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MECHANICAL AND PHYSICAL PROPERTIES OF PEA STARCH EDIBLE FILMS IN THE PRESENCE OF GLYCEROL

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26 ABSTRACT

27 The mechanical properties and moisture sorption at relative humidity (RH) range of 11–94%, water vapor permeability, solubility in water, and color of the pea starch films as a function of 28 glycerol were examined. The results showed that increasing the concentration of plasticizer 29 30 resulted in improvement of the tensile strength of the films at RH < 43%, the percent elongation as well as the deformation at break at RH < 84%. Increasing plasticizer content and RH also 31 resulted in films with lower Young's modulus, lower puncture force, but higher puncture 32 deformation. Furthermore, increasing plasticizer content led to the films with more opaque 33 appearance. Films prepared with 15 and 25% glycerol had lower water vapor permeability in 34 35 comparison with un-plasticized film. This study provides information regarding the advantageous or disadvantageous of possible application of pea starch films in food packaging industry. 36

37 PRACTICAL APPLICATION

Starch edible films have been utilized for packaging technologies and edible coatings. Pea starch has been found to produce the films with improved physical and mechanical properties in comparison with films prepared from other starches due to high amount of amylose. The development of pea starch film with improved functions affects its application. Pea starch edible films may find practical applications in the poultry, meat, seafood, fruit, vegetable, grains and candies industries.

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45 **KEYWORDS:** Pea starch; edible films; mechanical properties; water vapor permeability

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47 INTRODUCTION

Starch is the main source of stored energy in many cereal grains and besides their nutritive value, 48 starches and modified starches with functional properties have been used in various industries (Liu 49 2005a). Most starches such as wheat, corn, and potato contain about 25% amylose and 75% 50 amylopectin (BeMiller and Whistler 1996; Haase 1993) while most starches extracted from 51 legumes are characterized by high amylose content. Pea is an important grain legume which is 52 53 used as animal and human food and is cultivated in many regions of the world (Ratnayake et al. 2002). Pea seeds are rich source of starch, protein, dietary fiber and mineral components (Soral-54 Smietana et al. 2002). Pea starch is characterized by resistance to shear thinning, high 55 56 gelatinization temperature, fast retrogradation, high elasticity of the gel and a greater proportion of amylose than other starches (Singh et al. 2005). Van Soest et al. (2002) suggested that for 57 packaging applications, high amylose pea starch is a good material for the potential production of 58 starch-based bioplastics. High-amylose starch exhibits improved tensile strength (TS) and gas 59 barrier characteristics, due to the higher degree of crystallinity of amylose-rich regions after 60 61 dehydration (And and Han 2005; García et al. 2000), so it is a very suitable material for filmforming (Han et al. 2006; Palviainen et al. 2001). 62

63 The use of plastics for packaging has developed in recent years and the use of 64 biodegradable films is a practical solution for environmental conservation (Parris and Coffin 1997). Edible films can be prepared from proteins, polysaccharides, lipids or the combination of 65 these constituents. Starch has the ability to be used for edible films production because it can be 66 67 obtained from a large number of raw materials and is a renewable and biodegradable biopolymer (Liu 2005b). Starch based edible films generally do not change the taste, flavor and appearance of 68 food products because they are tasteless, odorless and generally transparent (Chiumarelli and 69 70 Hubinger 2012). The main advantages of using starch films are that they provide excellent barrier properties to gases (O₂ and CO₂) (Biliaderis *et al.* 1999). However a plasticizer is commonly obligatory for edible films to decrease film fragility. Plasticizers enhance the extensibility and flexibility of the films by decreasing the intermolecular forces and improving the mobility of polymer chains (Liu 2005b). Nevertheless, the addition of plasticizers generally increases gas and water vapor permeability (WVP) of the film and could possibly decrease the mechanical strength of the film (Gontard *et al.* 1993; Mali *et al.* 2004).

77 Starch films are often semicrystalline, containing both amorphous and crystalline phases, similar to starch granules (Liu 2005b). Amylopectin and parts of the amylose molecules form the 78 79 crystalline zones, which contains a specific amount of water molecules. When the films are being equilibrated, an increasing number of water molecules will be absorbed in the amorphous regions 80 81 depending on the water activity of the surrounding atmosphere (Perdomo et al. 2009). Therefore, 82 water has the ability to affect the amorphous structure of the starch films by acting as either 83 plasticizer or antiplasticizer (Chang et al. 2000a). Consequently, the physical properties of starch 84 edible films and fractural and textural properties of low-moisture foods can be influenced by 85 mechanical plasticization/antiplasticization effect of water molecules (Chang et al. 2000a; Chang 86 et al. 2000b). The properties of pea starch films have been widely studied (Chen et al. 2009b; 87 Corrales et al. 2009; da Matta et al. 2011; Han et al. 2006; Mehyar and Han 2004; Sun et al. 2013; Wu et al. 2010; Zhang and Han 2006; Zhang and Han 2008). However, there is still a lack of 88 89 information on the plasticizing-antiplasticizing effects of water and glycerol on the mechanical 90 properties of pea starch films, which provides better understanding of the role of water in edible biopolymer films. 91

92 This study investigated the effect of different concentrations of glycerol on the 93 physicochemical properties of 5% w/w pea starch films. Additionally, the effect of relative 94 humidity (RH) on the mechanical properties of the films was investigated.

95

96 MATERIALS AND METHODS

97 Chemical Composition of Starch. Canadian non-GMO yellow pea starch with 13.2% moisture,
98 0.2% protein, 0.5% fat and 0.3% ash, was kindly supplied by Yantai Shuangta Food Co., Jinling
99 Town, China. The amylose content of the starch was 36.25±0.32% that was determined after
100 defatting of starch with 80% methanol using the iodine method as described by Williams *et al.*101 (1970). All other chemicals were purchased from Merck Millipore Pty., VIC Australia.

Preparation of Film-forming Solution. Aqueous dispersion (5%, w/w) of pea starch was prepared, and plasticizer (glycerol) was added in to the dispersions at 0%, 15%, 25% and 35% of dry weight of starch (5:0.75, 5:1.25 and 5:1.75 w/w, starch/plasticizer). The dispersions were heated in a water bath at 90 °C for 20 min with agitation to allow complete gelatinization of the starch. After gelatinization, the starch solutions were chilled to 50 to 60 °C.

107 Viscosity of Film Forming Solutions. Viscosity measurement was carried out using a viscometer
108 (Brookfield, DV-II+S04, Brookfield Viscometers Ltd, Harlow, UK). Viscosity of each film
109 suspension was determined at 100 rpm and 25 °C by using spindle No. 2. Viscosity was measured
110 for three replicated samples for each type of film.

