Effect of skin wine pomace and sulfite on protein oxidation in beef patties during high oxygen atmosphere storage

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"The final publication is available at Springer via http://dx.doi.org/10.1007/s11947-015-1649-y"

ABSTRACT

Meat storage in high oxygen atmosphere has been reported to induce protein oxidation reactions decreasing meat quality. The incorporation of antioxidants has been proposed to reduce the extent of these reactions. In this study, the ability of red and white skin wine pomaces as well as sulfites to inhibit protein oxidation were tested in beef patties stored for up to 15 days at 4 °C in a high oxygen atmosphere (70 % O_2 and 30 % CO_2). SO_2 (300 ppm) effectively protected against protein oxidation measured as radical formation by electron spin resonance (ESR) spectroscopy, as thiol loss by the DTNB assay, and as myosin heavy chain (MHC) disulfide cross-linking by SDS-PAGE. Pomace from red wine production with total phenol of 9.9 mg gallic acid equivalent/g protected against protein radical formation and against MHC cross-linking, but not against thiol loss by addition of 2.0 % (w/w) to the beef patties. Pomace from white wine production with total phenol of 4.0 mg gallic acid equivalents/g only protected against MHC cross-linking. For both types of wine pomace, protein modifications not seen for sulfite addition were observed and were proposed to involve covalent phenol addition to proteins. Red wine pomace may be an alternative to sulfite as a meat additive for protection of beef patties against protein oxidation.

KEYWORDS

Beef patties, protein oxidation, protein radicals, sulfite, wine pomace, protein cross-linking

1. INTRODUCTION

Protein oxidation has recently attracted attention due to its consequences on protein solubility and functionality. Protein oxidation is accelerated by technological aspects such as high-oxygen modified atmosphere packaging (HiOx MAP), salting or exposure to light (Benjakul et al. 2012; Jongberg et al. 2014; Fu et al. 2015). As a consequence of protein oxidation, meat sensory properties are deteriorated, essential amino acids may be lost, and protein digestibility may decrease (Xiong 2000). These protein alterations are detrimental to the overall quality of fresh meat and meat products. In biological matrices such as meat, protein oxidation progress via free radical chain reactions, initiated by reactive oxygen species (ROS) generating protein radicals, which in turn may undergo different reactions resulting in formation of carbonyl derivatives, loss of thiol groups, and formation of protein cross-links. All reactions are widely used to evaluate the effect on meat quality (Lund et al. 2011).

Meat and meat products are basic components of Western diets and are excellent sources of proteins, vitamins or iron. However, the link between consumption of meat and meat products and some prevalent diseases such as cardiovascular disease, cancer, hypertension or obesity has affected consumers´ opinion about meat negatively (Jiménez-Colmenero et al. 2001). Consequently, the meat industry is seeking new alternatives in order to produce healthier meat products such as the replacement of artificial additives by natural plant extracts (Brewer 2011; Serrano and Bañón 2012), improving their fatty acid profile (Martínez et al. 2012) or fortifying the dietary fiber content (Martínez et al. 2011).

Natural plant extracts have been found to be effective antioxidants reducing the oxidation of meat and meat products (Brewer 2011). Previous studies show that numerous of these plant extracts can

inhibit or, at least delay lipid oxidation in meat products. However, the results reported on protein oxidation are more ambiguous. Jongberg et al. (2011b) showed that white grape extract was able to inhibit the formation of disulfide protein cross-links, but accelerated the loss of protein thiols. Nieto et al. (2013) found that the addition of oregano and rosemary essential oils to pork patties protected both against thiol loss and cross link formation. On the other hand, in the same study a prooxidative effect of garlic essential oil resulted in the complete depletion of protein thiols. Further, clear prooxidant activity was found by addition of aqueous potato peel extracts to minced horse mackerel, which increased the loss of tyrosine and tryptophan as measured by fluorescence spectroscopy (Sabeena Farvin et al. 2012). In contrast, significant reduction of carbonyl formation was found for addition of certain fruit and pine bark extracts to cooked porcine patties prior to cooking (Ganhão et al. 2010; Vuorela et al. 2005).

Wine pomace is a solid by-product generated in large quantities during the winemaking process after grape fermentation that contains mainly grape skin and grape seeds. Wineries have traditionally needed to handle and dispose this residue, incurring in new costs. However, it can be reutilized by the food industry because it still contains a wide range of interesting compounds such as dietary fiber, polyphenols, and minerals (García-Lomillo et al. 2014). Several studies have reported positive effects of increased fiber and antioxidant contents in bakery products (Mildner-Szkudlarz et al. 2011) resulting from the incorporation of processed wine pomace. Other studies have documented the ability of wine pomace to limit lipid oxidation in different fat systems (García-Lomillo et al. 2014), in yoghurt and in salad dressing (Tseng and Zhao 2013). The high content in bioactive compounds and the antioxidant activity suggest that wine pomace could effectively inhibit the oxidative processes affecting meat protein.

In southern European countries, sulfites are used to extend the shelf life of burger patties. The European Commission (Council Directive Nº 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners 1995) allows the incorporation of different sources of sulfur dioxide into products labeled as "burger meat". Sulfite is commonly used in agreement with this directive in burger meat in the Spanish meat industry due to its ability to delay the microbial spoilage and discoloration resulting from myoglobin oxidation (Serrano and Bañón 2012). Although sulfites possess exceptional good technological properties, the allergic and respiratory reactions resulting from exposure to sulfites, especially for sensitive individuals (Vally and Misso 2012), have led the meat industry to search for alternatives to sulfites, to extend the shelf-life of this type of products.

In the present study, the protective effects against protein oxidation in beef patties stored for 15 days in HiOx MAP of red and white skin wine pomaces were compared to the protective effect of sulfite. The progression of the protein oxidation was evaluated by the formation of protein radicals, loss of protein thiols, and the degree of protein cross-linking.

2. MATERIALS AND METHODS

2.1.Reagents

Reagent-grade chemicals and distilled-deionized (Milli-Q Plus, Millipore Coorporation, Bedford, MA) water were used throughout.

2.2.Preparation of wine pomace products

Red wine pomace was obtained from a selection of wineries situated in Burgos (Spain). White wine pomace was produced in the pilot plant at University of Burgos by fermentation of white grapes, obtained from different wineries situated in Burgos (Pérez-Magariño and González-San José 2001).

