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Foam inhibitors from specialty malts

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Abstract

Although there are product to product differences, the majority of the crystal and caramel malts tested have been shown to have a net destabilizing impact on foam. A foaming test using a model beer has been developed to quantify the magnitude of this inhibitory effect. Lipid analysis using thin layer chromatography reveals that there are elevated levels of triglycerides in problematic malts. There also appear to be oxidized lipids in the specialty malts, likely produced in the heating stage of malt production with higher levels in more intensely heated products. However some specialty malts, such as black malt, whilst containing such foam-negative entities, do display superior foaming properties, probably because they develop even more powerful foam-positive components in the severe heating events.

Key words

Foam; Inhibitor; lipid; malts; oxidation; triglyceride

Introduction

The stability of foam on beer is determined by a myriad of physical and chemical factors (1). Over the years there has been an extensive study of the materials in beer that stabilize foam, notably polypeptides and hop-derived bitter acids (2). However in recent times there has been an increasing realization that less than ideal foam performance of commercial beers may be due to the presence within them of foam-negative materials (3, 4).

Although ethanol itself is inhibitory to foam, it is obviously not a component that can be reduced in quantity for the simple expediency of improving head retention. Conversely, lipid materials in beer certainly can and should be eliminated, as not only do they include amongst them molecules that are profoundly foam-negative (5), but they also can contribute to the staling of beer (6).

Recently it was shown that certain specialty malts used in brewing are particularly significant sources of foam-negative substances (7). Experiments suggested that these inhibitors are likely lipid in nature. In the present study we have continued the investigation of such malts and have confirmed the lipid nature of these foaming inhibitors.

Materials and methods

Materials

Linoleic acid, monoolein, trilinolein, phosphotidylcholine, cholesteryl heptadecanoate and N,N-dimethyl-1,4-phenylenediamine were from Sigma Aldrich (St. Louis, MO). Heptadecanoic and diheptadecanoic acid were from Nu-chek Prep Inc. (Elysain, MN). Barley (cv Harrington and cv Stander) and malt of varying degrees of stewing and kilning were obtained from Great Western Malting Company (Vancouver, WA), Briess® Malt & Ingredients Co (Chilton, WI), Weyermann Specialty Malts (Bamberg, Germany), Crisp Malting Group (Norfolk, England), Muntons Malt (Stowmarket, Suffolk, UK), and Bairds Malt (Witham, Essex, UK). Tetrahop Gold was from Haas Hop Products, Yakima, WA, USA. Chloroform and ether were from Acros Organics (Morris Plains, NJ). Hexane, methanol and butylated hydroxytoluene were from Fisher Chemical (Hampton, NH). Acetic acid was from EM Science (Darmstadt, Germany)

Extraction Procedure

Wort production was performed according to the Method 4 of the American Society of Brewing Chemists (8) in which malt is extracted in four volumes of water over a temperature range from 45°C to 70°C followed by recovery of wort by filtration through paper. The wort was not boiled.

Assessment of foam stability

Foam stability was measured using a shaking method (9). The foam performance of malt extracts was evaluated as described previously (7). Assessment of foam inhibitors was performed using a model foaming system (10). The model beer consisted of a final concentration of 75µg/cm^3 barley albumin extract, 31.7g/kg ethanol, and 20mg/m^3 reduced iso-α-acid (Tetrahop Gold) in 0.1M acetate buffer pH 4.5. Linoleic acid was used as a standard for the foam inhibitor assay. Stock solution of model beer with the addition of 50ppm linoleic acid was made up and diluted down with model beer to concentrations between 0.1ppm and 2.5ppm linoleic acid. Wort produced from Crystal 75L was freeze dried using a VirTis BenchTop K freeze dryer (SP Scientific; Warminster, PA) at -41°C and 55mTorr. Stock solution of model beer with the addition of a final concentration of 5mg/ml freeze dried Crystal 75L was diluted until comparable levels of inhibition to linoleic acid was observed. Model solutions were made fresh for each experiment.

Thin layer chromatography of lipid extracts

Folch reagent (11) was prepared by mixing 2:1 (v/v) of chloroform: methanol with 0.05% (w/v) butylated hydroxytoluene. Wort (2.5mL) from each extraction were transferred into disposable glass test tubes (125mm x 16mm). Folch reagent (6 mL) and 1.8ml 0.01M potassium chloride were added to each test tube. The reaction mixture was then vortexed for 10 seconds and incubated at room temperature for 10

minutes. The test tubes were then centrifuged for 10 minutes at 210 RCF_{max} using an International Clinical Centrifuge Model CL (International Equipment Co.; Needham, MA). The bottom organic layer was transferred to a 45mm length x 15mm diameter amber, screw cap vial (Fisherbrand® ; Pittsburgh, PA) using a 9 inch glass transfer pipette. The vials were placed in a ReactiVap (Pierce; Rockford, IL) apparatus and evaporated to dryness under nitrogen, capped, and stored at -20°C.

Phosphotidylcholine, monoolein, diheptadecanoic acid, heptadecanoic acid, trilinolein, and cholesteryl heptadecanoate were used as the lipid class standards for phospholipid (PL), monoglyceride (MG), diglyceride (DG), free fatty acid (FFA), triglyceride (TG), and nonpolar lipids (NP) respectively. Each lipid was individually dissolved in chloroform to produce a stock solution at a final concentration of 10mg/ml. A lipid class standard was produced by introducing each individual stock lipid solution into a vial containing chloroform resulting in a mixture containing six lipids each at a final concentration of 1mg/ml.

An oxidized linoleic acid standard was produced by applying 0.25mg of linoleic acid to the inside wall of an amber vial and leaving it to undergo autoxidation in air at room temperature for 5 days (12). Chloroform was then added to the vial to bring the final concentration to 10mg/ml. The vial was flushed with nitrogen and stored at -20°C.

TLC Silica gel 60, 20cm x 20cm, glass plates (Merck, EMD Millipore; Darmstadt, Germany) were used for each experiment. TLC plates were prepared by drawing, with pencil, 2cm vertical lanes with 0.5cm between each lane and 0.25cm away from the edge. The origin as indicated by a horizontal line was drawn 1.5cm from the bottom of the plate. A horizontal line drawn 15cm above the origin was the targeted solvent front. A horizontal line 2cm from the top was used as a guide for labeling the lanes. Plates were stored in a desiccator until needed. Before spotting, TLC plates were placed into a preheated

Model 19 Thelco heating oven (GCA/PrecisionScientific; Chicago, IL) at 100°C for 30 minutes. Plates were then left to cool for 5 minutes immediately before spotting.

Lipid extracts were re-suspended in 60µl of chloroform and mixed by pipetting down the sides of the vials to ensure all remaining lipids were dissolved and briefly vortexed. The re-suspended lipid extracts (20µl. i.e. 10µl x 2) were plated as overlapping spots across the 2cm lane using a P10 Pipetman (Gilson Inc.; Middleton, WI). Reference samples (20µl) were pipetted in the same manner.

A solvent of 50:50:1 (v/v/v) of hexane: ether: acetic acid was used as the mobile phase for all experiments. Mobile solvent (101 mL) was transferred into a Kontes TLC developing tank (Fisher Scientific; Waltham, MA) lined with a 23cm x 57cm filter paper (Whatman; Buckinghamshire, UK) and was allowed to equilibrate for 30 minutes before introducing the spotted plates.