Film Preparation. All the films were obtained by casting method. Twenty g of filmogenic
suspensions were poured onto Petri dishes (10 cm in diameter). Films were formed by drying at

113 25 °C in an oven until reaching constant weight (about 24 h) (Hernández *et al.* 2008). The prepared
114 films were peeled-off from Petri dishes and equilibrated at 25 °C, 65% relative humidity for 72 h
115 prior to further examination.

Film Thickness and Density Measurements. Thickness of the films was determined using a digital micrometer (Mitutoyo Corp., Code No. 543-551-1, Model ID-F125, Japan). Ten measurements were randomly taken at different locations for each specimen and the mean value was reported and used in the calculations of the mechanical properties and WVP. Film density was evaluated by dividing the film weight by the film volume, where the film volume was calculated by multiplying the film area by the thickness (Larotonda *et al.* 2005).

Solubility in Water. Solubility of films in water was calculated according to the method of
Farahnaky *et al.* (2013). The percentage of the total soluble matter (% TSM) of the films was
calculated using the following equation:

125
$$\% TSM = \frac{initial \, dry \, weight - final \, dry \, weight}{initial \, dry \, weight} \times 100$$
 (1)

Moisture Content. Film moisture content was determined from the weight loss through which the
films go after a 24 h oven drying at 90 °C. The temperature was chosen to avoid loss of plasticizer.
Moisture content was calculated using the following equation:

129 Moisture content =
$$\frac{M_i - M_f}{M_i} \times 100$$
 (2)

where M_i and M_f are the masses of initial and dried samples, respectively. Three replicates were
obtained for each sample.

Water Vapor Permeability. The water vapor transmission of the films was determined
gravimetrically consistent with the ASTM procedure (E96-95 1995) with a 75% RH gradient at 25

134 °C. Permeation cells containing anhydrous calcium chloride (0% RH) were sealed by the test film (0.7065 mm² film area) using melted paraffin (leaving an air gap of 1 cm between the film and the 135 desiccant). To keep a 75% RH gradient across the film, a sodium-chloride-saturated solution (75% 136 RH) was used in the desiccators. The RH inside the cell was always lower than outside, and water-137 vapor transport was determined using the weight gain of the cell at a steady state of transfer. 138 Changes in the weight of the cell were recorded to the nearest 0.0001 g and plotted as a function 139 of time. The slope of each line was evaluated by linear regression ($r^2 > 0.99$), and the water-vapor 140 transmission rate was calculated through the slope of the straight line (g/s) divided by the test area 141 (m²). All values for water-vapor transmission rate (WVTR) were corrected for air-gap distance 142 between the calcium chloride and the film surface according to the equations of Gennadios et al. 143 (1994): 144

145 WVTR =
$$\frac{slope}{film \, area} = \frac{\Delta m}{\Delta t \times A}$$
 (4)

146 After the permeation tests, the film thickness was measured and water-vapor permeability (WVP) 147 (g $Pa^{-1}s^{-1}m^{-1}$) was calculated as:

148 WVP =
$$\frac{\Delta m}{A \Delta t} \frac{X}{\Delta P}$$
 (5)

where $\Delta m/\Delta t$ is the weight of moisture gain per unit of time (g/s), X is the average film thickness (mm), A is the area of the exposed film surface (m²), and ΔP is the water vapor pressure difference between the two sides of the film (Pa). WVP was measured for three replicated samples for each type of film (Khazaei *et al.* 2014).

Moisture Sorption. Prepared film specimens (40 mm \times 15 mm) were placed at 25±2 °C over saturated salt solutions in separated desiccators under desired relative humidity conditions including 11, 22, 43, 52, 65, 75, 84 and 94% RH, which were provided by LiCl, CH₃COOK,
K₂CO₃, Mg(NO₃)₂, NaNO₂, NaCl, KCl, and KNO₃ (Rizvi 2005). The samples were weighed at 24
h intervals until the equilibrium state was reached. The moisture content (MC) was calculated from
the increase in mass of the dried sample after equilibration at a given RH (Galdeano *et al.* 2009).
All tests were conducted in triplicate.

160 MC (%) =
$$((M_0-M)/M0) \times 100$$

where, M_0 was initial weight of dry sample and M was the weight after the test, respectively.

162 **Transparency (TR).** Films transparency (TR) was determined using a UV Vis Spectrophotometer 163 (Varian Australia Pty. Ltd., Melbourne, VIC Australia). The films were cut into rectangular shapes 164 (5 mm \times 40 mm) and placed inside the spectrophotometer cell at 560 nm. Three replicates of each 165 film were tested and tests were conducted at 25±2 °C and 50±5% RH. The transparency of the 166 films was calculated by the following equation:

167 Transparency =
$$\log T/x$$

where T is transmission (%) at each wavelength; x is film thickness (mm) (Han and Floros 1997). **Color.** The color of each film was determined with a Minolta colorimeter (CR-300 series, Radiometric instruments Operations, Osaka, Japan). A white color plate was used as a standard for calibration and as a background for color measurements of the films. The lightness ('L') and chromaticity parameters 'a' (red-green) and 'b' (yellow-blue) were measured. Measures the total color difference (ΔE) and whiteness (WI) indexes of samples were calculated:

174
$$\Delta E = \sqrt{(L^* - L)^2 + (a^* - a)^2 + (b^* - b)^2}$$
 (6)
175

176 WI =
$$100 - \sqrt{(100 - L)^2 + a^2 + b^2}$$
 (7)

177 YI=
$$\frac{142.86b}{L}$$
 (8)

where L*, a*, and b* are the standard color parameter values and 'L', 'a', and 'b' are the color
parameter values of the sample (Khazaei *et al.* 2014). The measurements were repeated six times
for each film.

181 Mechanical Properties

Tensile Test. Samples were equilibrated at different relative humidities (11-94%) until constant
weight at 25 °C before being tested. The mechanical properties of the films were determined using
a Texture Analyzer (LLOYD Instrument LTD, Fareham, UK) according to the method described
by Farahnaky *et al.* (2013) with crosshead speed of 1 mm/s and initial grip distance 40 mm. Eight
film specimens (40 mm × 15 mm) of each formulation were used for mechanical tests.