113 At the end of the alcoholic fermentation (reducing sugar content lower than 2 g/l), white wine
114 pomace was separated from liquids by draining and pressing.
115
116 Wine pomaces were dried in a conventional oven (P-Selecta, Barcelona, Spain) during 4 - 4.5 hours
117 at a temperature lower than 60 ºC. After dehydration, seeds were removed and the products
118 obtained were milled using a grinder (Ascaso, Barcelona, Spain), and sieved to obtain a skin wine
119 pomace product (SkWPP) with particles smaller than 0.25 mm. In order to assure microbial safety,
120 SkWPP were subjected to thermal processing (90ºC for 90 minutes) in a conventional oven before
121 storage in opaque packages at room temperature until patty preparation. Further details regarding
122 the preparation of wine pomace products and their characterization are described in a previous work
123 (García-Lomillo et al. 2014).
124
125 **2.3.Preparation, packaging, and storage of beef patties**
126 Beef meat (65.5 % water, 16.5 % protein, 13.4 % fat) was purchased from a local supplier,
127 chopped, ground using a food mincer (Cato, Sabadell, Spain), and divided in four portions. In the
128 control formulation, the ingredients per kilogram of burger patty were as follow: 920 g of meat, 50
129 mL of water, 15 g of sodium chloride, 12 g of potato starch, and 3.0 g of a commercially available
130 mix of food grade phosphates (Doscadesa, Murcia, Spain). In the samples added sulfite, water was
131 replaced by 50 mL of an aqueous solution of sodium metabisulfite ($Na_2S_2O_5$) to obtain a final
132 concentration of 300 mg of SO_2 per kg of burger (300 ppm) in agreement with the Council
133 Directive 95/2/EC. In the case of red and white grape pomace, water was replaced by a suspension
134 in water of the red or white SkWPP to obtain a final concentration of 20 g of SkWPP per kg of
135 burger (2 % w/w). The level of SkWPP was based on preliminary experiments including a sensory
136 analysis, and the same levels were found to reduce lipid oxidation in a previous study (García-

Lomillo et al. 2014). The meat was processed using a food mixer (Cato) for 5 minutes, and patties of 100 grams were formed by hand before packaging in polyethylene/ethylene vinyl alcohol/polystyrene trays (Sanviplast, Barcelona, Spain) with an oxygen permeability of 0.99 cm^3 m^{-2} atm^{-1}. Day-0 samples were vacuum packed and stored at -80 ºC until analysis. The trays were sealed with a PETPVdC/PE film (oxygen permeability = 7 cm^3 m^{-2} day^{-1} atm^{-1}) after filling with the gas mixture (70% O_2/30% CO_2) and using a gas mixer (WITT-Gasetechnik GmbH & Co KG, Witten, Germany). Samples were stored at 4ºC for up to 15 days. One sample refers to two beef patties packed together in one tray, and three trays of each sample were prepared resulting in three replicates of each sample for each time point, days 4, 8, 12 and 15. On the day of collection, the two patties were mixed and a portion of 10 g were collected and stored in vacuum at -80 ºC until preparation of myofibrillar protein isolates (MPI).

2.4.Extractable phenol contents of SkWPP

Two grams of SkWPP and 25 mL of methanol: formic acid (97:3) were left for extraction at room temperature for 24 hours and solid residues were removed by filtration. Total polyphenol content (TPC), total catechin content (TCC), total anthocyanin content (TAC) and total proanthocyanidin content (TPAC) were measured according the methods described by Barceló (1990). Briefly, TPC was determined by reaction with Folin–Ciocalteu reagent, and were expressed as mg/g of gallic acid. TCC was determined by the spectrophotometric vanillin method, and expressed as mg/g of D-catechin. TAC was quantified by measuring the absorbance at 525 nm of 1 mL of extract diluted in 10 mL of 1 N HCl, using 1 mL of the extract diluted in 10 mL of citrate phosphate buffer pH 3.5 as a blank, and expressed as mg/g of malvidin-3-glucoside. TPAC was determined from the increase of absorbance at 550 nm after acid hydrolysis at 95 ºC for 40 minutes, and expressed as mg/g of proanthocyanidin B1.

2.5.Isolation of myofibrillar proteins

The myofibrillar protein fractions were isolated from beef patties according to the method described by Jongberg et al. (2011a). The MPI were lyophilized and stored at – 20 °C until analysis.

2.6.Protein radicals by ESR Spectroscopy

The formation of protein radical was assessed according to the method proposed by Jongberg et al. (2013). Pulverized lyophilized MPI were transferred to quartz electron spin resonance (ESR) tubes (inner diameter = 4 mm, wall = 0.5 mm; Wilmad, Buena, NJ) to a height of 1 cm in the tube. The tubes were placed in the cavity of a JEOL JES-FR30X ESR spectrometer (JEOL Ltd., Tokyo, Japan) with the following settings: microwave power, 4 mV; center field, 336 mT; sweep width, 5 mT; sweep time, 2 min; modulation width, 0.125 mT; amplitude, 200; time constant, 0.1 s; accumulations, 1. The radical signal intensity of the ESR spectra were determined as follows:

$$Radical\ intensity = \frac{Peak\ height\ \cdot (peak\ width)^2 \Big/ Signal\ area\ (Mn(II))}{Weight\ of\ sample\ (g)}$$

Where the peak width is the peak-to-peak width in the ESR spectrum. Data are presented as mean ± SD of three replicates. Spectral manipulation using Savitzky-Golay signal processing (400 points were considered during the smoothing routine) was applied for the presentation of selected ESR spectra.

2.7.Protein thiol groups

Protein thiol groups were quantified after derivatisation with 5,5 dithiobis (2-nitrobenzoic acid) (DTNB) as recently described by Jongberg et al. (2014) though downscaled to using only 167 µL supernatant compared to 500 µL in the original method. The thiol concentrations in nmol thiol/mg protein are presented as means ± SD of three independent replicates.

Any potential interference caused by the presence of remaining sulfites and SkWPP in the DNTB assay was tested. Initially, free sulfite concentration was evaluated by dissolving 10 mg of MPI originating from patties added sulfite in 1 mL of 0.10 M TRIS buffer (pH 8.0) for one hour in an 80 °C water bath. Insoluble parts were removed by centrifugation and filtration and the supernatant was analyzed for sulfite content by derivatization with ThioGlo 1 and subsequent RP-HPLC separation of the fluorescent adducts according to Abrahamsson et al. (2012). Moreover, the interference from SkWPP was tested in a sample containing 10 mg of MPI originating from patties added red SkWPP. Further, the amount of red or white SkWPP in the MPI was estimated by spectrophotometric measurement of pure SkWPP compared with MPI containing SkWPP. Thus, a sample containing 0.57 mg red SkWPP and a sample containing 0.69 mg white SkWPP was prepared. All three samples (MPI_{SkWPP}, red SkWPP, and white SkWPP) were dissolved in 1 mL 5.0 % SDS in 0.10 M TRIS buffer (pH 8.0) for one hour in an 80 °C water bath, centrifuged and filtrated. An aliquot of 167 µL of each filtrate was added to the reaction mixture of L-cysteine and DTNB before or after the 30 minutes reaction time.

