

# Innovative gliadin/glutenin and modified potato starch green composites: chemistry, structure, and functionality induced by processing

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1	Innovative gliadin/glutenin and modified potato starch green composites:
2	chemistry, structure, and functionality induced by processing
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#### 15 Abstract

16 Designing green bio-based polymeric materials with controlled processing properties and versatile functionality of the final product is still a challenging task. In this study we combined 17 two natural components, wheat proteins; gliadin (Gli)/glutenin (Gt) and modified potato starch 18 19 (MPS) into a composite using extrusion processing. In the Gli/Gt MPS composites, we studied the structural dynamics of proteins and starch, protein-starch interactions, protein properties 20 and final material morphology in relation to mechanical and barrier properties. Different ratios 21 22 of Gli/Gt- MPS were plasticized using either glycerol, or glycerol-water and extruded at 110 and 130 °C. For the first time a hierarchical hexagonal structure was observed in the Gli-MPS 23 composite at both extruding temperatures. Higher temperature (130 °C) induced a high degree 24 of cross-links, an increase in the molecular weight of proteins and more  $\beta$ -sheets compared to 25 110 °C. Gt-MPS 50/50 sample extruded with glycerol and water showed large amount of  $\beta$ -26 sheets at 130 °C. Combination of plasticizers, glycerol and water, favored the macro-structural 27 morphology, improved gelatinization of starch, processability and tensile properties of Gt-MPS 28 composites. Highest amount of the oxidized protein was observed in the highest protein content 29 samples and at high extrusion temperatures. Gli- and Gt-MPS (30/70) samples showed very 30 31 good oxygen barrier properties under ambient testing conditions. These findings provide indepth information for tailoring the structural-functional relationship of the Gli/Gt-potato starch 32 composites for their promising use in designing environmentally friendly materials. 33

#### 35 **1. Introduction**

Naturally occurring plant based feedstocks have gained interest as "green" alternatives for 36 replacing petroleum based materials. Wheat gluten (WG) protein and potato starch are two 37 natural polymers known for their complex macromolecular structures in both un-processed and 38 temperature processed products (Lagrain et al., 2010, Yu et al., 2006). Both polymers have 39 shown attractive polymerization and thermoplastic properties resulting from both the protein or 40 starch chemical reactions when processed with a plasticizer (e.g. water or glycerol) and 41 42 temperature into a green material (Gonzalez et al, 2010). For example, the WG films demonstrated high viscoelasticity, extensibility and reasonable strength, while starch films 43 showed good processability, the properties suitable for making a number of biodegradable 44 products (Johansson et al., 2013, Wu et al., 2014, Blomfeldt et al., 2012, Ullsten et al., 2006, 45 Thunwall et al., 2006, Van Hung et al., 2006). 46

Wheat gluten proteins are available as a co-product of bio-ethanol industry. Two major types 47 of proteins from WG; high molecular weight glutenins and low molecular weight gliadin are 48 the main determinants of the functional properties in films, foams and adhesives (Chen et al., 49 2012, Blomfeldt et al., 2012, Kuktaite et al., 2012). In several studies on the thermo-processed 50 51 gliadin/glutenin materials, an importance of the protein structure in a close relation to the 52 mechanical properties of the final product has been observed (Rasheed et al. 2015a, Kuktaite et 53 al., 2014). Although, that the gliadin structure was assumed as an indicator for predicting 54 processing behavior of the protein film (Rasheed et al., 2015b, Rasheed et al., 2014), still more knowledge is needed to fully understand and steer this process. In particular, a better control of 55 the process chemistry and tuning of gliadin/glutenin protein in a blend with other components 56 57 like starch still remain unclear.

Native potato starch consists of 20-30% of amylose, a long chain linear glucose polymer, and 58 59 70-80% of amylopectin, with a highly branched molecular structure. Starch have been chemically or genetically modified to change amylose vs. amylopectin ratio, which led to 60 improvement of the functional properties of starch based materials (Van Hung et al., 2006). 61 Due to the modification, the macromolecular structure of the starch resulted into the linear 62 molecules of amylose with debranched amylopectin, which increased crosslinking and 63 improved the mechanical properties (Van Hung et al., 2006). However, more research is needed 64 in the area of the starch polymer chemistry and materials including starch processing with other 65 additives such as proteins. 66

67 In our previous study, we have showed that it is possible to extrude WG and modified potato starch composites with rather promising functional properties (mechanical and gas 68 permeability) for packaging materials (Muneer et al., 2015). The macromolecular structure of 69 WG-starch composites was under the influence of processing temperature (130 °C) and 70 plasticizer (45% glycerol or 30% glycerol and 20% water). The observed hexagonal structure 71 of WG proteins in the composites having 50% or more of protein was in favor for increased 72 73 extensibility of the materials. In WG based materials, proteins undergo irreversible aggregation 74 with the formation of disulphide cross-links, hydrogen and isopeptide bonds during thermo-75 mechanical and chemical treatment (Johansson et al., 2013, Domenek et al., 2003). The aggregation of proteins and formation of a great amount of  $\beta$ -sheets seem to be one of the 76 77 reasons why commercial WG protein arranges into hexagonal structure (Kuktaite et al., 2011, Kuktaite et al., 2012, Kuktaite et al., 2014). For the gliadin materials, protein plasticization with 78 glycerol during thermal processing was adequate to achieve the hexagonal structural 79 arrangement (Rasheed et al., 2014). 80

Although, a control of protein and starch structure during processing and in the final productremains a challenge when developing new plant polymer materials with desired functionalities.

In this work, we have combined modified potato starch and gliadin/glutenin into a composite using extrusion processing to study the structure and macromolecular properties, starch-protein network in relation to process-dependent modifications. The aim of this study was to monitor in detail the structural organization, polymerization and cross-linking of starch-protein macropolymers (in a blend) induced by various processing conditions. The macromolecular structure in relation to the functional properties (mechanical and barrier) of the composite was also investigated.

#### 90 **2. Experimental**

#### 91 2.1 Materials

Wheat gluten powder (protein 77.7%, starch 5.8%, moisture 6.9% and fat 1.2%) was graciously
supplied by Lantmännen Reppe AB, Lidköping, Sweden. Potato starch with long chain
amylopectin and amylose content of 27% was developed at the Department of Plant Breeding,
Swedish University of Agricultural Sciences, Alnarp, Sweden and was designated as modified
potato starch (MPS) in this study. Starch was processed at Lyckeby Starch AB, Kristianstad,
Sweden. The glycerol (99.5%) was supplied by Karlshamn Tefac AB, Karlshamn, Sweden.