Puncture Test. The puncture test was performed to realize the mechanical resistibility of films 187 under sharp stress by using a Texture Analyzer (LLOYD Instrument LTD, Fareham, UK). Films 188 189 were cut into a 4 cm-diameter disk and fixed in an annular ring clamp (3 cm diameter). A spherical probe of 1.0 mm diameter was moved vertically to the film surface at a constant speed of 1 mm/s 190 until the probe passed through the film. The force-deformation curves were followed. Force (N) 191 192 and deformation (mm) values at the puncture point were then recorded to represent the puncture strength (N) and deformation (mm) of the films. For each sample, eight replicates were performed 193 (Chen and Lai 2008). 194

Statistical Analysis. Glycerol was used in four levels of 0, 15, 25, and 35% and replicated three times. All experiments were performed in a randomized design. Analysis of variance was carried out and the results were separated using the Multiple Ranges Duncan's test (P < 0.05) using statistical software of Statistical Package for Social Science 16 (SPSS, Inc., NJ). All tests were performed at least in triplicate.

201 **RESULTS AND DISCUSSION**

202 Viscosity of Filmogenic Suspensions

Application of temperature (90 °C) for 20 minutes in preparation of 5% pea starch suspension is 203 generally thought to bring about an irreversible swelling, thus breaking the intermolecular 204 hydrogen bonds and leaching of amylose from granules, which results in a remarkable increase in 205 206 viscosity (Lagarrigue and Alvarez 2001; Li and Yeh 2001). The concentration of amylose, the architecture of the macromolecular components, mostly the size of the amylose and amylopectin 207 208 chains could change the film forming capacity of starches (Mali et al. 2004). The results in Table 1 show the apparent viscosity of the filmogenic suspensions at 5% w/w was decreased by the 209 210 addition of glycerol. Similar results have been reported for wheat starch (Farahnaky et al. 2013), 211 modified corn starch (López et al. 2008) and high amylose corn starch filmogenic suspensions 212 (Bertuzzi et al. 2007). Turhan et al. (2001) suggested that there is competition for hydrogen bonds 213 between starch chains and starch-plasticizer when glycerol is incorporated in the polysaccharide 214 network; direct interactions of starch chains and rigidity of the network are relatively reduced, 215 causing lower viscosity.

216 Solubility in Water

All films from pea starch produced from blends with glycerol were transparent, homogeneous andflexible. There was no sign of any opaqueness or inflexibility.

The water solubility of the pea starch films is presented in Table 1. The results show that the water solubility was higher in all films with glycerol compared to control (film without glycerol). The water solubility was 2.2% for the sample without glycerol and increased to 11.8 and 222 26.7% for the films containing 15 and 35% (w/w) glycerol, respectively. Control film showed limited tendency to interact with water molecules because the OH groups present in its structure 223 were more involved in the film network (Maran et al. 2013). Increasing glycerol concentration 224 gradually increased the film solubility due to the film structure changes. Since the total solid levels 225 in the cast pea starch film remained constant, the addition of glycerol increased the soluble matter 226 227 present in the films, leading to the formation of structures more sensitive to water (Kowalczyk et al. 2015). Increasing of water solubility might be because of an increase of the amorphous regions 228 (Flores et al. 2010) and increasing glycerol-starch interactions, which can interrupt the integrity of 229 230 polymeric network and increase water permeation into the film matrix (Basch et al. 2013).

Mehyar and Han (2004) reported that the water solubility of rice starch and pea starch films containing 50% glycerol based on starch weight was 44.4% and 32.0%, respectively. The presented results were comparable with previous investigations in high amylose corn starch (Bertuzzi *et al.* 2007), oxidized potato starch (Hu *et al.* 2009) and wheat starch films (Farahnaky *et al.* 2013).

236 Moisture Content and Moisture Sorption

The moisture content (%MC) of pea starch films containing different glycerol concentration (%w/w) are presented in Table 1 and show that the moisture content of pea starch films was significantly enhanced by the increase of glycerol (P < 0.05). It is often reported that the moisture content of hydrocolloid films increases with an increase in plasticizer concentration (Kristo and Biliaderis 2006; Mali *et al.* 2005a; Müller *et al.* 2008; Su *et al.* 2010; Zhang and Han 2006). In the absence of glycerol, the crystalline fraction of starch films holds a specific amount of water linked by hydrogen bonds, while amorphous regions have capacity to absorb relatively high amount of

water molecules (Flores et al. 2007). By incorporation of glycerol, the crystallization development 244 may be partially prevented, because it can disturb the configuration of polymeric chains by 245 interfering with amylose packing through the formation of glycerol-starch and glycerol-water 246 interactions (Famá et al. 2005). The increase of glycerol content resulted in weakening the 247 cohesiveness of the polymer structure, creating the polymer network with greater interchain 248 249 distances (Cerqueira et al. 2012), thus facilitating more water molecules immobilizing into the pea starch film matrix (Kowalczyk et al. 2015). Glycerol as a water holding agent with high number 250 of hydroxyl groups contributes to the formation of more hydrogen bonds in the film matrix and 251 252 increases the moisture content in the film.

253 The relationship between the water content of starch films and relative humidity is defined by moisture sorption isotherm (Srinivasa *et al.* 2003). The change in a sorption isotherm is a result 254 of some modification in the composition or structure of the films (Mali et al. 2005b) and the fact 255 256 that water is associated with the starch matrix with different mechanisms in different water activity 257 regions (Sebti et al. 2003). The moisture sorption in starch films can considerably change the barrier and physical characteristics of starch based films (Tongdeesoontorn et al. 2009). The mean 258 259 equilibrium moisture content (EMC) values of films are presented in Figure 1 and show that the 260 EM increased with increasing glycerol and % RH for each film. The sorption isotherm showed a sigmoidal shape (Type III) where the slope of the isotherms for pea starch films was smaller at 261 lower a_w (less than 0.60), with the raising in a_w the slope increased quickly, which brought about 262 large moisture adsorption with any increase in relative humidity (Coupland et al. 2000). At lower 263 264 relative humidities, water strongly adsorbed to the binding sites of the film surface, while by increasing moisture content, owing to the swelling of the hydrophilic network of films (Chen et 265 al. 2009a), more new sites for water were available to bind, causing higher equilibrium moisture 266

content (EMC) (Shih *et al.* 2011). At higher water activity, the accessibility of the hydroxyl groups
to the water molecules increases due to the swelling of the biopolymer and reduction of
crystallinity degree (Al-Muhtaseb *et al.* 2004). The molecular mobility and free volume in the film
network increased due to plasticization effect of water and high capacity of glycerol molecules to
interact with starch chains (Sothornvit and Krochta 2001).

