High lipid microalgae (*Schizochytrium* sp.) inclusion as a sustainable source of n-3 long-chain PUFA in fish feed – effects on the extrusion process and physical pellet quality

T.A. Samuelsen, Å. Oterhals, K. Kousoulaki

Nofima, P.O. Box 1425 Oasen, NO-5844 Bergen, Norway.

* Corresponding author. Tel.: +47 55112168; E-mail address: tor.a.samuelsen@nofima.no

Submitted to Animal Feed Science and Technology in August 2017

Revised and re-submitted in October 2017

Abstract

The aim of this research was to investigate effects on the extrusion process and physical pellet quality of high lipid microalgae (HL-microalgae) inclusion level as a source of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) in fish feed. The experiment was based on a 3-component mixture design on commercially relevant feed blends by varying the content of dried HL-microalgae *Schizochytrium* sp. meal, vital wheat gluten (WG), and soy protein concentrate (SPC). Mixture models with R^2 in the range of 0.881 to 0.998 ($P < 0.01$) were established for specific mechanical energy (SME), feed melt viscosity and temperature behind die, expansion parameters, and pellet hardness and durability. The mixture components affected SME, melt viscosity and viscous heat dissipation with the highest values observed for SPC followed by WG, and lowest for HL-microalgae rich blends. Increase in viscous heat dissipation and feed melt temperature increased the steam flashing rate and consequently pellet expansion and fat adsorption capacity. Lowest pellet hardness and durability were found for blends high in HL-microalgae or SPC level due to incomplete cooking. HL-microalgae addition up to 132 g/kg DM in SPC rich blends gave favourable processing effects with respect to improved plasticisation and physical pellet quality. This study documents that HL-microalgae has a potential to be used as an alternative and sustainable source of n-3 LC-PUFA in aquaculture feeds without compromising physical pellet attributes.

Keywords: fish feed, microalgae, pellet expansion, soy protein concentrate, viscosity, wheat gluten

Abbreviations: CGM, corn gluten meal; FM, fishmeal; HL-microalgae, high lipid microalgae; n-3 LC-PUFA, n-3 long-chain polyunsaturated fatty acids; SEI, sectional expansion index; SME, specific mechanical energy; SPC, soy protein concentrate; T_{die}, temperature behind extruder die; V_{die}, apparent viscosity in the extruder die; WG, vital wheat gluten; WSI, water holding capacity

1. Introduction

The inclusion level of fishmeal and fish oil in aquaculture feed formulations has significantly decreased the last decade, reflecting that further global growth of the aquaculture industry need to be based on more sustainable sources such as plant and single cell biomass, domestic animal by-products and insects (Tacon and Metian, 2008; Sørensen et al., 2011; Tacon et al., 2011). In salmonid diets, fishmeal has partly been replaced by plant proteins (e.g. wheat gluten, soybean and sunflower meal), and fish oil partly by rapeseed oil (Ytrestøyl et al., 2015). Recently, dried microalgae such as heterotrophic grown *Schizochytrium* sp. has been introduced as an alternative and sustainable source of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) in aquaculture feeds. *Schizochytrium* sp. contains high level of lipids (55-75% of dry matter), with as much as 49% docosahexaenoic acid (DHA) (Ren et al., 2010). The product appears to be a highly performing fish oil alternative in diets for major farmed fish species such as gilthead seabream (Ganuza et al., 2008) and Atlantic salmon (Miller et al., 2007; Kousoulaki et al., 2015, 2016, 2017).

The inclusion of a high lipid-containing *Schizochytrium* sp. ingredient in the dry feed recipe may introduce new challenges in the feed extrusion process. Extrusion is a thermomechanical process where the physical pellet quality and expansion rate is controlled by steam and viscous dissipation of mechanical energy (heat), moisture level, and the physicochemical and rheological properties of the feed ingredients (Blanche and Sun, 2004; Sørensen et al., 2009; Glencross et al., 2010; Kraugerud et al., 2011; Samuelsen et al., 2013, 2014; Samuelsen and Oterhals, 2016). Lipids act as a lubricant in the extruder barrel and reduce viscous heat dissipation and physical pellet quality. A total lipid level in the dry feed recipe below 120 g/kg is known to have negligible negative effect on the extrusion process and feed quality (Rokey, 1994). Consequently, for high-energy salmon feed with a total lipid level of

>30%, most of the oil has to be coated onto the expanded and dried feed pellet by use of vacuum coating technology (Strauch, 2005).

Recently, extruded pellets containing *Schizochytrium* sp. meal (50 g/kg dietary inclusion level) were found to have reduced pellet durability compared to a microalgae free diet (Kousoulaki et al., 2016). This may limit the use of high lipid microalgae (HL-microalgae) as an important source of n-3 LC-PUFA in aquaculture feeds. Due to the low lipid content in plant proteins compared to fish meal (Hill et al., 2010a, 2010b; Table 1) there may be a higher tolerance for high lipid ingredients in commercially relevant plant based feed recipes.

The use of bulk transport and pneumatic feed delivery systems demands consistent and high physical pellet quality to minimize product loss (Aarseth, 2004; Aarseth et al., 2006; Aas et al., 2011). To establish HL-microalgae as a sustainable source of n-3 LC-PUFA in the fish feed manufacturing process, it is of high importance to develop knowledge on techno-functional properties related to the extrusion process and physical pellet quality in plant based feed recipes. The objectives of this study were to quantify (1) the effect of HL-microalgae inclusion level in combination with vital wheat gluten (WG) and soy protein concentrate (SPC) on extruded feed melt viscosity, extrudate expansion and physical pellet quality; (2) in combination with WG and SPC, assess the maximum HL-microalgae inclusion level possible to use without compromising physical pellet quality.

2. Materials and methods

2.1 Materials

HL-microalgae, a spray dried heterotrophic grown *Schizochytrium* sp. was obtained from Alltech Inc. (Nicholasville, KY, USA). Corn gluten meal (CGM) and soy protein concentrate (SPC) was purchased from Agro Korn AS (Videbæk, Denmark). Fishmeal (FM) was of LT quality (Schmidtsdorff, 1995) obtained from Norsildmel AS (Bergen, Norway).

Vital wheat gluten (WG) was purchased from Tereos Syral (Aals, Belgium). Whole wheat flour (Wheat) was of bakery quality (falling number >200 s) produced by Norgesmøllene AS (Vaksdal, Norway). Proximate chemical composition of the ingredients are given in Table 1. All solvents and reagents for the analyses were of analytical grade. Food grade soybean oil was purchased locally.

2.2 Feed mix composition

Ten experimental feed mixes were prepared (Table 2). The level of HL-microalgae, WG and SPC was predetermined by the chosen range in the three component mixture design (Table 3). Constant levels of FM, CGM and Wheat were obtained. Prior to extrusion, the feed mixes were standardised to an equal moisture level by addition of water.

2.3 Extrusion and drying conditions.

The feed mixes, calibrated to 150 kg/h, were conditioned in an atmospheric double differential preconditioner (Wenger Manufacturing Inc., Sabetha, KS) prior to extrusion on a TX-52 co-rotating, fully intermeshing twin-screw extruder (Wenger). A standard shaft speed (220 rpm) and addition of steam (13.5 kg/h) and water (9.9 kg/h) in the preconditioner were used for all trials, giving a preconditioner outlet temperature of 86 ± 1.6 °C and a moisture content of 194 ± 6.1 g/kg (wet basis). Extruder water addition (18.0 kg/h), screw speed (400 rpm) and the temperature in the extruder barrel heads (83, 120, 140, 140, 60, 60 °C, head 2 to 7, respectively) were kept constant. The used screw configuration is described in Samuelsen and Oterhals (2016). The extruder outlet was restricted by two circular 7.3 mm dies. The wet extrudates were cut at the die surface with constant knife speed (1000 rpm, 2 knifes). Specific mechanical energy (SME) can be defined as (Akdogan, 1996):

$$SME = \frac{net\ motor\ torque \times screw\ speed}{mass\ flow\ rate} \quad (1)$$

The SME (Wh/kg wet extrudate) was calculated based on extruder torque (kW) recorded during processing and wet flow rate (kg/h) upstream the extruder die.

The feed mass temperature (T_{die}) and pressure were measured in the product stream behind the die. Sampling of pellets was conducted after achievement of steady state conditions in the preconditioner and extruder. From the sampling period of approximately 5 min a representative sample was collected in a closed bag and immediately dried in a laboratory hot air dryer (Dörrex, A. & J. Stöckli AG, Netstal, Switzerland) at 70 ºC to a moisture content of 81 ± 3.2 g/kg.

2.4 Chemical analyses

Dry matter (DM) was measured gravimetrically after drying at 103 ± 1 °C (ISO 6496). Crude protein (N x 6.25) was analysed by the Kjeldahl method (ISO 5983-2). Fat content was determined based on petroleum ether Soxhlet extraction before and after heating with hydrochloric acid (COMMISSION REGULATION (EC) No 152) and ash was determined by combustion of organic matter at 550 °C and gravimetric measurement of the residue remaining (ISO 5984). Total starch and degree of starch gelatinisation were measured utilising a modification of the glucoamylase methodology described by Chiang and Johnson (1977) and Samuelsen and Oterhals (2016). All chemical measurements were based on averages of duplicate analyses.

2.5 Analysis of extruded pellet properties

Hardness (peak breaking force) was measured on uncoated standing pellets by use of a texture analyzer (TA-HDi, Stable Micro Systems Ltd, Surrey, UK) as described in Samuelsen and Oterhals (2016). Pellets were treated individually and reported values based on the average of 20 analyses. Durability was measured on uncoated pellets using; 1) A Holmen pellet tester (Holmen Feed Technology, Berkshire, UK), where a sieved (8 mm screen) 100 g pellet sample was conveyed around in a closed circuit by a high velocity air stream as described in Samuelsen

et al. (2013); 2) A Doris tester (AKVAsmart, Bryne, Norway) where a sieved (8 mm screen) 350 g pellet sample was transported in a screw conveyor to a rotating fan. Impact with the fan and the walls downstream the fan generates cracks and dust, which were measured using different screen sizes (Aas et al., 2011). The following Doris parameters were determined: unbroken (8.0 mm), fracture (8.0-5.6 and 5.6-2.60 mm), and dust (<2.60 mm). All durability parameters were based on the average of duplicate measurements. Diameter and length were measured with an electronic caliper and based on averages of 20 pellets. Sectional expansion index (SEI) was calculated by dividing the square of the pellet diameter by the square of the die diameter. Bulk density was measured by loosely pouring the dry uncoated pellets from a funnel into a 1000 ml measuring cylinder. Reported values were based on the average of three replicates. Oil adsorption capacity (Max fat) was investigated using a lab-scale vacuum coater using food grade soybean oil and as described in Samuelsen and Oterhals (2016). Fat leakage was measured on the samples from the previous test. The sample was transferred into a closed plastic box with blotting paper and the pellet weight recorded. After incubation at 40 °C for 24 h in a heating cabinet the sample weight was registered and fat leakage calculated. Reported oil adsorption capacity and fat leakage were based on the average of duplicate measurements. Pellet water stability index (WSI) was determined utilising a slightly modified methodology described by Baeverfjord et al. (2006). Triplicate samples of each diet (20 g each) were placed in custom made steel-mesh buckets placed inside 1000 ml glass beakers filled with 500 ml distilled water. The beakers were incubated in a thermostat-controlled water bath (23ºC) and shaken (145/min) for 120 minutes, and the remaining amount of DM was determined.

2.6 Feed mass apparent viscosity and pellet pasting characteristics

The apparent wall shear stress (τ_{app}) and shear rate ($\dot{\gamma}_{app}$) of the melted feed mass in the extruder die was calculated based on:

$$\tau_{app} = \Delta P \frac{R}{2L} \quad (2)$$

176 $$\dot{\gamma}_{app} = Q\frac{4}{\pi R^3} \quad (3)$$

177 Where ΔP is the pressure drop over the die (Pa), R = die radius (0.00365 m), L = die length
178 (0.0064 m) and Q is the volume flow rate (m^3/s). Q was calculated based on the total flow
179 through the die and an approximate density of 1250 kg m^{-3} of the melted feed mass (Barron et
180 al., 2000; Ralston and Osswald, 2008).

181 Apparent viscosity of the feed mass in the die (V_{die}) was estimated by:

182 $$V_{die} = \frac{\tau_{app}}{\dot{\gamma}_{app}} \quad (4)$$

183 A Rapid Visco Analyser, (RVA; type RVA-4; Newport Scientific, Warriewood, NSW,
184 Australia; Whalen et al. 1997) was used to follow the pasting characteristics for uncoated feed.
185 The feed were ground on a Retsch ZM-1 centrifugal mill (Retsch GmbH, Haan, Germany) with
186 a ring sieve aperture of 0.5 mm. The measurement were based on 7.0 g DM of milled feed and
187 distilled water added to a total sample weight of 35.0 g. The following instrument profile was
188 used; hold at 25 °C in 5 min; ramp up to 95 °C from 5-10 min; hold at 95 °C in 7 min, cool-
189 down to 25 °C from 17-22 min; hold at 25 °C in 5 min. The paddle was run at 960 rpm for 10
190 seconds to disperse the sample and then constant at 160 rpm the rest of the test period. The
191 following RVA parameters were determined: Cold viscosity (maximum viscosity at 25 °C);
192 Peak viscosity (maximum viscosity at 95 °C); Hold viscosity (minimum viscosity at 95 °C)
193 and Final viscosity (maximum viscosity after cool-down to 25 °C). Reported figures were
194 based on the average of duplicate measurements.

195 *2.7 Experimental Design*

196 The experiment was based on a 3-component augmented simplex-lattice mixture design
197 by varying the content of HL-microalgae, WG, and SPC. The design represents a triangle with
198 a total of 10 experimental settings including three vertex points (pure blends), three edge points
199 (binary blends), three axial points (midway between the centroid and each vertex) and three

replications of the centroid point (a total of 12 trials; Table 3). A fraction of 45.5% DM was varied. The rest of the ingredients in the feed mix were held constant (Table 2). HL-microalgae was varied between 0.0-236.4 g/kg DM, WG between 36.4-272.8 g/kg DM and SPC between 181.8-418.2 g/kg DM (Table 3). There is a constant base level of WG (36.4 g/kg DM) and SPC (181.8 g/kg DM) in all feed mixes. This means that e.g. on the vertex of a pure WG blend or a binary HL-microalgae and WG blend there is a constant level of 181.8 g/kg DM of SPC. The experiments were run in random order. The responses were SME, T_{die}, V_{die}, starch gelatinisation, hardness, durability (Holmen and Doris), SEI, length, bulk density, Max fat, Fat leakage, WSI and RVA parameters (Table 4 and 5).