#### 98 **2.2 Methods**

#### 99 2.2.1 Separation of glutenin and gliadin from wheat gluten

Glutenin (Gt) and gliadin (Gli) were extracted from WG following the method described in (Blomfeldt et al., 2012). Briefly, WG powder (16 g) was slowly dispersed through a metal sieve in 200 ml ethanol (70%) solution and constantly stirred to avoid clump formation until the mixture was homogenized. The mixture was placed on a shaker, IKA-KS 500 (IKA, Germany) for 30 min at 300 rpm and thereafter centrifuged for 10 min at 26,000 g in Sorvall RC 6+ centrifuge (Thermo Scientific, USA). The ethanol soluble (70%) Gli were decanted and

106	collected in a rotary evaporator at 70±5 °C (Buchi, Switzerland). The ethanol non-soluble Gt
107	fraction was separated from the soluble Gli fraction. Gli and Gt were lyophilized and grounded
108	into powder using IKA A10 grinder (IKA, Germany) with 2 pulses of 10 sec.

#### 109 2.2.2 Starch modification

Starch with altered chain length distribution was developed by inhibition of SBE1 and SBE2 in potato using RNA interference (RNAi) as described by (Andersson et al., 2003). Potato cultivar Dinamo was transformed using *Agrobacterium Tumefaciens* strain AGLO harboring the RNAi construct. Transformation, regeneration and selection of lines were performed as described by Andersson et al., (2003) with the modification that 50 mg/l kanamycin was used as a selection agent. Starch consisted of 27% amylose and debranched amylopectin chains characterized by gel permeation chromatography according to Muneer et al. (2015).

#### 117 **2.2.3** Sample preparation and extrusion

The Gli and Gt were extruded with MPS in different compositions of 70/30, 50/50, 30/70 (wt. 118 %) with 45% glycerol (wt. % of the total wt. of the sample) at 110 and 130°C according to 119 Muneer et al. (2015). Sample names were abbreviated such as, Gli-MPS-45gly (30/70), in 120 which 30% Gli and 70% MPS blend was extruded with 45% glycerol. In two Gt-MPS (50/50) 121 122 samples a mixture of 20% water and 30% glycerol was used and samples were abbreviated as Gt-MPS-20W-30gly (50/50). Hand mixed samples were extruded at 110 and 130 °C using a 123 124 Haake Minilab twin screw extruder (Thermo Electron Corporation, Germany) at 30 rpm, in the form of strips of 3.9-4.1 mm wide and 1.1-1.6 mm thick. Temperature was kept constant 125 throughout the whole extruding barrel till the die exit according to Muneer et al. (2015). For 126 each specimen, a single strip of about 1 m was produced from which ten samples of 70 mm 127 each were cut for tensile testing and other analysis. 128

#### 130 **2.2.4** Protein solubility by size exclusion high performance liquid chromatography

Extruded sample strips were chopped into small particles (0.2 mm approx.) using scalpel for 131 the extraction of protein by SE-HPLC analysis in a three step extraction procedure according 132 to Newson et al. (2013). The extraction of the protein was done in triplicates and 16.5 mg ( $\pm 0.05$ 133 134 mg) of each sample was mixed with 1.4 ml buffer solution (0.5% SDS, 0.05M NaH2PO4, pH 6.9) in a 1.5 ml Eppendorf tube and vortexed for 10 sec in Whirli Vib 2 (Labessco, Sweden). 135 Thereafter, the samples were shaken for 5 min in IKA-VIBRAX VXR (IKA, Germany) at 2000 136 137 rpm and centrifuged in Legend Micro 17 (Sorvall, Germany) for 30 min (16,000 g). The supernatant was decanted and analyzed as first extraction (1Ex). Second extraction (2Ex) was 138 done by sonication for 30 sec at amplitude of 5 microns in Sanyo Soniprep 150 Ultrasonic 139 Disintegrator (Tamro, United Kingdom) and serially followed by a third extraction (3Ex) with 140 sonication for 30+60+60 sec. The samples were left to cool at room temperature during each 141 142 sonication interval in 3Ex step to avoid overheating.

The 1Ex, 2Ex and 3Ex were analyzed on Waters 2690 Separation Module with Waters 996 Photodiode Array Detector (Waters, USA) according to (Newson et al., 2013)). Twenty μl of each sample was injected onto SE-HPLC column (Biosep-SEC-S 4000, Phenomenex, USA) for analysis, with an isocratic flow of 0.2 ml/min (50% acetonitrile, 0.1% TFA; 50% H<sub>2</sub>O, 0.1% TFA). Empower Pro software (Waters, USA) was used for analysis of data (210 nm) and chromatograms were integrated and divided into two groups e.g. polymeric proteins (retention time, 7-14 min) and monomeric proteins (14-28 min).

#### 150 **2.2.5** Protein polymer size by asymmetrical flow field flow fractionation (A-FFFF)

151 The Gt polymer molecular weight (Mw) in the Gt-starch composites has been evaluated by 152 measuring the molecular size of extractable protein using asymmetrical flow field flow 153 fractionation (AFFFF further referred as A4F). Sample preparation has been conducted

