Effect of amino acid, pH and mineral salts on glass transition and flow behaviour of soy protein concentrate

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Abstract

The objective of this study was to assess the effect of proline, mineral salts (NaCl and Na_2SO_4) and pH combined with moisture content on the glass transition temperature (T_g) and flow-starting temperature (T_f) of soy protein concentrate (SPC). Initial screening of the variables based on fractional factorial design showed insignificant effect of NaCl on T_g and T_f. The design was extended to a face-centred central composite design (CCD) excluding NaCl and data evaluated by use of response surface methodology. The established model for T_g ($R^2 = 0.824$) shows significant negative first-order effects of moisture, proline and Na_2SO_4, and a positive interaction effect of moisture and Na_2SO_4. The T_f model ($R^2 = 0.937$) shows significant negative first-order effects of moisture and proline, a positive first-order and negative square effect of pH, and a negative interaction effect of moisture and proline. The main effect on T_g and T_f were 2.2 and 1.3 times higher, respectively, for moisture compared to proline. The study confirms that proline (or other free amino acids) can replace moisture as protein plasticizer in the extrusion process. Minor effects can also be obtained by reduction of pH.

Keywords: Amino acids, extrusion, plasticizer, protein hydrolysates, soybean products, thermal processing, viscosity.

Introduction

The extrusion process involves use of high temperature achieved by steam injection and mechanical energy dissipation, and transforms the biopolymers into a plasticized and flow-able melt with establishment of new intra- and intermolecular bindings. To reduce the plasticization temperature and avoid undesirable thermal degradation effects, a plasticizer is added (Matveev et al., 2000; Verbeek & van den Berg, 2010). Plasticizers are low molecular weight compounds that penetrate into the polymer matrix and reduce the glass transition (T_g) by weakening of the intermolecular forces and increase of the free volume and chain mobility (di Gioia & Guilbert, 1999; Abiad et al., 2009). Moisture is the universal plasticizer used in feed and food processing; however, there is a lack of information regarding the efficiency of other approved plasticizer in such systems. The reduction in and exchange of moisture by other plasticizers will be of advantage with respect to the extrusion process and reduce the need to remove water in subsequent drying operation.

The replacement of fishmeal with plant proteins in commercial fish feed formulations demands use of a higher moisture level (around 30%) and thermomechanical energy in the extrusion process to achieve target pellet durability and density specifications (Draganovic et al., 2011, 2014). A low effect of moisture addition on thermomechanical transformation in the extrusion processing of plant proteins compared to fishmeal may explain these observations (Bengoechea et al., 2007; Oterhals & Samuelsen, 2015). The addition of a higher moisture level in the extrusion process gives rise to increased drying cost before final oil coating of the pellet. Compared to plant proteins, fishmeal contains high levels of low molecular weight water soluble compounds (solubles) that act as plasticizers and give increased temperature effect on viscosity reduction in the rubbery phase (Oterhals & Samuelsen, 2015). Possible plasticizer candidates suggested by the authors include free amino acids, peptides, putrefaction products and mineral salts (Oterhals & Samuelsen, 2015). However, it is yet to be confirmed which of these are the most effective plasticizers for proteins.

Amphiphilic compounds have been shown to be effective plasticizers in biopolymer systems due to resemblance in chemical structure and good miscibility properties (Stein & Greene, 1997; di Gioia & Guilbert, 1999; Stein et al., 1999; Selmin et al., 2015).

Proline used in this study has been shown to be effective in starch-based, however, not documented in a protein-based systems. Starches are modified by breaking the intra- and intermolecular bonds, allowing the hydroxyl groups to engage with water or other plasticizers (Selmin et al., 2015). In comparison, proteins are destabilized by weakening of hydrogen bonds as well as hydrophobic interactions (Kokini et al.,1994; di Gioia & Guilbert, 1999; Matveev et al., 2000).

Mineral salts and pH might influence the glass transition temperature (T_g) of proteins by screening off and change of electrostatic interactions (Anker et al., 1999; Moreau et al., 2009). The combined effect of amino acids, low molecular peptides, mineral salts and pH have also been shown to impact the thermomechanical behaviour in the extrusion process and final physical product quality (Samuelsen et al., 2013).

The Phase Transition Analyser (PTA) has been used in several studies to assess the effect of recipe formulation and additon of plasticizers on polymer transformation in the extrusion process (Strahm et al., 2000; Bengoechea et al., 2007; Oterhals & Samuelsen, 2015; Samuelsen & Oterhals, 2016). The instrument enable to measure T_g, and a flow-starting temperature (T_f) at elevated moisture and high temperatures above T_g relevant for the extrusion process. The T_f can be defined as the temperature where the apparent viscosity of the biopolymer reaches a critical level initiating flow through a standardized die orifice at a defined pressure (Fujio et al., 1991; Hayashi et al., 1993; Igura et al., 1997). The viscosity in the glassy state is approximately 10^{12} Pa s and is dramatically reduced during transition from glassy to rubbery state (Baeurle et al., 2006; Abiad et al., 2009). The PTA flow starting temperature is equivalent to a viscosity of approximately 10^5 Pa s and reduced by the inclusion of plasticizers (Oterhals & Samuelsen, 2015).

Soy protein concentrate (SPC) used as a model system in this study is the most prominent vegetable protein alternative to fishmeal in fish feed formulations. Due to its high protein and low carbohydrate and fibre content, it can replace more than 75% of fishmeal without influencing on fish health and growth performance (Burr et al., 2012; Kousoulaki et al., 2012; Metochis et al., 2013). SPC contains 65-70 % crude protein and is produced by ethanol or acidic water extraction of defatted soybean meal (Lusas & Riaz, 1995). The extraction process removes soluble oligosaccharides (fiber) and saponins with negative impact on health and growth performance in salmonid fish

(Bureau et al., 1998; van den Ingh et al., 1996). The physicochemical properties differs from fishmeal and SPC is known to be a challenging ingredient requiring high moisture content to achieve proper thermomechanical transformation in the extrusion process (Bhattacharya et.,1986; Draganovic et al., 2011. 2014). Knowledge related to the optional use of amino acids, mineral salts and pH instead of moisture to obtain a satisfactory SPC plasticization might give the industry new formulation and processing tools.

The objectives of this study were to (i) assess the plasticizing effect of free amino acid (proline), pH and minerals salts (NaCl, Na_2SO_4) in a SPC model system, (ii) quantify the effect of the tested variables compared to moisture~~,~~ and (iii) assess the temperature effect on viscosity reduction in the rubbery phase. The results are interpreted and discussed in terms of thermal and rheological properties of SPC in the fish feed extrusion process, however, will be of general relevance for the processing of food and bioplastic formulations (Chinma et al., 2013).