2.8 Statistical Analyses

The main data structure was assessed by use of principal component analysis (PCA; Martens and Martens, 2001). The experimental data were fitted to Scheffe special cubic polynomial model (Cornell, 1990).

$$y = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 \qquad (5)$$

In the models y is the estimated response. The β's describe the shape of the response surface over the simplex region where β_i the expected response to the pure component i, β_{ij} the quadratic coefficient of the binary synergism or antagonism (if β_{ij} is negative) for component i and j and β_{ijk} represents the ternary blending coefficient of component i, j, k in the interior of the triangle. The PCA was carried out using Unscrambler 9.8 (Camo, Oslo, Norway) on mean centered and standardised variables. Design-Expert v10 (Stat-Ease, Inc. Minneapolis, USA) was used for mixture design modelling and numerical optimisation on pseudo scaled components. Basic statistics were carried out using STATISTICA v12.0 (StatSoft, Inc. Tulsa, USA).

3. Results

3.1 Data overview

The experimental conditions resulted in a large span in the measured extruder and pellet responses (Table 4 and 5) and different surface morphology of the dried pellet (Fig. 1). The PCA loading plot visualize the dominant structure of the design variables and responses (Fig. 2) with principal component (PC) 1 explaining 57% and PC2 24% of the variance. Effects of HL-microalgae and SPC were mainly explained by PC1, and effects of WG by PC2. HL-microalgae was positively associated with pellet length, bulk density, RVA hold and final viscosity and Doris 8.0-5.6 and 5.6-2.6 mm. HL-microalgae was strongly correlated to the lipid level in the feed mix (R^2 > 0.999). The degree of starch gelatinisation was positive associated with WG (Fig. 2). WG was also positively associated with hardness and Holmen durability. There was, however, poor correlation between starch gelatinisation and the two responses hardness and Holmen durability (R^2 = 0.224 and R^2 = 0.347, respectively). SPC was positively associated with SME, T_{die}, V_{die}, SEI, Max fat, Fat leakage, WSI, Doris >8.0 and <2.6 mm and RVA cold and peak viscosity. RVA cold and peak viscosity, SME, T_{die} and V_{die} were positively correlated (R^2 = 0.646-0.958). The feed samples showed different pasting characteristics where high SPC levels gave the highest RVA cold and peak viscosity and high HL-microalgae level the highest RVA hold and final viscosity (Table 5; Fig. 2 and 3). A strong positive correlation between SME and SEI was observed (R^2 = 0.933). The expansion parameters SEI, Max fat and Fat leakage were positively correlated (R^2 = 0.713-0.828). Bulk density and Max fat were strongly negatively correlated (R^2 = 0.987).

3.2 Mixture models

Based on the design variables, i.e. HL-microalgae, WG and SPC levels in the feed mix (Table 3), mixture models with acceptable (Lundstedt et al. 1998) to high explained variance for SME, T_{die}, V_{die}, hardness, durability (Holmen and Doris), SEI, length, bulk density, Max

fat and WSI were established (R^2 = 0.847 to 0.998, P = 0.002 to < 0.001; Table 6). Weaker models where found for Fat leakage (R^2 = 0.768, P = 0.001) and starch gelatinisation (R^2 = 0.602, P = 0.051). No outliers were detected. The responses SME, length, bulk density and Max fat showed significant lack of fit, indicating less variation in the three replicated centroid points compared to the other experimental design points.

The correlated responses SME, T_{die} and V_{die} were fitted to linear models with the highest values found on the vertex of the pure SPC blend and lowest value on the vertex of the pure HL-microalgae blend (Table 6, Fig 4A, B, and C). The observed variance in the degree of starch gelatinisation was between 76.3% and 96.9% of total starch (Table 4). Starch gelatinisation was fitted to a reduced quadratic model with highest values for binary blends of WG and HL-microalgae and with the lowest value found on the vertex of the pure HL-microalgae blend (Fig. 4D).

Hardness and durability responses were fitted to different models (Table 6). Hardness showed a linear fit, Holmen durability and Doris >8.0 and 8.0-5.6 mm were fitted to special cubic models and Doris 5.6-2.6 mm and <2.6 mm to reduced quadratic models. In general, pellet from all trials had satisfactory to high hardness (93.8-280.8 N; Table 4) and with the highest value found on the vertex of the pure WG blend (Fig. 5A). Increased level of both HL-microalgae and SPC in the blend reduced hardness. Durability (Fig. 5B) created a ridge downward sloping from the edge of the binary WG and HL-microalgae blend to the edge of the HL-microalgae and SPC blend. The lowest values were found on the vertex of the pure HL-microalgae and the pure SPC blend. A similar but opposite pattern was observed for Doris <2.6 mm (Fig. 5C). Doris >8.0 mm showed highest values on the edge of the binary SPC and HL-microalgae blend and with the lowest value found on the vertex of the pure HL-microalgae blend (Fig 5D).

The expansion parameters, SEI, Bulk density, Max fat and Fat leakage were all fitted to linear models, and length to a reduced quadratic model (Table 6, Fig. 6). Length was mainly affected by the HL-microalgae level in the three component blend (Fig. 6D). The linear model for SEI (Fig. 6A), Max fat (Fig. 6B) and Fat leakage (Fig. 6C) showed the highest value on the vertex of the pure SPC blend and consequently opposite for bulk density (Table 6). The linear model for WSI (Table 6) showed high values on the edge of the binary SPC and WG blend and with a reduction in WSI with increased level of HL-microalgae in the blends.

3.3 Optimisation

A numerical optimisation of the responses were performed using the following criteria; (1) Tdie > 100°C, (2) maximize HL-microalgae in the feed mix, Holmen durability, Doris >8.0 mm and Max fat and (3) minimize Doris <2.6 mm and Fat leakage. With the given desired goal for each factor, an optimal ternary blend space was suggested including a best local optimum. In Fig. 5D the suggested upper boundary level of HL-microalgae in the blends and the local optimum are shown. The optimum was found on the edge of the binary blend of HL-microalgae and SPC (HL-microalgae = 131.6, WG = 36.4, SPC = 286.6 g/kg DM; Fig. 5D). This corresponds to a lipid level in the feed mix of 128.2 g/kg DM.

4. Discussion

The effect of the inclusion levels of HL-microalgae, WG and SPC on the extrusion process, feed melt viscosity, physical pellet quality and expansion parameters is discussed below.

4.1 Effect on SME, T_{die}, and V_{die}

The response SME (Eq. 1) is a measure of the sum of the total mechanical energy dissipated over the total length of the extruder screw. The energy is mainly converted into heating of the material through viscous dissipation (Godavarti and Karwe, 1997; Della Valle

et al., 1989). In this study, screw design, screw speed, mass flow rate and moisture addition were standardised. An increase in motor torque and consequently an increase in SME and temperature will be a result of increased viscosity of the feed mass (Bhattacharya and Hanna, 1987; Akdogan, 1996), confirmed by the positive correlation between SME, T_{die} and V_{die} in this study. The highest SME values are found for the SPC rich blends followed by WG (Fig 4A). This is consistent with the findings of Bhattacharya and Hanna (1986) and Bengoechea et al. (2007) based on soy protein isolate/gluten blends and can be attributed to a lower glass transition temperature (T_g) for gluten and the mobilisation of more protein groups above T_g compared to soy protein (Bengoechea et al., 2007). HL-microalgae is a lipid rich ingredient (Table 1) and at the vertex of pure HL-microalgae blend, the lipid level is as high as 191 g/kg DM (Table 2). The reduced viscosity with increased HL-microalgae content can be attributed to the lubrication effect of lipids in the feed mix.

4.2 Effect on degree of starch gelatinisation and pellet pasting characteristic

In the high HL-microalgae region due to the high lipid content, the feed mass was poorly cooked with temperature <100 °C (Fig. 4B). This can explain the lower values of starch gelatinisation for blends high in HL-microalgae compared to WG and SPC rich blends (Table 4, Fig. 4D). The variation in starch gelatinisation showed a low degree of explanation in the PCA model (<50%) and was poorly correlated to the other responses in the design. The starch level was kept at similar level (Table 2) and the result supports our earlier observations that the applied glucoamylase methodology was not adequate for quantification of the binding properties of the starch paste (Samuelsen *et al*. 2013). The mixture design was based on commercial relevant levels of SPC and crude protein (Table 2; Ytrestøyl et al., 2015) and the feed mixes were only processed for technical evaluations. The design was restricted by the variation in the components HL-microalgae, WG and SPC, which had different chemical composition (Table 1), whereas micro ingredients (vitamins, minerals, amino acids,

astaxanthin), were not added, thus, the feed mixes were not nutritionally balanced. This approach gave somewhat higher starch content in the final feeds (Table 2) than commercial diets for salmonids (lower than ≈130 g/kg, calculated based on Ytrestøyl et al., 2015). A lower starch content may reduce the physical pellet quality, however, in a mixture design this will also increase the level of, and possible binding contribution from other constituents including proteins. The effect of lower starch level can only be determined based on new studies.

In the study of Samuelsen et al. (2013) there was a positive correlation between starch gelatinisation and SME, explained by increased melt viscosity with increasing starch gelatinisation, giving a positive contribution to specific mechanical energy. However, the result could not be confirmed in this study, probably caused by a dominating effect of the mixture components and with highest viscosities and SME's found for the SPC rich blends.

RVA is an instrument initially developed to characterize reduced paste viscosity due to sprout-damage of wheat, but can also be used as a rapid method to study starch pasting in general, viscosity contribution of pure ingredients or blends, and the degree of cook in the extrusion process (Whalen et al., 1997; Glencross et al., 2007, 2010; Tan et al., 2008). The method is based on biopolymer swelling, starch granule rupture, and solubilisation effects in excess moisture, and cannot directly be compared with biopolymer plasticisation and unfolding at low moisture and high temperature conditions in the extrusion process. However, a positive correlation of RVA cold and peak viscosity with the extruder responses SME, T_{die} and V_{die} in this study indicates that the two RVA parameters can be used as an indirect measure of the viscous heat dissipation and melt viscosity in the extrusion process. The lowest degree of starch gelatinisation (76.3%; Table 4) was observed at the vertex of the pure HL-microalgae blend and it was therefore expected a high peak viscosity for this blend. The opposite was observed, with a low and delayed peak viscosity relative to the other blends (Table 5; Fig. 3). Debet and Gidley (2006) observed a significant reduction in RVA viscosity onset temperature and the

highest peak viscosity for a pure wheat extracted for surface proteins and lipids. They concluded that both proteins and lipids on the surface of the starch granules affected the swelling characteristics. Although not directly comparable, this is consistent with the findings in this study where the viscosity profile is determined by the feed mix composition and with the lipids as the most important factor reducing the swelling rate of the starch granules. The lower hold and final viscosity found for the samples with the highest peak viscosities (Table 5; Fig 3) may be explained by a higher degree of granule swelling resulting in increased disruption and solubilisation (Debet and Gidley, 2006). The HL-microalgae product used in this study contain saturated fatty acids with melting point above 25 °C. Possible release of fat in the cooking cycle and formation of emulsions and/or crystalline lipid structures during cooldown might also have contributed to the high final viscosity observed in the high HL-microalgae blend.

The results from this study indicates that the RVA method can be applied as an indirect measure of the melt viscosity in the extrusion process. It can, however, not be used to quantify the degree of starch gelatinisation in extruded feed mixes containing different levels and compositions of proteins, lipids and starches.

4.3 *Effect on pellet hardness, durability and dust*

Different plasticisation temperatures may explain the higher physical quality (hardness and Holmen durability; Fig. 5A and B) and lower dust fraction (Doris <2.6 mm; Fig. 5C) found for WG rich compared to SPC rich blends. At comparable moisture level, Bengoechea et al. (2007) documented a T_g ~ 65 °C lower for gluten compared to soya protein isolate and a significantly lower temperature difference between T_g and the flow-transition temperature (~ 38 °C). Soya protein isolate is more refined than SPC and a direct comparison can therefore not be performed. However, based on the results it can be hypothesised that at the predetermined moisture level and the achieved temperature from steam and viscous dissipation,

375 the WG rich blends has been properly plasticised resulting in a high physical quality. At even
376 higher viscous dissipation for the SPC rich blends, the moisture level in the process has still
377 been too low for complete plasticisation. This may have resulted in increased level of non-
378 plasticised particles in the extrudate giving poor pellet durability and high dust fraction (Arêas,
379 1992; Mitchell and Arêas, 1992).

380 In contradiction to Holmen durability the highest values for Doris unbroken (>8.0 mm)
381 were found for SPC rich blends (Table 4, Fig. 5D) indicating a harsher treatment in the Holmen
382 tester compared to Doris when performed on uncoated pellet. The high values for Doris
383 unbroken and Doris dust for the SPC rich blends indicates that these pellets tend to crack into
384 dust rather than pieces, confirming the above hypothesis of high particulate matter in the SPC
385 rich pellets.

386 In the high HL-microalgae region poor physical pellet quality were expected and
387 documented through the low values for hardness, Holmen durability and Doris unbroken (Table
388 4; Fig. 5A, B and D). This is mainly due to the lubrication effect of the lipids giving low viscous
389 heat dissipation and undercooked melt with T_{die} <100 °C. For binary HL-microalgae and SPC
390 blends, increased Holmen durability and reduced dust were observed when partly replacing
391 SPC with HL-microalgae, and with an optimum near the edge point of the binary blend (Fig. 5
392 B and C). The protein fraction in the HL-microalgae consists of ~39% water soluble protein
393 (Kousoulaki et al., 2016). The oil fraction is mostly triglycerides but contains some free fatty
394 acids (~3%). Water soluble protein and free fatty acids are reported to have plasticising effects
395 (di Gioia and Guilbert, 1999; Pommet et al., 2003; Oterhals and Samuelsen, 2015; Samuelsen
396 and Oterhals, 2016). The plasticising effect of such constituents in combination with lower
397 SPC level may have improved overall plasticisation of the feed mass and physical pellet
398 quality. At higher HL-microalgae level, this is reversed due to the lubrication effect of the
399 lipids.