2.8.SDS-PAGE for analysis of protein cross-linking

Lyophilized MPI (1 µg protein per well) were analyzed by gel-electrophoresis using NuPAGE Novex 3-8 % TRIS-acetate gels according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) as described previously by Jongberg et al. (2014). The gels were scanned with a Thypoon

scanner (GE Healthcare, Freiburg, Germany) and the volume of the observed bands was determined after subtraction of background, using Phoretix 1D software, version 2003.02. Percent volume of myosin heavy chain (MHC) and cross-linked myosin heavy chain (CL-MHC) was calculated as follows:

$$\%\ volume = \frac{Volume\ of\ the\ studied\ band}{Sum\ of\ volume\ of\ all\ bands\ in\ the\ same\ lane} * 100$$

2.9.Statistical analysis

Statistical analysis was performed using StatGraphics ® Centurion XVI. Shapiro-Wilk tests were conducted to check the normal distribution of results with 95% confidence level. Fisher's least significant difference test was performed in order to identify significant differences between different formulations and at different days of storage with 95% confidence level.

3. RESULTS AND DISCUSSION

3.1.Extractable phenol contents of SkWPP

Results showed that red skin wine pomace product (SkWPP) contained more than two times the concentration of TPC (9.86 ± 0.24 mg gallic acid/g) as compared to the white SkWPP (3.97 ± 0.03 mg gallic acid/g). As expected, anthocyanins were only present in red SkWPP (1.23 ± 0.04 mg malvidin-3-glucoside/g). Anthocyanins are the characteristic pigments of red grapes and they are the main phenols in red grape skin (Pinelo et al. 2006). In contrast, levels of extractable catechins were similar in both white (2.10 ± 0.01 mg D-catechin/g) and red SkWPP (2.44 ± 0.15 mg D-catechin/g). Proanthocyanidins are oligomers of catechins and they are usually synthesized in higher quantities in red than in white grapes (Pinelo et al. 2006), and accordingly red SkWPP was found to have a higher content of proanthocyanidins (14.82 ± 0.50 mg proanthocyanidin B1/g) than

229 white SkWPP (8.23 ± 0.20 mg proanthocyanidin B1/g). Catechins and specially proanthocyanidins
230 are usually found to be highly reactive towards proteins (McManus et al. 1985).
231
232 **3.2.Protein radical formation**
233 The electron spin resonance (ESR) spectra of myofibrillar protein isolates (MPI) from beef stored
234 for 15 days in HiOX MAP indicates that the lowest accumulation of protein radicals had occurred
235 in the patties added sulfites (Figure 1, left panel). In contrast, addition of white SkWPP to the beef
236 patties resulted in higher radical intensity, whereas addition of red SkWPP was not found to affect
237 the protein radical intensity in the beef patties after 15 days of storage as compared to the control
238 (Figure 1, left panel). In order to evaluate whether the observed ESR signals from the different
239 samples were due to similar type of radicals, the ESR spectra were compared after augmentation,
240 meaning that the sample added sulfites spectra was amplified to the size of the control, followed by
241 comparison of the two spectra shapes (Figure 1, right panel). The shape of ESR spectra from beef
242 patties added sulfites did not deviate from the shape of the control spectrum, indicating that the
243 radical species formed were of similar nature (Figure 1, right panel, A). In the case of the samples
244 containing red and white SkWPP, the peak-to-peak width was narrower in comparison to the
245 control (Figure 1, right panel, B) indicating that the radical species may deviate from the radicals
246 generated in the control beef patties. It is expected that the water soluble free phenolic compounds
247 are removed during the MPI isolation process. Consequently, the difference in the shape of the
248 spectra is caused by the formation of other types of radicals incorporated into the protein structure.
249 Jongberg et al. (2013) ascribed these changes in the spectra to the formation of protein-bound
250 phenoxyl radicals, formed subsequently to covalent protein-phenol interactions. No relevant
251 differences in the radical spectra were observed between MPI from samples containing red or white

252 SkWPP, which suggests that the radicals are of similar nature in the two types of samples (Figure 1,
253 right panel, C).
254
255 The protein radical intensity was quantified during storage time, and it was found that the radical
256 intensity of the control patties and in those containing white SkWPP increased during storage
257 (Figure 2). Radical accumulation may lead undesirable changes in the structure, functionality and
258 digestibility of meat protein, decreasing the overall quality of meat products (Nissen et al. 2000;
259 Xiong 2000). Contrary, no significant increase over time was found in MPI from beef patties added
260 sulfites or red SkWPP. The observed difference between red and white SkWPP may be explained
261 by the higher phenolic content of red SkWPP in proanthocyanidins and anthocyanins. Grape
262 anthocyanins are water-soluble and well-known radical scavengers due to their more complete
263 conjugated structure, which better allows electron delocalization and formation of very stable
264 radicals (Rivero-Pérez et al. 2008). In the present study, water-soluble compounds, including
265 possible anthocyanins, are most likely removed during the MPI isolation process, and this may
266 explain the reduced radical intensity in the MPI from the beef patties added red SkWPP.
267
268 Sulfite was also able to inhibit formation of protein radicals throughout storage. There are several
269 plausible pathways which may explain this protective effect. Sulfites are be able to remove H_2O_2
270 (McFeeters 1998) (eq. 1), which otherwise is a common source of highly reactive radicals in meat
271 due to its participation in the pseudo-peroxidase cycle of myoglobin (Davies 1990) and in the
272 Fenton reaction (Stadtman 1990).
273
274 $HSO_3^- + H_2O_2 \rightarrow SO_4^{2-} + H^+ + H_2O$ (eq. 1)
275

Sulfites may also inhibit radical formation by reducing lipid and protein hydroperoxides to the corresponding alcohol without formation of radicals (eq. 2) (Serrano and Bañón 2012).

$HSO_3^- + ROOH \rightarrow SO_4^{2-} + H^+ + ROH$ (eq. 2)

In addition, sulfites may protect against protein radical formation by acting as a radical scavenger. (Andersen et al. 2000). Protein radicals may also be scavenged by sulfites (eq. 3) (Neta and Huie 1985), forming sulfite radicals that may undergo radical termination process (eq. 4) (Hayon et al. 1972).