Materials and methods

Materials

Soy protein concentrates (SPC; Table 1) was obtained from Sementes Selecta (Goiania, Brazil). Proline (purity, 98-101%) was purchased from Applichem (GmbH, Darmstadt, Germany). Sodium chloride (NaCl), sodium sulphate (Na_2SO_4), sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Merck KGaA (Darmstadt, Germany). All solvents and reagents for the sample preparation and analyses were of analytical grade.

Sample preparation

~~SPC~~ Soy protein concentrate was ground in a Retch SR-3 centrifugal mill (Retsch GmbH, Haan, Germany) with a ring sieve aperture of 0.5 mm. To secure an even distribution of plasticizers, the tested compounds (Table 2) were fully dissolved in water and mixed with SPC to a slurry before adjustment of pH by adding of NaOH or HCl. The slurry was frozen over night at -20 °C and lyophilized for 48 hours in a GAMMA 1-16 LSC dryer (MARTIN CHRIST GmbH, Osterode, Germany). The dried sample were conditioned against air at ambient temperature for two days before ground on a Retch ZM-1 Centrifugal mill (Retsch GmbH, Haan, Germany) with ring sieve aperture 0.5 mm. Moisture contents of dried samples were measured and samples rehydrated to the predetermined moisture level (Table 2) by the addition of finely crushed ice according to Oterhals & Samuelsen (2015). The samples were stored in closed containers at 4-5 °C overnight for conditioning and homogenized in a Warring MC3 container (Warring, Torrington, USA) before measurement of moisture contents. Prepared samples were stored in the freezer until used.

Chemical analyses

Moisture level in the samples were analysed according to ISO 6496. Crude protein (N x 6.25) was analysed by Dumas methods (ISO 16634-1) and water soluble protein by hot water extraction using Kjeldahl method (ISO 5983-2) as described in Oterhals & Samuelsen (2015). The starch content was determined using a glucoamylase method (Chiang & Johnson, 1977) according to Samuelsen & Oterhals (2016). Total ash content was determined according to (ISO 5984) and salt (NaCl) content based on water soluble chloride using AOAC (2000) method 937.09. The fat content was determined based on chloroform-methanol extraction (Bligh & Dyer, 1959). The pH of the samples was measured in a dispersion based on 5 g sample in 45 g of distilled water at room temperature using a Mettler-Toledo Ag digital pH meter (Schwerzenbach, Switzerland). The chemical analyses were carried out at Nofima Bio-Lab (accredited according to ISO 17025) based on duplicate measurements. Moisture analyses were performed in triplicate.

Measurement of phase transition temperatures and apparent viscosity

A phase transition analyser (PTA; Wenger Manufacturing, Sabetha, KS) (Strahm et al., 2000) was used for T_g, T_g endpoint (T_{gEnd}) and T_f measurements as described in the study of Oterhals & Samuelsen (2015). The principle is based on the measurement of change in displacement (sample volume) with respect to temperature increase (8 °C/min) at constant pressure (100 bars). After T_g and T_{gEnd} measurements, a blank die is replaced with a die opening of 1.75 mm and T_f is defined as the temperature level initiating start of flow through the die opening. The standard deviation based on duplicate measurements for T_g, T_{gEnd} and T_f was calculated to be 0.22, 0.92 and 1.03, respectively. Apparent viscosity at T_f was calculated based on the applied pressure, capillary die radius (0.000875 m) and capillary die length (0.025 m) as described in the study of Oterhals & Samuelsen (2015).

Experimental design

A fractional factorial screening design (FFD) with resolution V (2^{5-1}) was used to assess the significance of the studied variables: moisture (MC), proline, NaCl and Na_2SO_4 content, and pH. In the design, the main factors were confounded with four-factor interactions and two-factor interactions were confounded with three-factor interactions (Myers & Montgomery, 2002). Nineteen experiments (ENo 1-19; Table 2) including three centre points were performed. The design was extended to a face-centred central composite design (CCD) based on four significant variables by adding 8 points (ENo 20-27), giving a total of twenty-seven experiments (Table 2).

Statistical analyses

The experimental data based on the FFD was fitted to a first order linear model with two-factor interactions (Eqn (1)) by means of multiple linear regressions (MLR). The experimental data based on the face-centred CCD was fitted to a second order model (Eqn (2)) by means of MLR and response surface methodology. The statistical analyses were performed using STATISTICA 12 (StatSoft, Tulsa, OK, USA).

$$y = B_0 + \sum_{i=1}^{5} B_i x_i + \sum_{i=1}^{4} \sum_{j=i+1}^{5} B_{ij} x_i x_j + E \qquad (1)$$

$$y = B_0 + \sum_{i=1}^{4} B_i x_i + \sum_{i=1}^{4} B_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} B_{ij} x_i x_j + E \qquad (2)$$

In the models, y is the response variable, B_0 is the intercept and B_i, B_{ij} and B_{ii} are regression coefficients of each variables (x_i, x_j), interaction between them and each squared term, respectively, and E is the residual error.

All design variables (predictors) were coded before performance of the statistical analysis (Myers & Montgomery, 2002):

$$X_c = \frac{X_0 - [(\mathrm{Max}\,(X_0) + \mathrm{Min}\,(X_0))/2]}{[(\mathrm{Max}\,(X_0) - \mathrm{Min}\,(X_0))/2]} \qquad (3)$$

Where X_c is the coded variable and X_0 is the variable in the natural scale. In case of moisture and pH, the max and min values in ~~Eq. (3)~~ Eqn (3) where defined based on the average of the respective experimentally obtained levels. The established response surface models with best subset of regressor variables were identified by backward elimination of insignificant variables (*P,* remove >0.05). The quality of the models was assessed by *F*-statistics and coefficient of multiple determinations (R^2). The general linear regression module in STATISTICA 12 was used to estimate the main effect of the predictor variables. Comparison of the relative difference between main effects was based on coefficients converted back to natural scale values based on Eqn (3).

Results and discussion

The present study reports for the first time the effect of proline, mineral salts (NaCl, Na_2SO_4) and pH in combination with moisture on T_g and T_f in a plant protein (SPC) matrix using PTA as a measuring tool. The used PTA instrument is able to measure phase transition temperatures in the range 5-180 °C. A moisture content of 194.7 to 324.5 g kg^{-1} dry matter (DM) (Table 2; equivalent to 163 to 245 g kg^{-1} wet basis) was selected based on initial trials to enable the measurements of both T_g and T_f in the same sample.

Proline was selected due to its high plasticizing effect in starch-based systems (Stein & Greene, 1997; Stein et al., 1999; Selmin et al., 2015). At comparable moisture contents, SPC has higher T_g and T_f than fishmeal (Oterhals & Samuelsen, 2015), probably attributed to globular structure of soy proteins, lower levels of water-soluble constituents and high level of non-starch polysaccharides (Table 1).