Plates were placed in the tank so that the silica side (spotted side) faced up and towards the center of the tank with the glass side leaning on the side of the tank making sure the origin was above the mobile phase. Each tank can accommodate two 20cm x 20cm TLC plates. Lids to the tank were placed on top and held down firmly by the placement of two 1kg weights on top. Plates were allowed to develop until the mobile phase reached the 15cm solvent front indicated by a horizontal line, approximately 30 minutes. After development, plates were taken out of the tank, placed on paper towels and allowed to dry for 5 minutes before visualizing.

Cupric sulfate solution (10% w/v) was used as a non-specific stain for all lipids and has a lower detection limit in the picomole range depending on the lipid class (13). $CuSO_4$ (50mL) was poured directly on each plate after development. Plates were then immediately placed on a metal rack above a preheated electric double burner set on high for 20 minutes. Plates were rotated 180° after 10 minutes

to ensure even plate heating. Lipids are identified as black spots on a white background and R_f values for each band were recorded. Pictures were taken of all TLC plates using an iPhone 4 (Apple; Cupertino, CA).

Visualization of hydroperoxides involved the use of N,N-dimethyl-1,4-phenylenediamine Reagent (Wurster's Red Reagent) which specifically stains peroxides (14). N,N-dimethyl-1,4-phenylenediamine (1g) was dissolved in a mixture of 50ml methanol, 50ml deionized water, and 1ml glacial acetic acid and stored at 4°C until needed. After development, TLC plates were held upright using a Styrofoam cuvette holder and sprayed with Wurster's red reagent using a 75ml flask-type sprayer (Sigma Aldrich; St. Louis, MO) until the plate looked wet. Peroxides exhibit reddish-pink to purple-red spots on a pale pink colored background. R_f values were recorded for each band and pictures were taken of all TLC plates as above.

Recovery of lipids from TLC plates

Lipid extracts were prepared as described above. 60µl of re-suspended lipid extract was loaded onto the TLC plate. A lane closest to the edge was used as a sacrificial lane and plated with 20µl re-suspended lipid extract. The plate was developed using a 50:50:1 hexane: ether: acetic acid mixture. The sacrificial lane was stained with 10ml $CuSO_4$ and held over a Bunsen burner until lipids were visualized. Other lanes were protected by covering with aluminum foil. Once visualized, corresponding areas of the plate were scraped off with a TLC plate scraper (Sigma Aldrich; St. Louis, MO) and transferred to a 2ml Eppendorf centrifuge tube (Fisherbrand; Pittsburgh, PA).

Ether (1.5 mL) was added to each tube, they were vortexed briefly and incubated at room temperature for 10 minutes. Tubes were then centrifuged at 15,000rpm for 5 minutes using a Microfuge E (Beckman Coulter Inc.; Indianapolis, IN). The supernatant was then pipetted off with a 9 inch glass pipette into an amber screw top vial and evaporated to dryness under nitrogen using the ReactiVap apparatus. A 10µl sample was taken from each vial before evaporation and loaded on to a TLC plate to confirm recovery of the lipid from the plate. The recovered lipid was re-dissolved with 200µl of ethanol and added to a modified model foaming system (model foam solution described earlier but without ethanol) so that the resulting final concentration of 37.1g/kg ethanol and final volume of 5ml was achieved. The shake test to determine inhibition of foam stability was then performed.

Results and Discussion

Comparison was made of the foam stability of extracts of a diversity of malts (Fig 1). When compared to the pale malt (2 row) only three of the samples displayed superior head retention. Clearly some of the crystal malts produced using a stewing process are, in keeping with received dogma, capable of enhanced foam performance. However the majority do not have enhanced foam quality, indeed the opposite, as first indicated by Combe et al (7). Several of the malts investigated displayed no foam stability at all. Absent an understanding of precise protocols for making these various commercial products, it can only be inferred that this variance in performance must in some way relate either to variety selection or to differences in process conditions. It may indeed be the case that the protein-polysaccharide complexes whose existence was predicted by Bishop (15) are present, perhaps in all the

malts, but for most of them there is an even bigger impact of foam negative entities. It is the latter that we have been searching for.

We have sought to develop a standard assay for quantifying the impact of different samples on foam stability. For this purpose we employed extracts of Crystal 75L which, while not featured in Fig1, was the main specialty malt focused upn in our previous work (7). Fig 2 illustrates the comparison of extracts of Crystal 75L malt with linoleic acid for their impact on the foam stability of a model beer. Side by side comparison indicates that the linoleic acid is three orders of magnitude more foam negative than is the malt extract – but of course the latter will comprise diverse molecular entities, not all of which will be foam-negative and some of which may, indeed, be foam-positive.

If we were to make the assumption that the inhibitor was specifically linoleic acid, then on the basis of this experiment, it would be concluded that the inhibitor represents approximately 0.5×10^{-3} of the total dry weight of the malt, which is a rather lower order of magnitude than the 1-3% lipid that such a malt typically contains. However only a small proportion of the total lipid (sometimes as little as 0.1% of that in malt) survives into wort (16).

Although the inhibitor is not necessarily lipid in nature, this is by far the likeliest category of material that would comprise the foam-negative entity. Lipid fractions from grain have been analyzed by thin layer chromatography. Fig 3 illustrates the reference lipid pattern as generated with defined standards. The grains were extracted by using a conventional hot temperature mash. The procedure is a

small-scale mimic of conventional brewery mashing techniques and analysis of the worts reveal changes that have taken place in both malting and mashing.

Fig 4 shows the lipid patterns seen in the extracts of a diversity of malts. Curiously, lipid bands from the Harrington barley (figure 4a, lane 2) are largely absent. Barley has been shown to contain lipids but they are lost throughout the malting process and wort filtration (16,17). Thus low detection of lipids in this wort was unexpected.

A band co-migrating with triglyceride in many of the specialty malts suggests that this may be a consequence of the inactivation of the hydrolytic enzyme, lipase, during the production of these malts, meaning that triglycerides are less extensively hydrolyzed in mashing. Lipase has been shown to be active in normal mashing conditions (18, 19). The tryglycerides present in the crystal and caramel malts as indicated by the bands (specified by the red arrow) in figures 4b-e suggests that lipase activity is limited in these stewed malts as compared to the lack of a triglyceride band (figure 4a) in 2-row and other specialty malts. With the exception of roasted barley, most of the worts containing triglycerides have been shown to be foam negative. The exceptional behavior of roasted barley is likely due to the increased amounts of high molecular weight protein-polysaccharide complexes formed during the intensive heating process involved in the production of this adjunct (20), such complexes out-competing the negative impacts of these lipids (7). It has been suggested that a mixture of triglycerides and phospholipids exhibits the greatest foam inhibition capacity (21, 22). Thus

triglycerides are suspected to be at least in part responsible for the poor foam performance seen in the specialty malts.

However many of the specialty malts depicted in Figures 4a-e display a generally more intense lipid staining pattern than does 2 row malt (figure 4a lane 4). This may be likely due to the inactivation of multiple enzymes involved in the degradation of lipids due to the higher temperatures involved in the productionprocesses of these specialty malts.

