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The application of metabolomics to ascertain the significance of prolonged maturation in the production of lager-style beers.

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Abstract

NMR focused metabolomic analysis has been employed to ascertain the extent to which a diversity of non-volatile substances change in level during maturation and storage on a pilot and commercial scale. No substantive changes were observed, leading to the conclusion that once materials such as vicinal diketones and acetaldehyde have been dealt with, there is no merit in prolonged storage of beer.

Key words: Beer, lagering, metabolomics, non-volatile substances, NMR, storage

Introduction

There is a widespread acceptance of the dogma that certain beers, notably lagers, require a degree of storage to effect their maturation (1). This practice presumably originated from the days when beers were perforce held through the summer months (lagered) as the brewing of new batches of beer was forbidden (2).

It is helpful to divide maturation into two separate requirements: the physical stabilization of beer and the refinement of flavor.

In terms of the former, which is frequently referred to as colloidal stabilization, we have long since arrived at a situation wherein there is clearly no necessity for prolonged treatment times. The availability of palliative treatments to remove haze-forming polypeptides, polyphenols, polysaccharides etc., together with fining agents, centrifuges, filters as well as a recognition that it is the lowness of the temperature that is more important than lengthy holding time that is the more relevant in cold conditioning all render extended processing irrelevant (3 ,4).

It is the matter of flavor maturation that remains controversial, with polar opinions ranging from those who insist that it is essential that lager-style beers have a prolonged aging period in the cellar through to those adamant that such periods can be very brief, especially as another of the

original purposes of lagering was an increase in carbonation, which of course can nowadays be effected in short order (5).

Two of the key volatile substances that historically were removed in lagering are the vicinal diketones (VDK, 6) and acetaldehyde (7). However, the scientific understanding of the origins and control of these substances is now thoroughly appreciated. VDK can be dealt with effectively by careful attention to primary fermentation conditions and even for those insistent that more needs to be done, there is a range of options to accelerate the removal of these molecules (8). Effective removal of acetaldehyde is even more straightforward.

The question is begged, then, if there are any other chemical entities that change in their levels, either increase or decrease, in maturation, thereby benefitting the flavor of beer. The only paper that the authors have located that dwells on this issue was by Masschelein (9). In it, he claims that amino acids, peptides, nucleotides and organic acids as well as inorganic phosphates are released by yeast when left in contact with the beer and he indicates that this is a desirable occurrence.

Here we have applied the tool of metabolomics to investigate whether any significant changes occur during the ageing of a lager, beyond the matter of removing vicinal diketones and acetaldehyde.

Materials and Methods

Brewing and sampling aged beer

The experimental beer was brewed on a 176 L automated system. Salts (5 g magnesium sulfate, 5 g Calcium sulfate, and 20 g calcium chloride) were added to 98.4 L of 57°C strike water in the mash tank. Thirty-five kg of pilsner malt was milled on a two-roll mill and added to the mash tank. The mash was then topped off with 6.8 L of water. Mechanical agitation was used during mash heating. After the grist was added, the mash was held at 55°C for 10 minutes, it was then raised to 60°C for 10 minutes, 65°C for 30 minutes, and 76°C for 10 minutes. The mash was then transferred to the lauter tun, where it was vorlaufed for 10 minutes. The wort was then transferred to the kettle. Sparging was with water (74.3°C) for 74 minutes until kettle full (212 L) was achieved. Once the wort level in the kettle reached 45 L, the lower internal calandria was initiated, followed by the upper calandria when the wort level reached 113 L. The wort was boiled for 90 minutes. Magnum hop pellets (70 g) were added 30 minutes after the start of the boil. This was followed by an addition of 340 g Kazbec hop pellets after 60 minutes. After 85 minutes, 17 g Protofloc and 15 g Yeast nutrients were added. Kazbec hop pellets (100 g) were placed in the whirlpool prior to the transfer of wort. After the boil concluded, 177.5 L of wort was transferred to the whirlpool. The wort was then cooled through a heat exchanger to 20.1°C and transferred to a cylindroconical fermenter. The beer was pitched with BSI Czech Lager Yeast at a rate of $2x10^6$ cells/mL/^{o}Plato. The fermentation was carried out at 10°C. It took 6 days to reach 6°Plato at which point the beer was allowed to free rise up to 15°C for a vicinal-diketone rest. After 11 days, the beer was cold crashed for three days, which