4.4 Effect on expansion parameters and water stability

The experimental conditions resulted in different expansion and surface morphology of the dried feed (Fig. 1). Steam pressure is the main driving force for feed melt expansion (Fan et al., 1994). Expansion can be increased with higher steam flashing rate, i.e. increase in T_{die}. The extrusion conditions and moisture content were kept constant in this study and an increase in T_{die} will thereby be a direct response to increased viscous heat dissipation, i.e. increased melt viscosity and SME. This was confirmed by the high correlation between the responses SME, $V_{die,}$ and T_{die}, which all are positively correlated to the expansion parameter SEI, Max fat and Fat leakage (Fig. 2). A higher expansion gives more pore volume available for fat adsorption, however, also larger pore structures with increased tendency for fat leakage. A higher lipid level in the dry feed mix (i.e. higher HL-microalgae inclusion level) demands less oil to be coated onto the dried expanded feed to meet the target final fat content. A lower SEI can thereby be accepted with a positive effect on fat leakage. Bulk density was negatively correlated to Max fat and reflects a less expanded and more dens pellet structure, in line with Samuelsen and Oterhals (2016). The above results indicate that all the measured expansion parameters are mainly controlled by viscous heat dissipation and directly linked to the ratio between the components HL-microalgae, WG and SPC in the mixture design.

Insignificant changes in longitudinal expansion were observed in a large composition region and with a sharp increase at high HL-microalgae content (Fig. 6D). The increase corresponds to low T_{die} (Fig. 4B) which gives low driving force for diametrical expansion (SEI, Fig. 6A) and resulting increased longitudinal mass flow rate. A low apparent viscosity (V_{die}) in this mixture region (Fig. 4C) may also give increased structure collapse after expansion (Fan et al., 1994) and contribute to the formation of dense and long pellets.

Water stability of a feed is most important for aquatic slow eaters but may also influence the degradation pattern in the gastrointestinal tract of the fish (Hilton et al., 1981; Baeverfjord

et al., 2006; Sørensen, 2012). The linear model for WSI (Table 6) showed that WSI was negatively affected by the HL-microalgae content in the three-component blend and to lesser degree the SPC and WG content. This observation indicates that water stability of a feed can be controlled by varying the HL-microalgae or fat content in a feed mix.

4.5 Optimisation study and impact on industrial feed processing

The numerical optimisation study were based on standardised extrusion conditions, HL-microalgae levels and the obtained responses, T_{die}, Holmen durability, Doris >8.0 and <2.6 mm, Max fat and Fat leakage. Pellet expansion and physical quality can also be controlled by change in extrusion parameters as water, steam and mechanical energy (Blanche and Sun, 2004; Samuelsen and Oterhals, 2016). In addition, starch level and in-between variation of the used ingredients due to different species, cultivars, agricultural practice, geographical area, environmental conditions, crop year, and processing methods and conditions have not been included (Hill et al., 2010a, 2010b; Samuelsen et al., 2014). Results from this type of optimisation approach has limitations and should only be used for indicative purposes and general recommendations. A large range of binary and ternary mixtures meets the criteria with the best local optimum found slightly above the edge point of the binary HL-microalgae and SPC blend (Fig. 5D). At this point the HL-microalgae content in the feed mix is 131.6 g/kg DM, corresponding to a lipid level of 128.2 g/kg DM and close to the maximum recommend lipid level consider to have limited negative effect on feed quality (120 g/kg; Rokey 1994). SPC rich diets tolerate higher HL-microalgae content compared to the WG rich blends. This may be attributed to the higher viscosity for these blends (Fig. 4C) giving higher viscous heat dissipation and improved physical quality. In practical terms, 131.6 g/kg DM HL-microalgae correspond to 109.0 g/kg HL-microalgae in a final coated feed with 30% of total lipid content; equivalent to 2.0% EPA+DHA. This is above the safe EPA+DHA levels for good growth and

health performance of salmon, i.e. 1.6% in the feed (Bou et al., 2017). A recent long term growth study documented that *Schizochytrium* sp. can replace fish oil in feeds without negative effects on growth performance of Atlantic salmon (Kousoulaki et al., 2017)

5. Conclusion

The ratio between the components HL-microalgae, WG and SPC in the mixture design had significant impact on all the measured extrudate and pellet responses. A high HL-microalgae content (132 g/kg DM) can be used in plant based feed mixes without compromising feed quality, indicating that dry HL-microalgae ingredient can be used as an alternative and sustainable source of n-3 LC-PUFA in aquaculture feeds. For high SPC diets, the HL-microalgae addition may in addition give favourable processing effects with respect to improved overall plasticisation and physical pellet quality.

Acknowledgements

The authors are grateful for the financial support from the Research Council of Norway through the project "Heterotrophic microalgae for future marine omega-3 rich salmon feeds" (grant no. 234057/E40), and Alltech Inc. for providing the HL-microalgae ingredient. We also want to thank the skilful and accurate employees at the Nofima Feed Technology Centre and laboratory personnel at Nofima BioLab.

References

Aarseth, K.A., 2004. Attrition of feed pellets during pneumatic conveying: the influence of velocity and bend radius. Biosyst. Eng., 89, 197-213.

Aarseth, K.A., Perez, V., Bøe, J.K., Jeksrud, W.K., 2006. Reliable pneumatic conveying of fish feed. Aquacult. Eng., 35, 14-25.

Aas, T.S., Oehme, M., Sørensen, M., He, G., Lygren, I., Åsgård, T., 2011. Analysis of pellet degradation of extruded high energy fish feeds with different physical quality in a pneumatic feeding system. Aquacult. Eng., 44, 25-34.

Akdogan, H., 1996. Pressure, torque, and energy responses of a twin screw extruder at high moisture contents. Food Res. Int., 29, 423-429.

Arêas, J.A.G., 1992. Extrusion of food proteins. Crit. Rev. Food Sci. Nutr., 32, 365-392.

Baeverfjord, G., Refstie, S., Krogedal, P., Åsgard, T., 2006. Low feed pellet water stability and fluctuating water salinity cause separation and accumulation of dietary oil in the stomach of rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 261, 1335-1345.

Barron, C., Buleon, A., Colonna, P., Della Valle, G., 2000. Structural modifications of low hydrated pea starch subjected to high thermomechanical processing. Carbohydr. Polym. 43,171-181.

Bengoechea, C., Arrachid, A., Guerrero, A., Hill, S.E., Mitchell, J.R., 2007. Relationship between the glass transition temperature and the melt flow behavior for gluten, casein and soya. J. Cereal Sci., 45, 275-284.

Bhattacharya, M., Hanna, M.A., Kaufman, R. E., 1986. Textural properties of extruded plant protein blends. J. Food Sci., 51, 988-993.

Bhattacharya, M., Hanna, M.A., 1987. Influence of process and product variables on extrusion energy and pressure requirements. J. Food Eng., 6, 153-163.

Blanche, S., Sun, X.Z., 2004. Physical characterization of starch extrudates as a function of melting transitions and extrusion conditions. Adv. Polym. Tech., 23, 277-290.

496 Bou, M., Berge, G.M., Baeverfjord, G., Sigholt, T., Østbye, T.-K, Romarheim, O.H., Hatlen,
497 B., Leeuwis, R., Venegas, C., Ruyter, B., 2017. Requirements of n-3 very long-chain
498 PUFA in Atlantic salmon (*Salmo salar* L): effects of different dietary levels of EPA and
499 DHA on fish performance and tissue composition and integrity. Br. J. Nutr., 117, 30-47.
500 Chiang, B.-Y., Johnson, J.A., 1977. Measurement of total and gelatinized starch by
501 glucoamylase and *o*-toluidin reagent. Cereal Chem., 54, 429-435.
502 Cornell, J.A., 2002. Experiments with Mixtures: Designs, Models, and the Analysis of
503 Mixture Data, third ed. John Wiley and Sons, Inc., NY.
504 Debet, M.R., Gidley, M.J., 2006. Three classes of starch granule swelling: Influence of
505 surface proteins and lipids. Carbohydr. Polym., 64, 452-465.
506 Della Valle, G., Kozlowski, A., Colonna, P., Tayeb, J., 1989. Starch transformation estimated
507 by the energy balance on a twin screw extruder. Lebensm. -Wiss. u.- Technol., 22, 279-
508 286.
509 di Gioia, L., Guilbert, S., 1999. Corn protein-based thermoplastic resins: effect of some polar
510 and amphiphilic plasticizers. J. Agric. Food Chem., 47, 1254-1261.
511 Fan J., Mitchell J.R., Blanshard J.M.V. 1994. A computer simulation of the dynamics of
512 bubble growth and shrinkage during extrudate expansion. J. Food Eng., 23, 337-356.
513 Ganuza, E., Benitez-Santana, T., Atalah, E., Vega-Orellana, O., Ganga, R., Izquierdo, M.S.,
514 2008. *Crypthecodinium cohnii* and *Schizochytrium* sp as potential substitutes to fisheries
515 derived oils from seabream (*Sparus aurata*) microdiets. Aquaculture, 277, 109-116.
516 Glencross, B.D., Booth, M., Allan, G.L., 2007. A feed is only as good as its ingredients – a
517 review of ingredient evaluation strategies for aquaculture feeds. Aquacult. Nutr., 13, 17-34.
518 Glencross, B., Hawkins, W., Maas, R., Karopoulos, M., Hauler, R., 2010. Evaluation of the
519 influence of different species and cultivars of lupin kernel meal on the extrusion process,
520 pellet properties and viscosity parameters of salmonid feeds. Aquacult. Nutr., 16, 13-24.

521 Godavarti, S., Karwe, M.V., 1997. Determination of specific mechanical energy distribution
522 on a twin-screw extruder. J. Agric. Eng. Res., 67, 277-287.
523 Hill, D., Hoke, J., Taylor, R., Sliffe, T., 2010a. Nutrient variation of common ingredients-Part
524 1. inform, 9, 582-586.
525 Hill, D., Hoke, J., Taylor, R., Sliffe, T. 2010b. Nutrient variation of common ingredients-Part
526 2. inform, 10, 648-652.
527 Hilton, J.W., Cho, C.Y., Slinger, S. J., 1981. Effect of extrusion processing and steam
528 pelleting diets on pellet durability, pellet water-absorption, and the physiological-response
529 of rainbow-trout (*Salmo Gaird-Neri* R.). Aquaculture, 25, 185-194.
530 ISO 5983-2, 2009. Animal Feeding Stuffs - Determination of Nitrogen Content and
531 Calculation of Crude Protein Content - Part 2: Block Digestion/Steam Distillation Method.
532 International Organization for Standardization, Geneva, Switzerland.
533 ISO 5984, 2002. Animal Feeding Stuffs - Determination of Crude Ash. International
534 Organization for Standardization, Geneva, Switzerland.
535 ISO 6496, 1999. Animal Feeding Stuffs - Determination of Moisture and Other Volatile
536 Matter Content. International Organization for Standardization, Geneva, Switzerland.
537 Kousoulaki, K., Østbye, T-K.K., Krasnov, A., Torgersen, J.S., Mørkøre, T., Sweetman, J.,
538 2015. Metabolism, health and fillet nutritional quality in Atlantic salmon (*Salmo salar*) fed
539 diets containing *n-3*-rich microalgae. J. Nutr. Sci., 4, e24, 1-13.
540 Kousoulaki, K., Mørkøre, T., Nengas, I., Berge, R.K., Sweetman, J., 2016. Microalgae and
541 organic minerals enhance lipid retention efficiency and fillet quality in Atlantic salmon
542 (*Salmo salar* L.). Aquaculture, 451, 47-57.
543 Kousoulaki, K., Carlehög, M., Mørkøre, T., Ruyter, B., Berge, G.M., 2017. Long term
544 supplementation of heterotrophic microalgae in Atlantic salmon diets. Aquaculture Europe
545 2017, October 17-20, Dubrovnik, Croatia.

546 Kraugerud, O.F., Jørgensen, H.Y., Svihus, B., 2011. Physical properties of extruded fish feed
547 with inclusion of different plant (legumes, oilseeds, or cereals) meals. Anim. Feed Sci.
548 Technol., 163, 244-254.
549 Martens, H., Martens, M., 2001. Multivariate Analysis of Quality. An Introduction, second
550 ed. John Wiley & Sons Ltd. West Sussex, UK.
551 Mitchell, J.R., Arêas, J.A.G., 1992. Structural changes in biopolymers during extrusion, in:
552 Kokini, J.L., Ho, C.-T., Karwe, M.V. (Eds.), Food Extrusion Science and Technology,
553 Marcel Dekker Inc., New York, NY, pp. 345-360.
554 Miller, M., Nichols, P., Carter, C., 2007. Replacement of fish oil with thraustochytrid
555 *Schizochytrium* sp L oil in Atlantic salmon parr (*Salmo salar* L) diets. Comp. Biochem.
556 Physiol., 148, 382-392.
557 Oterhals, Å., Samuelsen, T.A., 2015. Plasticization effect of solubles in fishmeal. Food Res.
558 Int., 69, 313-321.
559 Pommet, M., Redl, A., Guilbert, S., Morel, M. H., 2005. Intrinsic influence of various
560 plasticizers on functional properties and reactivity of wheat gluten thermoplastic materials.
561 J. Cereal Sci., 42, 81-91.
562 Ralston, B.E., Osswald, T.A., 2008. Viscosity of soy protein plastics determined by screw
563 driven capillary rheometry. J. Polym. Environ., 16, 169-176.
564 Ren, L.J., Ji, X.J., Huang, H. Qu, L.A., Feng, Y., Tong, Q.Q., Ouyang, P.K., 2010.
565 Development of a stepwise aeration control strategy for efficient docosahexaenoic acid
566 production by *Schizochytrium* sp. J. Appl. Microbiol. Biotechnol., 87, 1649-1656.
567 Rokey, G.J. 1994. Petfood and fishfood extrusion, in: Frame, N.D. (ed.), The Technology of
568 Extrusion Cooking, first ed. Blackie Academic & Professional, Glasgow, UK, pp. 144-189.