Hydroxy fatty acids have been shown to destabilize foam (23). Also, it has been suggested that di- and tri-hydroxy fatty acids are better able to survive into the final product due to their increase in solubility as compared to non-oxidized fatty acids (23, 24). Hydroperoxides in the lipid extracts were visualized using Wurster's Red Reagent. Lipid hydroperoxides are the precursors to hydroxy lipids and are relatively unstable, thus the presence of lipid hydroperoxides can be related to the potential for the presence of hydroxy lipids. Results are shown in figure 5a-e.

In figures 5b-e, a noticeable pink band with an R_f of 0.08 is apparent in the Munich, Victory, HD08, Crystal 75L, Crystal 120L, Black Patent, Roasted barley, Crystal 40-50L, Crystal 55-65, Crystal 72-82L, Caramel Pilsner 10-15L, Crystal 49-64L, Crystal 135-165L, Caramel 15-25L, Caramel 25-35L, Caramel 30-38L, Caramel 53-61L, Caramel Wheat 57-75L, and Caramel 110-150L lipid extracts. A pink band with an R_f of 0.28 is detected in the oxidized linoleic acid standard which is assumed to be linoleic acid hydroperoxide. It seems, therefore, that the pink staining band in these malts is not oxidized linoleic acid per se.

The increasing intensity of the pink color suggests an increase of hydroperoxide groups in malts kilned to a higher degree. This data is supported by current research that shows an increase in organic radicals and decrease in oxidative stability in worts produced by dark specialty malts (25, 26).

In order to determine if oxidation is occurring during the preparation of the wort or has already happened in malt production, TLC was performed on lipids extracted from malt in cold water (Fig 6a-b). 0.5g of Harrington barley, 2-row, Victory, Caramel Pilsner 1.5L, Crystal 75L, and roasted barley were mixed with 2.5mL of water. Lipid extraction was carried out on this malt suspension as described in Methods.

The pink bands exhibited on the plate indicate that hydroperoxides are present in malts prior to mashing (figure 6b). The intensity and quantity of pink bands also seem to increase as

the color of the malt increases, which agrees with the previous observations. These data suggest that oxidation is mainly occurring during the heating step of malting and not during the preparation of the wort in the mash. Recent research reveals that higher kilning temperatures result in an increase in stable organic radicals (25, 26). The more complex lipid patterns of malt lipid extracts as compared to wort lipid extracts may be for several reasons. Firstly, much lipid tends to be removed with the spent grain during separation of wort (16). Secondly, lipid degrading enzymes would more likely be able to survive the less intense kilning temperatures used to produce 2-row malt (18). It is likely that during the mash, these enzymes are degrading triglycerides and hydroperoxy lipids into their breakdown products. The lack of hydroperoxides in 2-row malt despite the fact that lipoxygenase is still active agrees with earlier findings (27).

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To determine which lipid classes are responsible for poor foam stability, lipid extracts of wort prepared from Crystal 75L were separated by thin layer chromatography, recovered off the plate, and dosed into the model foaming system. 100µg of oxidized linoleic acid was plated and recovered for use as a positive control. Three bands were observed and each was recovered to measure foam inhibition. A lane was left blank and an area was scraped off to use as a negative control. Blank areas of the plate were scraped off before development of the plate to determine if any of the solvents affected the model foaming system. Fig 7a illustrates where the scrapings were taken. Seven distinct bands were observed and recovered on this lane. Ether was used to extract the lipids from all recovered scrapings. A portion of the ether extract of the recovered

scrapings was developed on TLC plates to verify recovery (data not shown). Ethanol was used on one set of the undeveloped scrapings to determine if ether extraction affected the model system. The modified model foaming system, which did not include ethanol, was dosed with ethanol to bring up to a final concentration of 37.1g/kg and final volume of 5ml was used as a control to determine if the extraction procedure affected the foaming assay.

Interestingly, as compared to previous findings, the lipid profile of Crystal 75L in this experiment exhibited faint bands at R_f 0.23 and 0.37 corresponding to oxidized fatty acids and diglycerides respectively (Fig 8). These bands were not observed in the initial experiments. This is likely explained by the use of a more concentrated lipid extract in this study. In this experiment lipids were extracted from 5ml of wort as opposed to 2.5ml possibly increasing the amount of oxidized fatty acids and diglycerides to detectable levels.

Each blank displayed lowered foam stability as compared to the model foaming system control. This implies that there may be residues from the TLC plate that are transferred into the foaming solution to inhibit the foam. More work is needed to determine the exact cause of this phenomenon. However, each recovered lipid causes poorer foam stability as compared to the developed blank. This does suggest that each lipid class can inhibit foam. Unfortunately, it proved impossible to determine the relative influence of each recovered lipid on foam stability due to the fact that recovery of lipids from TLC plates seems to be highly inefficient.

Theoretically, 100% recovery of the plated 100µg of linoleic acid would result in a final concentration of 20ppm of linoleic acid in the model foaming system. This amount of lipid would kill the foam almost immediately since earlier experiments show no measurable foam at 2.5ppm. However, in this case we still observe a measurable amount of foam. In addition, due to the differences in polarity of each of the lipid classes, the ether solvent would be less efficient at extracting the polar lipids and more efficient as the lipid classes become non-polar. This may explain why poorer foam is observed as the R_f value of the lipids increase.

In our previous paper (7) we demonstrated that the inclusion in a brewing grist at typical levels of the specialty malts containing substantial levels of foam inhibitors did indeed lead to decreased foam performance in the beers produced. The present work confirms that these inhibitors are most likely lipid in nature. In vierw of the greater solubility of the oxidized lipids as compared to triglycerides, it is likely that the former may be the more significant inhibitors.

Conclusions

The distribution of lipid classes in a range of malts is much more complex and concentrated than in worts derived from such malts. In these latter extracts there appear to be higher levels of triglycerides and oxidized fatty acids in those malts receiving most intense heating. These are likely to be significant contributors to the foam negative impact which some of these specialty malts can have. However there

does not appear to be a simple correlation between the level of these lipid entities in extracts of the malts and the tendency of such malts to be foam-negative. It is presumed that the net impact of a malt on foam represents a balance arising from their contribution of foam-negative material and putative foam-positive elements that are formed on intense heating.

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Legends to figures

Figure 1. Foam stability of worts produced by a single malt mash. Horizontal line signifies the foam stability of wort produced from 100% 2-row malt.

Figure 2. Comparison of foam stability of a model beer dosed with different concentrations of linoleic acid or freeze dried crystal.

Figure 3. Separation of lipid standards using thin layer chromatography using a solvent of 50:50:1 (v/v/v) of hexane: ether: acetic acid. Nonpolar class (NP, R_f 0.83), triglycerides (TG, R_f 0.75), free fatty acids (FFA, R_f 0.47), diglycerides (DG R_f 0.37), monoglycerides (MG R_f 0.07), and phospholipids (PL R_f 0.0).

Figure 4a-e. Separation of lipid classes using thin layer chromatography of lipid extracts of worts produced from single malt mashes. Malt used indicated on top of each lane. Reference lane contains an equal concentration of Nonpolar class (NP), triglycerides (TG), free fatty acids (FFA), diglycerides (DG), monoglycerides (MG), and phospholipids (PL).

Figure 5a-e. Lipid hydroperoxide profiles of worts produced from single malt mashes of different specialty malts as visualized by Wurster's Red reagent after separation by thin layer chromatography.