272 Water Vapor Permeability

273 Variations of the thickness influence the mechanical and barrier characteristics of the packaging, 274 therefore control of the thickness in producing films by casting method should be taken into 275 account (Jansson and Thuvander 2004). The results presented in Table 1 show there was not much variation in the thickness of glycerol containing films which ranged between 0.14 and 0.21 mm. 276 277 The higher glycerol content, the more moisture absorbs resulting in increasing the thickness of the film because of swelling process (Jouki et al. 2013b). The similar behavior has been reported by 278 279 Jouki et al. (2013a) and Ahmadi et al. (2012). The density of the pea starch films was reduced by 280 addition of glycerol. This performance could be associated with the hydrophilic feature of glycerol causing modifications of the starch network structure that might become less dense (Alves et al. 281 2007). The decrease in density could be associated with the increased thickness (and volume) 282 regarding increasing plasticizer concentration. This is in agreement with those reported by Sevedi 283 et al. (2014) and Razavi et al. (2015). 284

The function of the film in food systems can be significantly affected by water vapor permeability (WVP) of the film. According to Gontard *et al.* (1994) the diffusion rate and solubility of water molecules in the film matrix determine the water vapor transmission through a hydrophilic film. The high plasticizer content can make the film less dense (Mali *et al.* 2004) and increase the

sorption of water (Sothornvit and Krochta 2000). Thus, water molecules are more readily absorbed 289 into the surface of the films (i.e. higher solubility) and penetrate through the film structure more 290 easily (i.e. higher diffusivity) resulting in the increase in WVP. However WVP reduced in films 291 with 15 and 25% glycerol. In lower concentrations of glycerol, sorption sites for water have been 292 proposed to be reduced due to formation of hydrogen bonds between hydroxyl groups of starch 293 294 and glycerol which resulted in decreasing diffusion of water molecules through films (Farahnaky et al. 2013; Talja et al. 2007). By increasing the glycerol content to 35%, WVP increased. Similar 295 results have been reported for whey protein (Sothornvit and Krochta 2000) and carboxymethyl 296 297 cellulose (Ghanbarzadeh et al. 2011), cassava starch (Alves et al. 2007), oat starch (Galdeano et al. 2009), high-amylose rice and pea starch (Mehyar and Han 2004), and yam starch films (Mali 298 299 et al. 2004).

300 Optical properties

301 Since starch films are used as a food coating or as food packaging, transparency (TR) is an essential 302 feature of edible films that influences product appearance and consumer acceptability (Gontard et al. 1992). The results presented in Table 2 show that film transparency was influenced by glycerol, 303 304 where an increase in glycerol content caused a reduction in film transparency. More transparent films correspond with high values of internal transmittance, while low values of internal 305 306 transmittance are related to more opaque films (Ortega-Toro et al. 2014). It has been reported that the increasing of the solid concentration in the film-forming dispersion causes films become 307 thicker owing to the increased total residual mass in the film (Mali et al. 2005a). There is a negative 308 309 relationship between the thickness and transparency of the films, signifying that the thinner films 310 were simply more transparent (Kim et al. 2014). Generally data revealed that the utilization of glycerol in pea starch film made the film thicker and less transparent. The hydroxyl groups of 311

312 glycerol forms higher polymeric chain compaction modifying the refractive index and restricts the passage of the light through the film matrix (Ortega-Toro et al. 2014). The decrease in transparency 313 may be associated with the modified network structure by starch/glycerol interaction. Packaging 314 films with low transparency can protect light sensitive food products from photodegradation (Bhat 315 et al. 2013). This means that addition of glycerol to the films improved the ability of the films to 316 317 protect package contents from light increasing the quality of the packaged food. Similar findings have been observed by Shaw et al. (2002) and Bertuzzi et al. (2007) in whey protein isolate and 318 corn starch films. 319

320 The Hunter 'L', 'a', and 'b' color values, total color difference (ΔE), and whiteness index 321 (WI) and yellowness index (YI), of the pea starch films are presented in Table 2. The main 322 differences in color values among the pea starch films were the increased 'a' value (redness) with increasing glycerol content, however there were no other differences in values in other color 323 324 characteristics detected among the films. The control sample was generally transparent and clear 325 (highest 'L' value) but films with 35% (w/w) glycerol showed the high positive 'a' and positive 'b' values (0.02 and 2.92, respectively). The increasing value of 'a' indicates films to be more 326 reddish, whereas increasing value of 'b' indicates yellow color of the films. Moreover, it should 327 be noted that the decrease in 'L' parameter with glycerol content is in agreement with the 328 transparency decrease. 329

330 Mechanical Properties

331 Tensile Test

After storing the films at 25 °C under different RH for 72 h, the tensile properties of the samples were tested (Tables 3a and 3b) and show that the effects of both RH and plasticizer content had significant effects on the tensile mechanical properties. Generally, the increase of RH and plasticizer content resulted in a decrease in the tensile strength (TS) of the films, representing thatboth water and glycerol plasticized the pea starch films.

337 Contrary to the conventional behavior of plasticization, over the lower range of humidification ($a_w=0.11$) tensile strength of films increased by increasing of glycerol content. 338 Therefore, it seems that glycerol can apply an antiplasticizing effect on tensile strength in drier 339 340 films. At a_w=0.22, maximum value in tensile strength were observed in films with 25% glycerol 341 incorporated. The films containing 0% and 15% glycerol presented an increase in tensile strength with increasing a_w, as the tensile strength was 6.2 MPa for the control (unplasticized) sample and 342 8.4 MPa for sample with 15% glycerol and enhanced significantly (P < 0.05) to 19.2 MPa and 343 27.5 MPa at 65% RH and 43% RH, respectively. Similar trends were observed for maximum 344 345 breaking force (N) and breaking factor (N/mm) of films (Table 3a). The increase in mechanical 346 properties at very low and intermediate a_w can be explained as mechanical antiplasticization by 347 water in biopolymer films, which has been previously reported by many other researchers (Chang 348 et al. 2006; Chang et al. 2000a; Mathew and Dufresne 2002). Retrogradation phenomenon and the water-induced crystallization of the material mainly govern the ultimate characteristics of the 349 350 glycerol plasticized starch films (Mathew and Dufresne 2002). Storing films at the range of low to 351 intermediate aw accelerates the development of crystallites in the starch films, inducing the 352 antiplasticization (Zhang and Han 2010). Under such conditions, water and plasticizer molecules 353 increasingly pushed aside from starch polymers because of the movement or vibration of the starch 354 polymer chains. Consequently, the separated D-glucosyl residues of the amylose or amylopectin linked to create strong hydrogen bonds triggering retrogradation or recrystallization, which affects 355 the degree of plasticity. Higher crystallinity leads to a polymer that is more rigid and less flexible 356 357 (Kowalczyk et al. 2014). Hydrogen bonds between the starch chains are thought to contribute to

the high tensile strength value where these bonds bring about the high cohesiveness and low
flexibility of the films (Bertuzzi *et al.* 2012).