569 Samuelsen, T.A., Mjøs, S.A., Oterhals, Å., 2013. Impact of variability in fishmeal
570 physicochemical properties on the extrusion process, starch gelatinization and pellet
571 durability and hardness. Anim. Feed Sci. Technol., 179, 77-84.
572 Samuelsen T.A., Mjøs S.A., Oterhals, Å., 2014. Influence of type of raw material on fishmeal
573 physicochemical properties, the extrusion process, starch gelatinization and physical
574 quality of fish feed. Aquacult. Nutr., 20, 410-420.
575 Samuelsen, T.A., Oterhals, Å., 2016. Water-soluble protein level in fishmeal affects extrusion
576 behaviour, phase transitions and physical quality of feed. Aquacult. Nutr., 22, 120-133.
577 Schmidtsdorff, W., 1995. Fish meal and fish oil-not only by-products, in: Ruiter, A. (ed.),
578 Fish and Fishery Products, Composition, Nutritive Properties and Stability. CAB
579 International, Wallingford, UK, pp. 347-376.
580 Strauch, W., 2005. Einflussgrößen beim vakuumcoating zur herstellung energiereicher
581 mischfutter. Mühle + Mischfutter, 142, 97-103.
582 Sørensen, M., Stjepanovic, N., Romarheim, O.H., Krekling, T., Storebakken, T., 2009.
583 Soybean meal improves the physical quality of extruded fish feed. Anim. Feed
584 Sci.Technol.,149, 149-161.
585 Sørensen, M., Berge, G.M., Thomassen, M., Ruyter, B., Hatlen, B., Ytrestøyl, T., Aas, T.S.,
586 Åsgård, T., 2011. Today's and tomorrow's feed ingredients in Norwegian aquaculture.
587 Nofima report 52/2011. ISBN 978-82-7251-943-7.
588 Sørensen, M., 2012. A review of the effects of ingredient composition and processing
589 conditions on the physical qualities of extruded high-energy fish feed as measured by
590 prevailing methods. Aquacult. Nutr., 18, 233-248.
591 Tacon, A.G.J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in
592 industrially compounded aquafeeds: Trends and future prospects. Aquaculture, 285, 146-
593 158.

Tacon, A.G.J., Hasan, M.R., Metian, M., 2011 Demand and supply of feed ingredients for farmed fish and crustaceans: Trends and prospects. FAO Fisheries and AquacultureTechnical Paper No. 564, Rome, Italy. ISBN 978-92-5-106933-2.

Tan, I., Torley, P.J., Halley, P.J., 2008. Combined rheological and optical investigation of maize, barley and wheat starch gelatinization. Carbohydr. Polym. 72, 272-286.

Whalen, P.J., Bason, M.L., Booth, R.I., Walker, C.E., Williams, P.J., 1997. Measurement of extrusion effects by viscosity profile using the Rapid Visco Analyser. Cereal Foods World, 42, 469–475.

Ytrestøyl, T., Aas, T.S., Asgard, T., 2015. Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. Aquaculture, 448, 365-374.

Figure legends

Fig. 1

Pellet surface morphology for the centroid and vertices: A) centroid (ENo 3), B) pure HL-microalgae blend (ENo 5), C) pure WG blend (ENo 11) and D) pure SPC blend (ENo 8). Detailed composition data and abbreviations are given in Table 3.

Fig. 2

Principal component analysis (PCA) correlation loading plot based on experimental design and response variables. The two ellipses represent 50 and 100% of explained variance. Abbreviations are explained in Table 3 and 4.

Fig. 3

Rapid Visco Analyser (RVA) profiles for the centroid (ENo 3), pure HL-microalgae blend (ENo 5), pure WG blend (ENo 11), and pure SPC blend (ENo 8). Detailed composition data and abbreviations are given in Table 3. RVA parameters can be found in Table 5.

Fig. 4

Contour plots for extrusion parameters and starch gelatinisation. Actual inclusion levels (g/kg DM) for the three components are given for the pure (vertex) and binary (edge) points. The contours are flagged with the actual unit of the respective response. Solid red circles represent the design points. A) Specific mechanical energy (Wh/kg), B) Temperature behind the die (°C), C) Apparent viscosity in the extruder die (Pa s), D) Degree of starch gelatinisation (% of total starch). Detailed composition data and abbreviations are given in Table 3.

Fig. 5

Contour plots for physical quality parameters. Actual inclusion levels (g/kg DM) for the three components are given for the pure (vertex) and binary (edge) points. The contours are flagged with the actual unit of the response. Solid red circles represent the design points. A) Hardness (N), B) Holmen durability (%), C) Doris <2.6 mm (%), D) Doris >8.0 mm (%). The dotted line defines the upper boundary for the numerical optimisation and the dotted circle marks the local optimum at HL-microalgae = 131.6, WG = 36.4 and SPC = 286.6 g/kg DM. Detailed composition data and abbreviations are given in Table 3.

Fig. 6

Contour plots for pellet expansion parameters. Actual inclusion levels (g/kg DM) for the three components are given for the pure (vertex) and binary (edge) points. The contours are flagged with the actual unit of the response. Solid red circles are the design points. A) Sectional expansion index, B) Oil adsorption capacity (g/kg) C) Fat leakage (g/kg), D) Length (mm). Detailed composition data and abbreviations are given in Table 3.

Tables

Table 1

Proximate chemical composition of the feed mix ingredients (g/kg)

	HL-microalgae	WG	SPC	FM	CGM	Wheat
DM	972	947	935	927	886	876
In DM						
Crude protein	154	845	641	726	660	142
Lipid	632	43	29	136	43	27
Total ash	36	8	65	165	14	17
Carbohydrate[a]	178	104	265	---	283	814

HL-microalgae, high lipid microalgae (*Schizochytrium* sp.); CGM, corn gluten meal; DM, dry matter; FM, fishmeal; SPC, soy protein concentrate; WG, vital wheat gluten; Wheat, whole wheat flour.

[a] Estimated by difference (1000 - protein - lipid - total ash).

Table 2

Formulation and proximate chemical composition of the experimental feed mixes fed to the extruder system (g/kg)[a]

	EX1	EX2	EX3	EX4	EX5	EX6	EX7	EX8	EX9	EX10
Formulation										
HL-microalgae				111	222	111	74	37	148	37
WG	263	149	35	35	35	149	111	73	73	187
SPC	177	293	408	293	177	177	254	331	216	216
FM	166	166	166	166	166	166	166	166	166	166
CGM	83	83	83	83	83	83	83	83	83	83
Wheat	308	308	308	308	308	308	308	308	308	308
Water[b]	3	1		4	9	6	4	2	6	3
Chemical composition										
DM[c]	912	912	912	912	912	912	912	912	912	912
In DM										
Crude Protein[c]	564	540	516	459	401	483	494	505	448	529
Lipid[c]	52	50	48	119	191	121	97	72	144	74
Total Ash[c]	48	55	62	58	55	51	55	58	55	51
Carbohydrate[c]	336	355	374	364	354	345	354	364	354	345
Starch	230	225	213	211	206	219	216	222	213	227

HL-microalgae, high lipid microalgae (*Schizochytrium* sp.); CGM, corn gluten meal; DM, dry matter; EX, experimental feed mix; FM, fishmeal; SPC, soy protein concentrate; WG, vital wheat gluten; Wheat, whole wheat flour.

[a] Proximate chemical composition calculated for diets at lipid level of 320 g/kg DM: Crude Protein, 371±18.3 g/kg; Total ash, 41±3.7 g/kg; Starch, 158±6.7 g/kg.

[b] Water was sprayed homogeneously into the feed mixes at least 24 hours before processing to secure even partitioning and adsorption into the feed matrix.

[c] Calculated based on raw material analyses (Table 1).

Table 3

Pseudo and actual units for the experimental design variables, high lipid microalgae (*Schizochytrium* sp.; HL-microalgae), vital wheat gluten (WG) and soy protein concentrate (SPC).

ENo	Feed mix	Pseudo units			HL-microalgae[a] (g/kg DM)	WG[a] (g/kg DM)	SPC[a] (g/kg DM)
1	EX8	0.167	0.167	0.667	39.4	75.8	339.4
2	EX4	0.500	0.000	0.500	118.2	36.4	300.0
3	EX7	0.333	0.333	0.333	78.8	115.2	260.6
4	EX2	0.000	0.500	0.500	0.0	154.6	300.0
5	EX5	1.000	0.000	0.000	236.4	36.4	181.8
6	EX7	0.333	0.333	0.333	78.8	115.2	260.6
7	EX9	0.667	0.167	0.167	157.6	75.8	221.2
8	EX3	0.000	0.000	1.000	0.0	36.4	418.2
9	EX7	0.333	0.333	0.333	78.8	115.2	260.6
10	EX6	0.500	0.500	0.000	118.2	154.6	181.8
11	EX1	0.000	1.000	0.000	0.0	272.8	181.8
12	EX10	0.167	0.667	0.167	39.4	194.0	221.2

DM, dry matter; ENo, experiment number; EX, experimental feed mix.

[a]Based on DM in feed mixes.

Table 4

Extruder and pellet responses.

	Extruder			Pellet												
ENo	SME (Wh/kg)	T_{die} (ºC)	V_{die} (Pa s)	Starch gel[a] (%)	Hardness (N)	Dur. (%)	Doris >8.0 (%)	Doris 8.0-5.6 (%)	Doris 5.6-2.6 (%)	Doris <2.6 (%)	SEI	Length (mm)	Bulk density (g/L)	Max fat[b] (g/kg)	Fat leakage (g/kg)	WSI (g/kg)
1	48.0	117.8	764	83.5	111.9	74.0	59.5	21.6	10.6	6.7	2.14	11.19	447	440	60.0	83.3
2	38.8	111.8	497	90.0	96.5	80.8	64.7	22.2	8.3	4.4	1.87	11.09	512	313	45.0	69.1
3	38.9	110.9	635	89.8	139.3	86.0	59.4	23.4	13.2	3.9	1.75	10.75	542	278	48.0	62.6
4	41.8	116.8	834	88.4	165.3	76.7	43.5	31.4	17.4	7.7	2.03	11.11	487	377	58.0	85.6
5	20.0	91.3	130	76.3	93.8	39.6	35.1	32.4	24.3	7.8	1.12	15.23	580	181	43.0	2.0
6	39.5	111.1	668	92.9	154.5	85.3	61.3	21.8	12.3	4.5	1.78	10.94	542	281	50.0	66.9
7	29.0	100.1	260	94.0	105.8	73.2	48.5	25.6	20.1	5.5	1.25	13.71	559	221	45.0	2.8
8	52.5	125.9	817	92.8	117.4	40.5	64.0	14.3	10.6	10.6	2.48	12.15	364	606	63.0	83.9
9	39.2	112.3	575	96.5	168.6	86.2	62.9	21.1	11.9	4.1	1.73	10.75	544	276	43.0	54.5
10	29.2	103.5	361	96.9	148.8	85.2	46.3	30.3	19.1	4.0	1.47	11.63	568	224	44.0	35.3
11	35.1	110.7	620	94.4	280.8	87.6	54.3	25.5	14.2	5.8	1.81	10.30	562	250	48.0	88.4
12	35.2	110.2	649	93.7	226.0	90.8	53.0	26.9	16.3	3.7	1.63	10.79	585	216	48.0	66.6

Doris, Doris parameters in mm; Dur., Holmen durability; ENo, experiment number; Max fat, oil adsorption capacity; SEI, sectional expansion index; SME, specific mechanical energy; Starch gel, degree of starch gelatinisation; T_{die}, temperature behind extruder die; V_{die}, apparent viscosity in the extruder die; WSI, water holding capacity.

[a] % of total starch.

[b] g/kg of total oil adsorbed.

Table 5

Rapid Visco Analyser parameters.

ENo	Cold viscosity (mPa s)	Peak viscosity (mPa s)	Hold viscosity (mPa s)	Final viscosity (mPa s)
1	1059	2638	325	1437
2	479	1512	337	1450
3	609	1662	454	1674
4	1036	3083	529	1724
5	15	1081	672	2167
6	711	1787	482	1695
7	137	1285	798	2302
8	2026	3389	382	1652
9	676	1578	491	1746
10	184	1264	694	2150
11	450	2438	617	1872
12	594	1909	726	2054

ENo, experiment number.

Table 6

Mixture models.