according to Lemelin et al. (2005). 10 mg of Ground samples were dispersed and incubated at 154 60 °C for 15 min with 1.0 mL of 0.05 M sodium phosphate buffer (pH 6.9) containing 2% (w/v) 155 SDS. The extracts were then sonicated for 20 sec at a power setting of 30% (full power 125W). 156 The supernatant (centrifugation at 12,500 g at 20 °C for 15 min), was filtered through 0.45 µm 157 filters (Gelman Sciences, France) before injection (30 µl) into the A4F/MALLS system. AF4 158 was accomplished with an Eclipse3 F System (Wyatt Technology, Santa Barbara, CA, USA) 159 160 serially connected to a UV detector (Agilent 1200, Agilent Technologies, Germany), MALLS detector (Dawn multi-angle Heleos TM, Wyatt Technology Corporation, Europe) and an 161 interferometric refractometer (Optilab rEX, Wyatt Technology Corporation, Europe). 162 163 Absorbance was measured at 214 nm. The channel had a trapezoidal geometry and a length of 286 mm. The thickness of the spacer used in this experiment was 350 mm. The ultrafiltration 164 membrane forming the accumulation wall was made of regenerated cellulose with a cut-off of 165 166 10 kDa (LC-10 Nadir reg. Cell, Wyatt Technology Europe Germany). An Agilent 1200 Series Isocratic HPLC Pump (Agilent Technologies, Germany) with an in-line vacuum degasser 167 delivered the carrier flow to the channel. A 0.45 µm in-line filter (Gelman Sciences, France) 168 was installed between the main pump and the Eclipse system. Sodium phosphate buffer (0.05 169 M, pH 6.9) containing 0.1% (w/v) SDS was used as mobile phase and was filtered through a 170 171 0.1 µm membrane (Gelman Sciences, France). For the fractionations using a gradient in the cross flow, the focus time was 0.5 min at a flow rate of 2 mL.min<sup>-1</sup>, followed by a 172 focus/injection time of 1.0 min at 0.2 mL.min<sup>-1</sup> and a relaxation/focusing time of 0.5 min. 173 Elution then followed at an outflow rate (F<sub>out</sub>) of 1.0 mL.min<sup>-1</sup> and with a cross-flow rate (F<sub>c</sub>) 174 decreasing exponentially from 3.0 to 0.0 mL.min<sup>-1</sup> for 14 min. Finally, elution at a cross-flow 175 rate of 0.0 mL /min was maintained for 9 min. All necessary constants for molecular weight 176 distribution calculation were determined previously (Lemelin et al., 2005). Bovine serum 177

albumin (BSA) standard was used to normalize the diodes of the MALLS detector. The calculated  $M_w$  of BSA was reasonably close to the nominal value.

#### 180 **2.2.6 Carboxymethyllysine quantification**

181 Carboxymethyllysine (CML) extraction and determination of its concentration from protein182 starch and protein powder samples were done according to the method developed by (Niquet183 Léridon and Tessier, 2011).

#### 184 2.2.7 Scanning electron microscopy

The microstructure of the horizontal cross-sections of the Gt-MPS and Gli-MPS composites were studied by scanning electron microscope (SEM) (LEO 435VP, Cambridge, UK) coupled with a secondary electron detector; acceleration voltage of 10 kV was used. Au/Pd (3:2) coating (JFC-1100, JEOL, Tokyo, Japan) was used for the samples prior to SEM analysis. Porteinstarch based samples processed with either glycerol or glycerol and water were studied to compare the effect of plasticizer content on microstructural morphology of the composites.

#### 191 2.2.8 Light microscopy

The Gli-MPS and Gt-MPS composite films were cut into 5x5 mm and approximately 1 mm thick pieces with scalpel. The samples were placed on a glass slide and stained with 0.1% Light green (Sigma, St. Louis, MO, USA) for protein and 50% Lugol solution (Fluka, GmbH, Germany). The excess amount of staining liquids was rinsed and samples were covered with a glass slip. The samples were then immediately observed under a DMLB light microscope (Leics Microsystems, Bensheim, Germany) and pictures were taken with Leica DC200 digital camera.

#### 198 2.2.9 Small angle X-ray scattering

199 Small angle X-ray scattering (SAXS) analysis was carried out using a monochromatic beam at 200 the beamline I911-4 ( $\lambda = 0.91$  Å; scattering vector range:  $q = 4\pi/\lambda \sin(\theta)$ ;  $2\theta$  is the scattering angle and q was  $0.008 < q < 0.35 \text{Å}^{-1}$ ) at the MAX IV Synchrotron laboratory, Lund, Sweden (Labrador et al., 2013). Two dimensional images were obtained using a bi-dimensional hybrid pixel X-ray detector (Pilatus 1M, Dectris) with sample to detector distance of 1901.7 mm and an exposure time of 2 or 5 min, depending on the intensity of the beam. The obtained data were normalized and accounted for incident integrated intensity, sample absorption, parasitic scattering and detector background, using softwares such as bli9114 and Fit2D (Labrador et al., 2013).

208 2.2.10 Wide angle X-ray scattering

Wide angle X-ray scattering analysis (WAXS) was carried out using beamline I911-2 at the MAX-IV Synchrotron laboratory, Lund, Sweden (Mammen et al., 2002). WAXS measurements were performed at wavelength of  $\lambda = 0.91$  Å and a distance from the detector of 290 mm using an area-CCD detector (active area having a diameter of 165 mm). The Fit2D software was used for data analysis and parasitic scattering was subtracted from the data. Silicon powder was used as a standard for calibration (Hammersley et al., 1996).

#### 215 **2.2.11 Oxygen barrier properties**

Oxygen transmission rate analysis was carried according to ASTM F1927-07, using Ox-Tran 2/21 (Mocon Inc., USA) and samples were conditioned 16 h prior to the test at 23°C and 50% relative humidity. Two replicates from each sample were analyzed and test was carried out at measured area of 5 cm<sup>2</sup> using a gas flow of 10 ml/min. On one side of the sample oxygen (99.5%) was at atmospheric pressure and oxygen transmission rate was normalized with thickness of the samples and oxygen pressure, in order to calculate the oxygen permeability.

222

#### 224 2.2.12 Tensile testing

All the samples were cut to a minimum length of 70 mm and conditioned for 48 hours at 50% (±2) relative humidity and 23°C temperature before testing. The width and thickness of the samples were measured using Mitutoyo IDC 112B (Mitutoyo, Japan) at five different points and average was used for calculations. For all samples, 100 N load cell with clamp separation distance of 40 mm and crosshead speed of 10 mm/min was used in an Instron 5566 universal test machine using a Bluehill software (Instron AB, Danderyd, Sweden) (ASTM-Standard-D638-08 1998).

#### 232 **3. Results and discussion**

#### 233 3.1 Protein solubility using SE-HPLC

234 The Gli-MPS and Gt-MPS composites extruded at 110 and 130 °C were studied for protein polymerization behavior using SE-HPLC (Fig. 1). For Gli-MPS samples, the solubility of the 235 polymeric (PP) and monomeric (MP) proteins decreased at 130 °C compared to 110 °C. As 236 expected, the Gli solubility increased with increasing concentration of Gli in the composite, but 237 only for the samples extruded at 110 °C. Very small increase in monomeric fraction, due to an 238 increase in the amount of the protein in the blend was observed for the Gli-MPS samples 239 extruded at 130 °C (Fig. 1). The decrease in the monomeric Gli solubility in Gli-MPS samples 240 processed at 130 °C suggests that some of Gli became a part of the aggregated/crosslinked 241 242 protein network.