360 Above the intermediate RH (\geq 65%), the tensile strength of all films decreased with increasing glycerol content. The 'glycerol threshold' or 'glycerol antiplasticizing range' appeared 361 to become gradually narrow with an increase in moisture content until the antiplasticizing effect 362 was no longer obvious and glycerol converted to its function as a typical plasticizer at $a_w > 0.65$ 363 (Chang et al. 2006). The role of glycerol is to attract the water molecules around itself, decrease 364 the intermolecular interactions between the starch molecules, and then increase the flexibility of 365 starch films (Ke and Sun 2001). The plasticization of starch is a process of overcoming the 366 brittleness in starch by softening the structure and by increasing the mobility of the 367 368 macromolecular chains. Three theories have been recommended to explain the mechanisms of plasticization. These are lubricity theory, gel theory, and free volume theory. Lubricity theory 369 suggests that the plasticizer performs as a lubricant and lubricates movements of the 370 371 macromolecules over each other. Gel theory presents that the plasticizer disturbs the interaction of 372 starch chain bonds. Free volume theory proposes that plasticizer increases free volume between 373 the starch chains and lowers its glass transition temperature (Tg). Generally, plasticizer is 374 considered to place itself between the starch chains and decrease the forces holding the chains 375 together (Di Gioia and Guilbert 1999). An increase in molecular mobility of amorphous and to 376 some extent crystalline polymers could be attributed to the low molecular weight of water 377 molecules and glycerol, resulting in a fragile starch matrix because of an increased free volume (Farahnaky et al. 2013). Other authors have also reported a similar tendency (Galdeano et al. 2009; 378 Mali et al. 2004; Mali et al. 2005a). 379

380 Table 3b showed the percent elongation at break and deformation at break of pea starch films depend on glycerol concentration and amount of RH in the storage atmosphere. The percent 381 elongation and deformation at break of pea starch films increased by increasing glycerol content 382 and RH at low and intermediate relative humidity (RH < 84%). The permeated glycerol and water 383 molecules could further reduce the interactions (intra and intermolecular) among the starch 384 385 macromolecules, as a result of the formation of hydrogen bonds between hydroxyl groups of starch macromolecules, water and small molecules of glycerol. These contribute to polymeric chain 386 connection assisting their slithering and accordingly improving the facility of polymer chains 387 388 mobility, so reducing the firmness of the network, and generating a less ordered film structure (Sothornvit and Krochta 2005). Consequently, the elongation value of starch films increased. The 389 increase of elongation at break with plasticizer content was also reported by several authors for 390 starch-based films (Alves et al. 2007; Chang et al. 2000a; Gaudin et al. 1999; Hu et al. 2009; 391 López et al. 2008; Myllärinen et al. 2002). At high levels of RH, the percent elongation and 392 deformation at break of glycerol-plasticized films decreased slightly with the increasing glycerol 393 content. The increasing of RH in the storage atmosphere and glycerol above a critical value (25%) 394 resulted in small elongation and deformation at break due to the weakness of the interactions 395 396 between the polymers and the reduction of the cohesive force of the polymer chains (Zhang and Han 2008). 397

Increasing glycerol content resulted in films with higher Young's modulus (Table 3b) at a_w=0.11. At a_w=0.22, the Young's modulus of films increased, reaching a maximum at 15% glycerol content, and then decreased dramatically. The plasticized films would be expected to become more cohesive at low moisture levels, thus causing greater resistance and higher Young's modulus. It can be explained that the increase in Young's modulus could be due to 403 antiplasticization effect of water and glycerol. It is probable at low a_w, applied force during test could produce much greater bond breaking/forming, increase orientation of polymer chains, and 404 enhance free volume by creating "holes" in the polymeric system (Chang et al. 2000a). Water 405 molecules in small amounts would promote polymer alignment as well as fill "holes" formed under 406 stress, reducing "hole free volume" and further increasing structural orientation, which would be 407 408 demonstrated by increased Young's modulus of the films. Reduction of free volume through "hole filling" is a theory for antiplasticization of synthetic polymers by diluents described by several 409 researchers (Anderson *et al.* 1995; Chang *et al.* 2000a; Vrentas *et al.* 1988). However, at $a_w \ge 0.43$, 410 411 increasing glycerol content resulted in a continuous decrease in Young's modulus. This can be explained by the over-plasticization of the polymers. The plastic effect of water and enlargement 412 of pores and cracks resulting in film swelling in the higher moisture environment created the 413 reduction in Young's modulus with the increasing of glycerol content from 0% to 35% (Bertuzzi 414 et al. 2007). The plastic effect of water on films was enhanced at high RH (Table 3b). Similar 415 results have been previously reported for hydroxypropyl starch films (Arvanitoyannis and 416 Biliaderis 1998), potato and barley starch films (Koskinen et al. 1996) and for oat starch films 417 (Galdeano et al. 2009). 418

419 **Puncture Test**

The maximum puncture force and deformation of pea starch films are presented in Table 4 and show that the puncture force and deformation were affected by glycerol concentration and RH. The lowest puncture force (1.1 N) and the highest puncture deformation (7.10 mm) values were obtained for films formulated with 35% glycerol (w/w) at 97% RH and 25% glycerol (w/w) at 75% RH, respectively. Under the storage RH of 75% and 52%, the addition of 15% glycerol significantly enhanced the puncture deformation and puncture force of pea starch films, 426 respectively, while higher concentrations of glycerol led to a decrease in puncture mechanical factors of films. The effects of glycerol are well-known where glycerol incorporated into the starch 427 network results in the reduction of intermolecular interactions, and the increase of the mobility of 428 polymer chains (Cuq et al. 1997; Mali et al. 2002; Mali et al. 2004; Mali et al. 2005a; Parris et al. 429 1995; Sobral et al. 2001; Sothornvit and Krochta 2001). Consequently the puncture strength 430 431 decreased and puncture deformation increased with increasing glycerol concentration in the studied starch films. Gontard et al. (1992) found that puncture deformation values ranged from 4.8 432 to 17.6 mm with increasing of plasticizer content in wheat gluten films. An increase in puncture 433 434 deformation of gelatin films from 1.2 to 5.3% with increasing of sorbitol content from 15 to 65 g/100 g gelatin was observed by Sobral et al. (2001). Tapioca starch/decolorized hsian-tsao leaf 435 gum and yam starch plasticized with glycerol showed puncture strength and deformation (1.86-436 5.10 N, 1.46–3.28 mm) and (6.03–15.96 N, 3.44–4.78 mm), respectively (Chen and Lai 2008; Mali 437 et al. 2004). 438