$Protein^{\cdot} + HSO_3^- \rightarrow Protein + S^{\cdot}O_3^-$ (eq. 3)

$2\ S^{\cdot}O_3^- \rightarrow S_2O_6^{2-}$ (eq. 4)

3.3.Protein thiol loss

Protein thiols are highly susceptible to oxidation by radicals and transition metal ions leading to loss of thiol groups. Thiol oxidation may serve as an antioxidant mechanism to prevent other substrates from oxidation, and is in general considered a marker of protein oxidation in meat (Lund et al. 2011). Protein thiol loss was evaluated in the MPI obtained from the samples stored for 15 days (Figure 3). At day 0, samples added red and white SkWPP contained approximately 40 % less protein thiols than the control samples and samples added sulfite. As there may be some SkWPP remaining in the MPI that could interfere in the result of the DTNB assay, a set of control experiments were conducted for the reaction mixture of DTNB and the thiol group of cysteine (Cys-SH). The thiol-exchange reaction to form the yellow thiolate anion ($NB\text{-}S^-$) from the weakly colored disulfide reagent (NB-SS-BN) is:

300

301 $Cys\text{-}SH + NB\text{-}SS\text{-}BN \rightarrow Cys\text{-}SS\text{-}BN + NB\text{-}S^-$ (eq. 5)

302

303 When pure SkWPP was added to the reaction mixture containing cysteine and DTNB before the 30
304 minutes incubation time, the formation of the thiolate anion ($NB\text{-}S^-$) decreased significantly.
305 However, adding pure SkWPP to the reaction mixture of DTNB and cysteine after 30 minutes
306 reaction, no loss in the level of the yellow thiolate anion was observed. This suggested that the
307 interference from components in SkWPP was a result of a reaction between the thiol group of the
308 cysteine to form an addition products "Cys-SkWPP" rather than with the yellow thiolate anion for
309 the quantification (eq. 6).

310

311 $Cys\text{-}SH + NB\text{-}SS\text{-}BN + SkWPP \rightarrow$ "Cys-S-SkWPP" + NB-SS-BN (eq. 6)

312

313 As a further control, instead of pure SkWPP, MPI obtained from meat samples added SkWPP
314 (MPI_{SkWPP}) was added to the reaction mixture of the thiol analysis, and it was found, that MPI_{SkWPP}
315 did not affect the reaction between cysteine and DNTB:

316

317 $Cys\text{-}SH + 2\ NB\text{-}SS\text{-}BN + MPI_{SkWPP} \rightarrow Cys\text{-}SS\text{-}BN +$ "$NB\text{-}S\text{-}MPI_{SkWPP}$" $+ 2\ NB\text{-}S^-$ (eq. 7)

318

319 The fact that pure SkWPP, in contrast to the SkWPP residues in the MPI, was reactive towards
320 cysteine demonstrated the presence of thiol-reactive components in the SkWPP. This indicates that
321 the lower protein thiol concentrations found in beef patties added SkWPP were the actual level of
322 protein thiols and the lowering in protein thiol concentration was most likely caused by reaction

323 between thiol-reactive compounds in SkWPP and the protein thiol groups in the beef patties, as also
324 previously described (Jongberg et al. 2011a).
325
326 Sulfites added to patties could be another source of interference with the DNTB resulting in an
327 overestimation of the thiol content due to sulfitolysis of the DNTB (Morel et al. 2000). However,
328 no residual sulfite remaining was detected in the MPI obtained from patties added sulfites and there
329 was no significant difference between control samples and samples added sulfite at day 0,
330 indicating that sulfite residues were completely removed by the extensive washing procedure during
331 the MPI preparation.
332
333 During storage, the protein thiol content decreased significantly in all samples (Figure 3). After 15
334 days of storage, the largest protein thiol loss was observed in the control samples (~ 15 nmol
335 thiol/mg protein), while the decrease in samples containing sulfites was only 8 nmol thiol/mg
336 protein. Significant differences between samples added sulfites and control were observed at day 8,
337 12 and 15, indicating a protective effect of sulfite against protein thiol loss in the beef patties added
338 sulfite. As mentioned earlier, sulfites are able to remove several oxidizing agents such as H_2O_2,
339 peroxides and radicals, and hence, will protect proteins against thiol loss. The protein thiol loss in
340 beef patties added red or white SkWPP during the 15 days storage was comparable to the loss
341 observed in the beef added sulfite, although the concentration at day 0 was considerable lower. The
342 reduced thiol loss in beef added SkWPP as compared to the control sample is concluded to result
343 from the antioxidant capacity of SkWPP against thiol oxidation. However, taking the low starting
344 level into consideration, conclusions should be drawn very carefully. Sun et al. (2011) showed that
345 thiols were only lost to a certain level during long-term drying of Cantonese sausages indicating
346 that some thiols in MPI are not available for oxidation. Thus, the low thiol loss in the beef patties

added SkWPP may rather be due to inaccessible thiols rather than to a true antioxidant activity. Addition of either red or white SkWPP resulted in similar low starting level and low thiol loss during storage, despite the difference between the two products observed in the formation of protein radicals. This stresses that the radical scavenging activity of the components in the individual SkWPP was negligible with regards to protection against thiol loss.

3.4.Characterization of protein cross-linking

Thiol groups are highly susceptible to oxidation, which among other reactions leads to formation of disulfides. As a result, protein thiol oxidation changes the structural properties of myosin heavy chain (MHC) due to disulfide cross-linking (Lund et al. 2011). This reaction can be assayed by gel electrophoresis, which allows separation of cross-linked MHC (CL-MHC) as a dimer from MHC. In the present study, both loss of myosin heavy chain (MHC) as well as formation of cross-linked MHC (CL-MHC) were investigated by gel electrophoresis (Figure 4), and the intensities of protein bands were analyzed by a semi-quantitative approach in order to compare the levels of MHC and CL-MHC in the meat samples (Figures 5 and 6).

Gel electrophoresis revealed that at the end of storage, the dimer (CL-MHC) was formed, and was especially visual for the control sample (Figure 4). The CL-MHC was not found in the samples run after reduction by DTT, indicating that the dimer CL-MHC is formed through reducible disulfide bonds, as also reported elsewhere for meat stored in HiOx MAP (Kim et al. 2010). The formation of CL-MHC is linked to a serious loss in sensory properties, especially tenderness and juiciness (Kim et al. 2010; Lund et al. 2011). In contrast, the presence of sulfite, or red or white SkWPP in beef patties partly prevented the formation of CL-MHC during storage, indicating that these additives all protected against protein cross-linking induced by oxidation.