The Gt-MPS composites showed quite low protein solubility of both the monomeric and polymeric proteins, as compared to the Gli-MPS samples at both processing temperatures (110 and 130 °C). Furthermore, Gt-MPS samples in general showed low protein solubility, in particularly for the polymeric proteins compared to monomeric proteins at both temperatures. The increase in the Gt content in the composite did not affect the Gt solubility at 110°C. Slightly higher amount of both polymeric and monomeric proteins was extracted in Gt-MPS 70/30
sample extruded at 130 °C.

The decrease in solubility of polymeric and monomeric protein types of Gt was observed 250 previously at higher temperatures (Muneer et al., 2014). In this study, the Gt even blended with 251 252 starch forms higher amount of cross-links, potentially covalent and non-covalent interprotein bonds, due to the rearrangement of high molecular weight Gt polymeric proteins in the presence 253 of plasticizer and high temperature. A decrease in the solubility of polymeric and monomeric 254 255 proteins is a result of higher degree of polymerization at higher temperature due to formation of the inter-protein disulphide bonds, as well as covalent and hydrogen bonds (Johansson et al., 256 257 2013, Kuktaite et al., 2004). A similar behavior was also observed in the extruded pasta samples (Barbiroli et al. 2013). Furthermore, in this study shear mixing and high temperature provided 258 suitable environment to form irreversible bonds reported previously (Pommet et al., 2003, 259 Ullsten et al., 2009, Redl et al., 1999, Ture et al., 2011). 260

#### 261 3.2 Molecular weight distribution of glutenins in Gt-starch composites by A4F

262 Molecular weight distribution of Gt proteins in Gt-MPS extrudates was analyzed by A4F and is presented in Fig. 2. The molecular size of Gt proteins in the Gt-MPS samples was within the 263 range of around 3000 x 10<sup>5</sup> up to 17000 x 10<sup>5</sup>. The molecular weight of the polymeric proteins 264 was found higher at 130 °C compared to 110 °C (Fig. 2). The increase in the polymer size was 265 much higher for the 30/70 Gt-MPS compared to 70/30 Gt-MPS (4.3 vs. 2.4 times, respectively). 266 No increase in the polymer size was observed for the 50/50 Gt-MPS samples with 30% glycerol 267 and 20% water (Fig. 2). Gt-MPS 30/70 samples showed the highest increase in the large 268 polymer, suggesting that protein reorganization is affected by the presence of the resistance 269 starch (crystalline structure) (Barbiroli et al. 2013). Part of the MPS starch is being present in a 270 271 compact native-like conformation as it evidenced by its limited gelatinization in WG-MPS

glycerol blends (Muneer et al. 2015), and similarly in the extruded rice pasta (Barbiroli et al. 272 273 2013). This fact may explain the observation that a part of the resistance starch swelled in the 274 50/50 Gt-MPS samples with 30% glycerol and 20% water, and in this way limited the Gt protein-protein interactions. This behavior suggests that with a careful selection of plasticizer 275 (a blend of glycerol and water) the cross-linking behavior of Gt protein can be regulated. 276 Previous studies have shown that excessive aggregation of proteins can be manipulated using 277 278 chemical additives such as urea and salicylic acid, which delay the aggregation reactions during extrusion (Ture et al., 2011, Kuktaite et al., 2014). The A4F results are complementary with the 279 results observed by SE-HPLC showing higher amounts of the extracted proteins (both 280 281 monomeric and polymeric) for 70/30 Gt-MPS sample extruded at 130 °C compared to 110 °C 282 (Fig. 1).

#### 283 **3.3** Determination of N<sup>ε</sup>-(Carboxymethyl)lysine content in Gli- and Gt-starch extrudates

In the present study the content of N<sup>ε</sup>-(carboxymethyl)lysine (CML) was determined to study glycation end product formed by combined non-enzymatic glycation and oxidation reactions on Gli- and Gt- proteins in the protein-starch composites (Fig. 3). The CML contents of Gliand Gt-MPS samples varied greatly between the studied samples are shown in Fig. 3.

The Gli-MPS (70/30 and 50/50) samples processed at 130 °C showed highest amount of CML content as compared to the samples processed at 110 °C (Fig. 3a). The amounts of CML increased with an increasing protein concentration in the Gli-MPS composites, and the highest amount of around 80  $\mu$ g/g was observed for the 70/30 Gli-MPS at 130 °C (Fig. 3a). High amount of CML was also observed in the Gli, starch free sample (extruded at 110 °C). This fact indeed, suggests that in Gli-MPS samples the protein-starch interactions resulting into a number of glycated proteins and Amadori Dione products (Ahmad, 2001) (Fig. 3b). Similarly, Gt-MPS (70/30 and 50/50) and Gt-MPS-30gly-20w (50/50) samples extruded at 130
°C showed higher CML content compared to 110 °C (Fig. 3c). Differently from Gli-MPS
samples an increase in CML due to an increase in protein concentration was observed in GtMPS samples extruded only at 130 °C (Fig. 3c). The highest amount of CML was found in Gtstarch free samples produced at 110 and 130 °C, and 70/30 Gt-MPS sample at 130 °C. A slightly
higher amount of CML was observed for 50/50 Gt-MPS with 30% glycerol and 20% water at
130 °C compared to 110 °C.

302 High processing temperature favors the formation of CML content in wheat proteins as explained by the formation of bread crust in baked bread products (Assar et al., 2009). 303 Oxidation of the proteins results in formation of disulphide and tyrosine bonds between the 304 protein molecules, which was evident in our samples. The Gli- and Gt- protein rich fractions 305 processed at 110 °C showed the higher amounts of CML content compared to protein-starch 306 composite systems explaining that the presence of starch affect the protein oxidation and result 307 into several chemical pathways and formation of glycation derived lysine peptides (Fig. 3b). In 308 conclusion, higher amounts of protein content in all the Gli-MPS samples and in Gt-MPS 309 samples at 130 °C favored the protein oxidation and formation of high CML content in the 310 311 samples.