brought the temperature down to 1.4°C. At this point, the beer was transferred into six 18 L kegs. Three of the kegs were then filtered into clean 18 L kegs using two polysponge cartridge type filters in series. The first filter was a 3-micron super high efficiency 1D by BevBright followed by an absolute rated 10" sterile 0.45 µm BevBright filter. The kegs of the three filtered beers were stored at -1°C for one month. This filtered beer was considered finished beer. Carbon dioxide was used to pressurize the filtered beer for the purpose of forced carbonation as well as for sample acquisition. The three unfiltered beers were conditioned at 2°C for one month. During the conditioning stage, forced carbonation was not desired; so nitrogen was used to pressurize the tank in order to retrieve samples from the keg. Samples were taken daily for the first week and then once a week for the following three weeks. All samples were immediately placed in a -20°C freezer and stored there until sample preparation for NMR analysis. Metabolites in frozen samples are assumed to be stable at -20˚C for short term storage and limited freeze-thaw cycles (10).

A commercial pilsner-style lager (4.9% ABV; starting yeast count $1.5x10^6$ cells/mL/ ^{o}Plato) was stored at 13.7°C for the first 14 days and then was lowered to -1.6°C during the following 16 days in tanks that were 8.2 m high, 2.9 m wide and with a cone angle of 70^o. Samples were collected at nine time points during the 30 days of maturation.

Sample handling and NMR spectroscopy

Frozen samples were de-frosted at room temperature. Once liquid, the samples were placed in Amicon Ultra-0.5 Centrifugal Filter Units with Ultracel-3 membranes, which were previously cleaned with deionized water. An internal standard containing 5 mmol/L of DSS-d_6 (3-(trimethylsilyl)-1-propanesulfonic acid-d6) and 0.2% NaN_3 (to prevent bacterial growth) in 99.8% D_2O (for instrument locking) was added to each sample in a ratio of 1:10. Following this step, the samples were adjusted to a pH of 6.8± 0.1, and NMR spectra were acquired using a Bruker Avance 600 MHz NMR spectrometer following the method laid out by Slupsky et al. (11). Metabolites were assigned using Chenomx NMRSuite Profiler v8.31 as described elsewhere (12). The compounds found in the Chenomx library have been verified against known concentrations of pure compounds and are shown to produce accurate and reproducible results (11, 12).

Statistical Analysis

Statistical analysis of variance (ANOVA) and correlation analyses were performed using R (R Development Core Team, 2014; http://www/RXproject.org). Regression analysis was performed using GraphPad Prism. Principal component analysis was performed using Umetrics SIMCA 13.0.3.

Results and Discussion

Comparing unfiltered beer during maturation to filtered beer during storage on the pilot scale

To examine the impact of yeast on beer during the maturation stage of beer production after fermentation, a lager brewed on a pilot system was divided into two streams: filtered and unfiltered. While the filtered beer was sent through two cartridge filters in series, the unfiltered beer was simply racked off the bulk of the yeast. Samples were taken at multiple time points for both treatments and the concentrations of the metabolites present in those samples are shown in Table 1. Figure 1 shows a PCA of the metabolite concentrations obtained from samples collected from three replicate kegs for the filtered and unfiltered lagers. Two samples were outliers. These samples corresponded to first sample taken on two separate days from the same keg (Figure 1A). Examination of the metabolite concentrations from these two samples (collected on days 2 and 3) revealed that the concentrations were approximately 1/3 smaller than the two replicate samples taken on the same day. Therefore, these samples were considered outliers and removed from further analysis. Comparison of filtered and unfiltered beers (Figure 1B) revealed no clustering based on filtering. Moreover, no clustering was observed based on day of sample collection. Additionally, comparison of the samples taken at day two from the filtered beer (light open circles) and samples taken at day 30 from the unfiltered beer (black closed circles), which represent beer conditioned on yeast, tend to cluster in the middle of the PCA.