Screening design

A screening experiment (FFD) was initially performed to eliminate possible insignificant variables before performance of the full face-centred CCD. The T_g model showed significant negative first-order effects of moisture content (MC; $P < 0.001$), proline ($P = 0.018$) and Na_2SO_4 ($P = 0.024$). Insignificant negative first-order effect of pH ($P = 0.501$) and insignificant positive first-order effect of NaCl ($P = 0.248$) were observed. Only a positive MC × Na_2SO_4 interaction effect was significant ($P = 0.028$). The T_f model showed significant negative first-order effects of MC (*P* < 0.001) and proline (*P* < 0.001), and positive effects of pH (*P* = 0.024) and Na_2SO_4 (*P* = 0.040). The negative effect of NaCl was not significant (*P* = 0.109). Only a negative MC × Proline interaction effect was significant (*P* = 0.004).

Based on the screening design, NaCl did not show significant effect on neither T_g nor T_f and was excluded from the face-centred CCD. The result confirms the hypothesis in Oterhals & Samuelsen (2015) that the plasticizing effect of fish solubles can be attributed to the content of low molecular N-compounds and not NaCl. The insignificant effect of NaCl on T_g at the chosen moisture content (>195 g kg^{-1} DM) is in line with other studies based on starch model system (Farahnaky et al., 2009; Moreau et al., 2009). Contradicting, Chuang et al. (2015) observed an increase in T_g of condensed potato starch at increased NaCl level. The mechanistic understanding of the plasticising effect of NaCl is limited and no relevant comparative studies in protein systems are known. Starch molecules contains hydroxyl groups and in potato starch also phosphorylated groups, giving stabilization by hydrogen bond and electrostatic interactions, respectively. Protein structures is more complex and contains in addition to the peptide (amide) bonds, hydrophobic and positive and negatively charged side groups giving hydrophobic and electrostatic interactions (di Gioia & Guilbert, 1999; Matveev et al., 2000; Verbeek & van den Berg, 2010). At the intermediate moisture level used in this study (195-325 g kg^{-1} DM), there will be no free water phase (i.e. water activity < 0.9; Pan, 2003). The addition of salts will reduce the water activity and the availability of water molecules for plasticising of proteins. According to lower moisture starch-based studies (Farahnaky et al.2009; Chuang et al., 2015), we assume no crystalline NaCl in the used model system and that some of the NaCl will not be dissociated to ions and act as other small molecules as plasticizer. Sodium ions can also be complexed to polar C-O-H and C-O-C groups of carbohydrates (Ma et al., 2007) and similar complexing segments might be possible in proteins. Combined, the complexing and reduced water activity caused by NaCl addition might give an anti-plasticizing effect that counteracts the possible plasticizing effect of un-dissociated NaCl.

Glass transition temperature

To explore in more detail any square effect of the significant variables MC, proline, pH and Na_2SO_4, the design was extended to a face-centred CCD by adding eight experiments (ENo 20-27; Table 2). A face-centred CCD was chosen instead of a rotatable CCD due to use of a low level of zero for the variables proline and Na_2SO_4 in the screening design (Myers & Montgomery, 2002).

The measured T_g values varied from 7.8 to 14.1 °C (Table 2), and the established response surface model shows significant negative first-order effects of MC, proline and Na_2SO_4 and a positive MC × Na_2SO_4 interaction effect (Table 3 and Fig. 1a-c). A good correlation between observed and predicted values was obtained with $R^2 = 0.824$ (Table 3 and Fig. S1). No significant square terms were observed, confirming a linear relationship between the variables and T_g. The main effects on weight basis shows that moisture was 2.22 and 1.24 times more effective than proline and Na_2SO_4 respectively, and Na_2SO_4 was 1.78 times more effective than proline. However, the positive MC × Na_2SO_4 interaction effect will null the Na_2SO_4 effect at higher moisture levels (Fig. 1b). The higher effect of water compared to proline and Na_2SO_4 on weight basis may be attributed to the low molecular weight and the ability to form a larger number of hydrogen bonds with protein molecules. di Gioia & Guilbert (1999) and Pommet et al. (2005) also reported higher effect of water compared to various plasticizers with different functional groups and degree of hydrophobicity. Proline is an amphiphilic cyclic amino acid with a secondary amine group embedded in the ring structure, a molecular conformation documented to be highly effective as plasticizer in starch-based blends (Stein et al., 1999). A lower effect of proline compared to water and Na_2SO_4 may be attributed to the amphiphilic nature of proline. Soy protein has a globular structure with most of the hydrophobic region buried inside the tertiary structure (Sun, 2005). At the observed T_g (7.8 – 14.1 °C; Table 2), the hydrophobic region of SPC may not be fully exposed resulting in lower hydrophobic interactions between proline and SPC molecules.

The positive MC × Na_2SO_4 interaction caused a reduced effect of Na_2SO_4 with increased level of moisture and became ineffective at moisture level of approx. 300 g kg^{-1} DM (Fig. 1b). The effect of moisture also decreased with increased concentration of Na_2SO_4, however to a lesser degree due to the higher first order effect (Table 3 and Fig. 1B). The results show that Na_2SO_4 only acts as a plasticizer at low moisture level. Compared to chloride ions, the sulphate ion has the ability to interact with water and amino acids through hydrogen bonding. At higher moisture contents, the sulphate ions (SO_4^{2-}) may preferentially interact with water (i.e. reduce the water activity), giving a reduced effect on protein plasticization.

The face-centred CCD (Table 3) confirmed the insignificant effect of pH in the screening design. Based on earlier observations (Samuelsen et al., 2013), it was hypothesized that pH induced change in electrostatic interactions may influence T_g and extruded pellet hardness. However, effect on T_g could not be confirmed in this study.

The T_{gEnd} - T_g represents half of the glass transition range. The observed range (8.9 to 40.8 °C; Table 2) is in agreement with the range reported for fishmeal (10.7 to 47.6 °C; Oterhals & Samuelsen, 2015) and food polymers (up to 50 °C; Yildiz & Kokini, 2001).

Flow transition temperature

The T_f model shows significant negative first order effects of MC and proline, a positive first order and negative pH square effect, and a negative MC × Proline interaction effect (Table 3 and Fig. 2a-c). Na_2SO_4 was found to be significant in the screening design (confer above); however, this was not confirmed in the CCD. The measured range of T_f was 111.4 -171.6 °C (Table 2). High correlation between observed and predicted values was obtained with $R^2 = 0.937$ (Table 3 and Fig. S2).

