Journal of Food Science and Technology

Thermodynamic sorption analysis and glass transition temperature of faba bean (Vicia faba L.) protein

--Manuscript Draft--

Manuscript Number:	JFST-D-16-01673R1
Full Title:	Thermodynamic sorption analysis and glass transition temperature of faba bean (Vicia faba L.) protein
Article Type:	Original Article
Corresponding Author:	A.Y. Guadarrama-Lezama Universidad Autonoma del Estado de Mexico Mexico, Toluca MEXICO
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universidad Autonoma del Estado de Mexico
Corresponding Author's Secondary Institution:	
First Author:	E. Alpizar-Reyes
First Author Secondary Information:	
Order of Authors:	E. Alpizar-Reyes
	J. Castaño
	H. Carrillo-Navas
	J. Alvarez-Ramírez
	R. Gallardo-Rivera
	C. Pérez-Alonso
	A.Y. Guadarrama-Lezama
Order of Authors Secondary Information:	
Funding Information:	Project PRODEP (DSA/ 103.5/15/7186) — PhD A.Y. Guadarrama-Lezama
	Program of Basal Financing Program for Scientific and Technological Centers of Excellence (PFB-27) — PhD J. Castaño
Abstract:	Freeze-dried faba bean (Vicia faba L.) protein adsorption isotherms were determined at 25, 35 and 40 °C and fitted with the Guggenheim-Anderson-de Boer model. The pore radius of protein was in the range of 0.87 to 6.44 nm, so that they were considered as micropores and mesopores. The minimum integral entropy ranged between 4.33 and 4.44 kg H2O/100 kg d.s., was regarded as the point of maximum of stability. The glass transition temperature of the protein equilibrated at the different conditions of storage was determined, showing that the protein remained in glassy state for all cases. The protein showed compact and rigid structures, evidenced by microscopy analysis
Response to Reviewers:	Response to Comments Made by Reviewer #1 Manuscript No. JFST-D-16-01673 “Thermodynamic sorption analysis and glass transition temperature of faba bean (Vicia faba L.) protein” GENERAL REVIEWER’S COMMENT: “Minor revision for acceptance. Below some comments:” REVIEWER’S COMMENT #1: “The authors need to review the keywords. Faba bean

protein, Sorption isotherms, Thermodynamic analysis, Glass transition temperature are not keywords."
RESPONSE: The keywords were changed to reflect more clearly our contribution.

REVIEWER'S COMMENT #2: "The introduction section could be separated in paragraphs to improve the text in general."
RESPONSE: The introduction section was improved as the reviewer suggested.

REVIEWER'S COMMENT #3: "Lines 30 to 33: When dry products are evaluated, the most correct method is to describe the adsorption isotherms due to mainly the water adsorption phenomena observed in powder products."
RESPONSE: The paragraph was re-written in the corrected manuscript.

REVIEWER'S COMMENT #4: "Lines 50 to 51: "Based on that, the objective of this work was five-fold..." In fact it was not only four?"
RESPONSE: The reviewer is right. The sentence was changed using a main objective rather than particularly objectives as Reviewer #2 also suggested.

REVIEWER'S COMMENT #5: "Section 2.5: The authors calculating the thermodynamic properties using Clausius-Clapeyron methodology. Why? This methodology doesn't evaluate the effects of temperature. It presents the thermodynamic properties as an average of the temperature while the Othmer methodology presents a correction for each temperature. I suggest that authors write about this question on the manuscript. Several articles use Othmer methodology due to this limitation of Clausius-Clapeyron methodology. See below some examples:

Silva, E. K., et al. (2014). "Water adsorption in rosemary essential oil microparticles: Kinetics, thermodynamics and storage conditions." Journal of Food Engineering 140: 39-45.

Viganó, J., et al. (2012). "Role of enthalpy and entropy in moisture sorption behavior of pineapple pulp powder produced by different drying methods." Thermochimica Acta 528: 63-71."
RESPONSE: In the corrected manuscript, we incorporated a brief discussion explaining the justification to use the Clausius-Clapeyron methodology.

Response to Comments Made by Reviewer #2
Manuscript No. JFST-D-16-01673
"Thermodynamic sorption analysis and glass transition temperature of faba bean (Vicia faba L.) protein"

REVIEWER'S COMMENT #1: "Regarding to the objectives: What is the main purpose? The general aim should be Thermodynamic sorption analysis and glass transition temperature of faba bean (Vicia faba L.) protein."
RESPONSE: The main objective of this work was included in the end of the introduction section in the corrected manuscript, as follows: "The objective of this manuscript was determined sorption thermodynamic properties and glass transition temperature of faba bean (Vicia faba L.) protein to establish optimal conditions of storage."

REVIEWER'S COMMENT #2: "In the material and methods section. Line 78-83: It was not reported the temperature used in the lyophilizer, only the pressure and time."
RESPONSE: The temperature used in the freeze drying process was -60 °C.

REVIEWER'S COMMENT #3: "Similarity index is very high (44%), reduce and resubmit. Report is attached."
RESPONSE: Following the reviewer's recommendation, many paragraphs, mainly in Materials & Methods section, were rephrased to avoid similarity with published papers, although these methods have been developed and established in several publications by our research group.

Response to Comments Made by Reviewer #1

Manuscript No. JFST-D-16-01673

"Thermodynamic sorption analysis and glass transition temperature of faba bean (*Vicia faba* L.) protein"

GENERAL REVIEWER'S COMMENT: "*Minor revision for acceptance. Below some comments:*"

REVIEWER'S COMMENT #1: "*The authors need to review the keywords. Faba bean protein, Sorption isotherms, Thermodynamic analysis, Glass transition temperature are not keywords.*"

RESPONSE: The keywords were changed to reflect more clearly our contribution.

REVIEWER'S COMMENT #2: "*The introduction section could be separated in paragraphs to improve the text in general.*"

RESPONSE: The introduction section was improved as the reviewer suggested.

REVIEWER'S COMMENT #3: "*Lines 30 to 33: When dry products are evaluated, the most correct method is to describe the adsorption isotherms due to mainly the water adsorption phenomena observed in powder products.*"

RESPONSE: The paragraph was re-written in the corrected manuscript.

REVIEWER'S COMMENT #4: "*Lines 50 to 51: "Based on that, the objective of this work was five-fold..." In fact it was not only four?*"

RESPONSE: The reviewer is right. The sentence was changed using a main objective rather than particularly objectives as Reviewer #2 also suggested.

REVIEWER'S COMMENT #5: "*Section 2.5: The authors calculating the thermodynamic properties using Clausius-Clapeyron methodology. Why? This methodology doesn't evaluate the effects of temperature. It presents the thermodynamic properties as an average of the temperature while the Othmer methodology presents a correction for each temperature. I suggest that authors write about this question on the manuscript. Several articles use Othmer methodology due to this limitation of Clausius-Clapeyron methodology. See below some examples:*

Silva, E. K., et al. (2014). "Water adsorption in rosemary essential oil microparticles: Kinetics, thermodynamics and storage conditions." Journal of Food Engineering 140: 39-45.

Viganó, J., et al. (2012). "Role of enthalpy and entropy in moisture sorption behavior of pineapple pulp powder produced by different drying methods." Thermochimica Acta 528: 63-71."

RESPONSE: In the corrected manuscript, we incorporated a brief discussion explaining the justification to use the Clausius-Clapeyron methodology.

Response to Comments Made by Reviewer #2

Manuscript No. JFST-D-16-01673

"Thermodynamic sorption analysis and glass transition temperature of faba bean (*Vicia faba* L.) protein"

REVIEWER'S COMMENT #1: "*Regarding to the objectives: What is the main purpose? The general aim should be Thermodynamic sorption analysis and glass transition temperature of faba bean (Vicia faba L.) protein.*"

RESPONSE: The main objective of this work was included in the end of the introduction section in the corrected manuscript, as follows: "The objective of this manuscript was determined sorption thermodynamic properties and glass transition temperature of faba bean (*Vicia faba* L.) protein to establish optimal conditions of storage."