Analysis of variance (ANOVA) was performed on the measured metabolites to determine if there were significant ($p<0.05$) differences between treatments. When comparing the average across kegs, fructose and ethanolamine were the only metabolites that differed significantly between the two treatments (Table 1). After multiple comparisons correction (with false discovery rate set at 5%), neither of these metabolites were significantly different. In both the filtered and unfiltered beer, fructose appeared to increase between each time point; however, regression analysis revealed a non significant increase. Ethanolamine remained consistent throughout the maturation.

It has been suggested that yeast autolysis could be a main factor in the increased mouthfeel described in beer aged in the presence of yeast. A number of autolysis products including amino acids, amino acid derivatives, nucleosides, and nucleoside derivatives were measured in this study. Interestingly, all of the amino acids and their derivatives were not different between the filtered and the unfiltered beer.

Previous research also found an increase in concentration of nucleosides, specifically, cytidine, uridine, guanosine, and adenosine during induced yeast autolysis (13). These metabolites were not different between the filtered and unfiltered beers.

Overall, the data collected does not suggest that yeast are undergoing autolysis or that lagers conditioned in the presences of yeast are markedly different from lagers conditioned without yeast or even lagers without prolonged maturation of any type.

Metabolomic changes during the maturation of a commercial lager

To examine the metabolomic trends during maturation with yeast on a commercial scale, samples were taken at nine time points during the maturation stage of a commercially produced lager (Table 2).

No significant changes in concentration were observed during the maturation of the commercial lager. For the vast majority of the metabolites measured during commercial maturation, non-significant fluctuations in concentration occurred.The concentrations appeared to fluctuate less after 23 and 30 days of maturation, but this may be misleading since the measurements are more spread out temporally. With this in mind, it is difficult to assign a weight to the importance of these small changes in metabolite concentrations.

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General discussion and conclusions

It has become a part of received wisdom that lager-style beers should be stored post-fermentation, although the rationale for this is less than clear (14, 15, 16, 17). Perusal of the justification for the lagering process highlight the need to carbonate, to cold-stabilize and, in respect of flavor, to deal with vicinal diketones, hydrogen sulphide and acetaldehyde. The simple reality is that all of these requirements can be achieved without prolonged beer storage, as was mentioned into the Introduction to this paper. Thus we are left with some nefarious mention of a lager being brought to some superior state of aroma and taste balance in a storage period. As stated above, the only paper to firmly refer to changes in the level of non-volatile materials derived from yeast is that of Masschelein (9). As reported in the present paper, we have been unable to show that there is a convincing change in the level of any flavour-relevant substance in maturation of pilot scale and commercial brews. The present paper has not dwelled on volatile substances, however it is amply documented that the key entities such as the esters, sulphur-containing molecules, vicinal diketones, carbonyl substances (such as acetaldehyde) etc should be controllable by competent fermentation and upstream process practices (18). For example there are those that say that lagering is necessary to remove undeirable sulphidic character, e.g. that arising from hydrogen sulphide. However ensuring vigorous fermentation causes this substance to be purged with the fermentation gases (19). In just the same way entities like diacetyl (6) and acetaldeyde (18) can be eliminated in the fermenter and without recourse to lengthy storage periods.

There is of course no question that the flavor of beer changes with time (flavor instability), an occurrence that is undesirable for most beers but potentially favorable for more alcoholic brews (19). However this is a very different matter from the maturation of beer in the brewery.

The present authors contend that whilst there may be a need for some brewers to address matters like diacetyl, H_2S, acetaldehyde and perhaps a few other volatile substances post primary fermentation it is simply a reflection of them not having sought to, or succeeded in, dealing with them earlier.

The authors suggest that perhaps there is but one area worthy of further investigation in the context of flavour maturation and that would be in respect of polyphenols. Are there changes in the polymerization of such materials which influences the character of beer? We also suggest the need for authoritative organoleptic investigations such that the changes (if any) that occur during lagering might be legitimately identified.