Variables	Pseudo Component model	P - value	Lack of fit (P)	R^2
SME[a]	22.15 A + 35.29 B + 54.35 C	< 0.001	0.017	0.957
T_{die}[a]	93.08 A + 111.04 B + 126.48 C	< 0.001	0.170	0.974
V_{die}[a]	116.70 A + 684.87 B + 900.71 C	< 0.001	0.357	0.929
Starch gel[b]	80.22 A + 91.65 B + 90.29 C + 48.68 AB	0.051	0.382	0.602
Hardness[a]	80.41 A + 268.08 B + 103.68 C	< 0.001	0.456	0.921
Dur.[c]	39.04 A + 87.85 B + 40.85 C + 89.79 AB + 162.59 AC + 51.81 BC - 91.51 ABC	< 0.001	0.110	0.998
Doris >8.0[c]	34.07A + 54.69 B + 63.65 C + 5.46 AB + 57.86 AC - 62.46 BC + 252.11 ABC	0.002	0.275	0.967
Doris 8.0-5.6[c]	32.25 A + 25.48 B + 14.58 C + 5.10 AB - 4.43AC + 46.72 BC -193.88 ABC	< 0.001	0.812	0.985
Doris 5.6-2.6[b]	25.15 A + 14.07 B + 10.59 C - 40.07 AC + 14.82 BC	< 0.001	0.110	0.917
Doris <2.6[b]	8.23 A + 5.23 B + 10.33 C -12.33 AB - 19.44 AC	< 0.001	0.176	0.941
SEI[a]	1.08 A + 1.72 B + 2.47 C	< 0.001	0.062	0.954
Length[b]	15.16 A + 9.78 B + 12.09 C – 10.15 AC	< 0.001	0.026	0.881
Bulk density[a]	597.67 A + 586.00 B + 389.33 C	< 0.001	0.002	0.903
Max fat[a]	147.58 A + 213.25 B + 554.92 C	< 0.001	0.004	0.919
Fat leakage[a]	39.03 A + 47.69 B + 62.03 C	0.001	0.567	0.768
WSI[a]	- 2.44 A + 81.93 B + 95.76 C	< 0.001	0.170	0.847

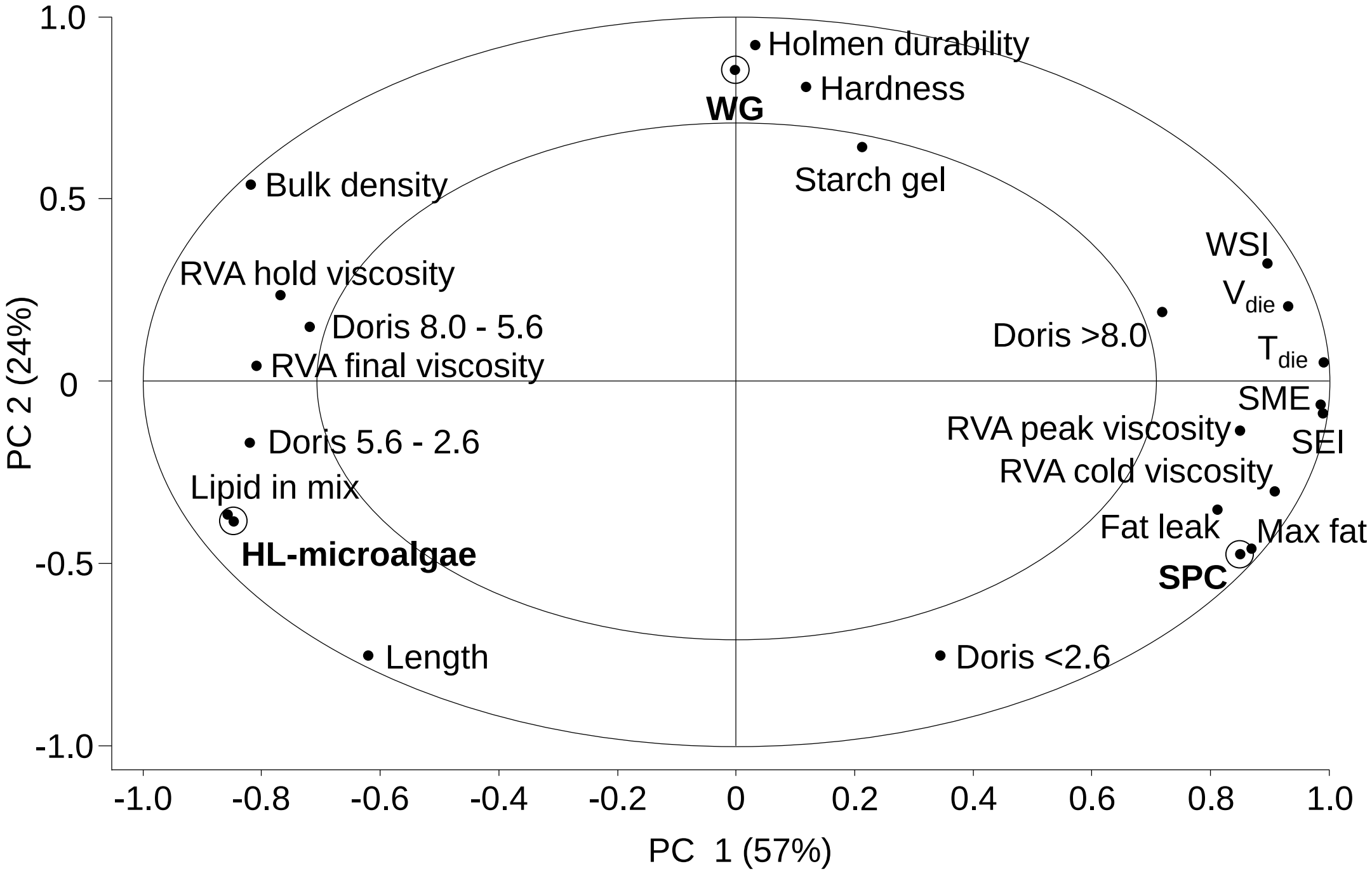
A = high lipid microalgae (*Schizochytrium* sp.), B = vital wheat gluten, C = soy protein concentrate.
Abbreviations explained in Table 4.
[a] Linear model.
[b] Reduced quadratic model, backward elimination of non-significant (P < 0.05) variables.
[c] Special cubic model.

