from pectin origin or from hemicellulose.

The purification of juice with ethanol or sodium caseinate can significantly reduce these levels. Ethanol precipitated 8.6%–9.1% of these sugars against 3.5%–6.6% with caseinate.

Whatever the extraction conditions, pectins purified by caseinates are characterized by a lower total of neutral sugar content. Moreover, their sugar composition is strongly different. In terms of Rhamnose, pectins purified by caseinate are characterized by a high content in this sugar than those precipitated with ethanol. An average of 61% rhamnose extract was recovered by caseinates against 37% by ethanol. In addition, whatever the purification method, the recovery rate was higher at 90 °C. This led to the assumption that in drastic extraction conditions, pectins rhamnogalacturonan type is recovered in large quantities by caseinate.

For arabinose, it was noted that this sugar is present in higher quantities in the pectin precipitated with alcohol. An average of 9.8% arabinose extract was recovered by alcohol against 5.8% by caseinate. This difference may be explained by the ethanol precipitation, which, contrary to the caseinate, may illicit the recovery of free sugar arabinose and neutral polymers such as arabinogalactan and arabinan.

For the galactose, the xylose and the glucose, it was observed that pectin purified by alcohol had higher content in these sugars than those purified by caseinate; 67%; 32.6% and 8,5% of these sugars were respectively recovered from juice- extracts by the ethanol against 31.8%; 22% and 2.3% by caseinate. These results showed a big difference between the two purification processes on their selectivity in the recovery of charged and neutral compounds.

Although there are no studies showing that glucose and mannose are part of pectin structure, they were present in the pectin purified by caseinate. This raised the following question: do these sugars make really part of pectin structure or are from other linked polymers to pectin or from coextracts?

3.4.4. Protein content

The determination of total nitrogen was done in order to determine the eventual presence of nitrogenous compounds such as proteins. As presented in Tables 5–8, small quantities (33.6 mg–109.8 mg) of protein were found in the extracts. These quantities were lower in the ethanol precipitate. About 1.1%–11% of protein extracted was recovered. The High content of proteins in the purified pectin by caseinate was generated by the purification process during the separation of pectins from caseinates by their precipitation at their isoelectric points pH (4.6). About 0.2%–0.6% of added protein for the precipitation of pectin at pH 3.5 remained soluble in the supernatant with purified pectin at pH 4.6.

4. Conclusion

ITC titration confirmed the existence of interactions between caseinates and pectin at pH 3 and 3.5. At pH 2, such interactions do not exist. The interaction depicts two interdependent steps, one attributed to an electrostatic interaction and another one related to a coacervation mechanism. However, it would be interesting to explore the physical presence of these coacervates by performing turbidity and dynamic light scattering measurements and transmission electronic microscopy (TEM) imaging. This would better elucidate the nature of the caseinate–pectin interaction.

The chemical characteristics of pectins are strongly dependent on the purification process used. This result shows that under certain extraction conditions, ethanol does not provide purity for the recovery of pectin since it causes the precipitation of other compounds along with this polysaccharide. However, comparing to the caseinate, it allows total precipitation of pectins extracted. It precipitates neutral pectic substances as well as charged pectin.

Caseinates have the advantage of being more specific for the charged polymers; in addition, this purification technique can be used as a concentration technique by re-dissolution of the complex formed at the first step of the process in a volume smaller than that of the juice to be purified. The major disadvantage of this method is the use a high quantity of caseinate and salt to recovery the pectin.

In addition, this purification technique can be used to obtain pectins with different characteristics from those of ethanol and

that can present others technofunctional properties such as emulsifying and stabilizing.

Acknowledgement

This research has been supported by the Ministère de la Région Wallonne, Direction Générale de l'Agriculture. Université de liège-Gembloux Agro-Bio Tech through a Post-Doctoral fellowship awarded to Dr. Thomas Happi Emaga.

References

AOAC (Association of Official analytical Chemist). (1990). *Official methods of analysis* (15th ed). (Washington, VA).

Burgess, D. J. (1990). Practical analysis of complex coacervate systems. *Journal of Colloid and Interface Science, 140*, 227–238.

Chou, T.C. (1989). *The role of shape and aggregation on rheological properties of dilute and concentrated pectin solutions*. Ph.D. dissertation, Rutgers University, New Brunswick.

Christensen, S. H. (1986). Pectins. Ch. 9 Glicksman, M.(Ed.). *Food hydrocolloids, Vol III* (pp. 213–214). Boca Raton, FL: CRC Press.

De Vries, J. A., Voragen, A. G. J., Rombouts, F. M., & Pilnik, W. (1986). Structural studies of apple pectins with pectolytic enzyme. In M. L. Fishman, & J. J. Jen(Eds.), *Chemistry and function of pectins* (pp. 41). Washington, DC: American Chemical Society.

Fang, Y. P., Al-Assaf, S., Phillips, G. O., Nishinari, K., Funami, T., & Williams, P. A. (2008). Binding behavior of calcium to polyuronates: comparison of pectin with alginate. *Carbohydrate Polymers, 72*, 334–341.

Fishman, M. L., Pfeffer, P. E., Barford, R. A., & Doner, L. W. (1984). Studies of pectin solution properties by high-performance size exclusion chromatography. *Journal of Agricultural and Food Chemistry, 32*, 372–378.