REVIEWER'S COMMENT #2: "*In the material and methods section. Line 78-83: It was not reported the temperature used in the lyophilizer, only the pressure and time.*"

RESPONSE: The temperature used in the freeze drying process was -60 °C.

REVIEWER'S COMMENT #3: "*Similarity index is very high (44%), reduce and resubmit. Report is attached.*"

RESPONSE: Following the reviewer's recommendation, many paragraphs, mainly in Materials & Methods section, were rephrased to avoid similarity with published papers, although these methods have been developed and established in several publications by our research group.

UAEM | Universidad Autónoma del Estado de México

August 1st, 2017

Professor Narpinder Singh

Editor-in-Chief

Journal of Food Science and Technology

RE: Manuscript No. JFST-D-16-01673. "Thermodynamic sorption analysis and glass transition temperature of faba bean (*Vicia faba* L.) protein"

Dear Professor Singh,

We are submitting the corrected version of the manuscript referred above. The manuscript includes 21 double-spaced pages, 5438 words, 36 references, 2 tables, 4 figures. Besides, the results contained in the corrected manuscript are original and have not been published elsewhere.

All the authors have read and approved the corrected manuscript and all are aware of the submission to Journal of Food Science and Technology. Furthermore, all authors have not declared conflict of interest.

The proposed highlights are the following:

- Sorption isotherms of FD-FBP presented a sigmoidal shape.
- The GAB equation was suitable for modeling moisture sorption of FD-FBP.

- The pore radius of FD-FBP varied from 0.87 to 6.44 nm.
- The minimum integral entropy was in the range ranged of 0.24 to 0.40.
- Glassy state was exhibited by protein regardless its a_W and moisture conten.

By submitting the corrected manuscript the corresponding author (A.Y. Guadarrama-Lezama) recognized her compromise to review at least three manuscripts submitted to Journal of Food Science and Technology.

The reviewers have a positive opinion on our work, and provided minor recommendations oriented to improve the quality of our work. The actions taken to attend the reviewer's recommendations are detailed in the response letter.

We acknowledge the editor for giving us the opportunity of submitting our research work to Journal of Food Science and Technology.

Sincerely,

A.Y. Guadarrama-Lezama

Thermodynamic sorption analysis and glass transition temperature of faba bean (*Vicia faba* L.) protein

E. Alpizar-Reyes[a], J. Castaño[b], H. Carrillo-Navas[c], J. Alvarez-Ramírez[c], R. Gallardo-Rivera[d], C. Pérez-Alonso[a], A.Y. Guadarrama-Lezama[a, *]

[a] *Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón esq. Paseo Tollocan s/n, Col. Residencial Colón, C.P. 50120, Toluca, Estado de México, México.*

[b] *Unidad de Desarrollo Tecnológico, Universidad de Concepción, Avenida. Cordillera, C.P 2634, Coronel, República de Chile.*

[c] *Departamento de Ingeniería de Procesos e Hidráulica, Universidad Autónoma Metropolitana-Iztapalapa, San Rafael Atlixco No. 186, Col. Vicentina, C.P. 09340, México, D.F., México.*

[d] *Departamento de Biotecnología, Universidad Autónoma Metropolitana-Iztapalapa, San Rafael Atlixco No. 186, Col. Vicentina, C.P. 09340, México, D.F., México.*

[*] Corresponding author: Tel.: +52 722 2173890; Fax: +52 722 2175109. E-mail: ayguadarramal@uaemex.mx (A.Y. Guadarrama-Lezama).

(Corrected Manuscript No. JFST-D-16-01673R1)

ABSTRACT

Freeze-dried faba bean (*Vicia faba* L.) protein adsorption isotherms were determined at 25, 35 and 40 °C and fitted with the Guggenheim-Anderson-de Boer model. The pore radius of protein was in the range of 0.87 to 6.44 nm, so that they were considered as micropores and mesopores. The minimum integral entropy ranged between 4.33 and 4.44 kg H_2O/100 kg d.s., was regarded as the point of maximum of stability. The glass transition temperature of the protein equilibrated at the different conditions of storage was determined, showing that the protein remained in glassy state for all cases. The protein showed compact and rigid structures, evidenced by microscopy analysis.

KEYWORDS: Faba bean protein; Pore radius; GAB; Minimum integral entropy; Glassy state.

1. Introduction

Legumes are good resources of proteins, carbohydrates, fiber, vitamins and minerals with functional and health-promoting activities contributing to human nutrition. Faba bean is composed of proteins (~30%), carbohydrates (60%) and fats (10%). Among the identified proteins in faba bean, the main are globulins (79%), albumins (7%) and glutelins (6%). The study of faba bean protein is of special interest because of its nutritional value, availability, and health benefits (Vioque et al. 2012). The protein extracted from faba bean can be used within a powder product, so it is important to dry the protein in order to decrease its moisture content until the required level, delay its deterioration and consequently have a hydrocolloid in powder of easy use and application such as emulsifier or stabilizer in different food products (Karaca et al. 2011). Among the different drying processes to this purpose, the freeze drying is often used to dehydrate heat-sensitive foods because of the absence of heating in this process preserving nutrients and their sensory characteristics. Hence, freeze drying is a good alternative to remove free water from proteins (Ghribi et al. 2015).

The preservation and stability of faba bean protein (FBP) involve the knowledge of the appropriate conditions to store it in order to predict deteriorative reactions, and enhance its stability in function of moisture content, water activity and temperature. This task is achieved by relating the above three parameters in the sorption isotherms. Water sorption isotherms provide information about the stability of food powder, and is also helpful to understand the structural features, such as sorption properties and thermodynamic functions of vapor water sorption (Silva et al. 2014).

Thermodynamic functions such as enthalpy and entropy are indices that provide valuable information to understand the properties of water, calculate the energetical requirements associated with the adsorption phenomena, and describe how the water interacts with the powder. Specifically, the minimum integral entropy (ΔS_{int}) is the most suitable criterion to predict the maximum storage stability in powder foodstuffs (Pérez-Alonso et al. 2006). Under minimum integral entropy, the powder food product has the highest stability with the suitable ratio of moisture content/water activity. On the other hand, changes in enthalpy are associated with bonding of repulsive forces between water and the food powder. The process related with enthalpy involves the strength of the intermolecular interaction or bonding, while that related with entropy reflects the randomness and disorder of the system. The glass transition temperature (Tg) has used as parameter for establishing stability conditions in a food powder from a physical or structural point of view (Basu et al. 2013). This parameter allows knowing if the powdered foods exhibit changes in their physical properties during storage such as collapsibility, stickiness, caking, agglomeration and re-crystallization phenomena as effect of its water content.

The aim of this manuscript was determined sorption thermodynamic properties and glass transition temperature of faba bean (*Vicia faba* L.) protein to establish optimal conditions of storage.

2. Materials and methods

Faba bean (*Vicia faba* L.) seeds were purchased in a local market (Toluca, Mexico). All reagents were analytical grade and were purchased from Sigma Aldrich S.A. de C.V. (Toluca, Mexico). Deionized water was used in all the experiments.

2.1. Protein extraction

Faba bean (*Vicia faba* L.) seeds were sun dried until reaching 5% wt. Then it was crushed with a mill (Pulvex 200, Mexico City, Mexico). Faba bean seed flour was sieved using a 70 mesh (Tyler Standard Sieve Series, Mentor, Ohio, USA). Extraction of the FBP was carried out according to Vioque et al. (2012). The flour was defatted with hexane using soxhlet equipment. Hexane was evaporated of the sample by exposing it in a laminar flow hood, before carrying out protein extraction. 10g of defatted flour was added to 100 mL of a Na_2SO_3 solution (0.25% w/v) and its pH was adjusted to 10.5 with a NaOH solution. The solution was kept under stirring 24h at 4°C. Further coarse alkaline solids were separated by centrifugation (5415R Eppendorf AG, Hamburg, Germany) at 15000 × g during 10 min. The soluble fraction was transferred to a beaker and the pH adjusted to 4.0 (isoelectric pH of the FBP). The resulting solution was centrifuged again at 15000 × g by 10 min. The FBP was washed twice with deionized water and stored for subsequent freeze drying process.