The main effects on weight basis showed that moisture was 1.30 times more effective than proline. The comparatively higher effect of proline on T_f compared to T_g may be attributed to the difference of system temperature. Increase of temperature above T_g in the rubbery phase significantly increases the molecular chain mobility. This gives a weakening of intramolecular hydrogen bonds with unfolding of the biopolymers and exposure of embedded hydrophobic sites (Verbeek & van den Berg, 2010). This phenomenon enables a greater interaction of an amphiphilic amino acid like proline with increased plasticizing effect. The results are in agreement with observations reported in the literature (di Gioia & Guilbert, 1999; Pommet et al., 2005). The finding also confirms that the plasticizing effect of fish solubles can be attributed to the content of low molecular N-compounds (Oterhals & Samuelsen 2015; Samuelsen & Oterhals, 2016). Proline was used in this model study to assess the potential effect of amino acids as a protein plasticizer. In industrial manufacturing practice, it will not be cost effective nor compatible with product formulation constraints to use a single free amino acid to obtain the desired plasticization effect. Protein hydrolysates (e.g. fish silage and peptones) with a high degree of hydrolysation will contain high levels of free amino acids and small peptides (Aspevik et al., 2016). Such products can be utilized as both a nutrient and a plasticizer in food and feed formulations. The negative MC × Proline interaction gives an additional reinforcing effect on T_f-reduction at high level of moisture (Table 3 and Fig. 2a).

A reduction in T_f of SPC was observed when pH changed from neutral to acidic or alkaline environment as indicated by positive first order and negative square effect of pH (Table 3 and Fig. 2b,c). Soy protein concentrate has an isoelectric point of 4.5 to 5 at room temperature (Lee et al., 2003). This is equivalent to the lowest pH-level in this study (Table 2) and a minimum intermolecular electrostatic interaction between the proteins at ambient temperature. The pK values of ionisable residues in a protein change with temperature and peptide chain length and can be calculated based on the Gibbs-Helmholtz equation (Steinhardt & Beychok, 1964). A temperature change of 20 °C can shift the pK value of an amino acid group upward by 0.45 units (Adler-Nissen, 1986). It can be hypothesized that the isoelectric point of SPC might be changed to neutral pH at the high temperature observed for T_f in this study (Table 2), explaining the observed maximum T_f value around pH 6.8 (Fig. 2b,c). At level below and above this pH the change in inter and intramolecular electrostatic interactions might improve the mobility of the biopolymer and reduce the energy needed for unfolding and the T_f. The observed increased pellet hardness with decreasing pH reported by Samuelsen et al. (2013) may be attributed to such effects causing reduced viscosity and improved biopolymer network formation.

Apparent viscosity at T_f

The established response surface model for temperature difference between T_f and T_g (T_f - T_g) have the same significant variables as T_f with $R^2 = 0.922$ (Table 3). The model shows good correlation between observed and predicted values (Fig. S3).This is in good agreement with the study of Oterhals & Samuelsen (2015) who documented equal variable effects on T_f - T_g and T_f in a fishmeal based system.

The PTA instrument can be considered as a constant pressure capillary viscometer. Apparent shear stress, shear rate and viscosity of the material can be calculated based on the initial displacement speed at T_f when the rubbery material starts to flow through the die orifice at the applied pressure. The initial displacement speed at T_f was equivalent to a shear rate of 0.4 - 7 s^{-1}; giving an apparent viscosity in the range of 0.2 - 4 × 10^5 Pa s. This is in good agreement with the apparent viscosity of 3-8 × 10^5 Pa s for fishmeal reported by Oterhals & Samuelsen (2015) and confirms that the apparent viscosity at T_f does not depend on the type of material tested but is defined by the capillary die geometry and applied pressure. The T_f - T_g reflects the temperature required to reduce the viscosity of the material from 10^{12} Pa s at T_g to a critical level of approx. 10^5 Pa s at T_f. A material with high T_f - T_g requires higher temperature increase to reach this critical value. The observed large variation in T_f - T_g indicates a significant effect of the studied plasticizers on the viscosity reduction of temperature increase in the rubbery phase.

Conclusions

The plasticization effects of the amino acid proline, mineral salt (NaCl and Na_2SO_4), pH and moisture has been studied by factorial design experiments using SPC as a model system. Addition of proline has a plasticizing effect on SPC and reduces both T_g and T_f. Addition of Na_2SO_4 has a negative effect on T_g, however, no effect on T_f. A positive MC × Na_2SO_4 interaction null the effect on T_g at higher moisture level. Adjustment of pH has no effect on T_g, however, significant effect on T_f and T_f - T_g. NaCl has no plasticizing effect within the tested moisture range from 194.7 to 324.5 g kg^{-1} DM. The apparent viscosity at T_f (3-8 × 10^5 Pa s) is equivalent to levels observed in fishmeal. The study confirms that proline can replace moisture as protein plasticizer in the extrusion process.

The effect may be extended to other free amino acids, small peptides and organic acids. In industrial manufacturing practice a protein hydrolysates with a high degree of hydrolysation may be a cost effective processing aid that will act as both nutrient and plasticizer in food and feed formulations. A reduced pH will give some additive effects. The studied mineral salts are inherent constituents in protein ingredients, however, less suited as a processing aid in feed applications due to formulation constraints.

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Table 1 Chemical composition of soy protein concentrate on dry matter (DM) basis.

Constituent	g kg^{-1} DM
Crude protein	652
Salt (NaCl)	<1
Fat	28
Ash	67
Starch	14
Water soluble protein	74
Non-starch polysaccharides[a]	237

[a] Non-starch polysaccharides were calculated by difference.

Table 2 Natural and coded levels of variables in the experimental designs (FFD ENo 1-19; face-centred CCD ENo 1-27) and measured phase transition temperatures.

