Different Forms of Nitrogen and the Stability of Beer

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Nitrogenous compounds present in beer were determined by methods of chromatography, gel filtration, electrophoresis, and spectroscopy. The chill haze formation was shown to consist of α-hordein and β-globulin fractions to which the stability of the commercial product can be related. Effective means of removal of nitrogenous materials are described. The quality of beer is not determined by the content of total protein, but rather by the relative amounts of nitrogen that is soluble, that can be precipitated by $MgSO_4$, that is titratable by formaldehyde, and that is present as amino acid. All of these forms are found in beer after technological processing. With the use of appropriate filtering agents that have been covered in this study, it is possible to modify the nitrogen content in beer and improve the stability and hence the quality of beer. Particularly good adsorbents for this purpose were found to be kieselgel stabifix, diatomite, and bentonite.

The presence of nitrogenous and sulfurous compounds in beer and their chemical compositions have been reported by a number of researchers (Chen et al., 1973; Clapperton, 1971; Clark, 1960; Drawert et al., 1971, 1973; Drawert et al., 1973; Gorinstein, 1973a,b, 1974a; Heatherbell, 1976; Hebert and Strobbel, 1972; Hejgard and Boeg-Hansen, 1974; Hudson, 1973; Narziss and Roettger, 1973a,b; Phillips, 1972; Savage and Thompson, 1973; Sielicka, 1973; Sommer, 1974; Steiner, 1973; Ten Hoopen, 1973).

The levels of Lundin fraction nitrogen, amino acids, and microelements which are retained in a beer to maintain foam head formation, retention, palate fullness, flavor, and stability were also given (Bulgakov, 1959; Gorinstein, 1973b, 1974c, 1976).

In this study we will be interested in determining (a) the distribution of nitrogenous compounds in beer, (b) their effect on the chill haze stability of beer, and (c) effective means for their removal.

APPARATUS, REAGENTS, AND PROCEDURES

The present investigation was carried out on "Zhuguli" nonfiltered beer (for details, see Gorinstein, 1974a, b, 1975). For comparison we report results for brews clarified by filtering through cotton filter bed masses: control mass of 34 nephelos units and test mass of 55 nephelos units.

The chill haze was obtained by storing beer for 48 h at 0 °C, or by adding 0.5 mL of ammonium sulfate to 10 mL of beer and then cooling for 1 h (Bulgakov, 1959).

Total nitrogen was determined by the methods of Duma and Kjeldahl (AOAC, 1970; Bulgakov, 1958; Klimova, 1967). Then the nitrogen was multiplied by 6.25 to determine crude protein.

Organic nitrogen was determined using potassium persulfate (Gertner and Grdinic, 1965), and protein nitrogen alone after precipitation of the total protein with cupric hydroxide. Determination of formaldehyde titratable nitrogen was based on the addition of formaldehyde which binds with amino groups.

Amino nitrogen was determined by the ninhydrin method (Lie, 1973; Marinelli, 1975). Soluble and coagulable proteins were investigated according to the methods of Kolbach and Wilharm (Bulgakov, 1959).

The determination of amino acids was carried out on an amino acid analyzer (Arslanbekova et al., 1973; Hampel, 1973; Kurganova et al., 1974; Laszlo and Joth, 1972), or by means of ion-exchange chromatography using single and double columns (Maendl et al., 1974; Moll et al., 1972; Reiner and Piendl, 1974). This latter technique employed either cationic exchange resin KY-2 or stabifix. For chromatography on KY-2, the column was eluted with NH_4OH, and on stabifix with 70% formaldehyde solution, followed in both cases by hydrolysis with 6 N HCl. Nitrogen of the amino acids also produced was then determined by the Kjeldahl method. A sufficient quantity of the amino acids was isolated for UV, IR and NMR analyses.

Amino acids were analyzed on a Beckman Model 120/3 by the two-column procedure of Spackman et al. (1958), after hydrolysis of the protein samples with 6 N HCl at 110 °C for 24 h. Infrared spectra were taken on a Unicam SP 200G spectrophotometer in KBr and Nujol. Ultraviolet spectra were recorded on a ultraviolet spectrophotometer in the 200–450 nm region. Nuclear magnetic resonance spectra were measured on a Varian T-60 spectrometer with TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-d_4, $(CH_3)_3SiCD_2CD_2CO_2$) reference standard and deuterium oxide as solvent.

The nitrogenous substances were also fractionated according to Lundin (Bulgakov, 1959). The solubility of proteins in different solutions was the basis for selecting and utilizing the procedure of fractionation (for details, see Gorinstein, 1974b).

Colloid-protein (chill haze) stability of beer was judged using a limit of precipitation in milliliters of ammonium sulfate (Bulgakov, 1959) Moll and Vinh, 1973; Steiner, 1972). As the limit of precipitation increased, chill haze stability also increased.

Protein fractions were concentrated by the tannin–caffeine method (Gordon et al., 1950; Fertman and Gorinstein, 1968; Gorinstein, 1975). The tannin–caffeine method is based on the protein complex forming property of tannin. These complexes were then dissolved with caffeine. Glutelin and hordein, which are normally insoluble in milk aqueous conditions, were found to be soluble in caffeine.