However, in respect of the current study, the overall lack of trends differentiating beer matured in the presence of yeast from beer conditioned without yeast, suggests that prolonged contact with yeast is a nonessential step in lager production in respect of non-volatile compounds. Our conclusions concur with those drawn by Rennie and Wilson (20).

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298 Table 1. The average (n=3) concentration (μM) and standard error of metabolites sampled from
299 three kegs, sourced from one fermentation, during the maturation (2°C) of unfiltered beer and
300 the average (n=3) concentration (μM) and standard error of metabolites sampled from three
301 kegs, sourced from the same fermentation as the unfiltered beer, during cold storage (-1°C) of
302 the beer post filtration. Both beers were sampled at four time points over 30 days.
303

	Unfiltered Beer					Filtered Beer				
	Time (Day)					Time (Day)				
Metabolite	2*	3*	8	30	Average Standard Error	2	3	8	30	Average Standard Error
Sugars										
1,6-Anhydro-β-D-glucose	75	86	80	80	7	93	76	80	71	31
Fructose	496	639	529	599	58	510	522	513	557	213
Gentiobiose	376	515	350	392	62	512	466	395	376	120
Glucose	1227	1345	1103	1236	224	1250	1436	1243	1145	492
Isomaltose	1154	1276	907	1049	246	1211	1129	1082	1129	365
Isomaltotriose	209	259	244	275	29	249	248	274	275	89
Maltose	7082	8708	7006	6873	725	8478	7016	7805	6544	2525
Melibiose	222	240	285	244	53	258	288	289	316	98
Xylose	356	361	319	392	76	390	407	377	350	138
Amino Acids and Derivatives										
4-Aminobutyrate	606	688	593	633	81	716	697	647	631	193
Alanine	1675	1894	1584	1714	201	1898	1889	1673	1703	552
Asparagine	321	360	310	311	40	357	346	317	322	100
Aspartate	325	375	315	331	36	366	368	332	338	104
Betaine	766	863	735	790	97	833	874	782	804	264
Glutamate	527	641	523	603	79	649	523	463	636	251
Glutamine	113	131	107	153	22	133	129	122	108	46
Histidine	239	269	235	244	27	283	270	249	258	81
Isoleucine	281	313	266	287	30	335	314	270	284	85
Leucine	366	437	412	430	41	456	431	427	428	129
Lysine	217	226	206	207	28	237	225	216	219	74
Methionine	58	61	55	59	9	67	60	58	57	19
Phenylalanine	497	564	474	514	67	567	549	492	506	151
Proline	3308	3706	3229	3542	422	3899	3853	3646	3263	1084
Pyroglutamate	1153	1335	1119	1170	122	1276	1299	1138	1150	379
Threonine	123	138	77	114	27	108	100	96	99	34
Tryptophan	161	181	154	163	20	184	182	160	163	50
Tyrosine	494	560	446	485	53	492	529	464	461	158
Valine	713	807	676	709	77	818	789	716	717	224
Nucleotides and derivatives										
2'-Deoxyadenosine	178	202	165	183	18	201	199	180	186	58
2'-Deoxyguanosine	12	13	11	11	1	12	12	12	11	3