2.2. Freeze drying process

Freeze drying was carried out using a laboratory freeze dryer (Freezone 6, Labconco, MO, USA). The solution of FBP was poured into a glass recipient to form a layer of 15 mm. The samples were placed at -40 °C for 24 h before transferring to the freeze dryer using a Labconco Bench Lyph-lock 6 laboratory freeze-dryer (Labconco, MO, USA). The vacuum pressure of the dryer was set at 5 mmHg, and the condenser was at -60 °C for 48 h. Dehydrated product was stored in desiccators above P_2O_5, in order to prevent any increase in adsorbed moisture, until being required for experiments.

2.3. Sorption isotherms

Adsorption isotherms at 25, 35 and 40 °C (± 0.1 °C) were determined through the static gravimetric described by Lang et al. (1981) using eight saturated salt solutions (lithium chloride, potassium acetate, magnesium chloride, potassium carbonate, magnesium nitrate, sodium nitrite, sodium chloride and potassium chloride) with water activities (a_W) ranging from 0.11 to 0.85 (Mousa et al., 2014). Samples of approximately 0.5 g of freeze-dried powder faba bean protein (FD-FBP) were placed into small glass desiccators of 10 cm diameter on filter paper (Whatman No. 1) that was placed above the saturated salt solutions in a perforated plate used as a support for the samples to allow moisture transmission. Five desiccators with each sample of FD-FBP were placed into three forced convection drying ovens (Riossa, model E-51, Mexico City, Mexico) maintained the specified temperature until equilibrium was reached (between 20 and 25 days). Equilibrium was assumed when the difference between two consecutive measurements was less than 1 mg/g of solids. To prevent microbial spoilage of samples, 0.2 mL of toluene was placed in the desiccators where high water activities occurred (a_W > 0.5). Moisture content of the freeze-dried protein was determined by difference in weight after drying in a vacuum drying (Felisa model FE 100, Mexico City, Mexico) at 60 °C for 24 h in the presence of magnesium perchlorate desiccant. The water activity was measured with an Aqualab water activity meter with temperature compensation (model series 3 TE, Decagon Devices, Inc., Pullman, WA, USA). Longer drying times did not produce a sample weight decrease of more than 0.1 mg. The GAB (Guggenheim-Anderson-De Boer) equation is mathematically expressed as (equation 1):

$$M = \frac{M_0 C K a_W}{(1 - K a_W)(1 - K a_W + C K a_W)} \quad (1)$$

where M is the equilibrium moisture content (kg/100 kg of dry solids (d.s.)), M_0 is the moisture content of the monolayer (kg/100 kg d.s.), C and K are constants related to the energies of interaction between the first and distant adsorbed molecules at the individual sorption sites. The parameters were estimated by fitting the mathematical model to the experimental data, using non-linear regression with Origin Pro version 8.5 Scientific Software (Origin Lab Corp., Northampton, MA, USA). The mean relative deviation modulus (*MRD*) was used to evaluate the goodness of fit. The *MRD* value is given as a percentage and may be estimated as follows

$$MRD = \frac{100}{n}\sum \frac{\left|M_1 - M_{Ei}\right|}{M_i} \quad (2)$$

where M_i refers to the experimental value of moisture content at i; M_{Ei} is the predicted moisture content at that observation and N is the number of observations. It is generally assumed that a good fit is obtained when $MRD < 5\%$.

2.4. Sorption properties

The sorption surface area (S_0) was evaluated using the following equation (Moraes and Pinto 2012):

$$S_0 = M_0 \frac{1}{M_w} N_0 A_{H_2O} = 3.5\times10^3 M_0 \quad (3)$$

where M_w is the molecular weight of water (kg/mol), N_0 is the Avogadro number (6.0×10^{23} molecules/mol) and A_{H_2O} is the area of a water molecule (1.06×10^{-19} m^2). The moisture content of the monolayer to calculate the area was obtained from the GAB model.

The critical radius was calculated using Kelvin equation:

$$r_c = \frac{2\sigma V_M}{RT\ln(a_W)} \quad (4)$$

where r_c is the critical radius (m), σ is the surface tension (N/m), V_M is the molar volume of sorbate (m^3/mol), R is the universal gas constant (8.314 × 10^{-3} kJ/mol K), T is the temperature (K) and a_W is the water activity. This equation applies primarily in the condensation region of the isotherm (Moraes and Pinto 2012). On the other hand, the Halsey equation:

$$t = 0.354\left(\frac{-5}{\ln a_W}\right)^{1/3} \quad (5)$$

where t is the thickness of the adsorbed water multilayer (nm), was used to evaluate the thickness of the adsorbed water multilayer (Singh et al. 2001). Finally, pore radius (r_p) is the sum of the critical radius when the capillary condensation or evaporation occurs (r_c) and the multilayer thickness (t):

$$r_p = r_c + t \quad (6)$$

2.5. Thermodynamic functions

The integral (enthalpy and entropy) as thermodynamic functions were determinated as indicated by Velázquez-Gutierrez et al. (2015); these authors have provided a thorough description of the following procedures and equations used for this purpose. The pressure of diffusion (Φ) can be determined by Nunes and Rotstein (1991):

$$\Phi = \mu_{ap} - \mu_a = RT\frac{W_{ap}}{W_v}\int_0^{a_W} Md\ln a_W \quad (7)$$

$$\Phi = \alpha_1 T\int_0^{a_W} Md\ln a_W \quad (8)$$

where μ_{ap} is the chemical potential of the pure adsorbent; μ_a is the chemical potential of the adsorbent in the condensed phase; W_{ap} is adsorbent molecular weight; W_v is the water molecular weight; Φ/α_1 constant is similar to a process at constant Φ. Eq. (8) was stepwise evaluated. For $a_W < 0.05$, the computed values of the constant pressure of diffusion at any temperature were determined assuming a linear relationship (Henry's law) (Nunes and Rotstein, 1991):

$$M = k_w a_W \tag{9}$$

where k_w is the slope of the Eq. (9). When $a_W > 0.05$, the GAB model (Eq. (1)) was used. Therefore, Eqs. (1) and (9) are substituted in Eq. (8) to determine Φ/α_1. This parameter was calculated with the Runge-Kutta method using MatLab 2013b Software (Math Works Inc., Natick, MA, USA).

The integral enthalpy was calculated using the Clausius-Clapeyron equation at constant diffusion pressure or surface potential (Φ) (Nunes and Rotstein, 1991):

$$\left(\frac{\partial \ln a_W}{\partial (1/T)}\right)_{\Phi} = \frac{H_s - H_l}{R} = \frac{\Delta H_{\text{int}}}{R} \tag{10}$$

where H_s is the integral molar enthalpy of water adsorbed of the mucilage (kJ/mol), H_l is the partial molar enthalpy of adsorbed water at constant temperature and pressure (kJ/mol), R is universal gas constant (8.314×10^{-3} kJ/mol K) and ΔH_{int} is the integral enthalpy at a constant temperature (kJ/mol). A plot in the form ln a_W vs $1/T$, at a specific constant pressure of diffusion, ΔH_{int} is determined from the slope $\Delta H_{\text{int}}/R$.

The use of the Clausius–Clapeyron equation implies that the moisture content of the system under consideration remains constant and that the enthalpy of vaporization of pure

water (as well as the excess heat of sorption) does not change with temperature (Rizvi, 1986).