ENo	MC (g kg^{-1} DM)	pH	Proline (g kg^{-1} SPC DM)	Na_2SO_4 (g kg^{-1} SPC DM)	NaCl (g kg^{-1} SPC DM)	Coded values[a]					T_g (°C)	T_{gEnd} (°C)	T_{gEnd} - T_g (°C)	T_f (°C)	T_f - T_g (°C)
						MC	pH	Proline	Na_2SO_4	NaCl					
1	254.7	6.7	50	25	25	-0.055	0.123	0	0	0	10.8	31.3	20.5	149.9	139.2
2	317.5	8.3	100	50	50	0.959	0.989	1	1	1	7.8	19.4	11.5	122.0	114.2
3	197.6	4.7	0	50	0	-0.977	-0.995	-1	1	-1	11.2	51.0	39.9	164.8	153.7
4	319.3	4.7	100	0	50	0.988	-1.005	1	-1	1	7.8	16.7	8.9	112.3	104.5
5	194.7	4.7	0	0	50	-1.023	-1.011	-1	-1	1	14.1	46.5	32.4	162.3	148.2
6	324.5	8.3	0	50	0	1.072	0.962	-1	1	-1	9.4	25.3	15.8	152.5	143.1
7	196.2	4.7	100	0	0	-1.000	-1.000	1	-1	-1	11.4	36.8	25.3	153.2	141.7
8	321.0	4.7	0	50	50	1.016	-1.000	-1	1	1	9.5	24.7	15.1	136.6	127.0
9	196.2	8.2	0	50	50	-1.000	0.891	-1	1	1	10.1	50.9	40.8	171.6	161.5
10	319.3	4.7	0	0	0	0.988	-1.011	-1	-1	-1	9.1	21.6	12.5	147.1	138.0
11	194.7	8.3	100	0	50	-1.023	0.973	1	-1	1	12.5	37.9	25.4	159.7	147.2
12	321.0	8.3	0	0	50	1.016	0.989	-1	-1	1	9.9	22.5	12.6	143.7	133.8
13	196.2	8.4	0	0	0	-1.000	1.022	-1	-1	-1	11.6	51.8	40.2	168.1	156.5
14	253.1	6.7	50	25	25	-0.080	0.112	0	0	0	10.4	31.5	21.1	150.5	140.1
15	321.0	4.7	100	50	0	1.016	-0.967	1	1	-1	7.9	17.5	9.6	122.8	114.9
16	196.2	4.7	100	50	50	-1.000	-1.011	1	1	1	10.5	42.9	32.4	154.1	143.6
17	254.7	6.7	50	25	25	-0.055	0.123	0	0	0	10.6	30.6	20.0	149.8	139.2
18	196.2	8.5	100	50	0	-1.000	1.082	1	1	-1	10.7	40.7	30.0	161.1	150.3
19	317.5	8.6	100	0	0	0.959	1.109	1	-1	-1	8.3	17.7	9.4	111.4	103.1
20	197.6	6.8	50	25	-	-0.977	0.139	0	0	-	10.8	45.2	34.3	161.0	150.1
21	319.3	6.7	50	25	-	0.988	0.123	0	0	-	9.0	21.3	12.3	138.7	129.7
22	256.3	4.7	50	25	-	-0.029	-0.967	0	0	-	9.8	30.2	20.4	147.0	137.2
23	254.7	8.0	50	25	-	-0.055	0.820	0	0	-	10.1	31.2	21.1	155.2	145.0
24	257.9	6.7	0	25	-	-0.004	0.123	-1	0	-	11.6	37.9	26.3	168.3	156.7
25	261.0	6.7	100	25	-	0.047	0.117	1	0	-	10.1	28.9	18.8	147.7	137.6
26	256.3	6.7	50	0	-	-0.029	0.123	0	-1	-	10.8	28.8	18.0	155.9	145.1
27	254.7	6.8	50	50	-	-0.055	0.128	0	1	-	10.6	32.5	21.9	155.9	145.3

Eno, Experiment number; DM, dry matter; T_g, glass transition temperature; T_{gEnd}, Upper glass transition range; T_f, flow-starting temperature.

[a] The design was coded using Eqn 3. Variation in the coded levels for moisture content (MC) and pH is due the deviation from the theoretical design levels.

Table 3 Phase transition temperature (°C) response surface models based on coded variables (Table 2).

Parameter	T_g	T_f	T_f - T_g
Intercept	10.219***	152.991***	142.526***
MC	-1.345***	-14.896***	-13.549***
MC × MC			
pH		2.263*	2.315*
pH × pH		-6.497**	-6.129**
Proline	-0.534**	-9.444***	-8.917***
Proline × Proline			
Na_2SO_4	-0.414*		
MC × Proline		-4.667***	-4.384**
MC × Na_2SO_4	0.426*		
Multiple R^2	0.824	0.937	0.922

Regression coefficients of significant variables and R^2 after backward elimination of insignificant ($P > 0.05$) variables. MC, moisture contents; T_g, glass transition temperature; T_f, flow starting temperature.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Legends to Figures

Fig. 1 Glass transition temperature (T_g) response surface plots based on model given in Table 3: (A) Moisture content (MC) vs. proline; (B) Moisture content (MC) vs. Na_2SO_4; (C) Proline vs. Na_2SO_4. The level of the respective constant design variable represents the average value in the design.

Fig. 2 Flow starting temperature (T_f) response surface plots based model given in Table 3: (A) Moisture contents (MC) vs. proline; (B) Moisture content (MC) vs. pH; (C) pH vs. proline. The level of the respective constant design variable represents the average value in the design.

Fig. S1 Observed vs predicted values for T_g based on MLR model given in Table 2 and 3.

Fig. S2 Observed vs predicted values for T_f based on MLR model given in Table 2 and 3.

Fig. S3 Observed vs predicted values for $T_f – T_g$ based on MLR model given in Table 2 and 3.

Fig. 1a

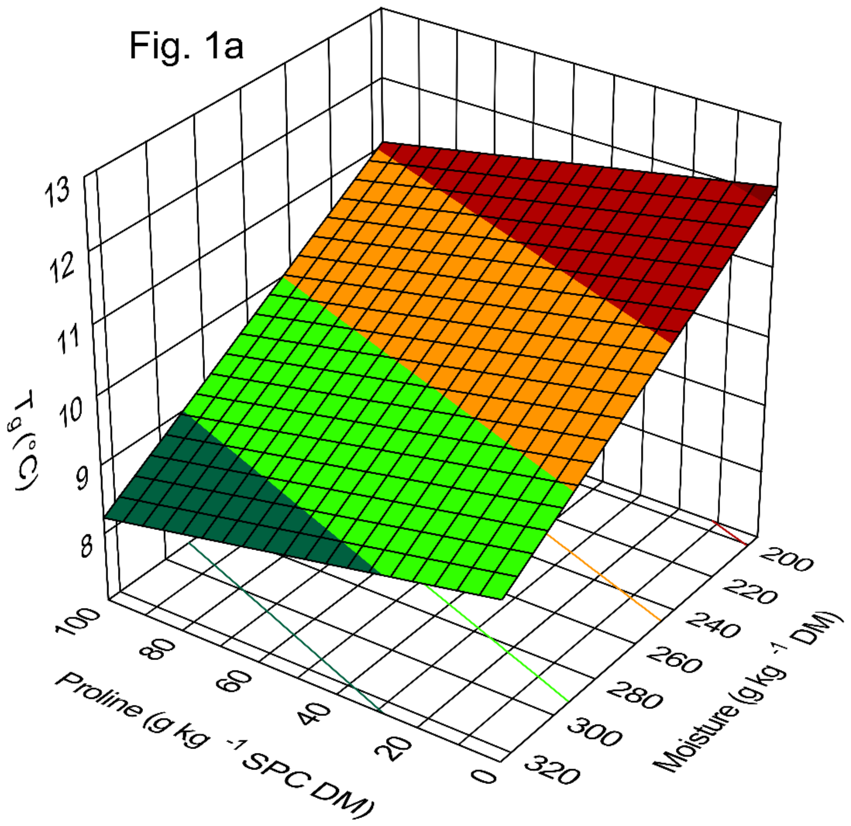

13
12
11
10
9
8
T_g (°C)
100
80
60
40
20
0
Proline (g kg^{-1} SPC DM)
200
220
240
260
280
300
320
Moisture (g kg^{-1} DM)