Adenine	7	9	7	8	2	7	7	6	7	4
Adenosine	101	114	96	104	13	115	112	102	103	31
Cytidine	150	169	144	152	16	171	170	151	153	48
Cytosine	7	7	8	7	1	8	8	7	7	2
Guanosine	222	251	258	254	11	255	246	250	244	69
Hypoxantine	19	20	15	18	2	19	20	17	17	6
Inosine	30	33	29	30	4	35	32	31	32	10
Oxypurinol	19	17	20	17	2	28	23	18	25	11
Thymidine	55	64	53	59	7	65	65	56	58	18
Uracil	45	47	42	42	6	51	48	44	44	13
Uridine	249	277	234	252	30	283	281	248	254	77
Energy related metabolites										
2-Methylglutarate	22	22	20	21	5	23	20	20	22	7
2-Oxoglutarate	36	37	32	35	7	42	39	35	38	13
Ethanol	661510	750148	630240	691894	102555	797279	746897	648956	671466	222031
Fumarate	36	41	34	37	4	41	40	36	37	11
Lactate	1033	1038	973	984	128	1119	1187	987	976	322
Malate	177	151	143	168	19	165	163	155	164	54
Pyruvate	395	442	369	393	52	467	434	390	411	132
Succinate	462	515	445	481	46	531	511	466	479	144
trans-Aconitate	16	19	15	18	2	19	19	17	18	6
Fatty acid associated metabolites										
Acetate	1222	1369	1152	1232	163	1359	1369	1184	1203	390
Acetoacetate	17	18	14	17	3	17	16	15	17	6
Choline	618	708	583	637	80	686	709	621	624	210
Ethanolamine	112	129	107	115	13	149	152	135	136	51
Glycero-3-phosphocholine	531	584	491	538	58	598	587	527	549	173
Glycerol	10357	11321	10188	11089	1099	12016	11834	10517	10752	3323
O-Phosphocholine	13	15	14	13	1	14	14	16	17	6
Vitamins										
4-Pyroxidate	18	14	13	14	3	15	17	14	14	5
Nicotinate	19	22	19	20	3	23	22	20	20	7
Pyroxidine	17	19	16	18	2	20	20	17	17	5
Plant associated metabolites										
Ferulate	11	10	10	11	1	13	11	11	11	4
Trigonelline	20	25	19	21	2	24	24	20	21	7
Miscellaneous metabolites										
Acetoin	6	7	5	6	0	7	7	6	7	2
Formate	68	79	66	71	7	78	76	68	71	22
Methanol	38	40	33	37	6	43	42	36	36	12
Propylene glycol	882	1039	688	778	288	833	739	800	618	270
3-Hydroxyisobutyrate	24	26	22	21	4	27	28	28	23	10

Critonellol	58	56	51	55	8	60	56	55	52	18
Dimethyl sufone	5	6	3	5	1	4	4	4	4	2
o- Cresol	8	8	4	8	2	7	6	5	6	4
Theophylline	4	4	4	4	0	4	4	4	4	2
*Average of 2 replicates.										

304

305

306 Table 2. Concentration (μM) of metabolites measured during the maturation process of a commercial

307 lager. The beer was stored at 13.7°C for the first 14 days and then was lowered to -1.6°C during the

308 following 16 days. Samples were collected at 9 time points during the 30 days of maturation

Metabolite	Time (Days)									% Net Change	Pearson's r	P-Value
	0	1	2	3	4	5	6	23	30			
Sugars												
1,6-Anhydro-β-D-glucose	211	189	208	206	170	197	189	125	210	0	-0.251	0.515
Fructose	1528	889	917	739	676	916	901	479	712	-53	-0.562	0.115
Gentiobiose	425	316	333	309	278	294	290	176	347	-18	-0.327	0.390
Glucose	3017	189	223	237	214	221	195	141	177	-94	-0.375	0.320
Isomaltose	993	749	986	957	644	921	995	484	1042	5	-0.058	0.882
Isomaltotriose	160	117	150	133	109	121	123	101	132	-18	-0.328	0.389
Maltose	12069	9115	6949	6118	5884	9107	6110	3566	5395	-55	-0.641	0.063
Melibiose	285	364	420	377	338	396	413	197	282	-1	-0.524	0.148
Xylose	715	684	898	863	673	867	882	394	688	-4	-0.424	0.256
Amino Acids and Derivatives												
4-Aminobutyrate	754	771	855	829	705	830	829	473	808	7	-0.290	0.450
Alanine	504	476	737	712	603	718	713	425	732	45	0.149	0.702
Asparagine	73	34	39	40	28	38	33	25	48	-34	-0.184	0.635
Aspartate	65	45	31	20	18	24	38	31	39	-40	-0.140	0.720
Betaine	919	952	1037	1008	883	1025	1003	578	966	5	-0.342	0.367
Glutamate	236	143	237	209	137	189	115	103	184	-22	-0.336	0.376
Glutamine	69	59	79	65	63	136	98	62	88	28	0.165	0.671
Histidine	90	78	98	95	85	102	98	62	103	14	0.021	0.956
Isoleucine	64	49	73	89	57	66	52	44	69	8	-0.110	0.778
Leucine	130	88	110	189	78	75	75	65	89	-32	-0.376	0.319
Lysine	105	125	114	103	109	115	97	68	105	0	-0.483	0.188
Methionine	20	15	17	17	14	19	19	11	20	0	-0.029	0.942
Phenylalanine	118	96	145	143	129	136	140	87	153	30	0.160	0.682
Proline	2828	2788	3313	3217	2625	3333	3186	1999	3120	10	-0.189	0.626
Pyroglutamate	1127	964	1294	1160	907	1154	1137	806	1055	-6	-0.346	0.362
Threonine	38	21	56	56	44	39	42	20	27	-29	-0.466	0.206
Tryptophan	86	85	105	100	89	105	99	63	103	20	-0.068	0.862
Tyrosine	209	196	287	277	231	268	286	184	286	37	0.186	0.633
Valine	189	185	268	281	245	305	287	170	287	52	0.187	0.630
Nucleotides and derivatives												
2'-Deoxyadenosine	148	157	163	167	139	155	172	94	159	7	-0.290	0.4487
2'-Deoxyguanosine	12	12	9	13	9	12	11	6	10	-17	-0.420	0.2597
Adenine	12	6	8	5	4	8	3	3	4	-67	-0.556	0.120
Adenosine	46	48	53	51	42	53	50	29	51	11	-0.236	0.541