Figure 6. Lipid (a) and lipid hydroperoxide (b) profiles of lipids extracted directly from selected barley or malt.

Figure 7. Thin layer chromatography of recovered lipids (a). Lipids areas were recovered based on visualized Crystal 75L and oxidized linoleic acid lanes (b).

Figure 8. Foam stabilities of model beer after introducing separated and recovered lipids from Crystal 75L wort and oxidized linoleic acid.

Figure 1

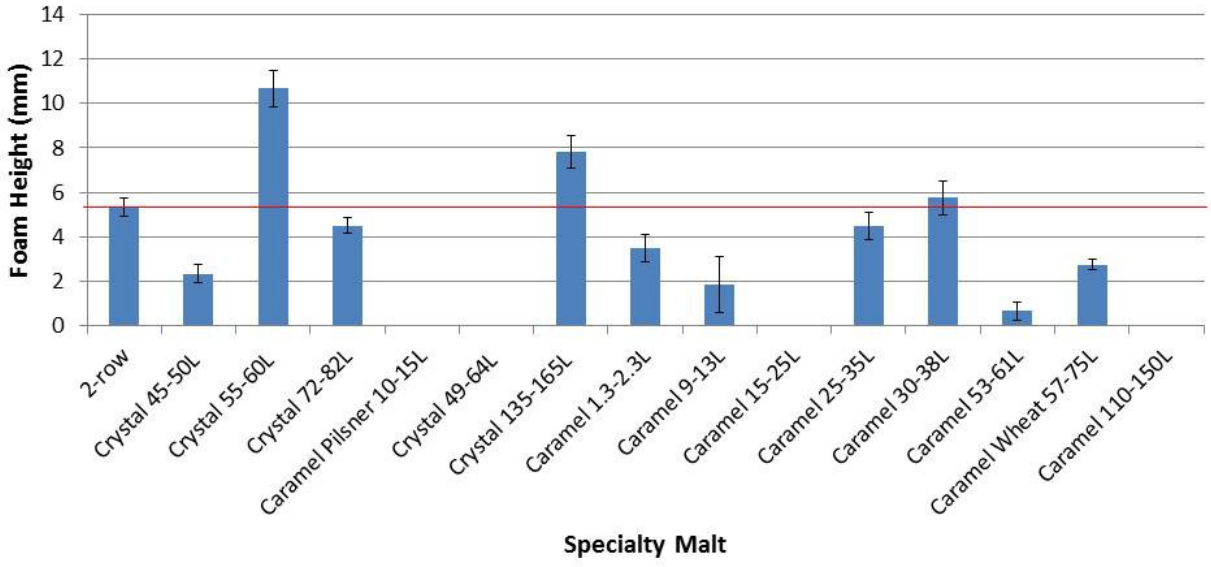