Fig. 1b

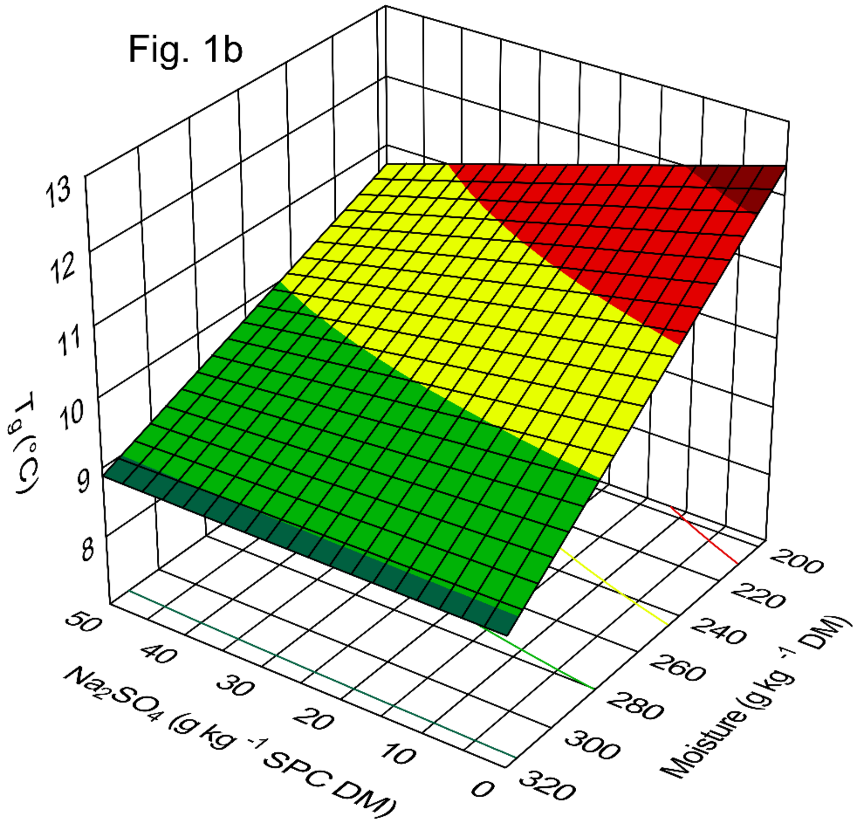

T_g (°C)
8
9
10
11
12
13
Na_2SO_4 (g kg $^{-1}$ SPC DM)
0
10
20
30
40
50
Moisture (g kg $^{-1}$ DM)
200
220
240
260
280
300
320

Fig. 1c

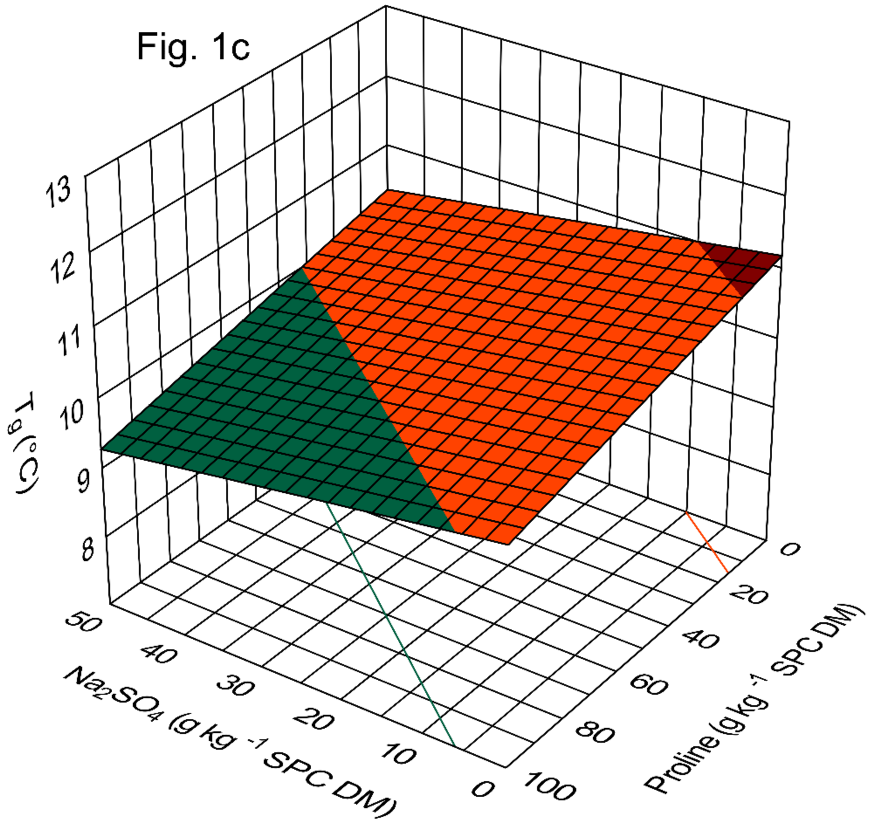

13
12
11
10
9
8
T_g (°C)
50
40
30
20
10
0
Na_2SO_4 (g kg $^{-1}$ SPC DM)
0
20
40
60
80
100
Proline (g kg $^{-1}$ SPC DM)