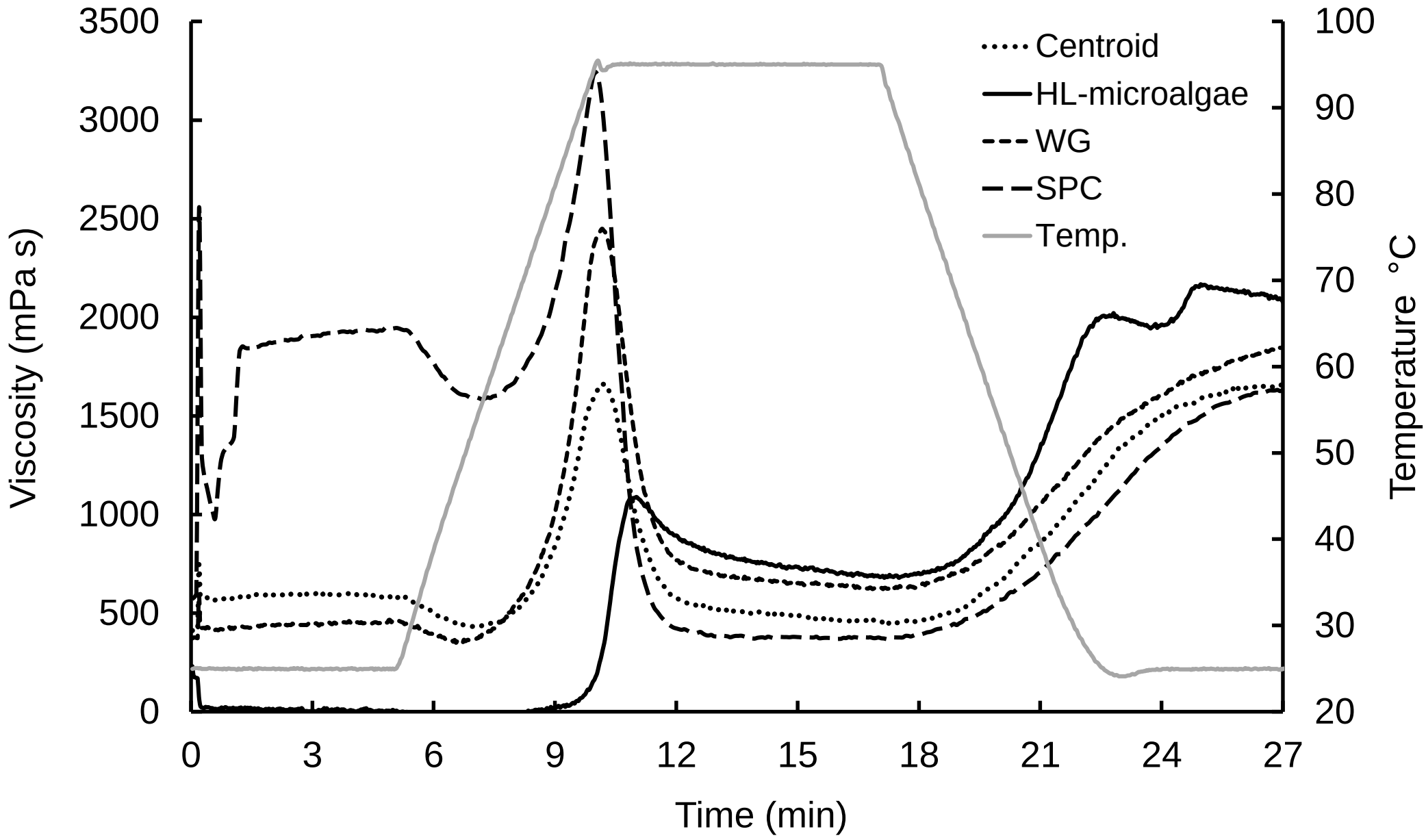
A

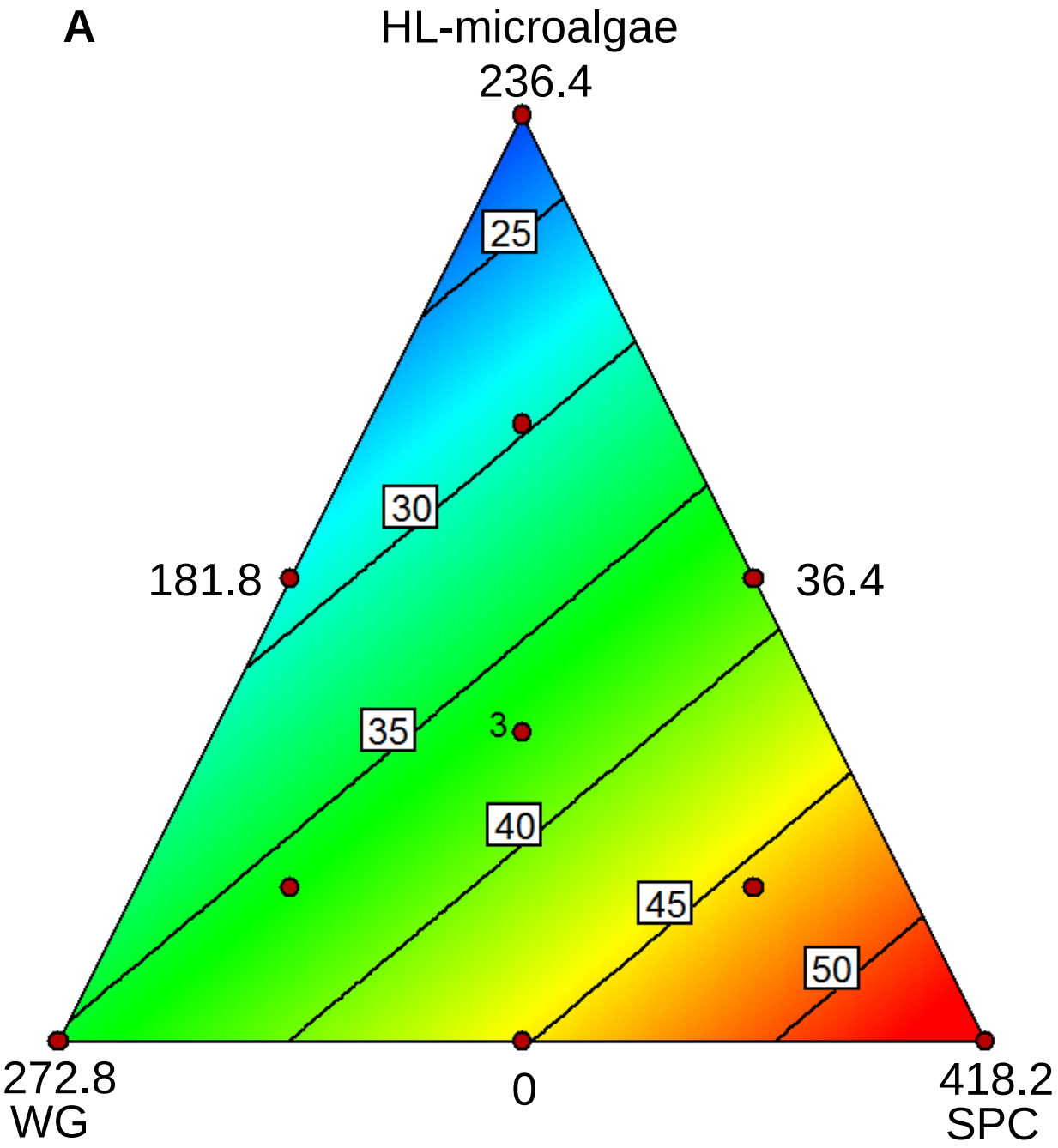
B

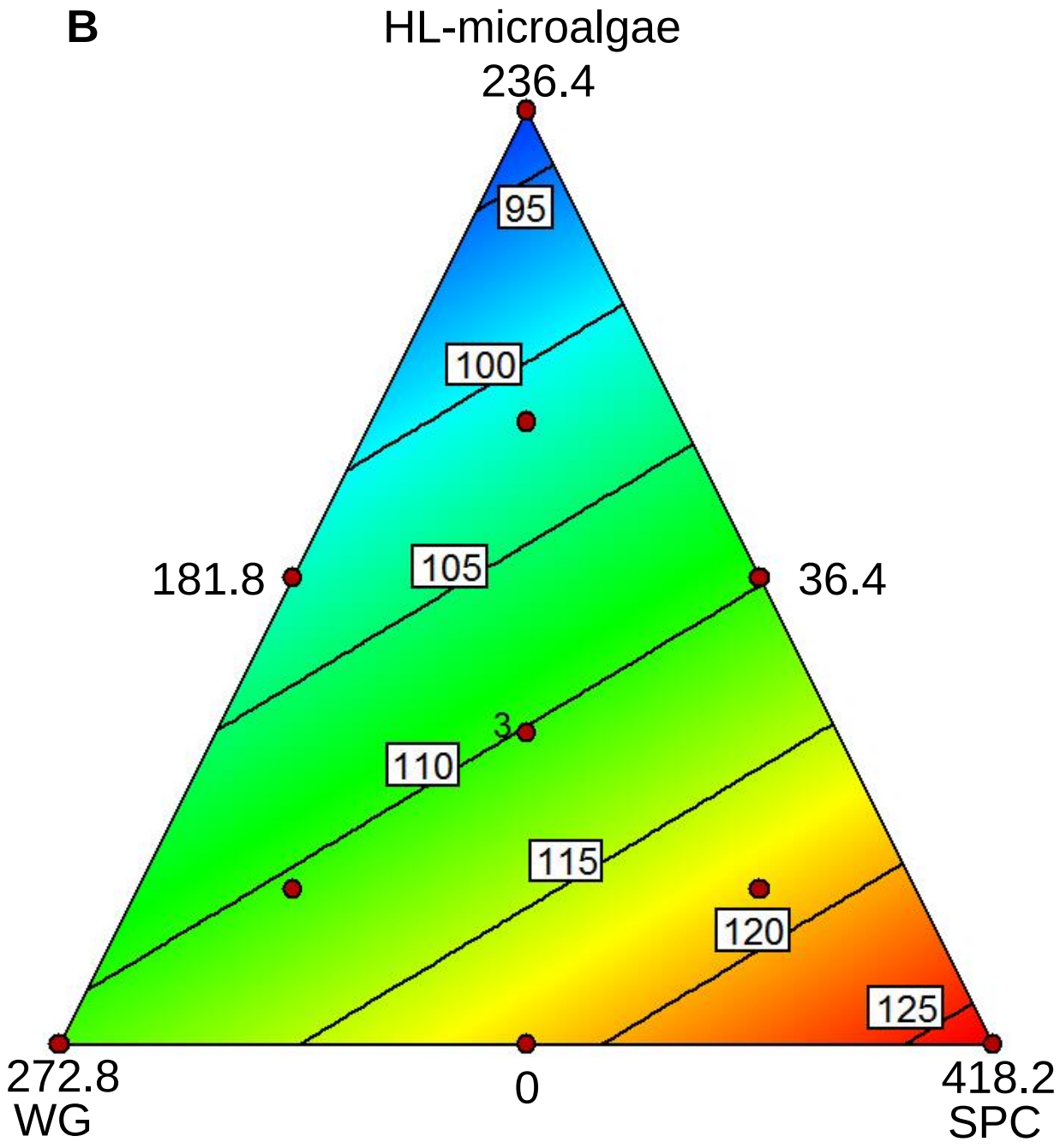

C

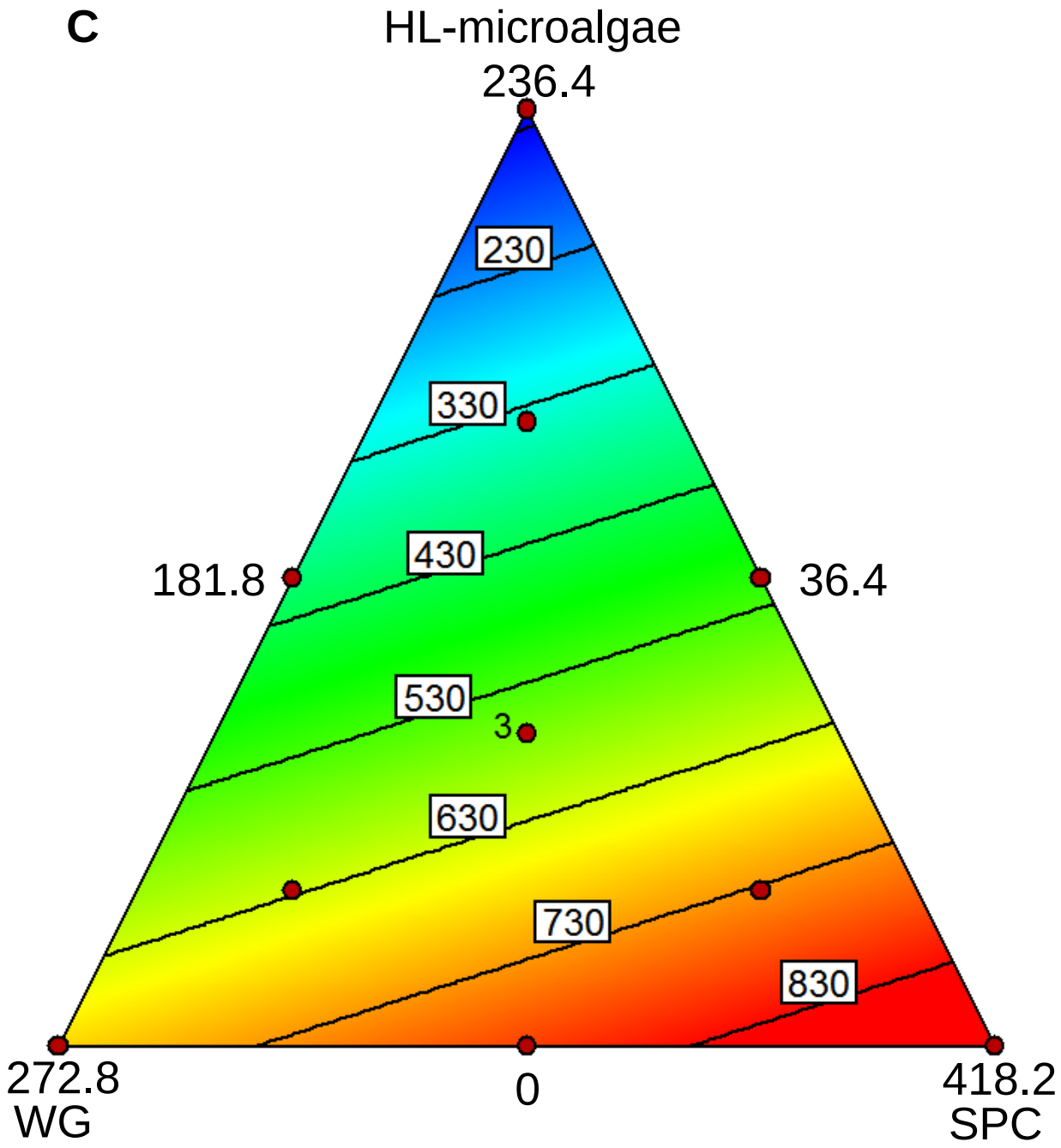

D

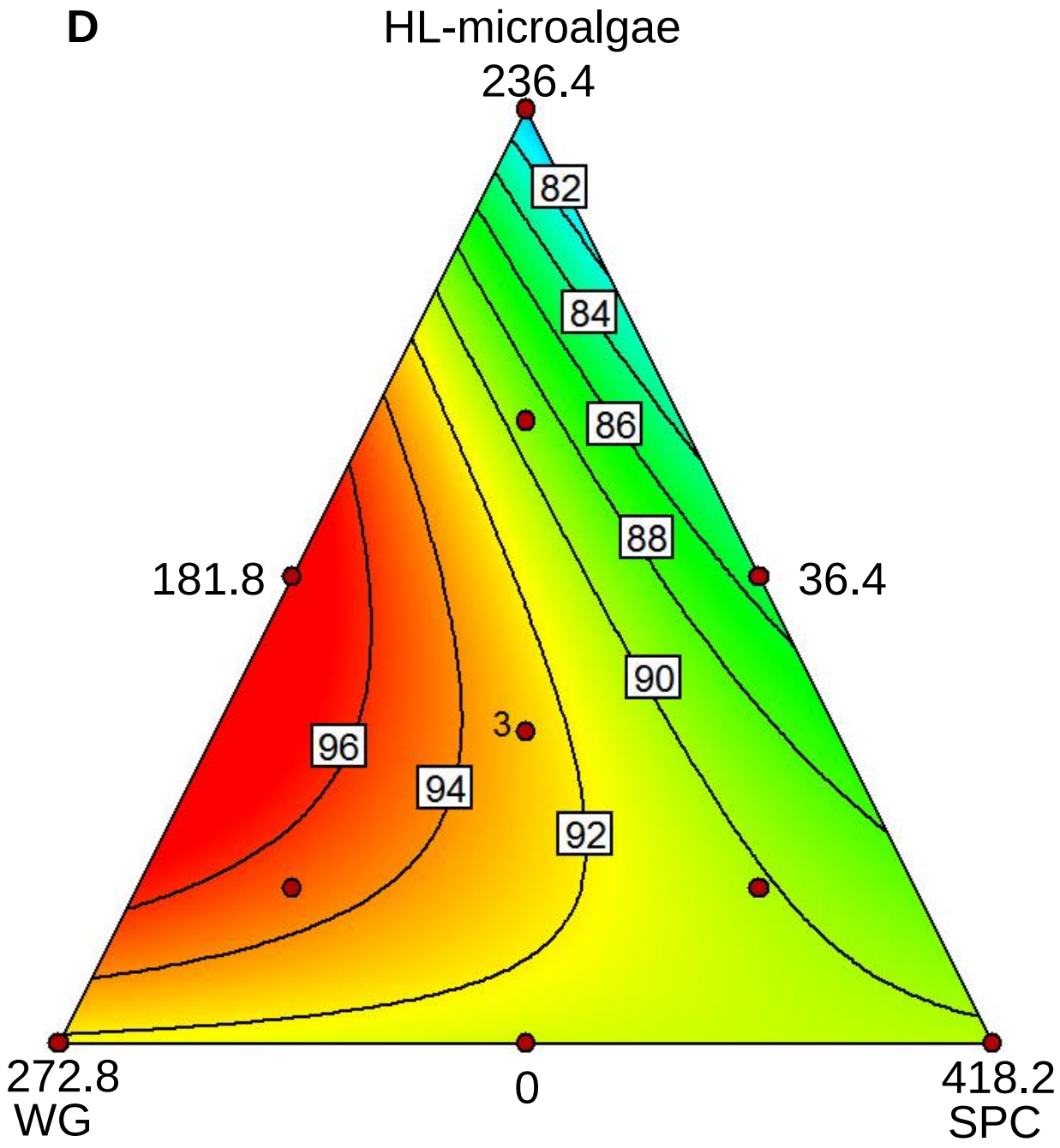

Holmen durability
Hardness
WG
Starch gel
Bulk density
WSI
RVA hold viscosity
V die
Doris 8.0 - 5.6
Doris >8.0
T die
RVA final viscosity
SME
SEI
RVA peak viscosity
Doris 5.6 - 2.6
RVA cold viscosity
Lipid in mix
Fat leak
Max fat
HL-microalgae
SPC
Length
Doris <2.6
PC 2 (24%)
PC 1 (57%)
1.0
0.5
0
-0.5
-1.0
-1.0
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0

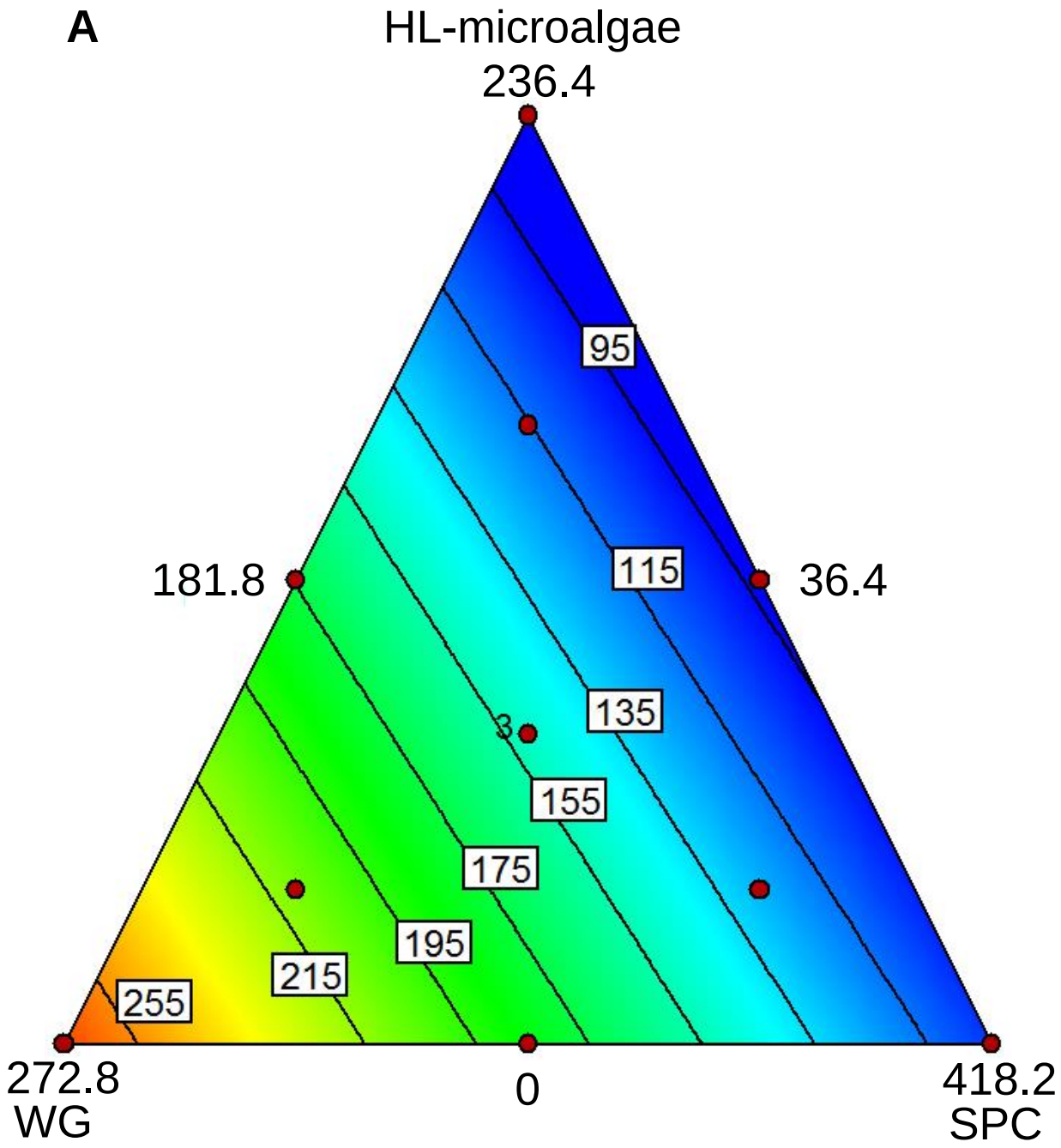

Viscosity (mPa s)
Temperature °C
Time (min)
Centroid
HL-microalgae
WG
SPC
Temp.
0
500
1000
1500
2000
2500
3000
3500
20
30
40
50
60
70
80
90
100
0
3
6
9
12
15
18
21
24
27