Figure 2

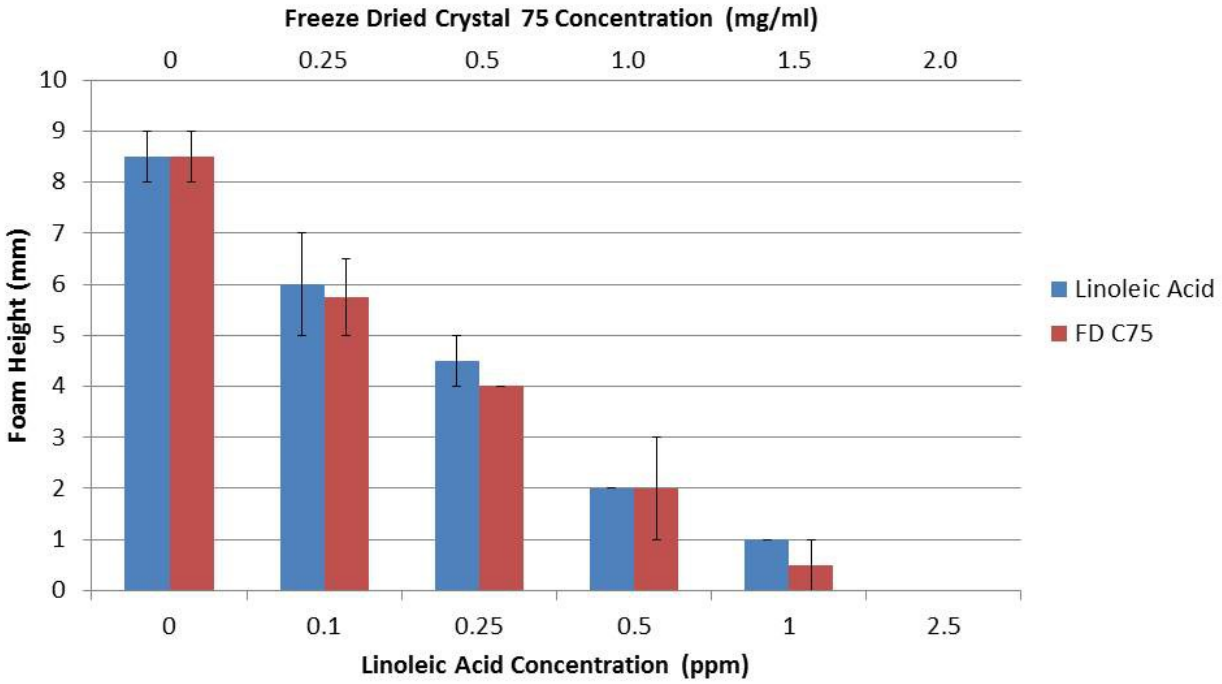


Figure 3

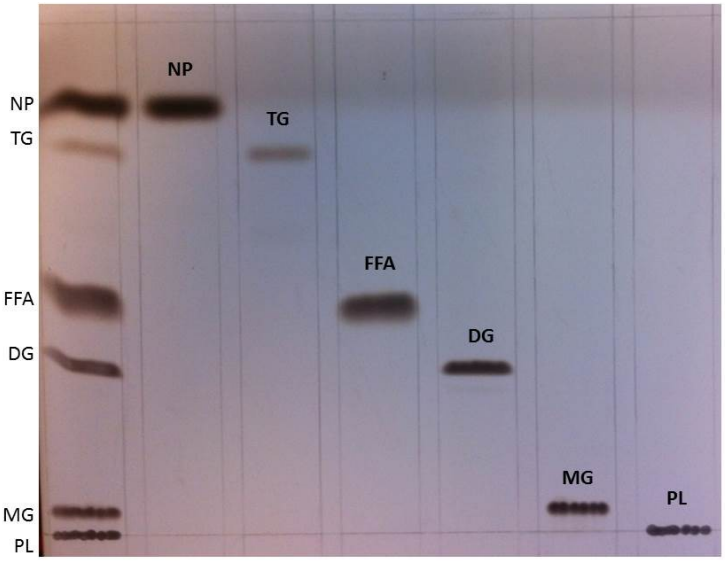


Figure 4a

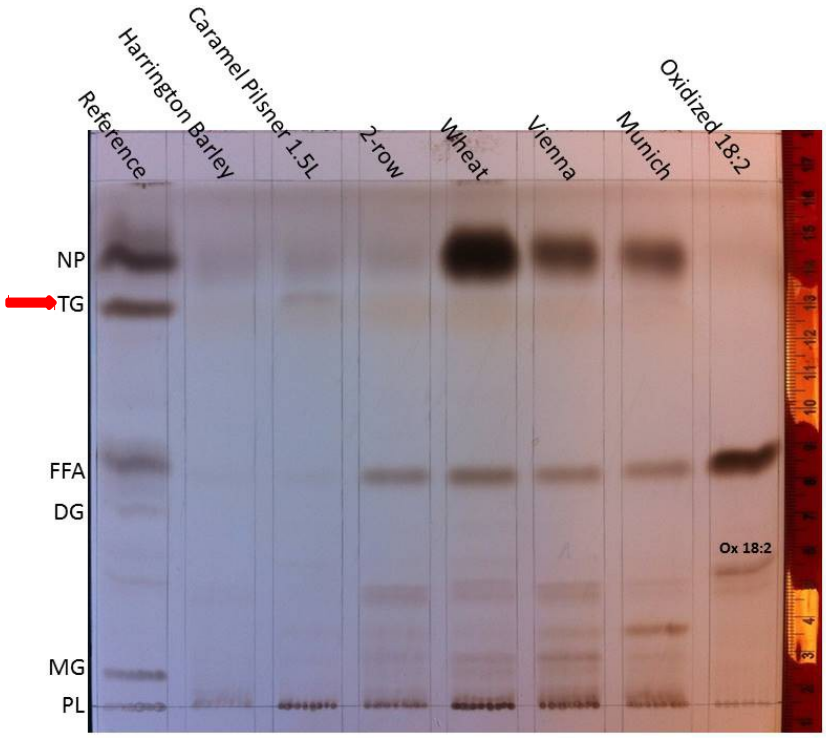


Figure 4b

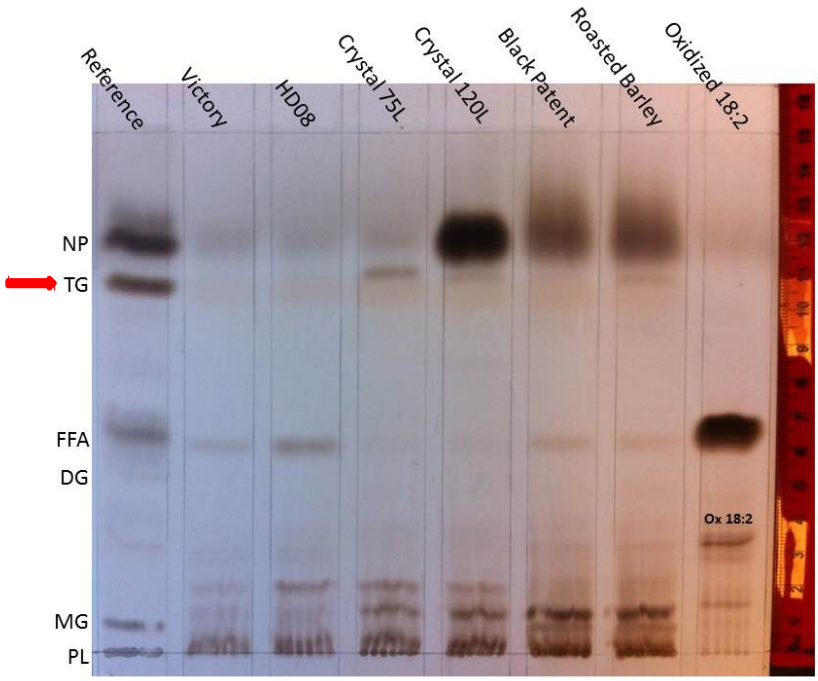


Figure 4c

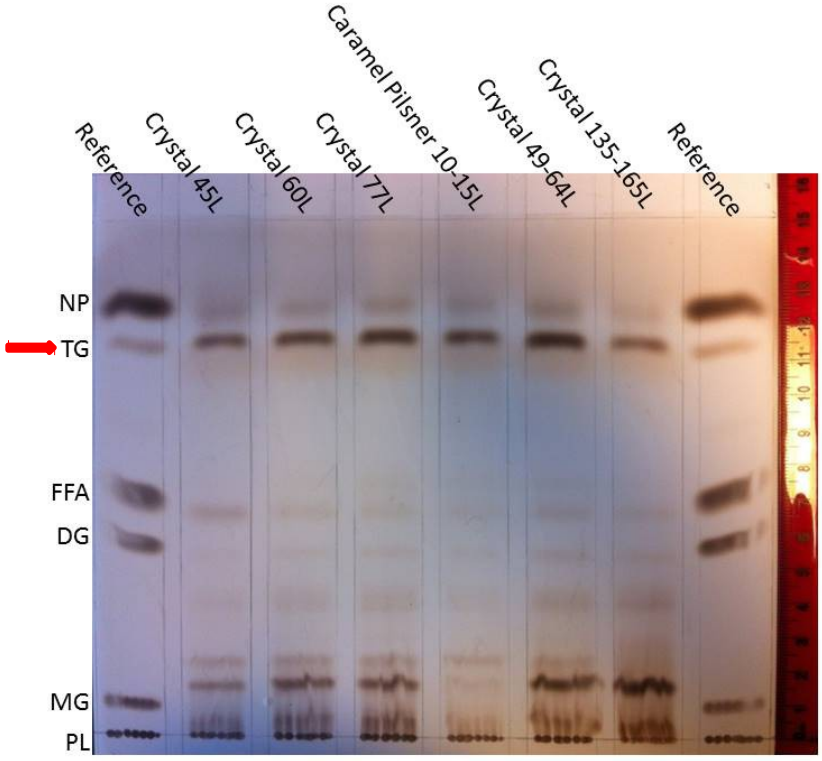


Figure 4d

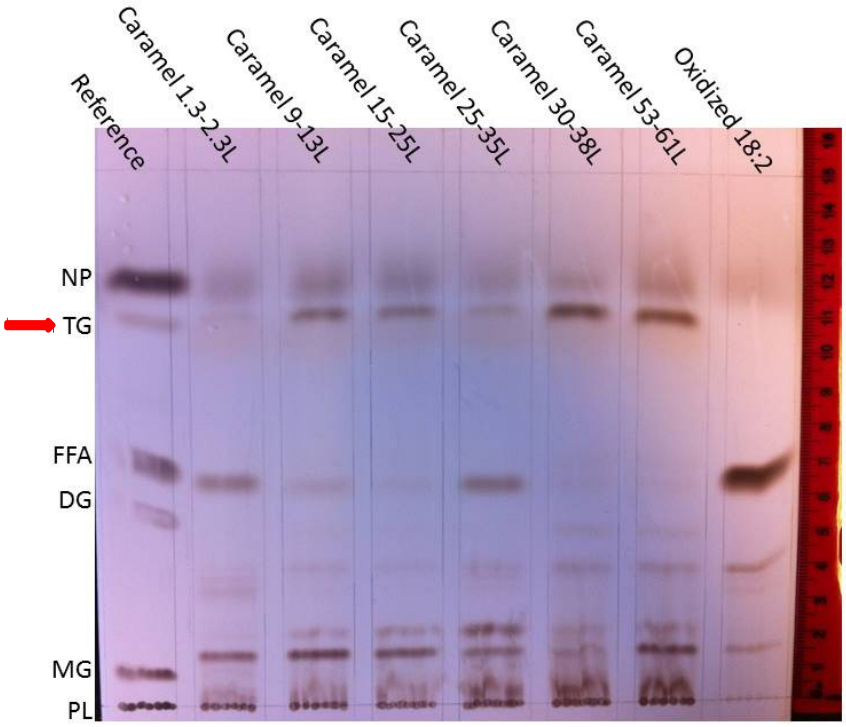

Reference
Caramel 1.3-2.3L
Caramel 9-13L
Caramel 15-25L
Caramel 25-35L
Caramel 30-38L
Caramel 53-61L
Oxidized 18:2
NP
TG
FFA
DG
MG
PL