Heat treatment of proteins and glucose lead to the formation of Maillard reactions which results in producing glycation end-products (AGE) shown in Fig. 2b (Niquet-Léridon and Tessier, 2011). CML is an AGE which was discovered to be present in most of the food products. CML is used to determine the protein damage in severely heated food items rich in proteins, sugars and glucose; calculating the reaction product of sugars and lysine amino acid (Niquet-Léridon and Tessier, 2011, Charissou et al., 2007, Delatour et al., 2009, Ames, 2008).

# 319 3.4 Microstructure of gliadin- and glutenin-MPS composites by scanning electron 320 microscopy and light microscopy

The Gli- and Gt- MPS samples (50/50 and 70/30) with either glycerol or glycerol-water 321 extruded at 130 °C were chosen for microstructural analysis using SEM and LM. The Gli- and 322 323 Gt-MPS samples showed a large variation in the microstructural pattern of the extruded composites (Fig. 4). A large amount of intact starch granules (violet stain) of various sizes was 324 embedded in the protein matrix (green stain) for the Gli-MPS-45gly samples observed by LM 325 326 (Fig. 4b indicated by arrows). The microstructure of the Gli-MPS composites (70/30) consisted of few large round and oval granules (around 10 microns), a number of smaller granules (around 327 328  $\leq$ 5 microns) embedded into the proteins, as well as few voids (dotted arrows) by SEM (Fig. 4a). Even though that the starch seemed well distributed in the Gli matrix, 45% glycerol and 130 °C 329 did not fully gelatinized the starch granules as observed by LM (Fig. 4b). A pre-gelatinization 330 step of the MPS, which we also tested in this study (unpublished data), did not give complete 331 gelatinization of the MPS. 332

The microstructure of the Gt-MPS (50/50) and (70/30) samples with either glycerol or glycerol-333 water differed from Gli-MPS composites. From the micrograph of the Gt-MPS containing 334 335 glycerol-water, there were clear patterns of homogenous protein-starch matrix as shown by SEM (Fig. 4c, white arrow). The structural difference of the Gt-MPS comparing to the Glia-336 337 MPS was that intact starch granules were presented in the protein-starch matrix by SEM, they 338 seemed better "attached" in the Gt-MPS blend with glycerol and water (dotted arrows; Fig. 4c). A large number of cracks in the microstructure of the Gt-MPS with glycerol-water were 339 detected by SEM (likely due to drying effect during measurement) (intact arrows; Fig. 4c). We 340 341 also observed that the microstructure of the Gt-MPS glycerol sample contained areas where the starch granules were hydrated, disrupted and incorporated in the Gt proteins (dotted arrows; 342 Fig. 4d). The combination of the plasticizers and temperature made a suitable environment for 343

achieving a homogenized matrix of the protein-starch polymers. Similar results on the use of
glycerol-water in WG-MPS blends have been previously reported in our study Muneer et al.,
(2015).

Previous studies on using modified potato starch for film formation indicated some challenges 347 regarding gelatinization of the semi-crystalline structure of the modified starch (Ältskar et al., 348 2008). Poor adhesion and interaction between the two polymers e.g. protein and starch, is one 349 of the main reasons for poor microstructure in the glycerol plasticized composites. Another 350 351 reason is excessive aggregation of proteins in the extruder barrel before exiting the die, as observed in SE-HPLC and AFFFF results (Fig. 1, 2) (Verbeek and van den Berg, 2010). 352 However, a promising combination of glycerol and water for protein-starch blends has a 353 potential to be developed further in improving the interaction between components and 354 gelatinization of starch. Higher processing temperatures (> 130 °C) and pre-gelatinization of 355 starch, as well as the use of other processing methods are next routes to explore. 356

### 357 **3.5** Secondary structure of the protein-starch composites by FT-IR spectroscopy

358 Secondary structure of the proteins in the protein-starch composites was analyzed using FT-IR spectroscopy in the amide I region (1600-1700 cm<sup>-1</sup>) (Fig. 5). The presence of  $\alpha$ -helices/random 359 coils and  $\beta$ -sheets were observed in the amide I regions 1645-1660 cm<sup>-1</sup> and 1620-1635 cm<sup>-1</sup>, 360 respectively. All the Gli-MPS-45gly extruded samples showed the formation of  $\alpha$ -helices and 361  $\beta$ -sheets at both 110 and 130 °C temperatures compared to pristine Gli powder where a small 362 peak in the region 1645-1660 cm<sup>-1</sup> showing the presence of  $\alpha$ -helices and random coils was 363 observed (Fig. 5a). The Gli-MPS-45gly (70/30 and 50/50) samples processed at 130 °C showed 364 higher intensities of  $\beta$ -sheets formation as compared to Gli-MPS 30/70 sample, most likely due 365 to greater concentration of protein in the sample (Fig. 5a). However, at 110 °C, the Gli-MPS-366

45gly samples showed the formation of β-sheets with rather low intensities and flat shoulders in 1620-1635 cm<sup>-1</sup> region compared to 130 °C (Fig. 5a).

The Gt-MPS-45gly samples extruded at 110 °C showed the formation of both  $\alpha$ -helices and 369 random coils in the 1645-1660 cm<sup>-1</sup> region for all the protein-starch ratios and a small shoulder 370 peak in 1620-1635 cm<sup>-1</sup> region, for 70/30 and 50/50 samples (Fig. 5b). Somewhat the intensity 371 of  $\beta$ -sheet peak was slightly greater compared to the intensity of  $\alpha$ -helices and random coils 372 which means that at 110 °C there is only a small fraction of  $\alpha$ -helices to  $\beta$ -sheets conversion 373 374 due to the effect of temperature (Fig. 5b). However, at 130 °C, the Gt-MPS samples were more aggregated as shown by the clear formation of  $\beta$ -sheets in the 1620-1635 cm<sup>-1</sup> region (Fig 5b). 375 The conversion of  $\alpha$ -helices to  $\beta$ -sheets (changed ratio) at 130 °C suggests that the higher 376 temperature favors the aggregation of Gt to more organized secondary structures. The 377 reorganization of the protein secondary structures *i.e.* the conversion of  $\alpha$ -helices to  $\beta$ -sheets at 378 higher temperatures, pressure and addition of plasticizers such as glycerol has been reported in 379 the previous studies (Ullsten et al., 2009, Cho et al., 2011). 380

The Gt-MPS-30gly20w (50/50) samples showed higher amount of  $\beta$ -sheets when processed at 381 130 °C compared to 110 °C (Fig. 5b). Important to mention, that the Gt-MPS sample with 382 383 glycerol-water at 110°C showed a rather "balanced"  $\alpha$ -helices to  $\beta$ -sheets ratio with a small increase in  $\beta$ -sheets. This suggests that the presence of water in the composite blend favored 384 385 the formation of hydrogen bonds and disulphide bonds, and 110 °C was a suitable temperature 386 to process a composite. Higher temperature (130 °C) induced the Gt protein aggregation and resulted in the formation of even higher  $\beta$ -sheets vs.  $\alpha$ -helices ratio. In a previous study, similar 387 effect of temperature was observed when Gt protein was modified with 30% glycerol and 388 389 processed at 130 °C (Rasheed et al., 2014).