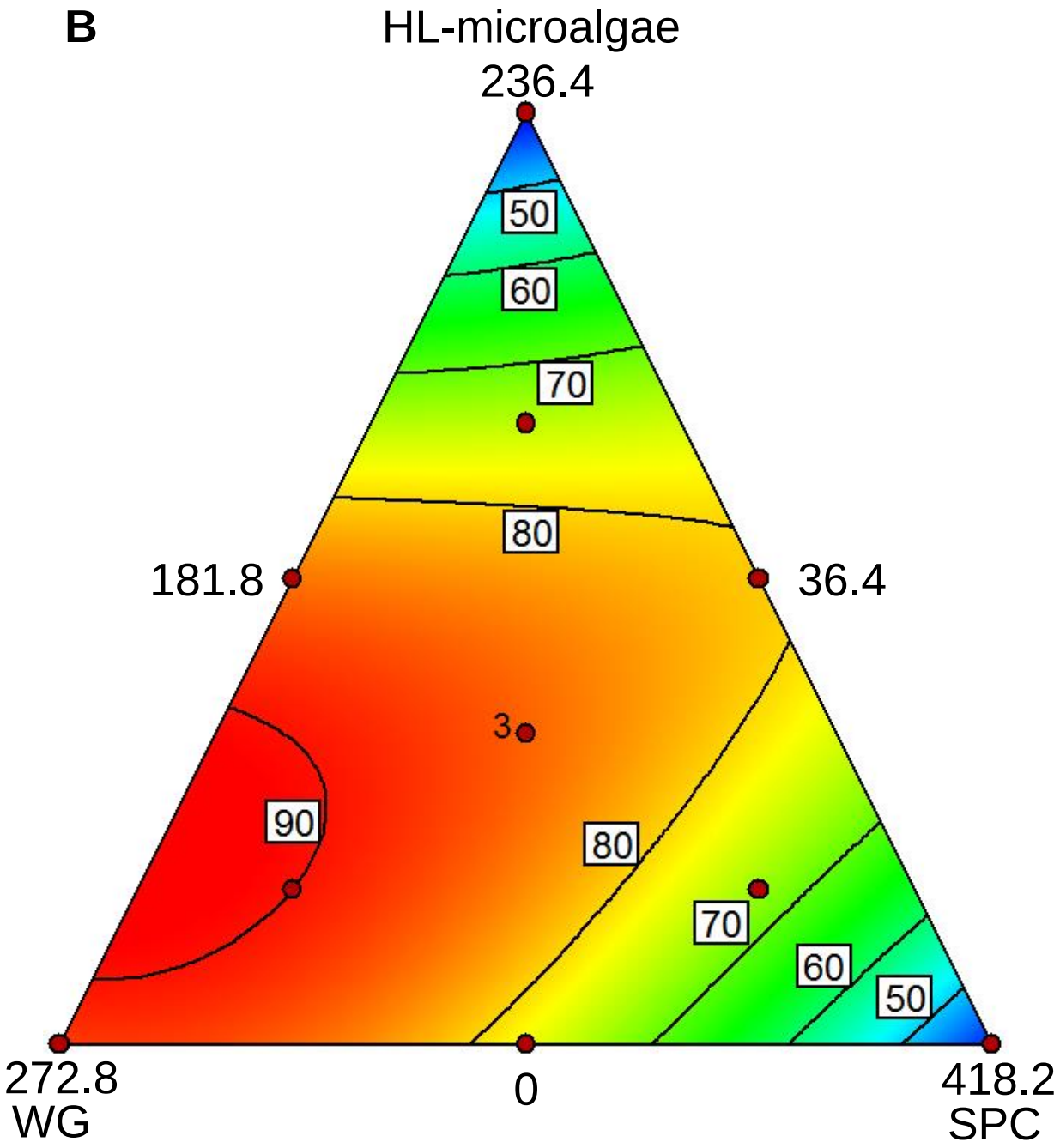

A
HL-microalgae
236.4
25
30
181.8
36.4
35
3
40
45
50
272.8
WG
0
418.2
SPC

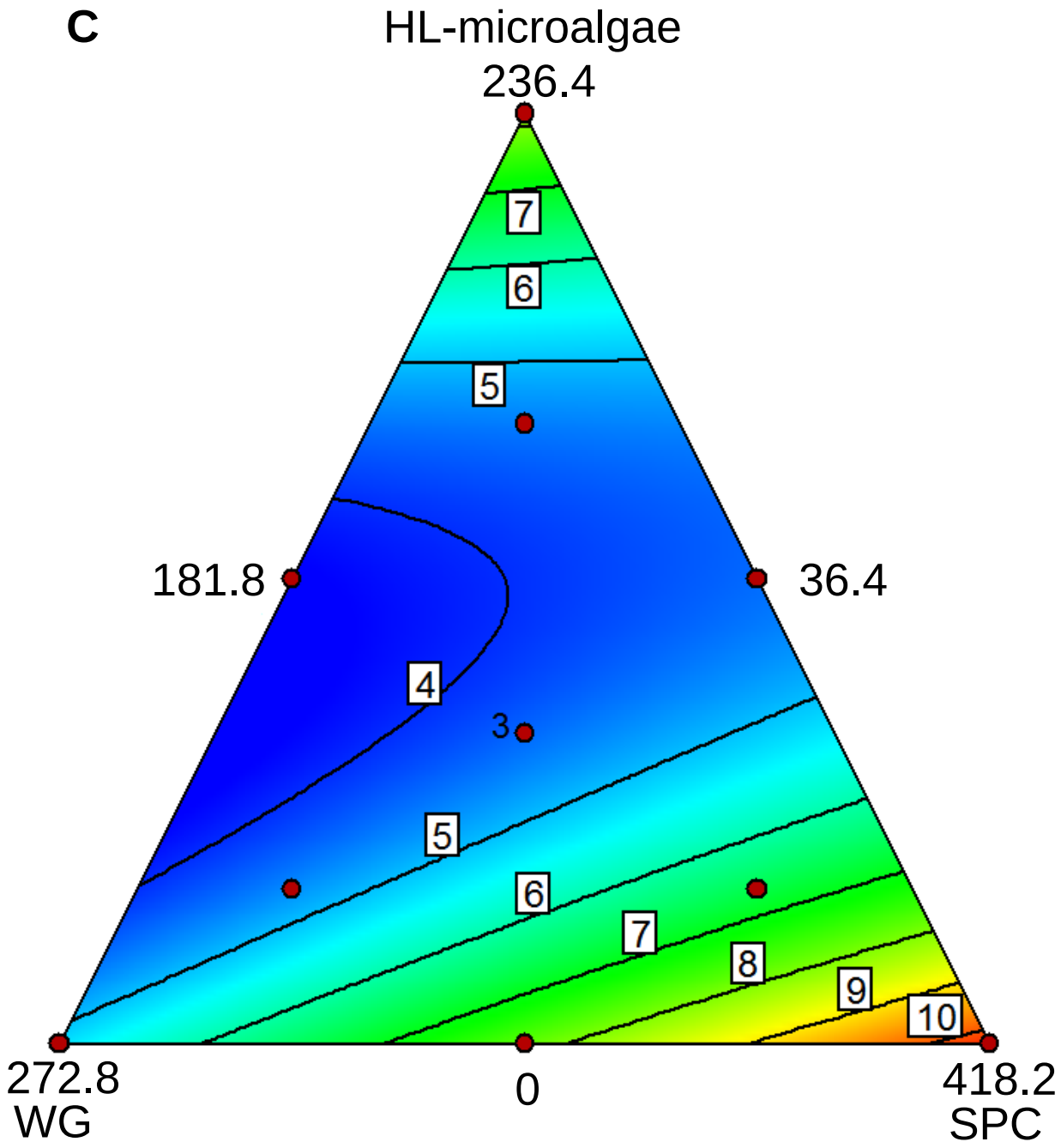

B
HL-microalgae
236.4
95
100
105
181.8
36.4
3
110
115
120
125
272.8
WG
0
418.2
SPC

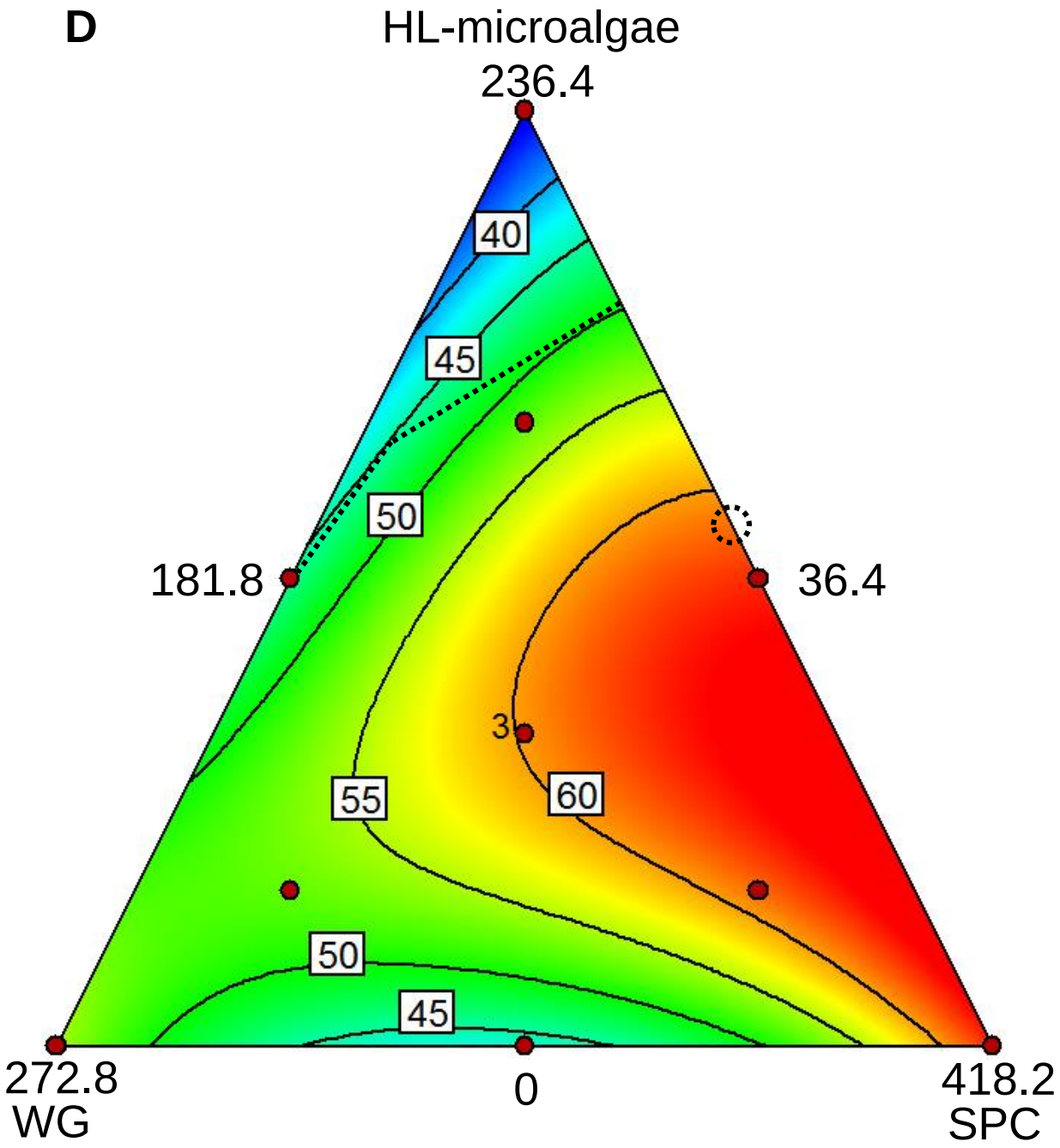

C
HL-microalgae
236.4
230
330
430
181.8
36.4
530
3
630
730
830
272.8
WG
0
418.2
SPC