Figure 4e

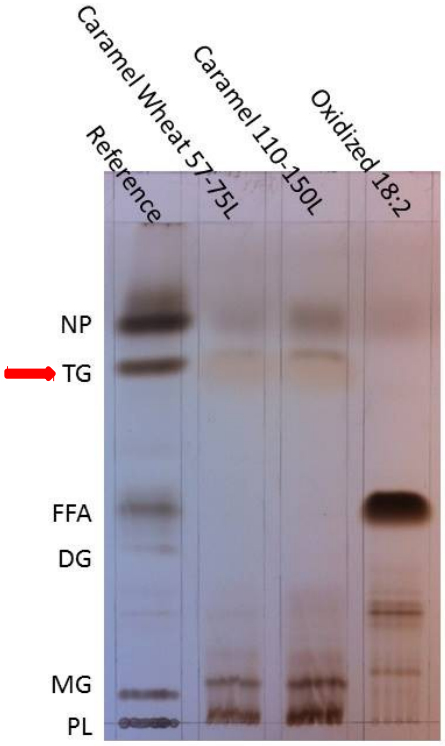

Reference
Caramel Wheat 57-75L
Caramel 110-150L
Oxidized 18:2
NP
TG
FFA
DG
MG
PL

Figure 5a

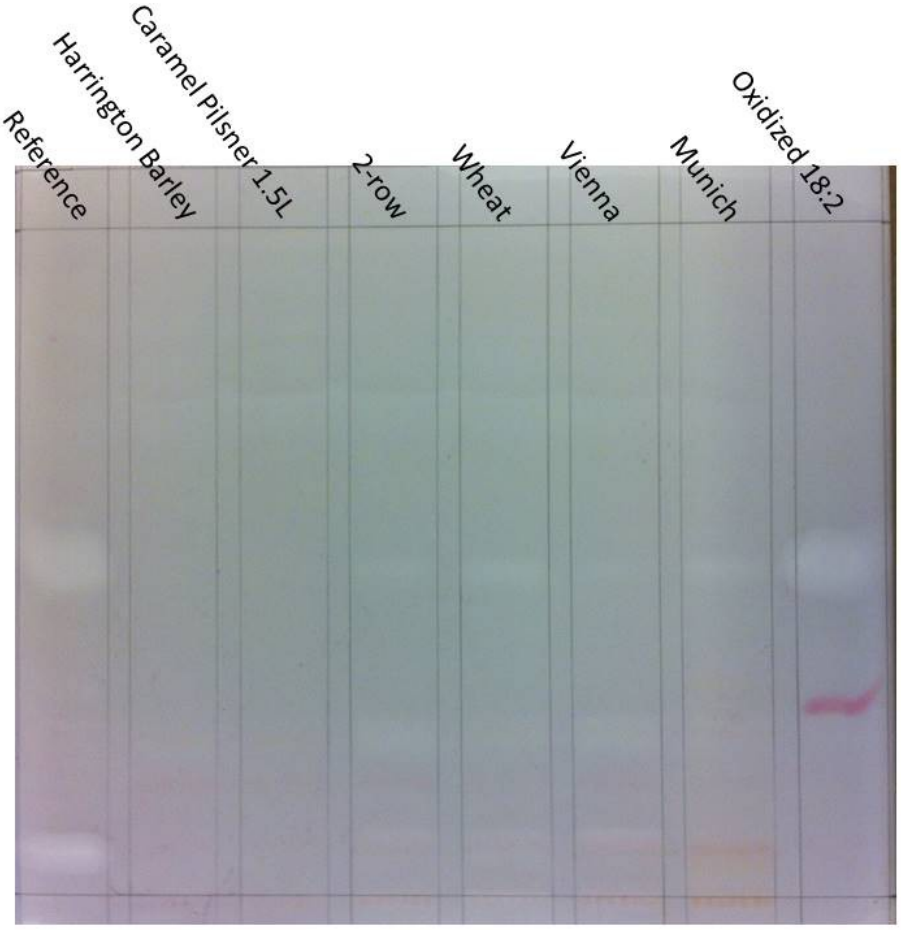

Reference
Harrington Barley
Caramel Pilsner 1.5L
2-row
Wheat
Vienna
Munich
Oxidized 18:2

Figure 5b

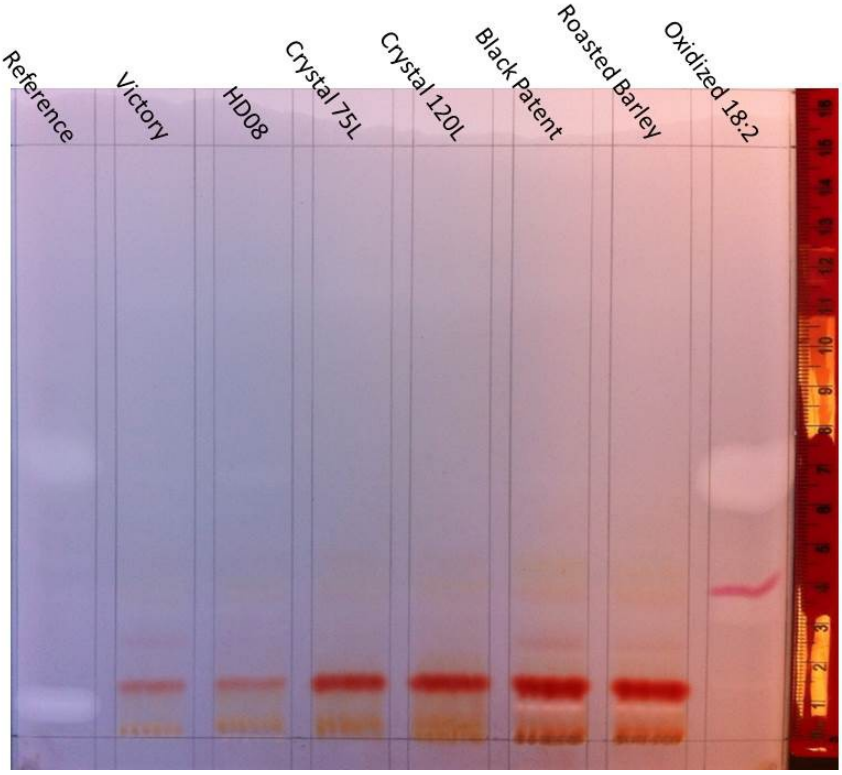

Reference
Victory
HD08
Crystal 75L
Crystal 120L
Black Patent
Roasted Barley
Oxidized 18:2