The integral enthalpy is needed to determine the integral entropy associated with the sorption process. The integral entropy can be calculated as follows:

$$\Delta S_{\text{int}} = S_s - S_l = \frac{\Delta H_{\text{int}}}{T} - R \ln a_W \quad (11)$$

where S_s = S/N_1 is the integral entropy of water adsorbed in the protein; S_l is the partial molar entropy of adsorbed water at constant temperature and pressure (kJ/mol) and ΔS_{int} is the integral entropy at a constant temperature.

2.6. Glass transition temperature

A differential scanning calorimetry (Q1000, TA-Instruments, New Castle, DE, USA) equipped with a mechanical cooling system (RCS-refrigerated cooling accessory) was used to perform experiments. The calorimeter was calibrated according to the instructions provided by TA instruments user manual by checking temperature and enthalpy of fusion of indium and sapphire as standards. An empty aluminum hermetic pan was used as a reference. The instrument was purged with nitrogen at a flow rate of 100 cm^3/min. Freeze-dried faba bean protein samples (4-5 mg) equilibrated at different water activities were in the range of the 0.11−0.85 and temperatures (25, 35 and 40 °C) were placed in aluminum hermetic pans and were cooled to -50 °C at 2.5 °C/min, and equilibrated for 2 min. After equilibrated it was scanned from -50 °C to 150 °C at a rate of 2.5 °C/min. Each thermogram was analyzed for the glass transition temperature (*Tg*), and midpoint values were used. The data were analyzed using Universal Analysis 2000 software, version 4.7a (TA Instruments, New Castle, USA).

2.7. Morphology by scanning electron microscopy (SEM) analysis

A Scanning Electron Microscope (JSM-7600F, Jeol Co. Ltd., Tokyo, Japan) of high vacuum with the GB-H mode at 1 kV accelerating voltage, was used to investigate the microstructural properties of the FBP samples previously kept under controlled atmospheres such as described in section 2.5. The samples were mounted on carbon sample holders using double-side sticky tape. Micrographs at 2000× magnification are presented.

3. Results and discussion

3.1. Sorption isotherms

The experimental sorption isotherms at 25, 35 and 40 °C of FBP are shown in Figure 1. The amount of sorbed water increased as temperature increased, at a constant value of water activity. This could be explained by the degree of exposure of hydrophobic regions of protein. It is possible that when the protein is elongated, the water adsorption on the specific sites is being promoted and, when the protein is short, the water adsorption on the specific sites becomes more difficult. Therefore, when temperature increases, the degree of exposure of hydrophobic regions also increases. It improves the amount of water strongly adsorbed to specific sites. According to Brunauer's classification (Brunauer et al. 1938), FBP isotherms had typical type II sigmoid shape. This type of isotherms is commonly observed in vegetal and animal derived proteins as cottonseed protein isolate (Tunç and Duman 2007), soy protein (Cassini et al. 2006), whey protein powder (Zhou and Labuza 2007), acid casein from buffalo skim milk (Sawhney et al. 2011), gelatin (Kasapis and Sablani 2005). The experimental sorption data were fitted to GAB model. The model parameters M_0, C and K were determined by non-linear regression procedure (Table 1). The

mean relative deviation modulus (*MRD*) values were less than 2% and the coefficients of determination (R^2) were over 0.998 for all temperatures. The value of the monolayer (M_0) is of particular interest. It shows the amount of water that is strongly adsorbed to specific sites and is considered as the optimum value at which a food is more stable. The values of the monolayer of FBP were in the range of 4.32–5.52 kg H_2O /100 kg d.s. and decreased as temperature increased from 25 to 40 °C. These results can be attributed to reductions in the number of available sites for water binding due to excitation state of molecules, an increase in kinetic energy leading to an increase in the distance between them. Therefore, water molecules with a low motion at low temperatures bound more easily to suitable binding sites on surfaces (McLaughlin and Magee 1998). The water adsorption in other proteins extracted from legumes such as soy protein and cowpea, has been reported in the literature, where the monolayer moisture content values has been found in a range between 1.39 and 7.4 kg H_2O/100kg d.s. (Cassini et al. 2006; Ayranci and Duman 2005). These values are in the range where the monolayer moisture content is found for the FBP (Table 1). There is not a clear tendency in *Mo* values depending on the chemical nature of the protein (vegetal or animal) and its chemical structure (globular, fibrillary and others). It has been stated that the amount of sorbed water in proteins depends essentially on the number and availability of two kinds of hydrophilic groups that enhance the binding water through hydrogen bond formation. These are the polar side chains and the carbonyl and amid groups of peptide bonds. Water sorption by proteins occurs into polar side chains at low humidities, spreads to peptide linkages and then leads to multilayer formation at higher humidities (Singh et al. 2001). On the other hand, it can be seen that the maximum moisture content reached for the FD-FBP was about 12 kg H_2O/100kg d.s., while for others hydrocolloids and biopolymers the moisture contents values are higher, in the same range of studied water activities

(Włodarczyk-Stasiak and Jamroz 2008). This is due to the protein having hydrophobic groups in their structure, which does not favors the adsorption of moisture in the surface of the hydrocolloid. The parameter *C* obtained from the GAB model is related to the heat of adsorption of water by the FD-FBP. It is assumed that strong adsorbent-adsorbate interactions are favored at lower temperatures, resulting in an increase in *C* with increasing temperature (Diosady et al. 1996). In this work the value of *C* decreased with increasing temperature suggesting that interactions between the freeze-dried FBP and water vapor were lower at 25 ºC than at 35 ºC and 40 ºC. Alternatively, it is possible that *C* lacks any physical meaning being the result of mathematical compensation among parameters during the curve-fitting process.

The values of *K* involve interactions between water molecules and the adsorbent (protein) in the multilayer. $K<<1$ indicates a structured state of the adsorbate in the adjacent layers to the monolayer (Lewicki 1997). In this study, *K* values were in range of 0.71-0.74 for the FD-FBP, which implies that there were fewer interactions between water molecules and the protein in the multilayer, and a tendency was not found as the temperatures increase or decrease.

3.2. Sorption properties

The sorption properties are physical properties described as pore radius, critical radius pore, surface area of sorption and multilayer thickness, which determine the water adsorption rate on solids. They have been studied using the GAB model. The rate and extent of hydration of the food materials are decided by the surface properties of pores. Furthermore, the temperature at each pore is likely to affect the rate of entry and exit of water molecules (Kapsalis 1981). The specific surface area values for the FBP were 193.30, 159.41, and

151.29 m^2 / g for 25, 35 and 40 °C, respectively. The sorption surface area values decrease as temperature increases. This behavior is attributed to the availability of active sites for hydrophilic binding energies, which decreased as consequence of induced changes by temperature, modifying physical and chemical properties of the adsorbent (Rakshit et al. 2014). Labuza (1968) indicated that S_0 values of food products are within the range of 100-250 m^2/g. Some authors reported similar values of S_0 for vegetable proteins. For instance, Tunç and Duman (2007) reported values between 115.3-137.4 m^2/g at 15-45 °C for cottonseed protein isolate. Moreover Cassini et al. (2006) analyzed texturized soy protein finding values between 162.75 and 260.05 m^2/g at 10 and 40 °C.

Pore radius (r_p) of FBP was determined at different moisture contents and temperatures (Table 2). The pore radius increased as moisture content and temperature increased and ranged in values between 0.75-6.68 nm. In agreement with the International Union of Pure and Applied Chemistry (IUPAC) pore radius less than 2 nm are classified as micropores, and pore radius between 2 and 50 nm are denoted as mesopores (Moraes and Pinto 2012). Therefore, at moisture contents between 1 and 6 kg H_2O/100 kg d.s., the FBP presented micropores and when the moisture content is ≥ 6 kg H_2O/100 kg d.s mesopores were observed. The diffusion mechanisms depend on the properties within the local structure of the porous matrix. In micropores, diffusion is dominated by interactions between the water molecules and the pore walls, i.e., steric, and other effects associated with the proximity of the pore walls (entropic effects) become important, and barriers control the process. In mesopores, surface forces and capillary forces become important, whereas for the macropores, very little is contributed by the pore characteristics to the adsorption capacity (Viganó et al. 2012). According to this description, the adsorption mechanism is controlled by diffusion and capillarity phenomena in the FBP.