371

372 Sulfite is able to reduce disulfides leading to the formation of S-sulfonates (eq. 8) (Cecil and
373 McPhee 1955).

374

375 $P\text{-}S\text{-}S\text{-}P + HSO_3^- \rightarrow PS\text{-}SO_3^- + PSH$ (eq. 8)

376

377 In the present study, sulfite was found to reduce the level of protein radicals and to partly prevent
378 thiol loss, which complies with the low level of protein cross-linking as observed by the gel
379 electrophoresis.

380

381 Even though SkWPP partly prevented CL-MHC formation (Figure 4), samples added SkWPP was
382 found to generate new protein bands which were not seen in control samples or in samples added
383 sulfite. The three unidentified protein bands (Unidentified 1, 2, and 3) are indicated in Figure 4. The
384 unidentified bands 1 and 3 were most prominent at day 0 and seemed to disappear during storage,
385 whereas the unidentified band 2 was most prominent at day 15 and seemed to be formed during
386 storage. The molecular weights were estimated to be 220 KDa, 155 KDa and 90 KDa, respectively.
387 When the samples were run in their reduced state, the unidentified bands disappeared, suggesting
388 that these bands were formed through reducible protein cross-linking. An increase in protease
389 activity may also explain the unidentified bands, as increased proteolysis could lead to the
390 formation of new peptides that could become subsequently cross-linked and form new protein
391 bands. However, this seems unlike since polyphenols have been traditionally considered enzyme
392 inhibitors due to the conformational changes produced in the enzyme structures as well as the
393 modification produced in their substrate (Sartor et al. 2002).

394

3.5.Quantification of protein-cross-linking

The band intensities were determined and used as a semi-quantitative measure of the degree of protein cross-link formation. Control beef patties had significant higher CL-MHC levels as compared to the other samples (Figure 5, upper panel). As already seen from the gel, red and white SkWPP was able to prevent formation of CL-MHC to a similar level as by addition of sulfite. However, in contrast to both the control beef patties and beef patties added sulfite, addition of SkWPP resulted in a significant decrease in the MHC band intensity at day 8 and 15 as compared to day 0 (Figure 5, lower panel). This indicates that the MHC in the beef patties added SkWPP was modified during storage.

As mentioned, treatment by DTT significantly reduced the band intensity of the dimer CL-MHC formed in the control beef patties (Figure 6, upper left panel) indicating that the CL-MHC were formed through reducible disulfide protein cross-links. The MHC band intensity increased significantly in beef patties added SkWPP stored for 8 or 15 days after treatment by DTT, indicating that the low MHC level observed in these samples were partially due to reducible modifications or polymerization reactions generated during storage (Figure 6, lower right panel). Meanwhile, the MHC level in the control beef patties did not change significantly after treatment by DTT (Figure 6, lower left panel) indicating that no reducible modifications were generated. By comparing the levels of MHC in the control beef patties and beef patties added red SkWPP after treatment by DTT it becomes clear that MHC in the beef patties added red SkWPP was only partially recovered by treatment with DTT, suggesting that some protein polymers in the beef patties added SkWPP were generated through non-reducible cross-links. Jongberg et al. (2013) found that addition of green tea extract to Bologna type sausages modified the myofibrillar proteins through covalent thiol-quinone adduct formation causing phenol-mediated protein cross-linking.

419 Both reducible and non-reducible protein cross-links were reported in pork patties added essential
420 oil of rosemary or oregano (Nieto et al. 2013), and Hagerman et al. (1998) observed that the
421 addition of DTT did not increase the solubility of the precipitate formed by reaction between two
422 different tannins and BSA, indicating that a considerable proportion of protein-phenol interactions
423 may be resistant to strong reducing agents such as DTT.
424
425 **3.6.Protein-phenol interactions**
426 The results of the present study suggests that protein cross-links generated through reaction with
427 quinones from the SkWPP may be responsible for the unidentified protein bands observed on the
428 gel (Figure 4). Quinones are extensively formed during the vinification process and the subsequent
429 processing. Since the SkWPP are obtained from the by-product of vinification, the phenolics may
430 already have been subjected to oxidation leading to the formation of quinones. As mentioned,
431 quinones reacts rapidly with nucleophiles in meat to generate thiol-quinone adducts (Jongberg et al.
432 2011a).
433
434 Protein-phenol interactions have been suggested not only to change the protein structure but also to
435 play a key role in the protection against deteriorative reactions of proteins (Viljanen et al. 2005).
436 Phenols incorporated into the protein structure may exert their antioxidant protection locally on the
437 protein structure (Satué-Gracia et al. 1997). In the present study, proanthocyanidins were found in
438 both products in relatively high concentrations, and may be responsible for the reduced protein thiol
439 concentration due to thiol-quinone interactions, and subsequently reduced CL-MHC levels. The
440 difference between the molecular weight of MHC and the unidentified band 1 was ~20 KDa.
441 Seventy units of catechin could produce the observed increase in the molecular weight of MHC.
442 Similar polymerization degrees have previously been detected in grape skin (Pinelo et al. 2006),

443 and the reaction between such structures and the myofibrillar proteins may have caused the
444 formation of the unidentified protein bands. Moreover, polyphenols in wine pomace are usually
445 associated with an elevated content of fiber (García-Lomillo et al. 2014). The incorporation of large
446 polymers of fiber into the protein structure could also contribute to the increase in the molecular
447 weight of MHC due to addition reactions.
448
449 ## 4. CONCLUSIONS
450 Red SkWPP protected against protein radical formation, unlike white SkWPP, and may be an
451 interesting antioxidant in meat products. During storage red and white SkWPP protected against the
452 formation of the dimer CL-MHC, but resulted in loss of native MHC and in formation of
453 unidentified protein structures, presumably MHC modified through protein-phenol interactions.
454 However, both red and white SkWPP were found to result in an instant drop in protein thiol
455 concentration at day 0. Sulfite added to beef patties was found to be an effective antioxidant
456 towards protein oxidation in beef patties stored in high-oxygen atmosphere, since not only thiol loss
457 was reduced, but also the formation of protein radical and cross link were inhibited.
458
459 ## ACKNOWLEDGEMENTS
460 Authors thank the financial support of the Autonomous Government of Castilla y León through the
461 research projects (BU268A11-2 and BU282U13) and The Danish Council for Independent
462 Research | Technology and Production within The Danish Agency for Science Technology and
463 Innovation for granting the project entitled: "Antioxidant mechanisms of natural phenolic
464 compounds against protein cross-link formation in meat and meat systems" (11-117033). The PhD
465 grant of J. García–Lomillo (FPU grant) is funded by the "Ministerio de Educación, Cultura y
466 Deporte" of the Spanish Government.