Figure 5c

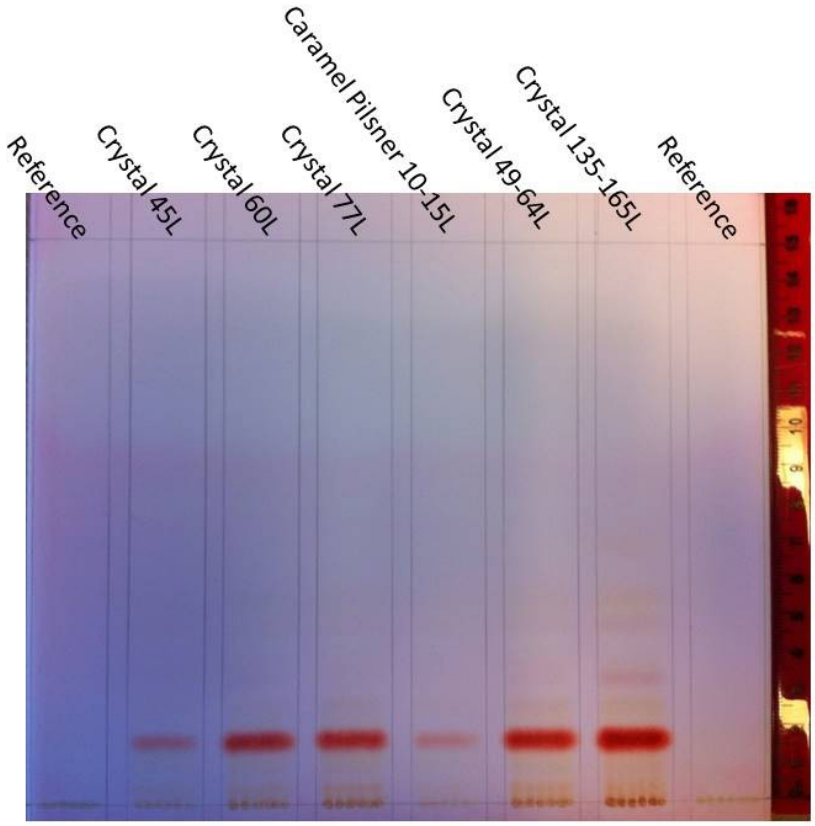

Reference
Crystal 45L
Crystal 60L
Crystal 77L
Caramel Pilsner 10-15L
Crystal 49-64L
Crystal 135-165L
Reference

Figure 5d

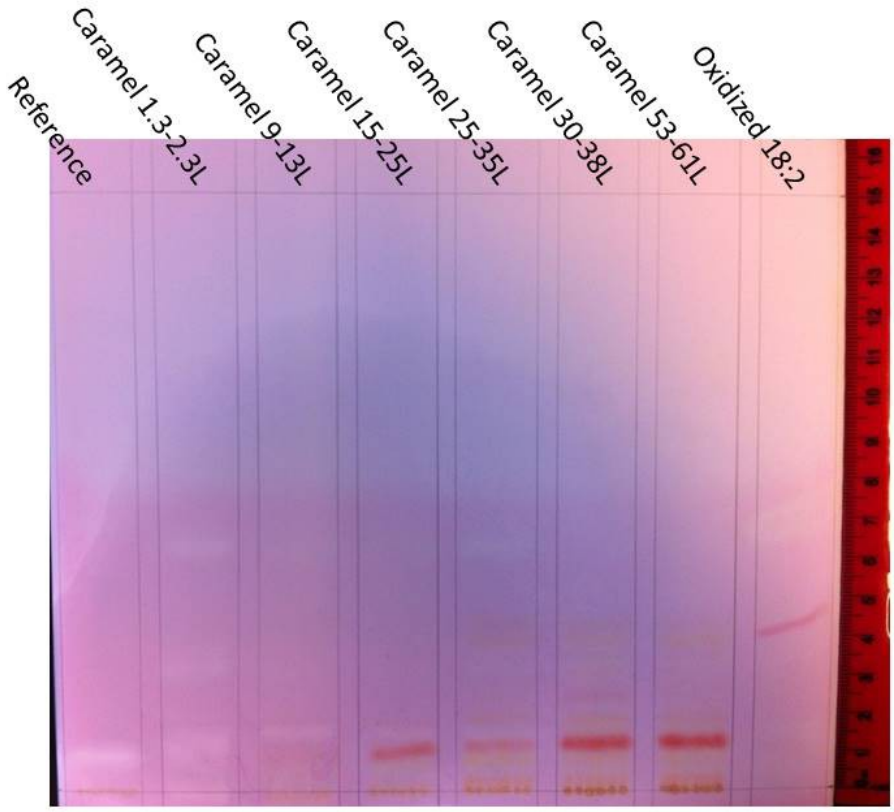

Reference
Caramel 1.3-2.3L
Caramel 9-13L
Caramel 15-25L
Caramel 25-35L
Caramel 30-38L
Caramel 53-61L
Oxidized 18:2

Figure 5e

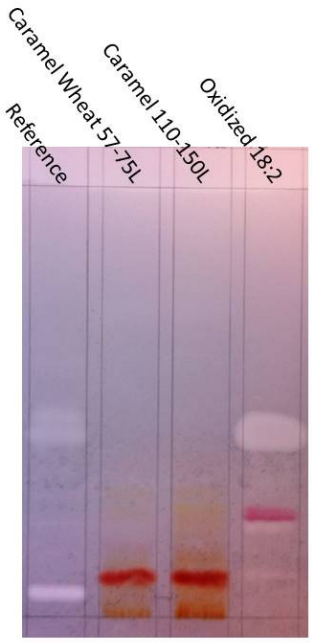

Reference
Caramel Wheat 57-75L
Caramel 110-150L
Oxidized 18:2

Figure 6a

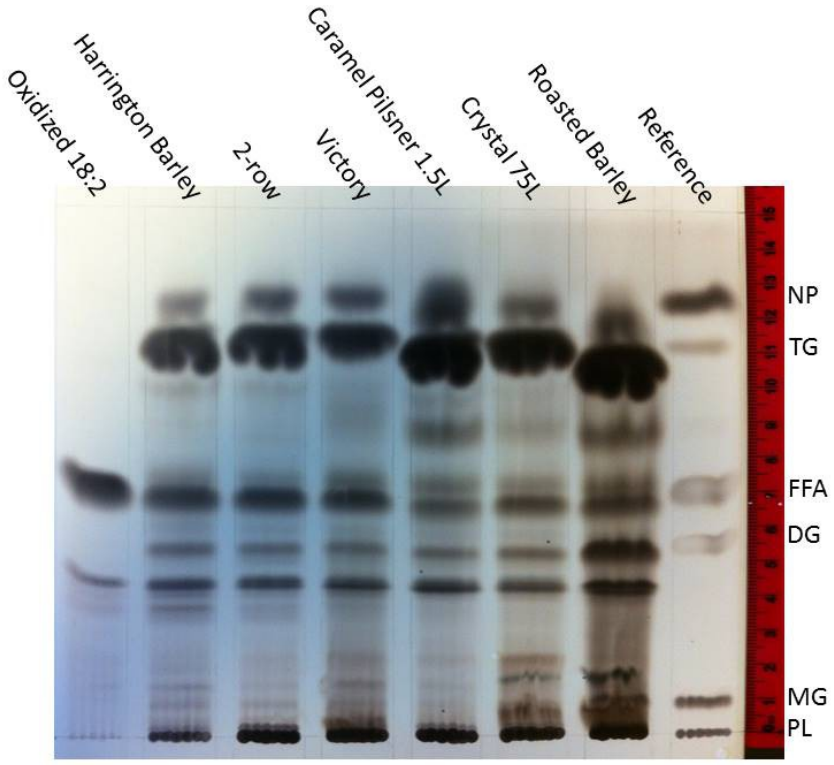

Oxidized 18:2
Harrington Barley
2-row
Victory
Caramel Pilsner 1.5L
Crystal 75L
Roasted Barley
Reference
NP
TG
FFA
DG
MG
PL