390

#### 391 **3.6** Nano-morphology of protein-starch composites by Small angle X-ray Scattering

The SAXS curves of Gli-MPS and Gt-MPS composites, Gli and Gt samples are shown in Fig. 392 6. Several distinct scattering peaks were observed in the Gli-MPS samples extruded at 110 and 393 130 °C both at low and high q values (Fig. 6a, b). Three Bragg peaks such as  $d_1$ ,  $d_2$  and  $d_3$  were 394 clearly observed in all the Gli-MPS samples (except the 30/70 sample at 130 °C; with very low 395 intensity) at both processing temperatures (indicated by arrows; Fig. 6a, b) The positional ratios 396 of the observed peaks (d<sub>1</sub>, d<sub>2</sub> and d<sub>3</sub>) followed 1:  $\sqrt{3}$ :  $\sqrt{4}$  ratio indicating a hierarchical hexagonal 397 398 structure of Gli protein for the first time in the protein-starch composite system (Fig 6, Table 1). The formation of hexagonal structure in the WG and Gli films with either urea-glycerol 399 400 (Kuktaite et al., 2012, 2014) or glycerol (Rasheed et al 2014), has been reported in the previous studies. For the WG-MPS composites only similar to hexagonal structure morphology has been 401 observed in our previous study (Muneer et al. 2015). The intensity of the peaks representing the 402 hexagonal structure decreased with an increase in the starch content in the samples as observed 403 in Gli-MPS (30/70) films (Fig. 6a, b). 404

Beside the hexagonal structure, additional peaks were observed in the Gli-MPS samples 405 extruded at 130 °C (represented by d\*; Fig. 4b, Table 1). The reason for the appearance of these 406 407 peaks might be the aggregated structure of the protein without a specific structural 408 conformation. Similar peaks were reported at same q-values in pristine Gli powder and Gli-409 glycerol compression molded films (Rasheed et al., 2014). At low q-values we observed the 410 additional peak (d\*\*) in the Gli-45gly (without starch) samples at both 110 and 130 °C. In a previous study by Rasheed et al., (2015) the additional peak observed in WG-glycerol material 411 was referred to the presence of lamellar structural arrangement (Rasheed et al., 2015a). 412

The d<sub>BROAD</sub> peak in this study was superimposed reflection of the scattering objects from lamellar
structure of starch and a broad correlation distance of Gli proteins (Fig. 6a, b; Table 1) (Rasheed

et al., 2014, Muneer et al., 2015). The d<sub>100</sub> peak found in this study was also observed in the
WG-MPS composites (Muneer et al., 2015). This peak represent the reflection of the B-type
crystalline structure of starch (scattering distance between two double helixes of the
amylopectin fraction in a hexagonal unit cell) (Nishiyama et al., 2009). The scattering intensity
of the 100 reflection of the starch decreased with the decrease in starch content of the sample
regardless of the temperature difference (Fig. 6a, b).

The Gt-MPS composites did not show any specific nano-structural arrangements, as shown in 421 422 Fig. 6c, d and e. Two broad peaks  $d_{BROAD}$  and  $d_{100}$  were observed in all the protein-starch samples processed at 110 and 130 °C. The dBROAD peak is a combined reflection of the scattering 423 424 distances from starch lamellar structure and amorphous morphology of aggregated Gt proteins (Fig. 6c, d; Table 2). The scattering distance seemed to increase with increase in the MPS 425 content in Gt-MPS samples, which was obvious as observed in Table 2. The addition of water 426 in the Gt-MPS samples did not affect the structural morphology of the composites (Fig. 6e; 427 Table 2). 428

#### 429 3.7 Atomic structure of protein-starch composites by wide angle X-ray Scattering

The WAXS profiles of the Gli-MPS and Gt-MPS composites are shown in Fig. 7. The x-ray 430 diffraction profile of MPS powder was also included in this study (Fig. 7a). The WAXS profile 431 432 of MPS powder showed a large number of peaks indicating the crystalline behavior of starch and scattering reflections of these peaks were indicated by their Miller index as reported 433 previously (Nishiyama et al., 2009) (Fig. 7a). A comparison of protein-MPS extrudates and 434 MPS powder also showed that the B-type crystalline (100 reflection) structure of starch is 435 present in all the studied samples regardless of the processing conditions used (Fig. 7). As 436 expected, the Gli-MPS samples showed a decrease in the starch crystallinity at higher 437 temperature (i.e. 130 °C) with less intense crystalline peaks in the WAXS profile (Fig. 7a, b). 438

This decrease was more profound in the composites with the highest amount of Gli such as, 70/30. The most dominating crystalline starch peak in Gli-MPS samples at 110°C was 311.
Similarly, in Gt-MPS samples with higher protein content a small decrease in starch crystallinity was observed at 130°C compared to 110°C (Fig. 7c, d). For the Gt-MPS composites the scattering crystalline peak was 220 (Fig 7c).