Fig. 2a

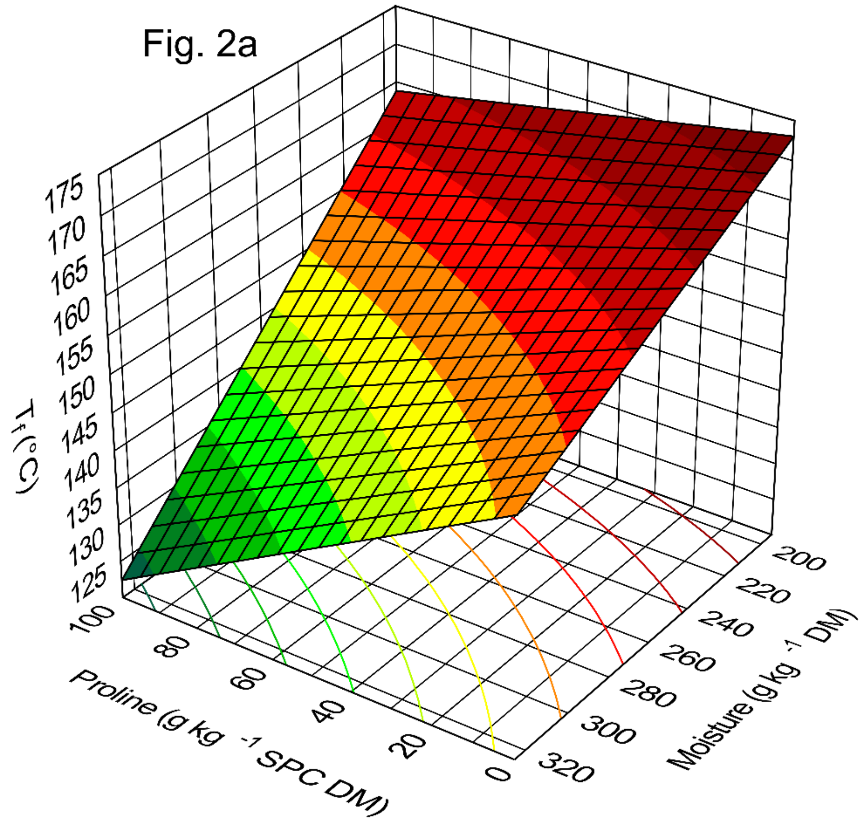
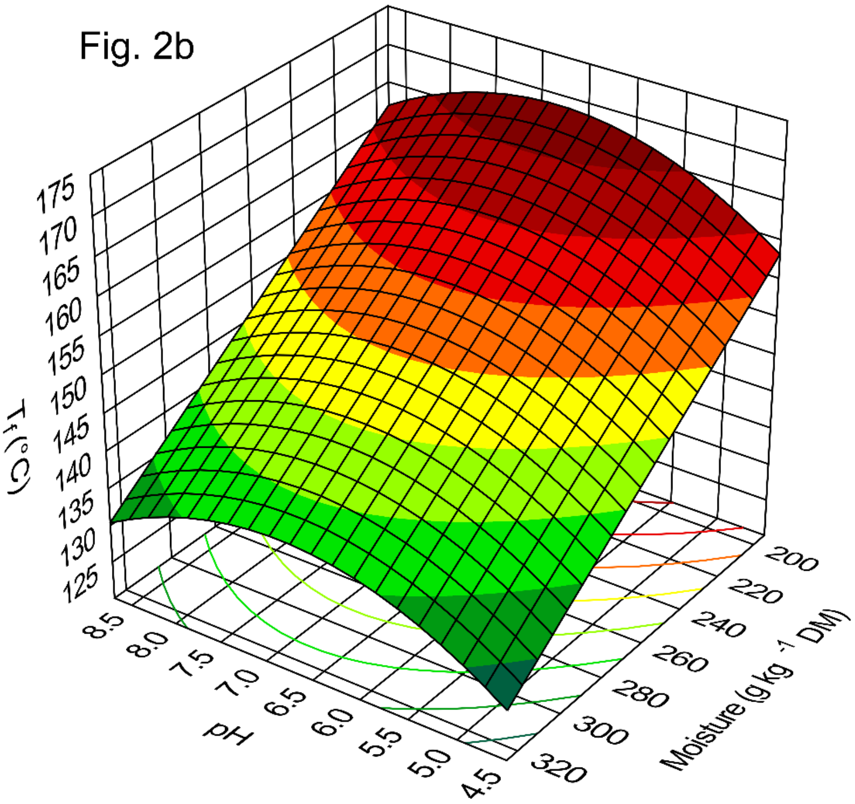
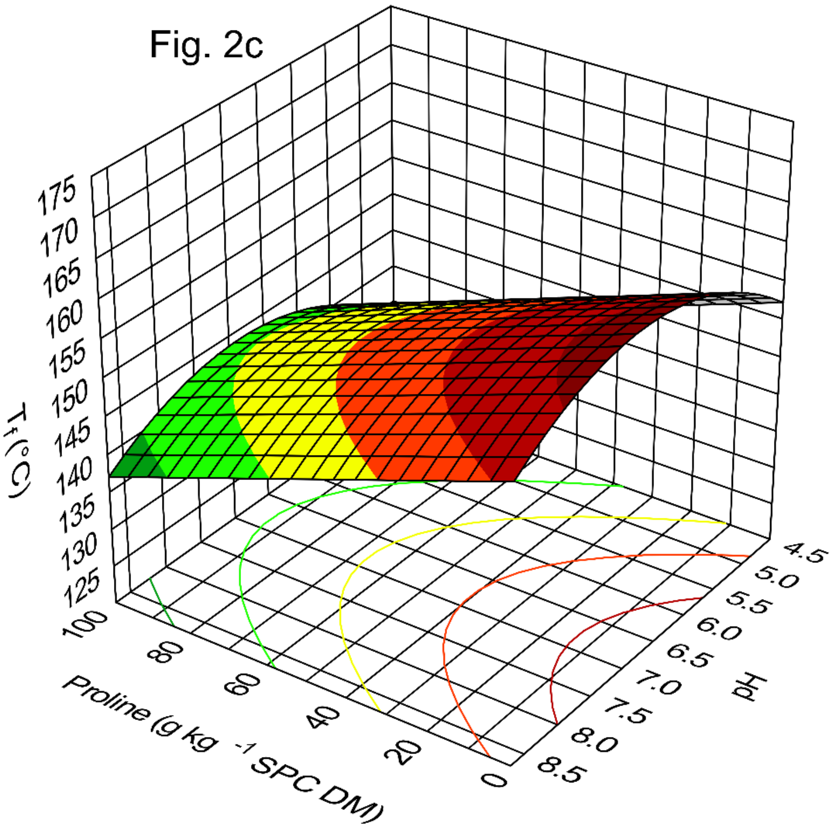

175
170
165
160
155
150
145
140
135
130
125
T_f (°C)
100
80
60
40
20
0
Proline (g kg $^{-1}$ SPC DM)
200
220
240
260
280
300
320
Moisture (g kg $^{-1}$ DM)

Fig. 2b



Tf (°C)
175
170
165
160
155
150
145
140
135
130
125
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
pH
200
220
240
260
280
300
320
Moisture (g kg -1 DM)

Fig. 2c


175
170
165
160
155
150
145
140
135
130
125
T_f (°C)
100
80
60
40
20
0
Proline (g kg $^{-1}$ SPC DM)
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pH

Supplementary information

Effect of amino acid, pH and mineral salts on glass transition and flow behaviour of soy protein concentrate