439

440 CONCLUSION

441 The pea starch films produced from yellow pea starch (5% w/w) formulated with glycerol as 442 plasticizer were easy to handle and were not sticky. The effect of glycerol on the physical and 443 mechanical properties of plasticized pea starch films was dependent on its concentration. The increase of storage RH and glycerol content decreased the tensile and puncture mechanical 444 445 properties of the films. The optical properties of the films did not noticeably change by increasing glycerol content. However with increasing the plasticization effect of glycerol, the water solubility, 446 water sorption and WVP of pea starch-based edible films significantly increased. WVP was lower 447 448 for the films with 15 and 25% w/w glycerol compared with the films with 35% w/w. Films from

- 449 yellow pea starch formulated with 15 and 25% glycerol contents showed good performance of
- 450 flexibility and low water permeability, indicating potential application as edible films in food and
- 451 pharmaceutical industries.

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454 **CONFLICT OF INTREST**

455 The authors declare no conflict of interest.

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728 FIGURE CAPTION

FIGURE 1. EQUILIBRIUM MOISTURE CONTENT (% EMC, G WATER/G DRY MATTER)

730 OF PEA STARCH FILMS WITH DIFFERENT GLYCEROL CONTENTS (% W/W) AT 25 $^{\circ}$ C.

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PS	Glycerol (%w/w)			
film	0	15	25	35
Apparent Viscosity	960.857±38.033ª	902.286±36.358 ^{ab}	833.333±28.024 ^{cb}	783.500±53.642°
(IIIPa.s) Solubility in water				
(%)	2.206 ± 1.106^{d}	11.784±0.115°	19.354±1.828 ^b	26.704±2.228ª
Moisture Content	0.493 ± 0.134^{d}	1.549±0.361°	3.290±0.209 ^b	5.193±0.702 ^a
Thickness $\times 10^{-3}$ (m)	0.143±0.010 ^a	0.164±0.024 ^a	0.166±0.033 ^a	0.206±0.051ª
Density $\times 10^{-2}$ (gmm ⁻³)	2.135±0.002 ^a	1.282±0.002 ^b	1.129±0.003 ^b	0.985 ± 0.002^{b}
WVTR ×10 ⁻⁵ (gs ⁻¹ m ⁻²)	2.165±0.261 ^{ab}	1.604±0.206 ^b	1.751±0.236 ^b	2.792±0.116 ^a
WVP ×10 ⁻⁹ (gs ⁻¹ m ⁻¹ Pa ⁻¹)	2.051±0.247 ^b	1.129±0.167°	1.859±0.592 ^{bc}	3.281±0.136 ^a

* Values are the means of triplicates \pm standard deviations. Means at same line with different super manuscript letters are significantly different (*P* < 0.05).

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Table 2. THE TRANSPARENCY AND HUNTER COLOR VALUES ('L', 'a' AND 'b'),
TOTAL COLOR DIFFERENCES (ΔΕ), YELLOW INDEX (YI), AND WHITNESS INDEX (WI)
OF PEA STARCH FILMS AS A FUNCTION OF GLYCEROL CONTENT.*

Glycerol	(%w/w)	0	15	25	35
Transp	arency	14.538±0.121 ^a	12.274±0.193 ^b	10.199±1.62 ^c	8.952±0.327 ^d
	L	96.542±0.068ª	96.440±0.134ª	96.298±0.310 ^a	96.171±0.273 ^a
	а	-0.264±0.013 ^d	-0.231±0.007°	-0.149±0.002b	0.020 ± 0.003^{a}
Color	b	2.828 ± 0.007^{a}	2.867 ± 0.095^{a}	2.897±0.137 ^a	$2.918{\pm}0.058^a$
value	ΔΕ	0.811 ± 0.076^{a}	0.916 ± 0.050^{a}	1.031±0.312 ^a	$1.159{\pm}0.284^{a}$
	WI	95.525±0.051ª	95.421 ± 0.148^{a}	95.297±0.324 ^a	95.181±0.247 ^a
	YI	4.184±0.008 ^a	4.247±0.145 ^a	4.298±0.218 ^a	4.335±0.095 ^a

* Values are the means of triplicates ± standard deviations. Means at same line with different super manuscript letters

772 are significantly different (P < 0.05).