3.3. Thermodynamic functions

Integral enthalpy ΔH_{int} is the parameter that indicates the degree of water-solid interaction. The net equilibrium heat for the FBP was determined. It increased to maximum value of 31 kJ/mol at 3.5 kg H_2O/100 kg d.s. of moisture content. At this point the strongest binding sites and the greatest water-protein powder interaction occurs. From this point, integral enthalpy gradually decreased as the moisture content increased. An increase on the moisture content indicates the covering of fewer favorable sites promoting the formation of multi-layers for which the enthalpy is reduced. Besides, low enthalpies values (less than 60 kJ/mol) are associated with physical sorption (Smith et al. 2005). In this work, the integral enthalpy values in the range of moisture content studied were <60 kJ/mol and therefore it can be assumed that a physisorption phenomenon is occurring.

The integral entropy is a thermodynamic function used to describe the degree of arrangement of water molecules during the sorption process. The ΔS_{int} is considered as the maximum stability point. It is here where water molecules have more ordered arrangement with the solid (FBP) and strong bonds between the adsorbate and the adsorbent occur. Thus, water is less available to carry out spoilage reactions (Bonilla et al. 2010; Viganó et al. 2012). Figure 2 shows the integral entropy as a function of moisture content at 25, 35 and 40 °C of FBP. The integral entropy decreased until reaching a minimum point and then increased as moisture content increased. The decrease in integral entropy represents a diminution on mobility of water molecules promoting the saturation of available sites. Higher energy is required to continue the adsorption phenomena. Thus the increase implies that water molecules are able to form multilayers. At higher moisture contents, entropy will be approximately the same as liquid water (McMinn and Magee 2003). The conditions for maximum stability of FBP were 4.33 kg H_2O/100 kg d.s. (a_W = 0.40) at 25 °C, 4.37 kg

H_2O/100 kg d.s. (a_W = 0.36) at 35 °C, and 4.44 kg H_2O/100 kg d.s. (a_W = 0.24) at 40 °C, as it can be observed in Figure 2. As temperature increased, the moisture content and water activity in the FBP increased. Besides, the integral entropy can be directly related to the order-disorder of water molecules sorbed on protein and, therefore, it is a useful function on the stability food.

3.4. Glass transition temperature (Tg) and morphology by Scanning Electron Microscopy (SEM)

The moisture content, water activity (a_W) and glass transition temperature (*Tg*) are parameters used in order to predict the stability of a food during processing and storage, because its physical state is affected by these factors. The glass transition temperature (*Tg*) of the FBP at different water activities is shown in Figure 3. It can be observed that *Tg* decreases as water activity increases due mainly to the plasticization effect of the water. Also it can be appreciated that at the same level of water activity, the *Tg* is the highest at the temperature of 40°C of storage. The *Tg* values ranged from 9.78 to 47.10 °C.

Values of *Tg* for the FBP were lower than those reported from other proteins. Protein isolated from barley with *Tg* values from 48 °C to 63 °C at a moisture content of 0.12% (van Donkelaar et al. 2015), gelatin with *Tg* of 100 °C in anhydride conditions (Kasapis and Sablani 2005), whey protein isolate (WPI, with water content of 6-16 %), and whey protein hydrolysates (WPH, with water content of 50–26 %) with a *Tg* between 119-75 °C, and 99-15 °C, respectively (Zhou and Labuza 2007), and soy, casein and gluten protein (with moisture contents less than 30 %) with *Tg´*s ranged approximately from 50-105, 20-105 and 10-140 °C, respectively (Bengoechea et al. 2007). It is possible that the variation of *Tg* among proteins may be due to the nature of them, such as source (vegetal or

animal, or modification method such as hydrolysis, unfolding or denaturation). In addition, the values of glass transition temperature for the different kind of proteins also may be due to the existence of hydrophilic-hydrophobic sites in its molecular arrange, total apparent charge, the presence of sulfhydryl groups and disulphites-sulfhydryl bonds (Caurie 1981).

SEM micrographs of the FBP at 25 °C and different water activities are shown in Figure 4. The micrographs of the FBP showed that not structural changes were noticeable, all the samples showed dense, continues, compact and rigid structures regardless of their water activity. Thus, it can be inferred that the protein was in glassy state even at high water activities. SEM micrographs of the protein equilibrated at 35 and 40 °C, at a_W's of 0.2, 0.4 and 0.6 respectively, showed a similar trend (not shown here). Therefore, the FBP appeared to be stable in the range of tested temperature (25, 35 and 40 °C) even at high water activity values, and there was not evidence of stickiness, caking or collapsing. Within literature, it has been reported that different drying methods (freeze drying and convective drying) used for the preparation of protein concentrates in powders, influence its thermal and functional properties (Ghribi et al. 2015). It seems to be that the drying method by which the protein concentrates are obtained, has influence on the microstructure and the changes that it presents during storage. Other hydrocolloids obtained by freeze-drying have remained in glassy state in the water activity range of 0.11–0.85 such as the chia mucilage (Velázquez-Gutiérrez et al. 2015; Capitani et al. 2013), while those obtained by spray drying have presented coalescence, agglomeration, stickiness and collapsing phenomena, such as maltodextrin and skim milk (Malafronte et al. 2016).

4. Conclusions

Sorption isotherms for FBP presented a sigmoidal shape. Experimental data of moisture content and water activity of the FBP were well adjusted to the GAB model. The pore radius of the freeze-dried FBP presented increasing values as moisture content and temperature increased so that they were considered as micropores and mesopores, which have an effect in the adsorption mechanism. Not only micropores but also mesopores are able to allow the physisorption mechanism and form the monolayer. The minimum of integral entropy was found at low water activities (between 0.24-0.40) for the temperature range between 25 and 40 °C. It means that at these water activities there exist the most suitable conditions for storing the FBP. The FBP showed dense, continuous, compact and rigid structures, and was inferred that it remained in glassy state in the range of water activity at which the protein was equilibrated. The FBP properties suggest that it could be stored under conditions of temperature, moisture content and water activity determined in this study and used as additive in powder as possible emulsifier or stabilizers.

No conflict of interest is declared by the authors

Acknowledgements

The authors thank the financial support for this work from the Autonomous Mexico State University (UAEM-Mexico), through the Project PRODEP DSA/ 103.5/15/7186, as well as from the Program of Basal Financing Program for Scientific and Technological Centers of Excellence (PFB-27).

References

Ayranci E, Duman O (2005). Moisture sorption isotherms of cowpea (*Vigna unguiculata* L. Walp) and its protein isolate at 10, 20 and 30 °C. J Food Eng 70:83–91

Basu S, Shivhare US, Muley S (2013). Moisture adsorption isotherms and glass transition temperature of pectin. J Food Sci Technol 50(3):585-589

Bengoechea C, Arrachid A, Guerrero A, Hill SE, Mitchell JR (2007). Relationship between the glass transition temperature and the melt flow behavior for gluten, casein and soya. J Cereal Sci 45(3):275–284

Bonilla E, Azuara E, Beristain CI, Vernon-Carter EJ (2010). Predicting suitable storage conditions for spray-dried microcapsules formed with different biopolymer matrices. Food Hydrocolloid 24:633–640

Brunauer S, Emmett PH, Teller E (1938). Adsorption of gases in multimolecular layers. J Am Chem Soc 60:309–319

Capitani M I, IxtainaVY, Nolasco SM, Tomás M C (2013). Microstructure, chemical composition and mucilage exudation of chia (*Salvia hispanica* L.) nutlets from Argentina. J Sci Food Agr 93:3856–3862