The use of glycerol-water in Gt-MPS samples extruded at 110 and 130 °C slightly changed the 444 morphology of the composite compared to the samples with glycerol (Fig 7c-e). The X-ray 445 446 diffraction profile of Gt-MPS glycerol-water samples was somewhat "smoothen" containing only few crystalline starch peaks (Fig. 7e). Very small morphological difference was observed 447 between the two temperatures studied. The Gt-MPS composite at 130 °C showed a more intense 448 shoulder for the 311 peak (Fig. 7e). So, the use of water-glycerol in the Gt-MPS composites 449 affected the morphology of the starch granules by the partial gelatinization of starch granules 450 (Lai and Kokini, 1991, Muneer et al., 2015). The gelatinization of starch happens due to the 451 swelling of amorphous regions of starch which results in melting of amylopectin crystals 452 (Waigh et al., 2000). 453

#### 454 **3.8 Mechanical properties**

The mechanical properties of Gli-MPS and Gt-MPS composites are shown in Fig. 8. For the 455 456 Gt-MPS 50/50 samples with 30gly-20W the highest E-modulus and the maximum stress were observed at both 110 and 130 °C (Fig. 8). In addition, the highest extensibility was observed in 457 the Gt-MPS 50/50 with 30gly-20W, but only in 110 °C sample (Fig. 2c). For Gt-MPS-45gly 458 30/70 samples the increase in both the maximum stress and extensibility, and no change in E-459 modulus was observed with increasing temperature (from 110 to 130 °C) (Fig. 2). In general, 460 an increase in Gt content in the sample resulted in a decrease of E-modulus and maximum stress 461 at both temperatures, and decrease in extensibility at 130 °C sample. None of the mechanical 462

tests were possible for the Gt-MPS-45gly 70/30 composite extruded at 110 °C (disrupted
appearance after processing).

The Gli-MPS-45gly composites showed different mechanical properties compared to Gt-MPS composites. Only the Gli-MPS-45gly samples extruded at 130 °C were mechanically tested. The Gli-MPS-45gly 70/30 showed the second highest elongation between the samples produced in this study (Fig. 2c). The increase in Gli content in the composites did not affect either of the E-modulus or maximum stress. The Gli-MPS-45gly samples extruded at 110 °C were not testable after 24 h conditioning due to glycerol migration to the surface of the samples (very sticky and difficult to handle).

In this study the best mechanical performance of Gt-MPS-30gly-20W composites was achieved 472 when a high amount of plasticizer (50%) was used, and sample was processed at 110 °C. 473 474 Possible explanation is that higher temperature and higher plasticizer content induced the Gt protein aggregation (decreased solubility), which further increased the protein-protein 475 interactions. These protein-protein interactions in the Gt-MPS-30gly-20W were in favor for 476 achieving rather high E-modulus, but negatively affected the maximum stress and extensibility 477 478 at 130°C (Fig. 2). The Gt protein fraction is mainly dominated by glutenins made of polymers 479 connected by disulphide bonds (low polymer solubility due to large their size, Fig. 1 and 2). 480 When these Gt proteins and potato starch are in a blend, which further is blended with water 481 and glycerol, starch acts as filler in the protein polymeric matrix. From our previous study 482 (Muneer et al, 2015), we observed that a combination of two plasticizers e.g. water and glycerol, led to higher tensile properties as compared to only glycerol used in the WG-potato starch 483 composites. In this study, the added water even more plasticized Gt-MPS blend, and favored 484 485 the gelatinization of starch granules (see Fig. 4c, and improved homogenization of the 486 composite).

In protein based systems the addition of water favors the hydrogen bonding which ultimately 487 488 help to improve the strength and elongation of the protein based materials (Gontard et al., 1993). The potato starch used in the present study contained longer chain-length of amylopectin and 489 higher amylose content than normal potato starch (27 vs 20%) (Muneer et al 2015). Therefore, 490 the pasting properties of the potato starch used in this study were expected to have higher 491 viscosity (around 1.7 folds) compared to normal potato starch (Thunwall et al., 2006). 492 493 Processing conditions of the protein-starch composites were chosen on the basis of both polymers with the main focus of using glycerol as plasticizer. The use of both water and glycerol 494 for the Gt-MPS composites improved the tensile properties significantly. No such effect was 495 496 observed for Glia-MPS composites.

#### 497 **3.9 Oxygen permeability of protein-starch composites**

The oxygen permeability (OP) of selected Gli-MPS and Gt-MPS composites was measured at two conditioning environments i.e. 23 °C and 38 °C (50% and 90% relative humidity (RH), respectively) are shown in the Table 3. The Gt-MPS-45gly (30/70) sample showed the lowest OP values when measured at 23 °C and 50% RH, however at higher temperature (38 °C) and RH (90%) the values were over the range (OR) (Table 3). Similar OP was observed previously for MPS-45gly sample (Muneer et al. 2015). In this study, the OP properties for the Gt-MPS composite were greatly determined by the large amount of potato starch in the blend (Table 3).

The Gli-MPS-45gly 70/30 and 30/70 samples showed the higher OP values compared to Gt-MPS and MPS samples (Table 3). In this study the OP properties of the Gli-MPS composites at higher temperature and RH showed reasonable properties and were still measureable; the OP increased up to 23 folds compared to values measured at 23 °C and 50% RH. The attractive OP properties observed in the Gli-MPS materials showed a great potential to be further explored in packaging as for example, paper coating, multilayered films as well as in medical sector e.g. 511 wound healing textiles, where specific oxygen permeability is required. The OP values 512 observed in this study are comparable to the other WG-starch and WG-clay based nano 513 composites (Muneer et al., 2015, Kuktaite et al., 2014).

514 Conclusions

515 In this study, the structural morphology of plasticized the Gli-MPS and Gt-MPS composites extruded at 110 and 130 °C was studied in relation to protein properties (solubility, polymer 516 size and protein oxidation), macromolecular interactions, oxygen barrier and mechanical 517 behavior. For the first time we have showed the hierarchically arranged hexagonal structure of 518 519 Gli proteins at 110 and 130 °C observed in the protein-starch composite system. The crystalline 520 structure of MPS was also observed in the Gli/Gt-MPS composites. The Gt-MPS samples did not show any specific hierarchical structure at the used processing conditions. The analysis of 521 522 secondary structure of proteins indicated the large amounts of  $\beta$ -sheets in the samples processed at 130 °C temperatures and also with higher protein content. The Gt-MPS samples with water 523 and glycerol extruded at 110 °C showed a more "balanced" amount of  $\alpha$ -helices and  $\beta$ -sheets 524 compared to all other samples. The higher extrusion temperature (130 °C) favored the 525 crosslinking of the proteins (decreased solubility and increased polymer size) compared to the 526 527 samples processed at 110 °C, which contributed to excessive aggregation of the proteins. . The largest extracted Gt polymers were formed in the 70/30 Gt-MPS-45% glycerol extrudate 528 529 processed at 130 °C. The most oxidated proteins (CML content) were found in 70/30 Gli-MPS 530 and Gt-MPS samples plasticized with 45% glycerol at 130 °C. For Gli/Gt-MPS samples the Maillard reactions were important. 531

The microstructural analysis of the Gli-MPS and Gt-MPS with 45% glycerol showed rather inhomogenous structural pattern with large amount of intact starch granules and few voids. The use of plasticizers blend consisting of 30% glycerol and 20% water improved the microstructure

of Gt-MPS compared to only glycerol plasticized protein-starch samples. In addition, this blend of plasticizers improved the mechanical properties of the Gt-MPS (50/50) composites (especially at 110 °C) with the highest E-modulus, maximum stress and extensibility compared to glycerol samples. Also, a decrease in MPS crystallinity and improved processability was observed in the protein-starch and glycerol-water composites.