Figure 6b

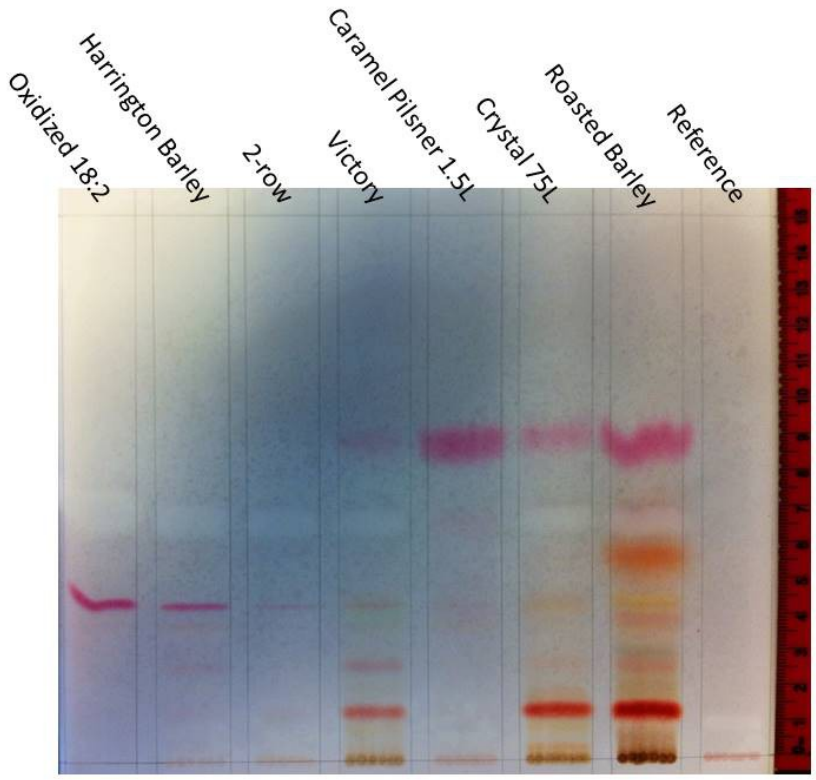


Figure 7a

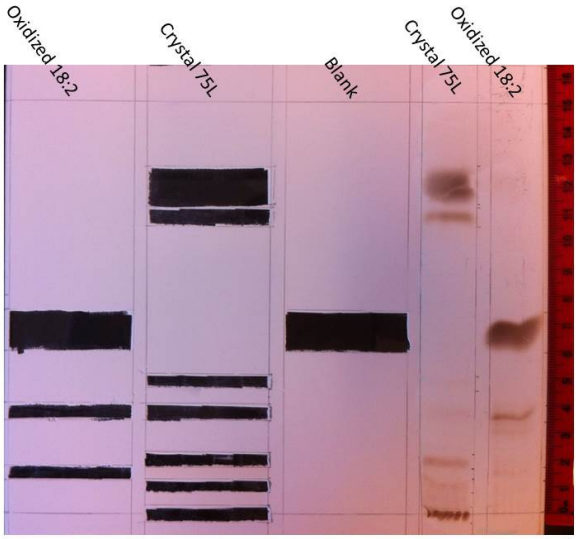


Figure 7b

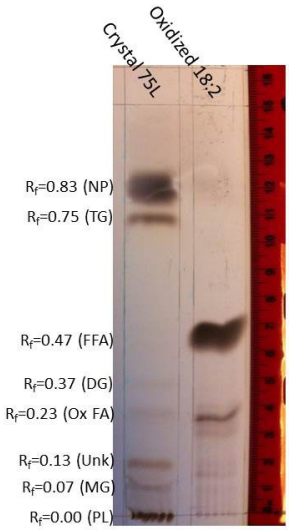
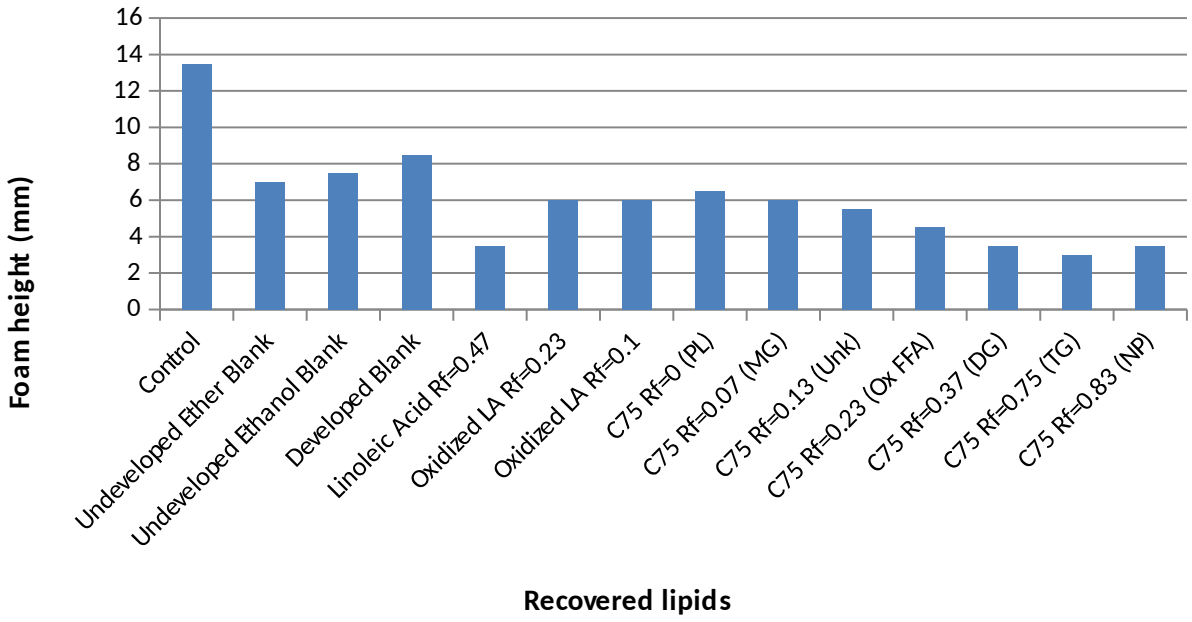


Figure 8


Foam height (mm)
16
14
12
10
8
6
4
2
0
Control
Undeveloped Ether Blank
Undeveloped Ethanol Blank
Developed Blank
Linoleic Acid Rf=0.47
Oxidized LA Rf=0.23
Oxidized LA Rf=0.1
C75 Rf=0 (PL)
C75 Rf=0.07 (MG)
C75 Rf=0.13 (Unk)
C75 Rf=0.23 (Ox FFA)
C75 Rf=0.37 (DG)
C75 Rf=0.75 (TG)
C75 Rf=0.83 (NP)
Recovered lipids