Cassini AS, Marczak LDF, Noreña CPZ (2006). Water adsorption isotherms of texturized soy protein. J Food Eng 77:194–199

Caurie M (1981). Derivation of full range moisture sorption isotherms. *Academic Press Publishers* 63-87

Diosady LL, Rizvi SSH, Cai W, Jagdeo DJ (1996). Moisture sorption isotherms of canola meals, and applications to packaging. J Food Sci 61(1):204–208

Ghribi AM, Gafsi IM, Blecker C, Danthine S, Attia H, Besbes S (2015). Effect of drying methods on physico-chemical and functional properties of chickpea protein concentrates. J Food Eng 165:179–188

Hill TL, Emmet PH, Joyner LG (1951). Calculation of thermodynamic functions of adsorbed molecules from adsorption isotherm measurements: nitrogen on graphon. J Am Chem Soc 73:5102–5107

Kapsalis JG (1981). Moisture sorption hysteresis. In: Rockland LB and Stewart GF (Eds). Water activity: influences on food quality. Academic Press, New York

Karaca AC, Low N, Nickerson M (2011). Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. Food Res Int 44:2742–2750

Kasapis S, Sablani SS (2005). A fundamental approach for the estimation of the mechanical glass transition temperature in gelatin. Int J Biol Macromol 36(1):71–78

Labuza TP (1968). Sorption phenomena in foods. Food Technol 22(3):263–272

Lang KW, McCune, TD, Steinberg MP (1981). A proximity equilibration cell for rapid determination of sorption isotherms. J. Food Sci. 46:936–938

Lewicki PP (1997). The applicability of the GAB model to food water sorption isotherms. Int J Food Sci Tech 32(6):553–557

Malafronte L, Ahrné L, Robertiello V, Innings F, Rasmuson A (2016). Coalescence and agglomeration of individual particles of skim milk during convective drying. J Food Eng 175:15–23

McLaughlin CP, Magee TRA (1998). The determination of sorption isotherm and the isosteric heats of sorption for potatoes. J Food Eng 35(3):267–280

McMinn WAM, Magee TRA (2003). Thermodynamic properties of moisture sorption of potato. J Food Eng 60:157–165

Moraes K, Pinto LAA (2012). Desorption isotherms and thermodynamics properties of anchovy in natura and enzymatic modified paste. J Food Eng 110:507–513

Mousa W, Ghazali FM, Jinap S, Ghazali HM, Radu S (2014). Sorption isotherms and isosteric heats of sorption of Malaysian paddy. J Food Sci Technol 51(10):2656-2663

Nunes RV, Rotstein E (1991). Thermodynamics of the water-foodstuff equilibrium. Dry Technol 9:113–117

Pérez-Alonso C, Beristain CI, Lobato-Calleros C, Rodríguez-Huezo ME, Vernon-Carter EJ (2006). Thermodynamic analysis of the sorption isotherms of pure and blended carbohydrate polymers. J Food Eng 77(4): 753–760.

Rakshit M, Moktan B, Hossain A, Sarkar PK (2014). Moisture sorption characteristics of *wadi*, a legume-based traditional condiment. J Food Sci Technol 51(2):301–307

Rizvi SSH (1986). Thermodynamic properties of foods in dehydration. In: Rao NA, Rizvi SSA (eds) Engineering properties of food. Marcel Dekker, Inc., New York, pp. 133–214

Sawhney IK, Sarkar BC, Patil GR (2011). Moisture sorption characteristics of dried acid casein from buffalo skim milk. LWT-Food Sci Technol 44:502–510

Singh RRB, Rao KH, Anjaneyulu ASR, Patil GR (2001). Moisture sorption properties of smoked chicken sausages from spent hen meat. Food Res Int 34:143–148

Silva EK, de Barros Fernandes RV, Borges SV, Botrel DA, Queiroz F (2014). Water adsorption in rosemary essential oil microparticles: Kinetics, thermodynamics and storage conditions. J Food Eng 140: 39–45

Smith JM, Van Ness HC, Abbott MM (2005). Introduction to Chemical Engineering Thermodynamics. Mc Graw Hill, Ney York

Tunç S, Duman O (2007). Thermodynamic properties and moisture adsorption isotherms of cottonseed protein isolate and different forms of cottonseed samples. J Food Eng 81:133–143

van Donkelaar LH, Martinez JT, Frijters H, Noordman TR, Boom RM, van der Goot AJ (2015). Glass transitions of barley starch and protein in the endosperm and isolated from. Food Res Int 72:241–246

Velázquez-Gutiérrez SK, Figueira AC, Rodríguez-Huezo ME, Román-Guerrero A, Carrillo-Navas H, Pérez-Alonso C (2015).Sorption isotherms, thermodynamic properties and glass transition temperature of mucilage extracted from chia seeds (*Salvia hispanica* L.) Carbohyd Polym 121:411–419

Viganó J, Azuara E, Telis VRN, Beristain CI, Jiménez M, Telis-Romero J (2012). Role of enthalpy and entropy in moisture sorption behavior of pineapple pulp powder produced by different drying methods. Thermochim Acta 528:63–71

Vioque J, Alaiz M, Girón-Calle J (2012). Nutritional and functional properties of *Vicia faba* protein isolates and related fractions. Food Chem 132:67–72

Włodarczyk-Stasiak M, Jamroz J (2008). Analysis of sorption properties of starch-protein exudates with the use of water vapour. J Food Eng 85:580–589

Zhou P, Labuza TP (2007). Effect of water content on glass transition and protein aggregation of whey protein powders during short-term storage. Food Biophys 2:108–116

Table 1. Estimated GAB parameters of FD-FBP.

T (°C)	M_0 (kg H_2O/100 kg of dry solids)	C	K	R^2	MRD (%)
25	5.52 ± 0.18^{b}	9.300 ± 0.111^{b}	0.714 ± 0.012^{a}	0.999	1.65
35	4.55 ± 0.15^{a}	6.320 ± 0.178^{a}	0.742 ± 0.019^{a}	0.999	1.82
40	4.32 ± 0.13^{a}	5.989 ± 0.234^{a}	0.723 ± 0.021^{a}	0.998	1.59

Values are means ± standard error, of three replicates. Superscripts with different letters in same column indicate significant differences ($P \leq 0.05$).

Table 2. Pore radius of FD-FBP at different moisture contents and temperatures.

Pore Radius (nm)			
M **(kg H_2O/100 kg of dry solids)**	**25 °C**	**35 °C**	**40 °C**
1	$0.75 \pm 0.05^{a,x}$	$1.02 \pm 0.03^{a,y}$	$0.78 \pm 0.02^{a,x}$
2	$0.85 \pm 0.06^{a,b,x}$	$0.92 \pm 0.04^{a,x,y}$	$0.98 \pm 0.05^{a,b,y}$
3	$0.99 \pm 0.01^{b,c,x}$	$1.10 \pm 0.07^{a,b,x,y}$	$1.23 \pm 0.07^{b,c,y}$
4	$1.18 \pm 0.04^{c,x}$	$1.40 \pm 0.06^{b,y}$	$1.55 \pm 0.09^{c,y}$
5	$1.43 \pm 0.05^{d,x}$	$1.76 \pm 0.11^{c,y}$	$1.96 \pm 0.10^{d,y}$
6	$1.74 \pm 0.08^{e,x}$	$2.17 \pm 0.09^{d,y}$	$2.47 \pm 0.11^{e,z}$
7	$2.12 \pm 0.11^{f,x}$	$2.66 \pm 0.17^{e,y}$	$3.12 \pm 0.14^{f,z}$
8	$2.58 \pm 0.14^{g,x}$	$3.29 \pm 0.18^{f,y}$	$3.97 \pm 0.19^{g,z}$
9	$3.14 \pm 0.11^{h,x}$	$4.13 \pm 0.15^{g,y}$	$5.10 \pm 0.17^{h,z}$
10	$3.84 \pm 0.08^{i,x}$	$5.19 \pm 0.16^{h,y}$	$6.68 \pm 0.20^{i,z}$

Values are means ± standard error, of three replicates. Superscripts with different letters in same column and line indicate significant differences ($P \leq 0.05$).