Cytidine	107	104	116	109	96	113	105	67	106	-1	-0.382	0.311
Cytosine	3	5	5	4	3	4	3	3	5	67	0.204	0.599
Guanosine	202	202	216	217	188	226	212	143	210	4	-0.300	0.434
Hypoxantine	6	6	6	3	6	4	7	4	8	33	0.352	0.353
Inosine	58	60	71	65	57	63	66	38	65	12	-0.244	0.526
Oxypurinol	36	35	42	30	30	40	32	28	31	-14	-0.454	0.220
Thymidine	66	64	74	69	61	73	72	41	70	6	-0.274	0.476
Uracil	13	8	11	10	6	7	7	8	12	-8	0.144	0.711
Uridine	263	280	317	301	272	314	304	183	297	13	-0.242	0.530
Energy related metabolites												
2-Methylglutarate	11	11	14	11	11	13	7	11	12	9	0.024	0.950
2-Oxoglutarate	26	31	34	40	30	31	30	28	31	19	-0.110	0.777
Ethanol	532122	579855	622805	643788	564022	656840	667088	363097	614220	15	-0.213	0.582
Fumarate	47	47	53	51	44	52	51	27	49	4	-0.340	0.370
Lactate	914	876	843	784	718	946	862	740	853	-7	-0.205	0.596
Malate	151	171	228	202	141	196	176	125	119	-21	-0.628	0.070
Pyruvate	837	878	929	913	795	905	889	476	813	-3	-0.486	0.185
Succinate	393	399	457	405	353	443	445	278	405	3	-0.305	0.425
trans-Aconitate	14	15	18	15	15	17	18	12	17	21	-0.047	0.904
Fatty acid associated metabolites												
Acetate	292	173	560	549	469	567	566	370	632	116	0.449	0.225
Acetoacetate	11	14	14	18	12	16	17	11	16	45	0.116	0.766
Choline	974	973	1107	1074	915	1066	1067	604	1033	6	-0.302	0.430
Ethanolamine	124	146	100	102	104	177	174	93	189	52	0.390	0.300
Glycero-3-phosphocholine	20	22	21	15	14	16	21	10	22	10	-0.089	0.820
Glycerol	9960	11843	12565	11885	10346	11625	11681	7465	11171	12	-0.339	0.372
O-Phosphocholine	270	291	295	290	262	297	294	175	286	6	-0.331	0.384
Vitamins												
4-Pyroxidate	6	8	7	11	8	9	8	6	9	50	0.134	0.731
Nicotinate	5	3	4	4	5	4	4	3	4	-20	0.126	0.746
Pyroxidine	12	13	16	11	11	15	14	6	16	33	0.023	0.954
Plant associated metabolites												
Ferulate	8	13	9	11	10	13	11	10	13	63	0.410	0.273
Trigonelline	19	20	21	21	18	23	25	13	19	0	-0.303	0.428
Miscellaneous metabolites												
Acetoin	12	15	17	15	14	17	16	9	14	17	-0.332	0.383
Formate	49	30	38	38	30	34	35	20	32	-35	-0.514	0.157
Methanol	47	51	51	56	45	54	51	30	46	-2	-0.488	0.182
Propylene glycol	917	464	532	456	625	732	700	468	774	-16	0.104	0.789
3-Hydroxyisobutyrate	45	27	37	57	35	31	31	21	25	-44	-0.560	0.117
Critonellol	58	57	68	62	39	54	40	39	47	-19	-0.536	0.137
Dimethyl sufone	12	14	15	15	13	13	14	8	13	8	-0.365	0.334