The Gt-MPS (30/70) samples showed excellent oxygen barrier properties under ambient testing conditions which can easily compete with several synthetic or biobased polymer films/composites, and shows good potential for packaging applications (see Fig. 9). The Gli-MPS samples also showed good barrier properties and were rather extensible (70/30 Gli-MPS), properties potentially suitable for reactive extrusion in 3D printing of similar to textile products (Fig. 9), although more testing are needed in this area.

With this study we provide useful insights into the protein-starch structure and polymer biochemistry during extrusion processing and in the final Gli- and Gt- MPS composites for various potential end-uses. We significantly improve current molecular-level understanding of the factors affecting the physicochemical properties of the protein-starch composites. This knowledge could lead for better tuning of the most promising protein-starch materials and their best functional use in sustainable applications.

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Fig. 1. Protein solubility Gli-MPS-45gly, Gt-MPS-45gly and Gt-MPS-30gly samples extruded
at 110 and 130 °C as studied by SE-HPLC.

- Fig. 2. Estimation of the protein polymer size of Gt protein in Gt-MPS extruded samples byAFFFF method.
- **Fig. 3.** CML content in Gli-MPS and Gt-MPS composites extruded at 110 and 130 °C.
- **Fig. 4.** SEM and LM micrographs of Gli-MPS and Gt-MPS samples extruded at 130 °C with

either glycerol or glycerol-water; (a) Gli-MPS-45gly (70/30), (b) Gli-MPS-45gly (50/50), (c)

- 710 Gt-MPS-30gly20W (70/30), (d) Gt-MPS-30gly20W (50/50). Bars correspond to 30 for SEM
- 711 and 100  $\mu$ m for LM.
- **Fig. 5.** FT-IR spectra of Gli-MPS and GT-MPS samples extruded at 110 and 130°C; (a) Gli-

713 MPS-45gly, and (b) GT-MPS-45gly samples. Spectra range 1645-1660 cm<sup>-1</sup> corresponds to  $\alpha$ -

- helices and 1620-1635 cm<sup>-1</sup> to  $\beta$ -sheets.
- 715 Fig. 6. SAXS profiles of Gli-MPS-45gly (a, b) and GT-MPS-45gly (c, d) and (e) GT-MPS-
- 716 30gly-20W (50/50) composites extruded at 110 and 130  $^{\circ}$ C.
- Fig. 7. WAXS analysis of (a,b) Gli-MPS-45gly and (c,d) GT-MPS-45gly and (e) GT-MPS30gly-20W (50/50) composites extruded at 110 and 130 °C.
- **Fig. 8.** Tensile properties of Gli-MPS-45gly, GT-MPS-45gly and GT-MPS-30gly samples extruded at 110 and 130 °C; (a) E-modulus, (b) Maximum stress and (c) elongation.
- Fig. 9. A graphical representation of the effect of processing on the structure of the materialsand their possible use.
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- **Table 1.** SAXS scattering distances of Gli-MPS-45gly and Gli-45gly extruded at 110 and 130
- °C samples.

Samples	Temperature	d-broad (Å)	d* (Å)	d1 (Å)	d** (Å)	d2 (Å)	d3 (Å)	d100 (Å)
Gli-MPS45gly (70/30)	110°C	96.3		56.6	51.8	32.8	28.4	15.9
Gli-MPS45gly (50/50)	110°C	92.1		57.3		33.2	28.8	16.0
Gli-MPS45gly (30/70)	110°C	94.6		57.9	52.4	33.6	29.2	16.1
Gli (100)	110°C	106.7		55.9	51.4	32.3	28.1	
Gli-MPS45gly (70/30)	130°C	96.6	65.6	57.9	52.3	33.7	29.2	16.0
Gli-MPS45gly (50/50)	130°C	99.2	65.8	59.3	53.3	34.4	29.8	16.2
Gli-MPS45gly (30/70)	130°C	93.8	62.9	58.2	52.4	33.8	29.3	16.3
Gli (100)	130°C	97.1	77.8	56.1	50.8	32.5	28.2	

**Table 2.** SAXS scattering distances of Gt-MPS-45gly, Gt-MPS-30gly-20W and Gt-45gly

729 extruded at 110 and 130  $^{\circ}$ C samples.

Samples	Temperature	d broad (Å)	d <sub>100</sub> (Å)
Gt-MPS45gly (70/30)	110°C	80.1	16.0
Gt-MPS45gly (50/50)	110°C	86.9	16.1
Gt-MPS45gly (30/70)	110°C	89.7	16.1
Gt (100)	110°C	67.5	
Gt-MPS45gly (70/30)	130°C	73.3	16.1
Gt-MPS45gly (50/50)	130°C	87.2	16.3
Gt-MPS45gly (30/70)	130°C	90.6	16.4
Gt (100)	130°C	68.8	
Gt-MPS-30gly-20W (50/50)	110°C	88.5	16.1
Gt-MPS-30gly-20W (50/50)	130°C	69.9	16.5

730

**Table 3.** Oxygen permeability values of Gli-MPS-45gly, Gt-MPS-45gly and MPS-45gly
samples. Standard deviations are in brackets.

Samples	23°C, 50% RH	38°C, 90% RH		
	$(mm mL/m^2 24h atm)$	$(mm mL/m^2 24h atm)$		
Gli-MPS-45gly 70/30	10.62 (1.37)	226.3 (170.69)		
Gli-MPS-45gly 30/70	12.01 (0.24)	260.4 (2.82)		
GT-MPS-45gly 30/70	3.02 (0.19)	OR>2000		
MPS-45gly*	3.29 (1.30)	OR>2000		
WG-clay <sup>**</sup>	6 (0.4)			

\*Muneer et al., 2015, \*\* Kuktaite et al., 2014, OR-over range

734















ОН

CML







748 Figure 5







**Figure 7** 