Figure Captions

Figure 1. Sorption isotherms of FD-FBP.

Figure 2. Integral entropy at 25, 35 and 40°C of FD-FBP.

Figure 3. Glass transition temperature as function of water activity of FD-FBP at 25, 35 and 40°C.

Figure 4. Morphology of FD-FBP at a) 0.2, b) 0.4 and c) 0.6 of water activity and 25°C.

The graphic program used was: Origin Pro 8.0

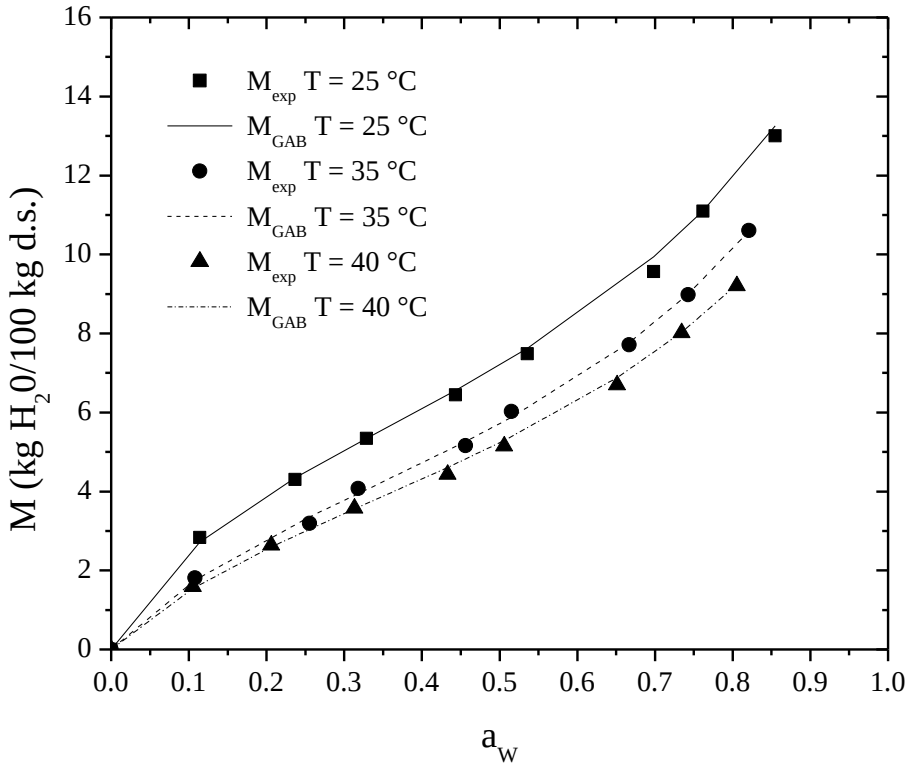


Figure 1

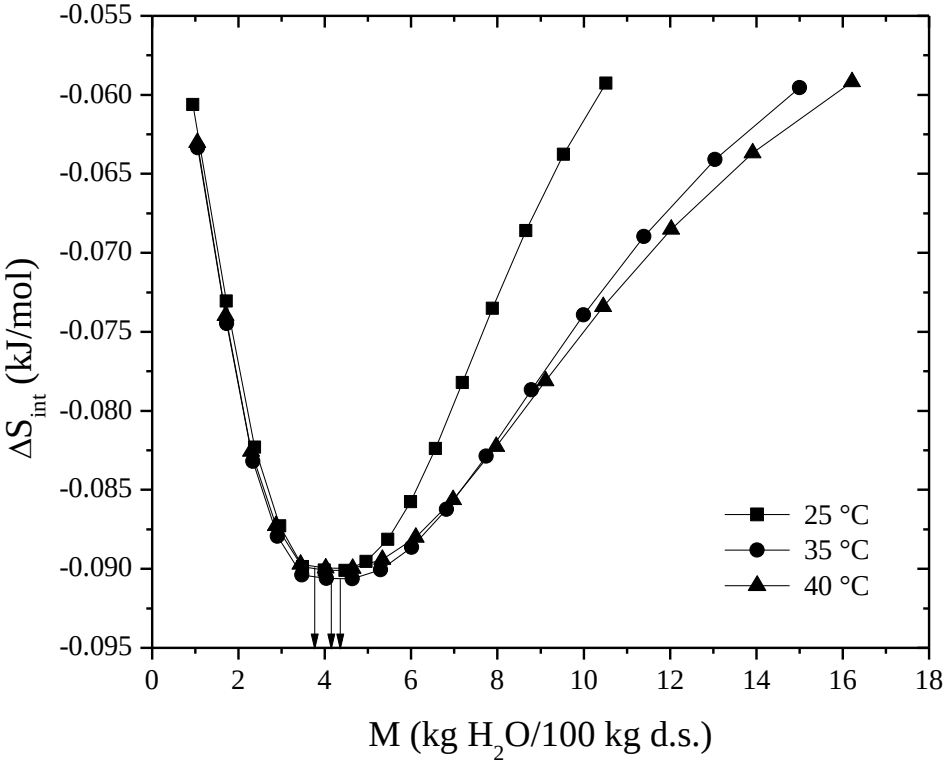

-0.055
-0.060
-0.065
-0.070
-0.075
-0.080
-0.085
-0.090
-0.095
ΔS_int (kJ/mol)
0
2
4
6
8
10
12
14
16
18
M (kg H2O/100 kg d.s.)
25 °C
35 °C
40 °C