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FIGURE CAPTIONS

Fig. 1 ESR spectra of myofibrillar protein isolates (MPI) extracted from beef patties without addition of any antioxidant (Control) or with addition of sulfites (300 ppm SO_2), red or white skin wine pomace product (SkWPP, 2 % w/w) stored in high-oxygen atmosphere for 15 days at 4 °C. **Left panel**: representative ESR spectra obtained from freeze dried myofibrillar protein isolate. **Right panel**: ESR spectrum of MPI from A) control beef patties compared to patties added sulfites (spectra amplified by 1.7). B) beef patties added red SkWPP compared to control beef patties (spectra amplified by 1.3). C) beef patties added white SkWPP compared to beef patties added red SkWPP (spectra amplified by 1.7).

Fig. 2 Protein radical intensity (mean ± SD, n = 3) of myofibrillar protein isolates (MPI) extracted from beef patties without addition of any antioxidant (Control), with addition of sulfites (300 ppm SO_2), or with addition of red or white skin wine pomace product (SkWPP, 2% w/w) stored for 15 days in high-oxygen atmosphere at 4 °C as determined by ESR spectroscopy.

Fig. 3 Protein thiol concentration (mean ± SD, n = 3) in myofibrillar protein isolates (MPI) extracted from beef patties without addition of any antioxidant (Control) or with addition of sulfites (300 ppm SO_2), red or white skin wine pomace product (SkWPP, 2% w/w) stored in high-oxygen atmosphere for 15 days at 4 °C.

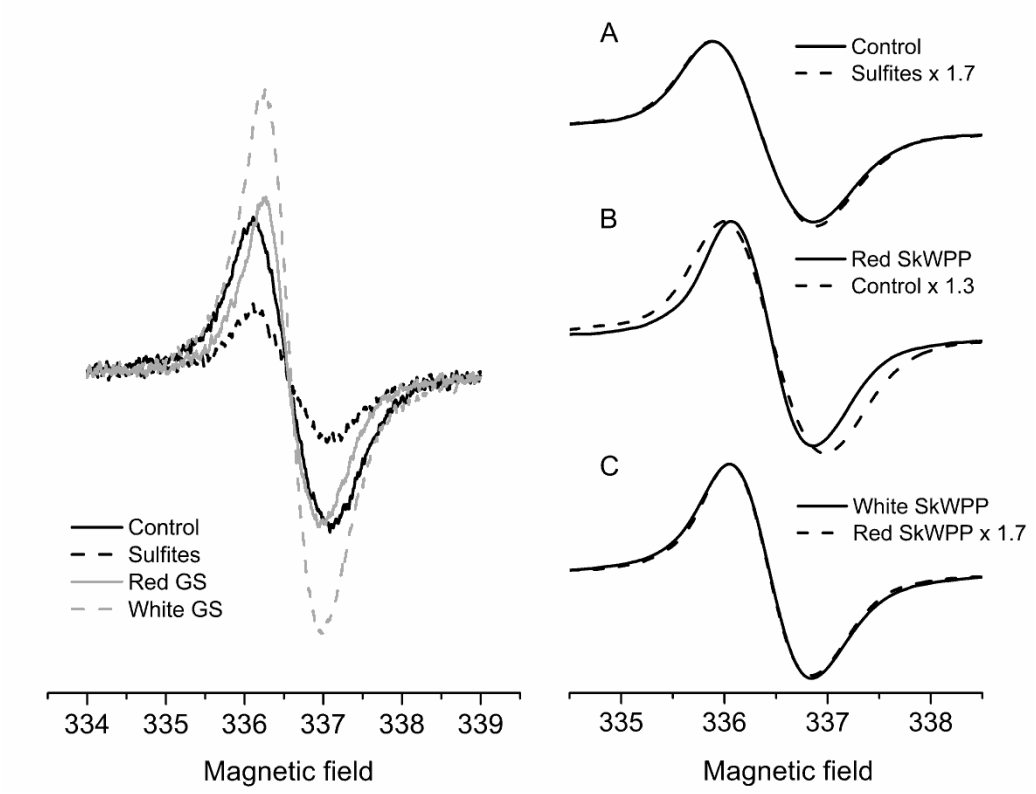
Fig. 4 Representative SDS-Gel of MPI from beef patties (C), added sulfites (S), red (R) and white (W) skin wine pomace product packed in high-oxygen atmosphere (70% O_2/30% CO_2) and stored for 0, 8 and 15 days at 4 °C. Myosin heavy chain (MHC), cross-linked MHC (CL-MHC), and actin are indicated on the gel, as well as unidentified proteins 1, 2, and 3. Samples from day 0 and 15

were run in the central lanes of the gel to ensure the highest quality in protein separation of these samples.

Fig. 5 Percentage volume (mean ± SD, n = 3) of cross-linked myosin heavy chain (CL-MHC) (upper panel) and myosin heavy chain (MHC) (lower panel) separated by SDS-PAGE in control beef patties (C), and beef patties added sulfite (300 ppm SO_2), red or white skin wine pomace product (SkWPP, 2% w/w) stored for 0, 8 and 15 days in high-oxygen atmosphere packaging (70% O_2/30% CO_2) at 4 °C. Values are means of three independent replicates.

Fig. 6 Percentage volume (mean ± SD, n = 3) of cross-linked myosin heavy chain (CL-MHC) (upper panels) and myosin heavy chain (MHC) (lower panels) separated by SDS-PAGE in control beef patties (left panels), and beef patties added red skin wine pomace product (SkWPP, 2% w/w) (right panels) stored for 0, 8 and 15 days in high-oxygen atmosphere packaging (70% O_2/30% CO_2) at 4 °C. Solid lines correspond to non-reduced samples and dotted lines correspond to samples previously reduced by DTT. Values are means of three independent replicates.