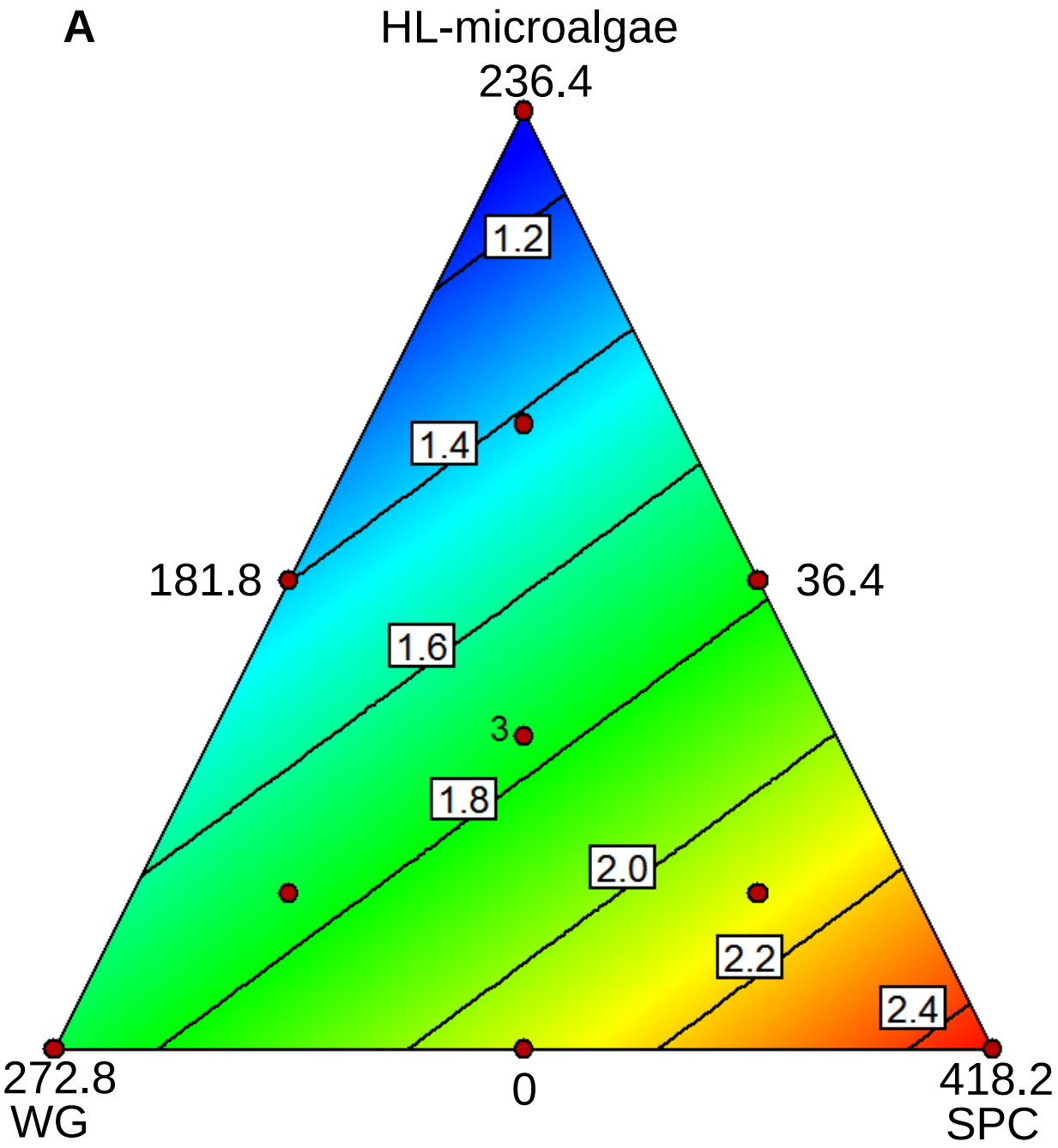

D
HL-microalgae
236.4
82
84
86
88
90
92
94
96
3
181.8
36.4
272.8
WG
0
418.2
SPC

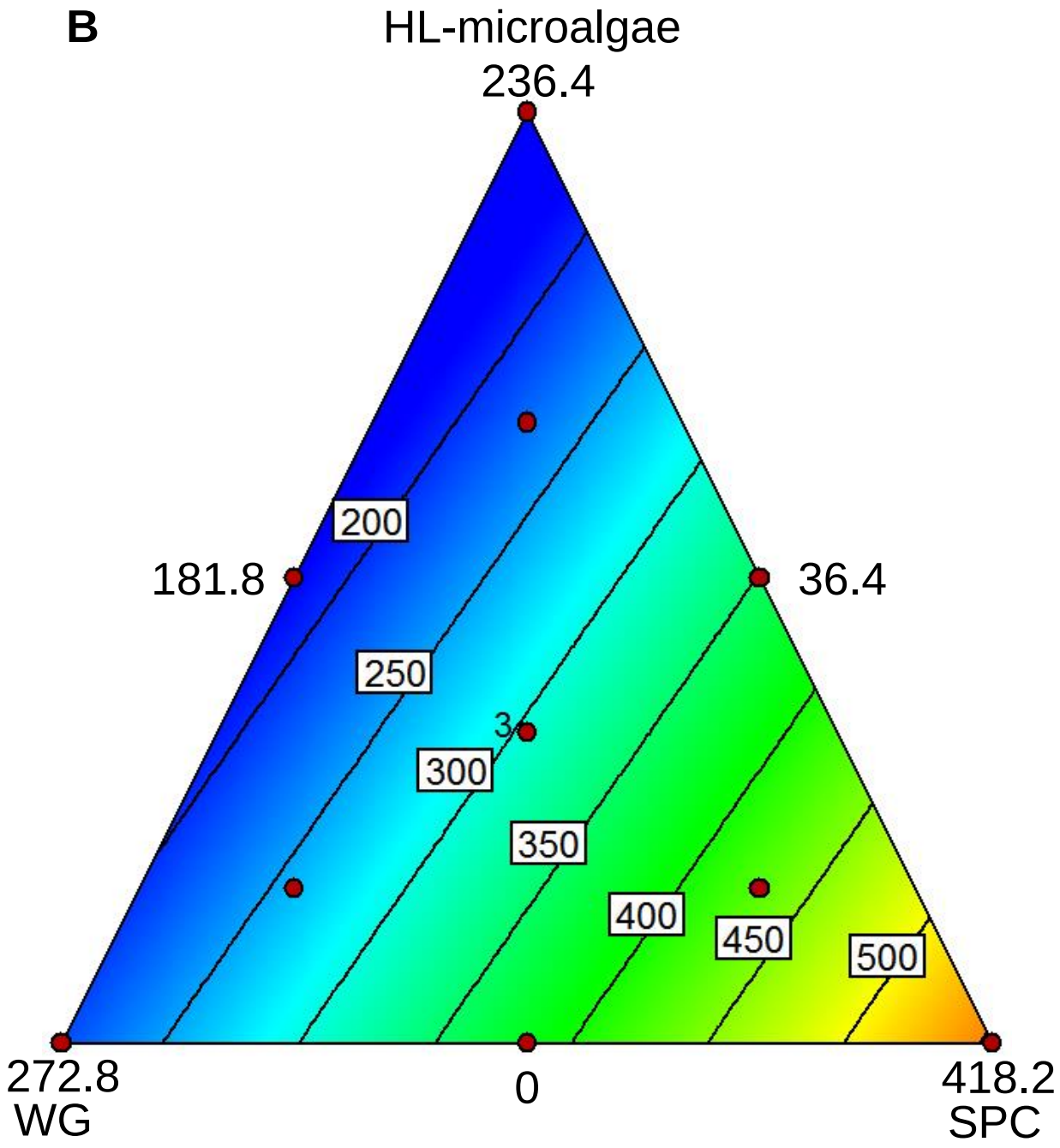

A
HL-microalgae
236.4
181.8
36.4
272.8
WG
0
418.2
SPC
95
115
135
155
175
195
215
255
3

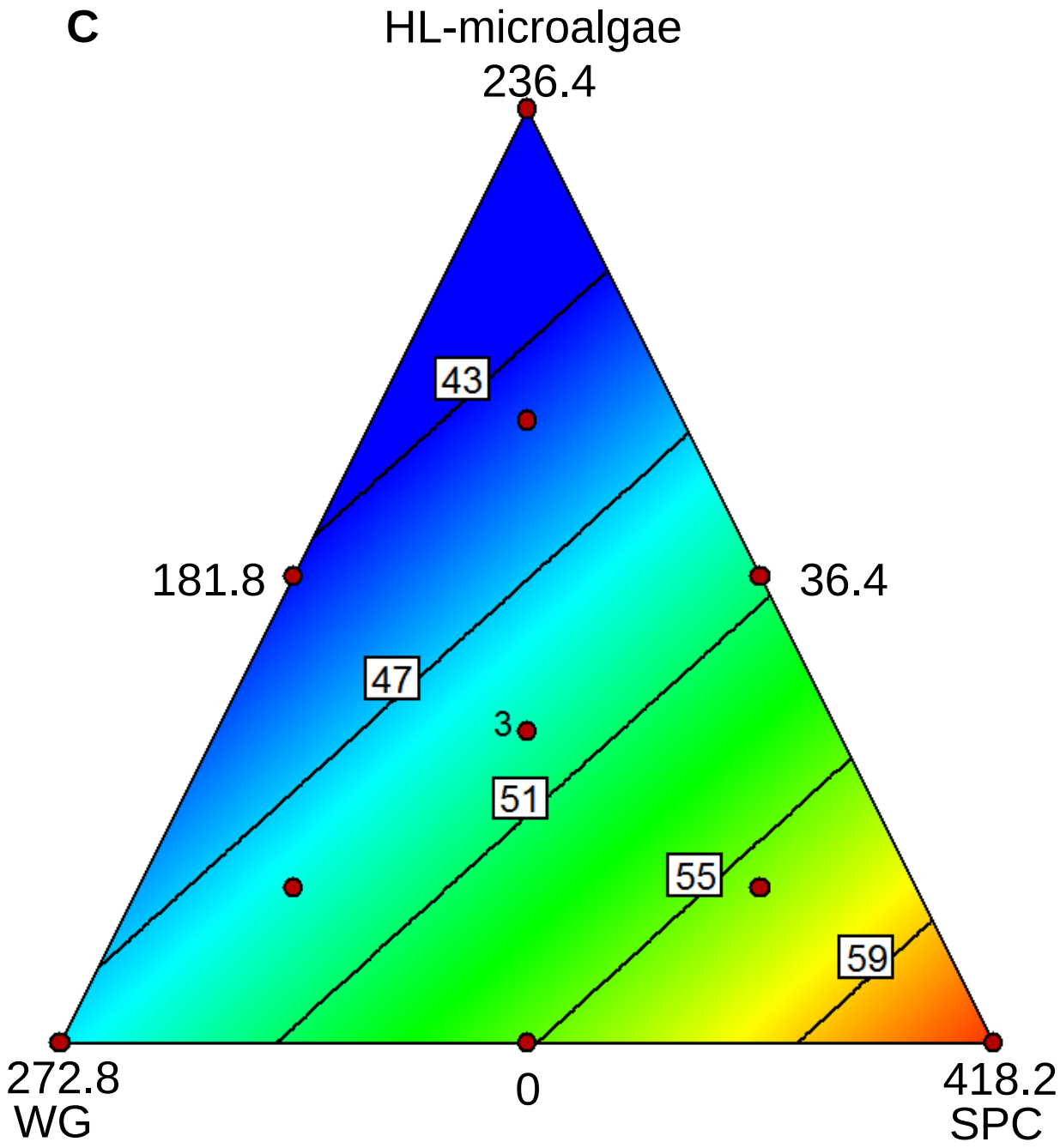

B
HL-microalgae
236.4
50
60
70
80
181.8
36.4
3
90
80
70
60
50
272.8
WG
0
418.2
SPC

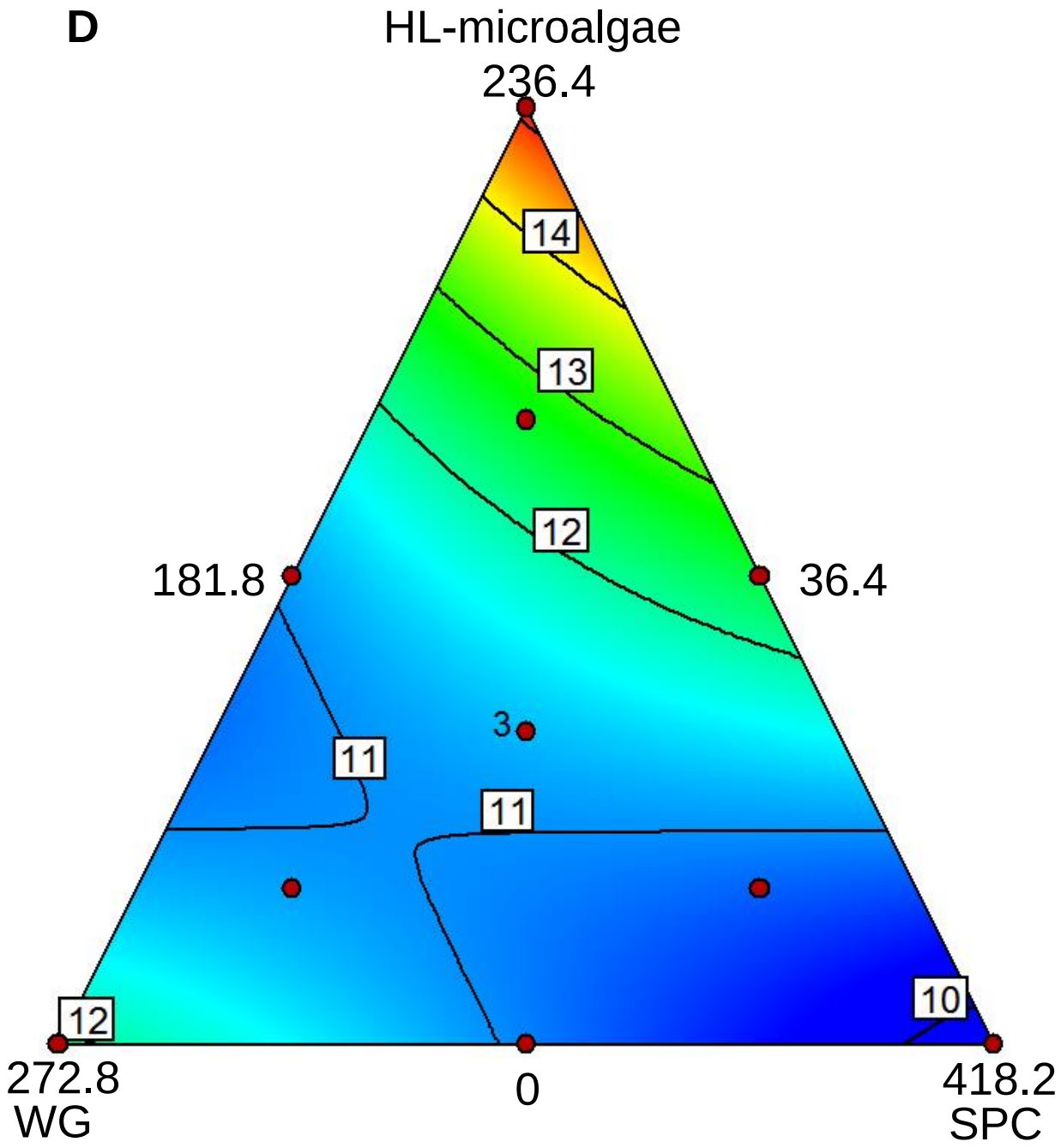

C
HL-microalgae
236.4
181.8
36.4
272.8
WG
0
418.2
SPC
7
6
5
4
3
5
6
7
8
9
10


D
HL-microalgae
236.4
40
45
50
181.8
36.4
3
55
60
50
45
272.8
WG
0
418.2
SPC


A
HL-microalgae
236.4
1.2
1.4
181.8
36.4
1.6
3
1.8
2.0
2.2
2.4
272.8
WG
0
418.2
SPC


B
HL-microalgae
236.4
181.8
36.4
272.8
WG
0
418.2
SPC
200
250
300
350
400
450
500
3


C
HL-microalgae
236.4
181.8
36.4
272.8
WG
0
418.2
SPC
43
47
51
55
59
3


D
HL-microalgae
236.4
14
13
12
181.8
36.4
3
11
11
12
10
272.8
WG
0
418.2
SPC