Figure 2

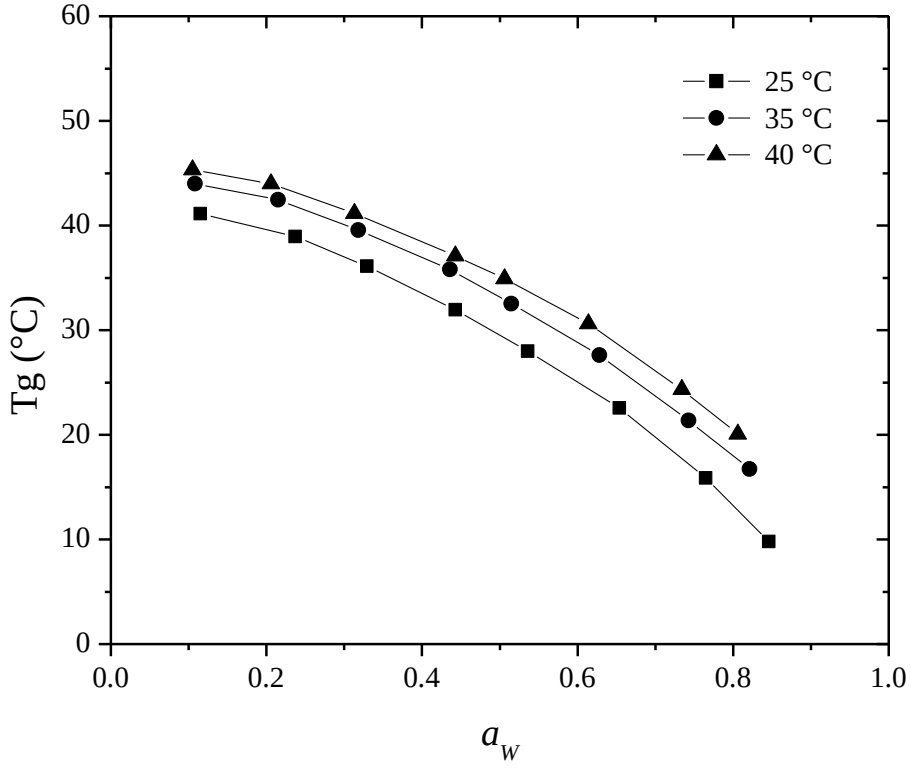


Figure 3

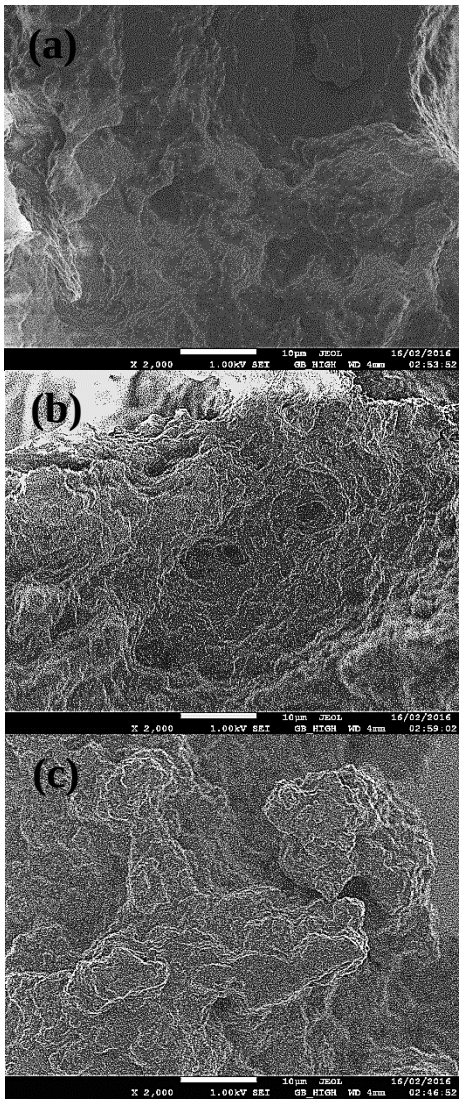

(a)
10μm JEOL 16/02/2016
X 2,000 1.00kV SEI GB HIGH WD 4mm 02:53:52
(b)
10μm JEOL 16/02/2016
X 2,000 1.00kV SEI GB HIGH WD 4mm 02:59:02
(c)
10μm JEOL 16/02/2016
X 2,000 1.00kV SEI GB HIGH WD 4mm 02:46:52

Figure 4

Journal of Food Science and Technology

Copyright Transfer and Financial Disclosure/Conflict of Interest Statement

Title of the article

Thermodynamic sorption analysis and glass transition temperature of faba bean (Vicia faba L.) protein

Author (s)

E. Alpizar-Reyes, J. Castaño, H. Carrillo-Navas, J. Alvarez-Ramírez, R. Gallardo-Rivera, C. Pérez-Alonso, A.Y. Guadarrama-Lezama

Author(s)' signature (s)

Date

August 1st, 2017

Empowering the Food Professional

Association of Food Scientists and Technologists of India (AFSTI)

Please sign this form, scan and upload to:
https://www.editorialmanager.com/jfst while submitting your manuscript.

SPRINGER NATURE

Change of authorship request form

Please read the important information on page 4 before you begin

This form should be used by authors to request any change in authorship. Please fully complete all sections. Use black ink and block capitals and provide each author's full name with the given name first followed by the family name.

Section 1 Please provide the current title of manuscript

Manuscript ID no. JFST-D-16-01673

Thermodynamic sorption analysis and glass transition temperature of faba bean (*Vicia faba* L.) protein

Section 2 Please provide the current authorship, in the order shown on your manuscript.

	First name(s)	Family name	ORCID or SCOPUS id, if available
1st author	E.	Alpizar-Reyes	
2nd author	J.	Castaño	
3rd author	H.	Carrillo-Navas	
4th author	R.	Gallardo-Rivera	
5th author	C.	Pérez-Alonso	
6th author	A.Y.	Guadarrama-Lezama	
7th author			

Please use an additional sheet if there are more than 7 authors.

Section 3: Please provide a justification for change. Please use this section to explain your reasons for changing the authorship of your manuscript. Please refer to the journal policy pages for more information about authorship. Please explain why omitted authors were not originally included on the submitted manuscript.

J. Alvarez-Ramírez was omitted in the manuscript because her contributions were made in the corrected manuscript.

SPRINGER NATURE

Change of authorship request form

Section 4 Proposed new authorship. Please provide your new authorship list in the order you would like it to appear on the manuscript.

	First name(s)	Family name (this name will appear in full on the final publication and will be searchable in various abstract and indexing databases)
1st author	E.	Alpizar-Reyes
2nd author	J.	Castaño
3rd author	H.	Carrillo-Navas
4th author	J.	Alvarez-Ramírez
5th author	R.	Gallardo-Rivera
6th author	C.	Pérez-Alonso
7th author	A.Y.	Guadarrama-Lezama

Please use an additional sheet if there are more than 7 authors.

Section 5 Author contribution, Acknowledgement and Disclosures. Please use this section to provide revised Author Contribution, Acknowledgement and/or Disclosures of your manuscript, ensuring you state what contribution any new authors made and, if appropriate acknowledge any contributors who have been removed as authors. Please ensure these are updated in your manuscript.

New Disclosures (potential conflicts of interest, funding, acknowledgements):

New Author Contributions statement: J. Alvarez-Ramirez improved Introduction section and rephrased many paragraphs, mainly in the Materials & Methods section.

New Acknowledgement Section:

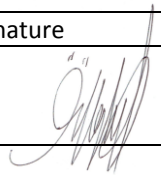
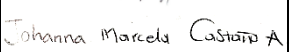
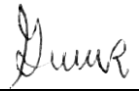
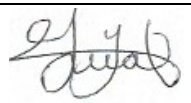
State 'Not applicable' if there are no new authors.

SPRINGER NATURE

Change of authorship request form

Section 6 Declaration of agreement. *All* authors, unchanged, new and removed *must* sign this declaration.

***please delete as appropriate. Delete all of the bold if you were on the original authorship list and are remaining as an author**

	First name	Family name		Signature	Affiliated institute	Date
1st author	E.	Alpizar-Reyes	I agree to the proposed new authorship shown in section 4		Universidad Autónoma del Estado de México	01/08/17
2nd author	J.	Castaño	I agree to the proposed new authorship shown in section 4		Universidad de Concepción	01/08/17
3rd author	H.	Carrillo-Navas	I agree to the proposed new authorship shown in section 4		Universidad Autónoma Metropolitana-Iztapalapa	01/08/17
4th authors	J.	Alvarez-Ramírez	I agree to the proposed new authorship shown in section 4 and the addition of my name to the authorship list		Universidad Autónoma Metropolitana-Iztapalapa	01/08/17
5th author	R.	Gallardo-Rivera	I agree to the proposed new authorship shown in section 4		Universidad Autónoma Metropolitana-Iztapalapa	01/08/17
6th author	C.	Pérez-Alonso	I agree to the proposed new authorship shown in section 4		Universidad Autónoma del Estado de México	01/08/17
7th author	A.Y.	Guadarrama-Lezama	I agree to the proposed new authorship shown in section 4		Universidad Autónoma del Estado de México	01/08/17

Please use an additional sheet if there are more than 7 authors. *please delete as appropriate. Delete all of the bold if you were on the original authorship list and are remaining.

Important information. Please read.

- Please return this form, fully completed, to the editorial office. We will consider the information you have provided to decide whether to approve the proposed change in authorship. We may choose to contact your institution for more information or undertake a further investigation, if appropriate, before making a final decision.

SPRINGER NATURE

Change of authorship request form

- Please note, we cannot investigate or mediate any authorship disputes. If you are unable to obtained agreement from all authors (included those who you wish to be removed) you must refer the matter to your institution(s) for investigation. Please inform us if you need to do this.

- If you are not able to return a fully completed form within **30 days** of the date that it was sent to the author requesting the change, we may have to reject your manuscript. We cannot publish manuscripts where authorship has not been agreed by all authors (including those who have been removed).

- Incomplete forms will be rejected.