			Glycerol (%)			
_	RH (%) -	0	15	25	35	
	11.3	6.268±0.346 ^{Fd}	8.369 ± 0.527^{Ec}	16.228±1.306 ^{Cb}	22.272±1.545 ^{Aa}	
	22.5	9.606±0.593 ^{Ed}	15.098 ± 0.465^{Cc}	20.790±0.806 ^{Aa}	17.877 ± 0.930^{Bb}	
	43.2	12.289±0.717 ^{Cc}	27.537±1.391 ^{Aa}	18.365 ± 0.774^{Bb}	13.117±0.871 ^{Cc}	
Tensile	52.9	16.529 ± 0.720^{Bb}	22.174 ± 0.374^{Ba}	11.755±0.921 ^{Dc}	$8.770 \pm 0.717^{\text{Dd}}$	
strength	65.4	19.202±0.357 ^{Aa}	$12.199 \pm 0.743^{\text{Db}}$	8.327±0.553 ^{Ec}	5.058 ± 0.349^{Ed}	
(MPa)	75.3	10.567 ± 0.598^{Da}	$7.887 \pm 0.569^{\text{Eb}}$	$5.548 \pm 0.434^{\text{Fc}}$	3.099 ± 0.480^{Fd}	
	84.3	7.118 ± 0.508^{Fa}	$4.626 \pm 0.402^{\text{Fb}}$	3.343±0.350 ^{Gc}	$2.112 \pm 0.170^{FG,d}$	
	93.6	4.038±0.471 ^{Ga}	$2.704 \pm 0.253^{\text{Gb}}$	2.048 ± 0.212^{Hc}	1.097 ± 0.148^{Gd}	
	97.3	2.483 ± 0.289^{Ha}	1.530 ± 0.321^{Hb}	$1.319 \pm 0.320^{H,bc}$	0.883 ± 0.067^{Gc}	
	11.3	12.919+0.714 ^{Fd}	17.249+1.087 ^{Ec}	33.446+2.692 ^{Cb}	45.902+3.185 ^{Aa}	
	22.5	19.798 ± 1.221^{Ed}	31.118 ± 0.959^{Cc}	42.847±1.660 ^{Aa}	36.845+1.917 ^{Bb}	
	43.2	25.328 ± 1.478^{Cc}	56.754+2.867 ^{Aa}	37.851+1.596 ^{Bb}	27.033 ± 1.795^{Cc}	
Maximum	52.9	$34.067 \pm 1.485^{\text{Bb}}$	45.701 ± 0.772^{Ba}	$24.227 \pm 1.899^{\text{Dc}}$	$18.074 \pm 1.477^{\text{Dd}}$	
breaking	65.4	39.579±0.736 ^{Aa}	25.143±1.532 ^{Db}	$17.162 \pm 1.139^{\text{Ec}}$	$10.424 \pm 0.720^{\text{Ed}}$	
force (N)	75.3	21.779 ± 1.232^{Da}	$16.256 \pm 1.172^{\text{Eb}}$	11.435 ± 0.894^{Fc}	$6.387 \pm 0.989^{\text{Fd}}$	
	84.3	14.671 ± 1.047^{Fa}	9.533±0.829 ^{Fb}	6.890 ± 0.722^{Gc}	4.352±0.351 ^{FG,d}	
	93.6	8.322 ± 0.972^{Ga}	$5.573 \pm 0.522^{\text{Gb}}$	4.221±0.437 ^{Hc}	2.262 ± 0.380^{Gd}	
	97.3	5.116 ± 0.596^{Ha}	3.154 ± 0.661^{Hb}	$2.718 \pm 0.660^{H,bc}$	1.820 ± 0.138^{Gc}	
	113	90 531+5 001 ^{Fb}	105 432+6 644 ^{Eb}	201 363+16 208 ^{Ca}	222 717+15 453 ^{Aa}	
	22.5	138 736+8 559 ^{Ec}	190 205+5 862 ^{Cb}	257 960+9 996 ^{Aa}	$17870+9302^{Bb}$	
	43.2	$177 488 \pm 10 357^{Cc}$	$346\ 907+17\ 526^{Aa}$	227 881+9 608 ^{Bb}	131 167+8 707 ^{Cd}	
Breaking	52.9	238.733 ± 10.405^{Bb}	$279.344+4.716^{Ba}$	$145.856+11.433^{\text{Dc}}$	87.696+7.167 ^{Dd}	
factor	65.4	277.336+5.158 ^{Aa}	153.685+9.365 ^{Db}	$103.322+6.857^{\text{Ec}}$	$50.580 + 3.491^{\text{Ed}}$	
(N/mm)	75.3	152.623+8.635 ^{Da}	99.365+7.166 ^{Eb}	68.846+5.382 ^{Fc}	30,990+4,801 ^{Fd}	
(1 ())	84.3	102.808±7.334 ^{Fa}	58.272±5.067 ^{Fb}	41.481 ± 4.348^{Gc}	$21.116 \pm 1.704^{FG,d}$	
	93.6	58.317±6.810 ^{Ga}	$34.063 \pm 3.190^{\text{Gb}}$	$25.410\pm2.628^{\text{Hc}}$	10.974 ± 1.842^{Gd}	
	97.3	35.855±4.179 ^{Ha}	19.276±4.038 ^{Hb}	16.361±3.974 ^{Hb}	8.829±0.670 ^{Gc}	

 Table 3a. TENSILE MECHANICAL PROPERTIES OF PEA STARCH FILMS.*

* Values are the average of triplicates \pm standard deviation. Means at same row with different lower case are significantly different (P < 0.05); means at same column with different upper case are significantly different (P < 0.05).

	$\mathbf{DII}(0/)$	Glycerol (%)			
	KH (%)	0	15	25	35
	11.3	1.374 ± 0.172^{Hd}	4.052 ± 0.942^{Gc}	7.092±1.062 ^{Fb}	$11.355 \pm 0.751^{EF,a}$
	22.5	4.390±1.066 ^{Gc}	6.422 ± 0.444^{Fc}	$10.305 \pm 1.080^{\text{Eb}}$	15.670 ± 1.996^{Da}
	43.2	7.526±0.490 ^{Fd}	11.294±1.227 ^{Ec}	$15.450 \pm 2.005^{DE,b}$	20.185±1.305 ^{BC,a}
Percent	52.9	$11.718 {\pm} 0.803^{\text{Ed}}$	14.765±0.749 ^{Dc}	18.947±2.057 ^{Cb}	22.872±0.935 ^{Ba}
elongation at	65.4	14.292±1.684 ^{Dc}	16.834±1.956 ^{BCD,c}	$21.967 \pm 0.884^{AB,b}$	26.851±2.410 ^{Aa}
break (%)	75.3	$16.163 \pm 1.856^{\text{CD,c}}$	20.451 ± 0.979^{Ab}	24.503 ± 2.388^{Aa}	23.203±2.111 ^{B,ab}
	84.3	$22.384 \pm 1.697^{BC,a}$	$18.916 \pm 1.155^{AB,a}$	20.953±1.379 ^{BC,a}	19.381 ± 2.460^{Ca}
	93.6	20.359±2.230 ^{AB,a}	$17.831 \pm 2.416^{BC,ab}$	$16.142 \pm 1.357^{D,bc}$	13.727±1.756 ^{DE,c}
	97.3	18.555 ± 1.217^{Ea}	$15.748 \pm 0.719^{\text{CD,b}}$	$12.984 \pm 1.405^{\text{EF,c}}$	10.095 ± 2.007^{Fd}
	11.3	0 550+0 069 ^{Hd}	1 621+0 379 ^{Gc}	2 837+0 425 ^{Gb}	4 542+0 301 ^{EF,a}
	22.5	1.756 ± 0.427^{Gc}	$2569\pm0.178^{\text{Fc}}$	$4 122 \pm 0.432^{\text{Fb}}$	6 268+0 799 ^{Da}
	43.2	3.010 ± 0.196^{Fd}	$4518+0.491^{\text{Ec}}$	$6.180\pm0.802^{\text{DE,b}}$	8 074+0 522 ^{BC,a}
	52.9	4.687 ± 0.321^{Ed}	$5,906\pm0,299^{\text{Dc}}$	7 579+0 823 ^{Cb}	9 149+0 374 ^{Ba}
Deformation at	65.4	5 717+0 674 ^{Dc}	6 734+0 782 ^{BCD,c}	8 787+0 354 ^{AB,b}	$10.740+0.964^{Aa}$
break (mm)	75 3	$6465\pm0743^{CD,c}$	8 181+0 391 ^{Ab}	9 801+0 955 ^{Aa}	9 281+0 $844^{B,ab}$
	84 3	8 954+0 679 ^{Aa}	7 566+0 462 ^{AB,a}	8 381+0 552 ^{BC,a}	7 752+0 984 ^{Ca}
	93.6	8,144+0,892 ^{AB,a}	$7.132+0.966^{BC,ab}$	6.457+0.543 ^{D,bc}	5.491+0.702 ^{DE,c}
	97.3	7.422±0.487 ^{BC,a}	6.299±0.288 ^{CD,b}	5.194±0.562 ^{EF,c}	4.038±0.803 ^{Fd}
	11.3	177 184+14 752 ^{Cd}	216 884+6 231 ^{Cc}	263 321+16 623 ^{Ab}	358 669+30 279 ^{Aa}
	22.5	226 745+15 666 ^{Bb}	285 675+14 005 ^{Aa}	$172.688+13.632^{Bc}$	$115\ 197+14\ 446^{Bd}$
	43.2	273 392+5 224 ^{Aa}	245 144+19 668 ^{Bb}	$120.685+20.968^{Cc}$	65 018+2 892 ^{Cd}
Young	52.9	$150.483 \pm 11.473^{\text{Da}}$	110.357+5.134 ^{Cb}	$62.310+5.066^{\text{Dc}}$	38.414+4.023 ^{Dd}
modolous	65.4	135.906+19.726 ^{Da}	72.849+5.721 ^{Db}	37.951+2.971 ^{DE,c}	$19.021+3.093^{\text{Ed}}$
(MPa)	75.3	65.735±5.066 ^{Ea}	38.580±2.476 ^{DE,b}	22.778 ± 2.693^{Ec}	13.444 ± 2.587^{Ed}
()	84.3	31.811±0.570 ^{EF,a}	$24.463 \pm 1.647^{\text{Eb}}$	$16.003 \pm 2.005^{\text{Ec}}$	$11.069 \pm 2.086^{\text{Ed}}$
	93.6	19.851±1.355 ^{Fa}	15.505±3.788 ^{Eb}	12.750 ± 1.796^{Eb}	7.970±0.347 ^{Ec}
	97.3	13.348±0.703Fa	9.753±2.256 ^{Eb}	10.069±1.638 ^{Eb}	8.914±1.311 ^{Eb}