Garna, H., Happi Emaga, T., Robert, C., & Paquot, M. (2011). New method for the purification of electricallycharged polyssacharides. *Food Hydrocolloids, 25*, 1219–1226.

Garna, H., Mabon, N., Nott, K., Wathelet, B., & Paquot, M. (2004). New method for two-step hydrolysis and chromatographic analysis of pectin neutral sugar chains. *Journal of Agricultural and Food Chemistry, 52*, 4652–4659.

Garna, H., Mabon, N., Robert, C., Cornet, C., Nott, K., Legros, H., et al. (2007). Effect of extraction conditions on the yield and purity of apple pomace pectin precipitated but not washed by alcohol. *Journal of Food Sciences, 72*, 1–9.

Girard, M., Turgeon, S. L., & Gauthier, S. F. (2003). Thermodynamic parameters of beta-lactoglobulin-pectin complexes assessed by isothermal titration calorimetry. *Journal of Agricultural and Food Chemistry, 51*, 4450–4455.

Happi Emaga, T., Ronkart, S., Robert, C., Wathelet, B., & Paquot, M. (2008). Characterisation of pectins extracted from banana peels (*Musa AAA*) under different condition using an experimental design. *Food Chemistry, 108*, 463–471.

Harnsilawat, T., Pongsawatmanit, R., & McClements, D. J. (2006). Characterization of beta-lactoglobulin-sodium alginate interactions in aqueous solutions: a calorimetry, light scattering, electrophoretic mobility and solubility study. *Food Hydrocolloids, 20*, 577–585.

Kaussar, P., & Nomura, D. A. (1980). A new approach to pectin manufacture by copper method. Part 1. Preparation of pectin pomace, pectin extraction and concentration by copper salt. *Journal of the Faculty of Agriculture Kyushu University, 26*, 61.

Kaussar, P., & Nomura, D. A. (1981). A new approach to pectin manufacture by copper method. Part 2. Preparation of drymetal complexes. *Journal of the Faculty of Agriculture Kyushu University, 26*, 1.

Kaussar, P., & Nomura, D. A. (1982). A new approach to pectin manufacture by copper method. Part 3. Demineralization of dry Cu-Al-pectin complex and characteristics of the pectin produced thereby. *Journal of the Faculty of Agriculture Kyushu University, 26*, 111.

Kim, W. J., Soluski, F., & Lee, S.c.k. (1978). *Chemical and gelation characteristic of ammonia6demethylated sunflower pectins*.

Levigne, S., Ralet, M. C., & Thibault, J.-F. (2002). Characterization of pectins extracted from fresh sugar beet under different conditions using an experimental design. *Carbohydrate Polymers, 49*, 145–153.

Lucey, J. A., Srinivasan, M., Singh, H., & Munro, P. A. (2000). Characterization of commercial and experimental sodium caseinates by multiangle laser light scattering and size-exclusion chromatography. *Journal of Agricultural and Food Chemistry, 48*(5), 1610–1616.

Mañas, E., & Saura-Calixto, F. (1993). Ethanolic precipitation: a source of error in dietary fiber determination. *Food Chemistry, 47*, 351–355.

Miyamoto, A., & Chang, K. C. (1992). Extraction and physicochemical characterization of pectin from sunflower Head Residues. *Journal of Food Science, 57*, 1439–1443.

Perrozzo, R., Folkers, G., & Scapozza, L. (2004). Thermodynamics of protein-ligand interaction: history, presence, and future aspects. *Journal of Receptors and Signal Transduction Research, 24*, 1–52.

Pfeffer, P. E., Doner, L. W., Hoagland, P. D., & McDonald, G. G. (1981). Molecular interactions with dietary fiber components. Investigation of possible association of pectin and bile acids. *Journal of Agricultural and Food Chemistry, 29*, 455–461.

Phatak, L., Chang, K. C., & Brown, G. (1988). Isolation and characterization of sugar-beet pulp. *Journal of Food Sciences, 53*, 830–833.

Pintauro, ND. (1967). *Pectinous material from cranberry fruit isolation, purification and characterization*. Ph.D. dissertation, Rutgers University, New Brunswick.

Ralet, M.-C., Bonnin, E., & Thibault, J.-F. (2002). Pectins. InDe Baets, S., Vandamme, E. J., & Steinbuchel, A.(Eds.). *Polysaccharides II— Polysaccharides from eukaryotes, biopolymers, Vol. 6* (pp. 345–380). Weinheim: Wiley–VCH Verlag.

Rediguieri, C. F., de Freitas, O., Lettinga, M. P., & Tuinier, R. (2007). Thermodynamic incompatibility and complex formation in pectin/caseinate mixtures. *Biomacromolecules, 8*, 3345–3354.

Renard, C. M. G. C., & Thibault, J.-F. (1993). Structure and properties of apple and sugar-beet pectins extracted by chelating agents. *Carbohydrate Research, 244*, 99–114.

Shi, X. Q., Chang, K. C., Schwarz, J. G., & Wiesenborn, D. (1995). Effect of countercurrent ethanol washing on sunflower pectin quality. *Carbohydrate Polymers, 27*, 171–175.

Shrimanker, S.H. (1989). *Evaluation of the Doi-Edwards theory for predicting viscoelastic properties of food biopolymer*. M.S. Thesis, Rutgers University, New Brunswick.

Stevens, B. J. H., & Selvendran, R. R. (1984). Structural features of cell-wall polysaccharides of the apple. *Carbohydrate Research, 135*, 155–166.