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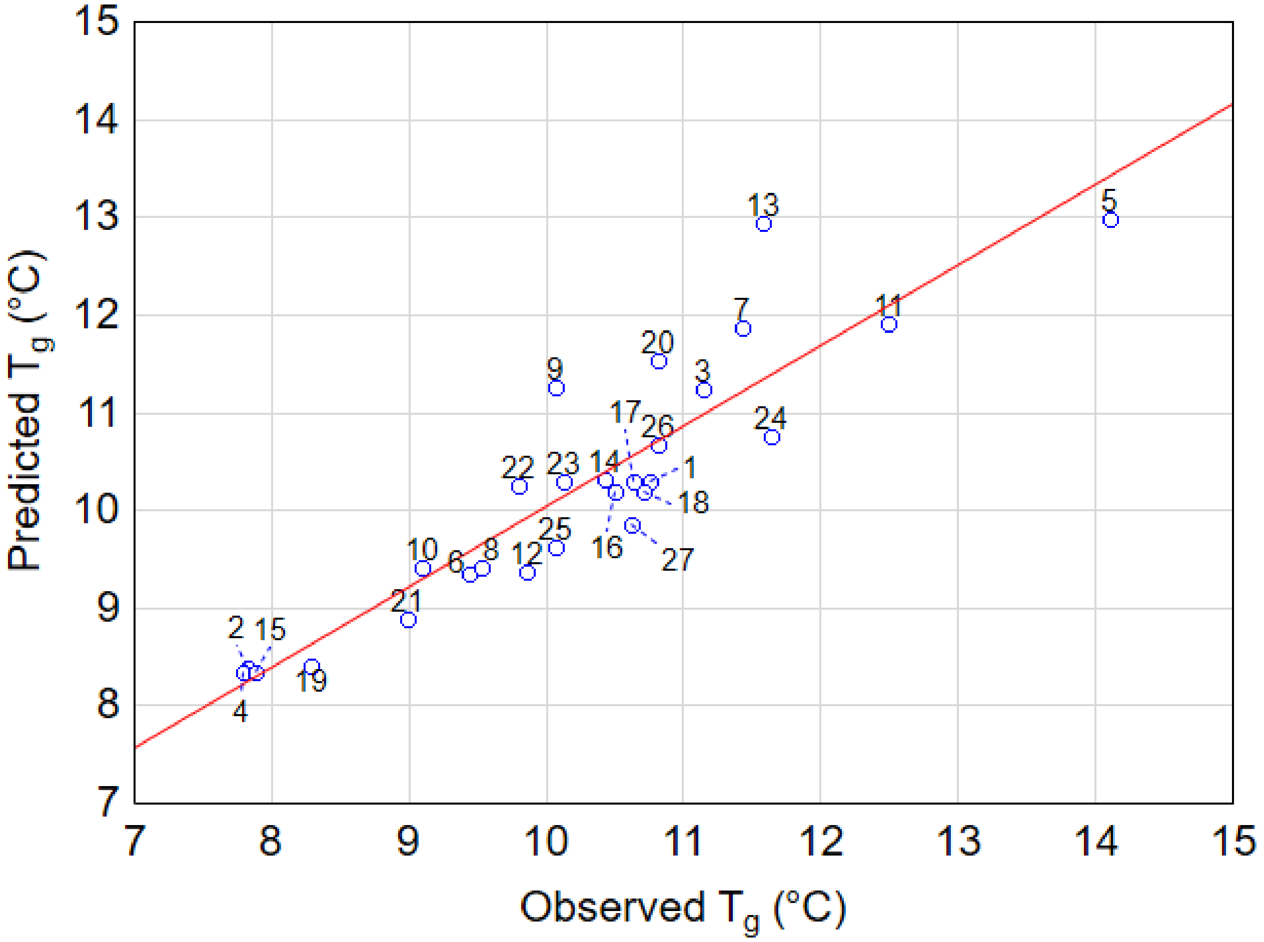
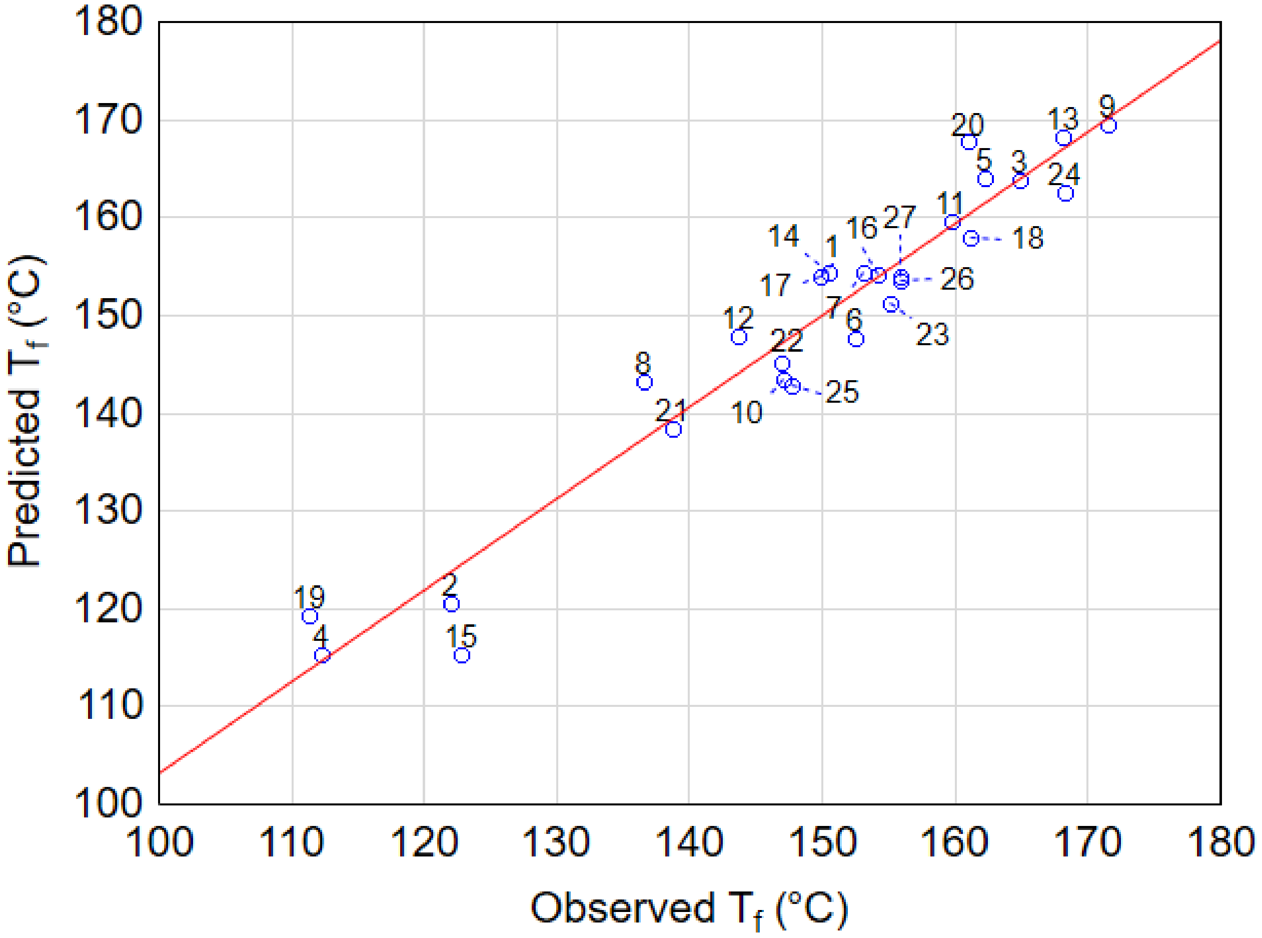
Supplementary Fig 1. Observed vs Predicted values for T_g based on MLR model given in Table 2 and 3.



Supplementary Fig 2. Observed vs Predicted values for T_f based on MLR model given in Table 2 and 3.



Supplementary Fig 3. Observed vs Predicted values for T_f - T_g based on MLR model given in Table 2 and 3.