 Table 3b. TENSILE MECHANICAL PROPERTIES OF PEA STARCH FILMS.*

* Values are the average of triplicates \pm standard deviation. Means at same row with different lower case are significantly different (P < 0.05); means at same column with different upper case are significantly different (P < 0.05).

Table 4. PUNCTURE MECHANICAL PROPERTIES OF PEA STARCH FILMS WITH DIFFERENT GLYCEROL CONTENTS

(% W/W) AT 25 °C AND DIFFERENT RELATIVE HUMIDITIES.*

	RH (%) -	Glycerol			
		0	15	25	35
	11.3	$0.333 {\pm} 0.075^{Gd}$	1.296±0.058 ^{Fc}	2.443±0.283 ^{Db}	3.699±0.477 ^{Ca}
	22.5	1.321±0.712Fc	$2.269 \pm 0.475^{\text{Eb}}$	3.505 ± 0.364^{Ca}	4.207 ± 0.335^{Ca}
	43.2	$2.618 \pm 0.497^{\text{Ec}}$	3.525±0.294 ^{CD,bc}	4.267 ± 0.209^{Cb}	5.384 ± 0.990^{Ba}
Puncture	52.9	3.404 ± 0.223^{Dc}	4.039±0.770 ^{Cc}	5.198 ± 0.667^{Bb}	6.562±0.413 ^{Aa}
deformation	65.4	4.603±0.231 ^{Cd}	5.327 ± 0.282^{Bc}	6.386 ± 0.426^{Ab}	7.031±0.281 ^{Aa}
(mm)	75.3	$5.805 \pm 0.509^{B,bc}$	6.734±0.451 ^{A,ab}	$7.094{\pm}0.550^{Aa}$	5.271 ± 0.448^{Bc}
	84.3	7.037±0.542 ^{Aa}	5.636±0.140 ^{Bb}	6.770 ± 0.643^{Aa}	4.595±0.262 ^{BC,c}
	93.6	5.689±0.591 ^{Ba}	4.946±0.614 ^{B,ab}	$4.047 \pm 0.549^{\text{Cb}}$	2.243±0.523 ^{Dc}
	97.3	3.922±0.213 ^{CD,a}	3.175 ± 0.498^{Da}	2.229±0.393 ^{Db}	$1.483 \pm 0.703^{\text{Db}}$
	11.3	3.169±0.537 ^{Fd}	4.990±0.746 ^{Fc}	6.522 ± 0.609^{Eb}	15.571±1.074 ^{Aa}
	22.5	$7.987 \pm 0.636^{\text{Ed}}$	16.298 ± 0.668^{Cb}	$21.021{\pm}1.701^{Aa}$	11.525±0.728 ^{Bc}
	43.2	14.275±0.686 ^{Cc}	22.400 ± 0.850^{Aa}	15.879 ± 0.577^{Bb}	9.585 ± 0.502^{Cd}
Maximum	52.9	16.763±1.833 ^{Bb}	$18.938 {\pm} 0.755^{\text{Ba}}$	11.811±0.789 ^{Cc}	$7.860 \pm 0.641^{\text{Dd}}$
puncture	65.4	18.402±0.395 ^{Aa}	13.255±1.376 ^{Db}	9.732±0.531 ^{Dc}	5.244 ± 0.461^{Ed}
force (N)	75.3	13.233±1.626 ^{Ca}	$10.204 \pm 0.388^{\text{Eb}}$	7.761 ± 0.864^{Ec}	4.164 ± 0.883^{Ed}
	84.3	10.634±1.009 ^{Da}	7.207 ± 0.483^{Eb}	4.801±0.395 ^{Fc}	$2.530 \pm 0.572^{\text{Fd}}$
	93.6	6.886 ± 0.652^{Ea}	4.933±0.753 ^{Fb}	2.324 ± 0.342^{Gc}	1.679±0.327 ^{FG,c}
	97.3	$4.325{\pm}0.474^{Fa}$	2.450 ± 0.552^{Gb}	1.619±0.113 ^{Gc}	1.101±0.213 ^{Gc}

* Values are the average of triplicates \pm standard deviation. Means at same row with different lower case are significantly different (P < 0.05); means at same column with different upper case are significantly different (P < 0.05).