633 **Figure 1**



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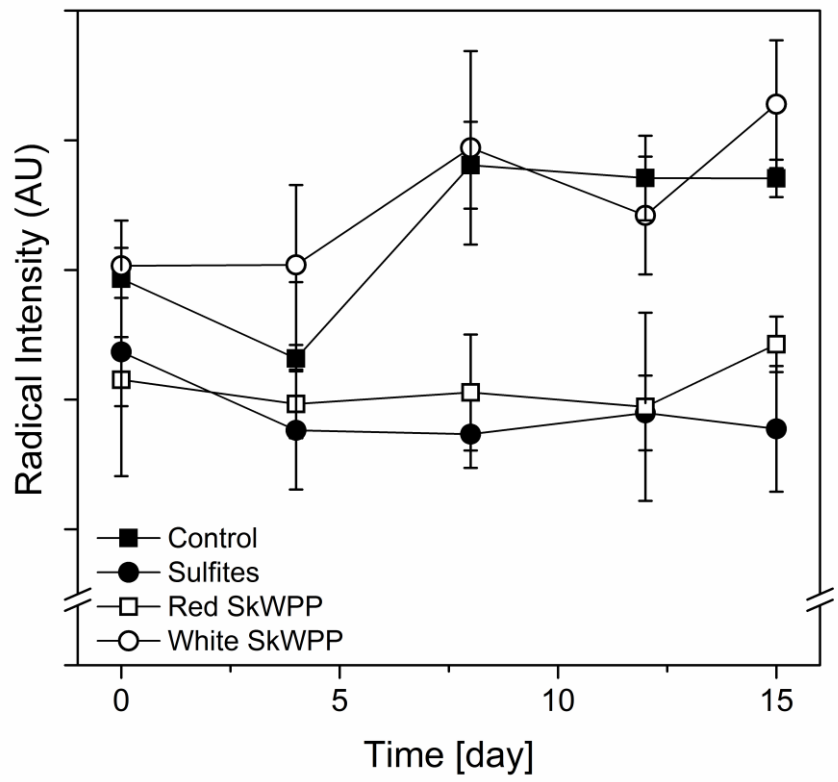
636 **Figure 2**



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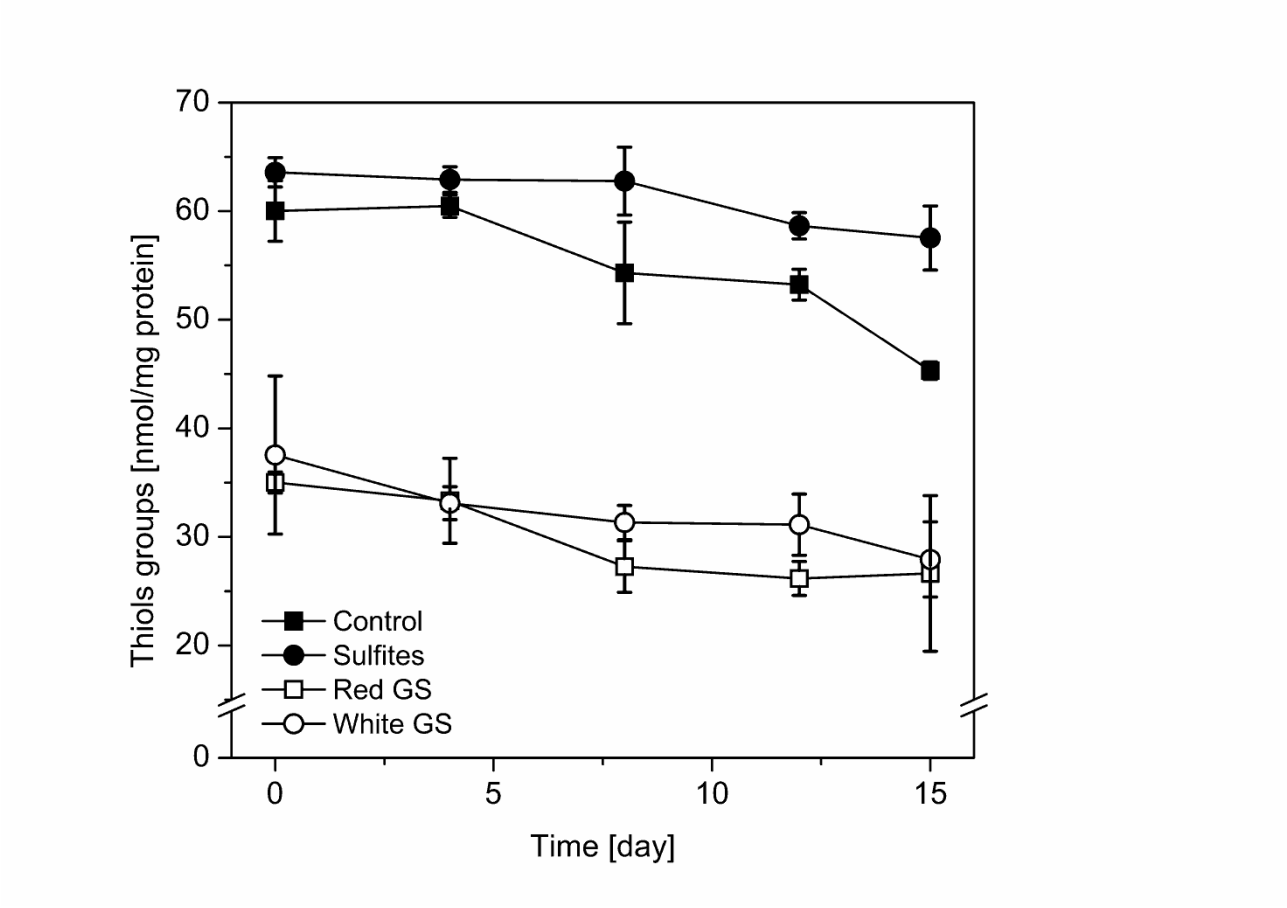
639 **Figure 3**



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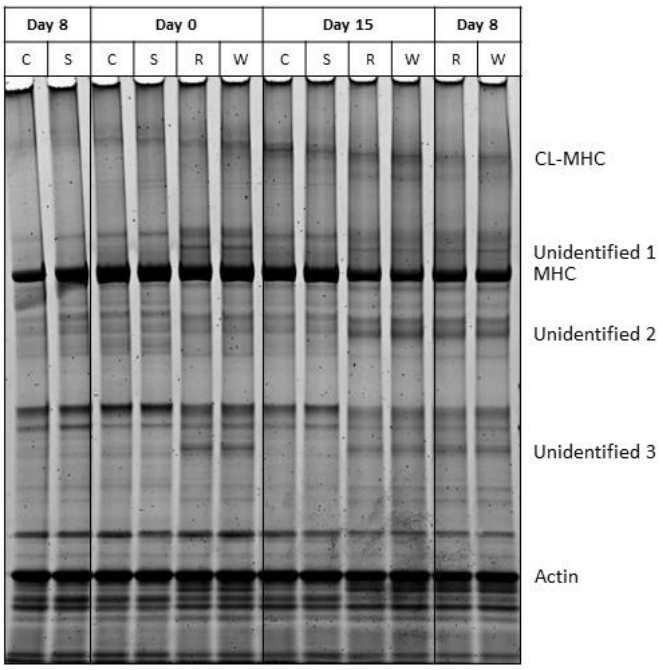
642 **Figure 4**