o- Cresol	10	9	14	11	11	12	12	7	15	50	0.251	0.515
Theophylline	3	3	6	3	4	4	3	2	3	0	-0.288	0.452

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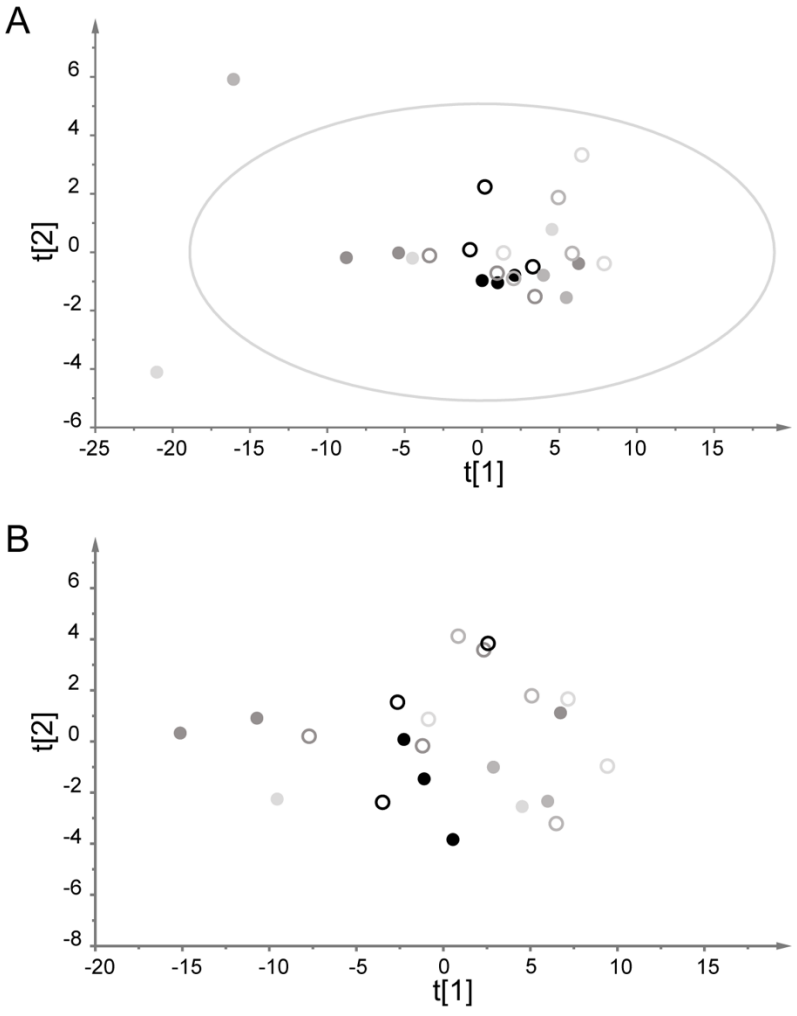
313 Fig 1. PCA of filtered and unfiltered beer at various time points during maturation. (A) All samples. The

314 ellipse represents Hotellings T2. (B) Removal of two outliers (outside of the Hotellings T2 limit).

315 Unfiltered beer closed circles; filtered beer, open circles. Day 2 (light grey), Day 3 (medium grey), Day 8

316 (dark grey), Day 30 (black).

317



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