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645 **Figure 5**

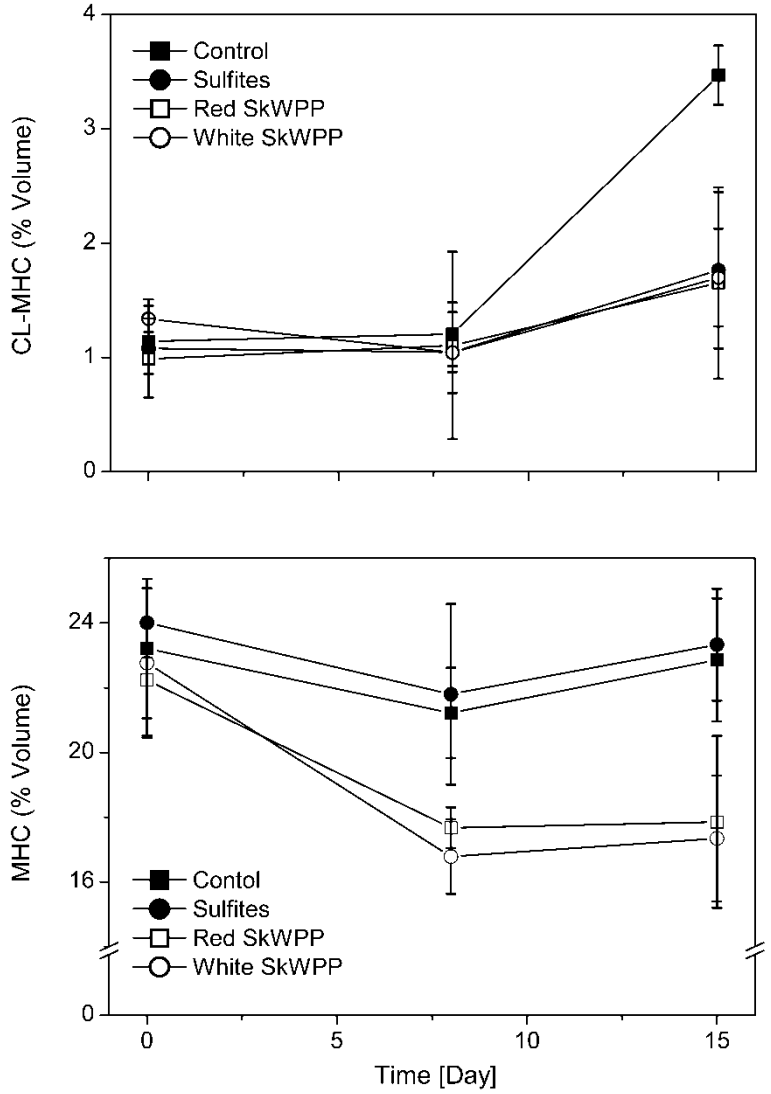

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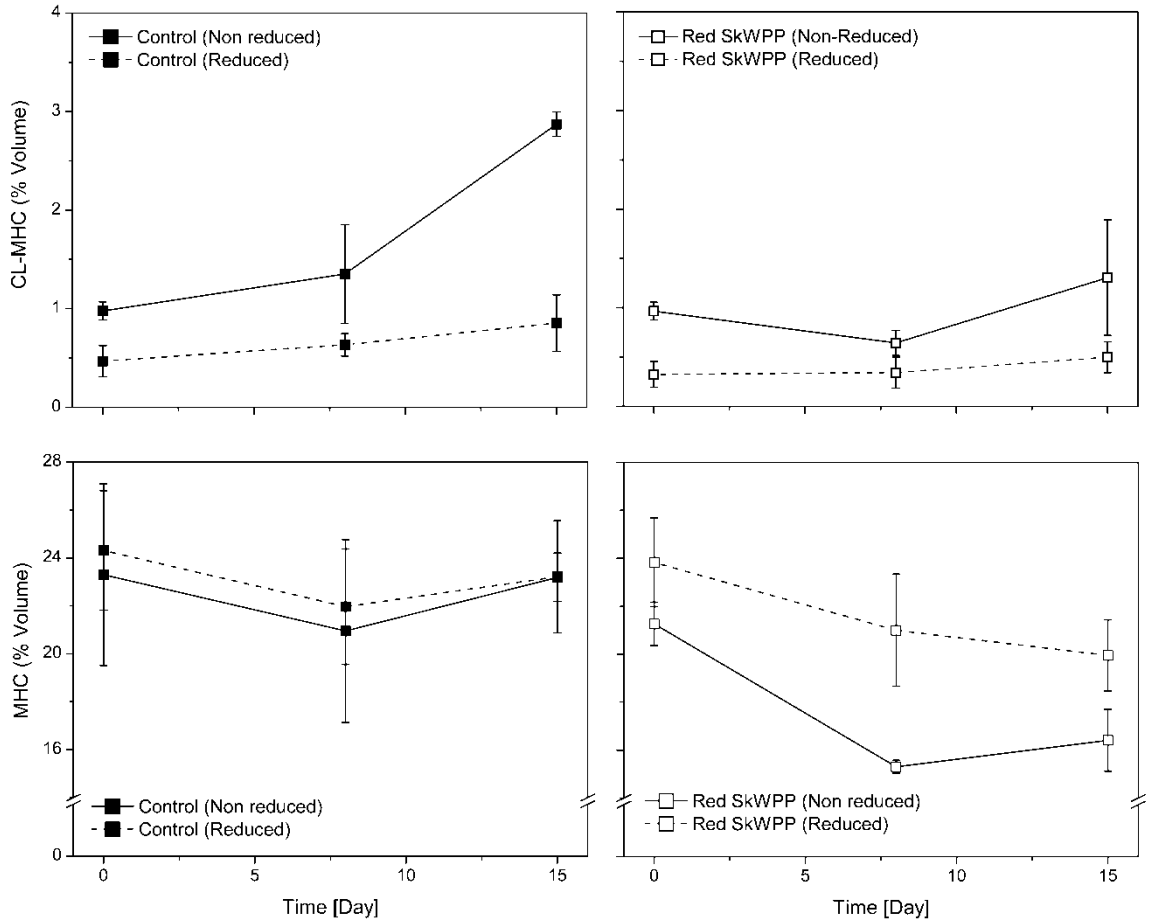
Figure 6


Control (Non reduced)
Control (Reduced)
Red SkWPP (Non-Reduced)
Red SkWPP (Reduced)
CL-MHC (% Volume)
MHC (% Volume)
Red SkWPP (Non reduced)
Red SkWPP (Reduced)
Time [Day]
Time [Day]