For horizontal electrophoresis a veronal-medinal buffer of pH 8.6 (Gordon et al., 1950; Fertman and Gorinstein, 1968; Gorinstein, 1975), containing agar gel in 1.5–2% concentration, was used. The sample of beer (400 mL) was treated with (a) 30 mL of 4% solution of tannin, followed by dissolving the precipitate by addition of 0.2 g of dry caffeine, or (b) with 200 mL of 4 M ammonium sulfate in order to separate out the protein fractions. The samples of the concentrated protein (0.04 mL) were placed on the agar gel and electrophoresis was carried out for 5 h at 40–80 V. Then the gels were strained for 2 h in 0.5% amido black in 7.5% acetic acid solution. They were then destained

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0021-8561/78/1426-0204$01.00/0

Table I. Distribution of Proteins (mg/100 mL), Removed from Beer by Various Filtering or Precipitating Agents

Protein fractions[a]	Tannin-caffeine	Ammonium sulfate	Sephadex G-25	Sephadex G-50	Sephadex G-75	Sephadex G-100	Acrylex P30	Stabifix	Bentonite
Total protein	280.00	271.48	25.18	100.00	120.00	31.40	170.16	134.27	145.18
Albumin	71.25	69.24	18.16	30.00	20.00		40.24	30.25	35.15
Glutelin-1	70.00	68.97		44.18	20.15		37.18	29.18	31.18
Glutelin-2	18.34	14.25		7.14	10.22		12.15	7.24	8.25
β-Globulin	51.44	49.34	4.15	18.18	40.15	7.10	30.25	23.14	27.40
α-Hordein	21.12	20.14			10.35	10.12	13.16	1.15	9.14
β-Hordein	16.24	15.45			6.18	12.15	10.24	21.18	17.27
γ-Hordein	26.38	23.27			10.18		22.35	11.12	16.37

Protein fractions[a]	Diatomite	Polyacryl-amide	Polyamide resin "Perlon"	Silica gel	Silicic acid	Aluminum oxide	Florisil	Perlite	Molecular sieve and molselect G25
Total protein	138.81	190.40	163.27	223.18	171.24	230.24	150.34	220.44	209.14
Albumin	32.24	47.20	40.14	52.14	43.25	57.24	36.25	50.24	50.18
Glutelin-1	30.18	44.18	38.17	50.18	41.18	53.18	32.18	48.16	48.14
Glutelin-2	8.27	12.15	10.27	16.25	10.24	14.25	9.14	12.14	12.15
β-Globulin	26.18	32.41	30.14	43.18	31.14	63.18	28.25	40.25	33.14
α-Hordein	7.12	15.25	12.16	20.14	14.15	20.18	12.15	18.27	16.18
β-Hordein	17.14	23.44	18.25	26.18	18.24	15.34	16.27	25.18	23.14
γ-Hordein	15.14	15.21	14.28	14.24	12.18	16.34	16.21	23.25	15.16

[a] As determined by electrophoresis of the filtered or adsorbed protein.

and stored in 7.5% acetic acid solution.

A standard for classification of the protein separation by electrophoresis was to use a molecular weight marker of human serum.

After electrophoresis, the agar gel, mentioned above, was cut and extracted. The nitrogen content of extracts was estimated by the Kjeldahl procedure. The dilute electrophoretic fractions were also dialyzed to remove interfering compounds and the protein content was estimated by the spectrophotometric method (Groves et al., 1968; Srikanta and Narasinga, 1974). The amount of protein substances was determined by adsorption. The adsorbent was washed with 0.05 N NaCl solution and packed into a 2.0 × 75 cm column. A known volume of sample solution (1–2 mL containing 25 mg/mL) was carefully layered on the gel column and allowed to be adsorbed. The column was then eluted with 0.5 N NaCl solution at 24 mL/h. The absorbance of the fractions at 280 nm was measured and the nitrogen contents of the dialyzed and freeze-dried column fractions were estimated by the micro-Kjeldahl procedure. This procedure was carried out on all of the following adsorbents: Sephadex G-25 (mol wt, 4000), G-50 (mol wt, 10000), G-75 (mol wt, 50000, and G-100 (mol wt, 100000), acrylex P-30, kieselgel stabifix, kieselguhr, bentonite, polyacrylamide, polyamide resin "Perlon", silica gel, silicic acid, aluminum oxide, Florisil, and perlite. All the above adsorbents were used in the volume ratio of adsorbents to sample 50:1, but for the adsorbents molecular sieve (mesh 44), Bio-Gel molselect G25 and sample the volume ratios were 10:40:1.

The sources of the adsorbents, mentioned above, were from USSR, Czechoslovakia, East Germany, Hungary, Poland, and Romania.

Diatomite is a commercial product (Abaryshev, 1972; Butterworth, 1976). Kieselgel stabifix (Stabifix) is a commercial form of silica gel, but in this investigation stabifix differs from the silica gel by its stabilizing effect (the stabilizing effect of silica gel is 50% that of stabifix).

In this study the results used were the averages of 30 different samples of beer.

RESULTS AND DISCUSSION

It was possible by employing different analytical and chemical methods to classify the various forms of nitrogen-containing compounds in beer, and with the use of filtering media to bring about their removal.

It can be seen from Table I that a number of means may be employed for the removal of protein from beer. Comparison shows that the most efficient removal is by precipitation using the tannin–caffeine method and slightly less efficient is precipitation by ammonium sulfate. Filtration through Sephadex is not so effective in removing protein in bulk but can be exploited in selective removal of protein according to size. From this part of the study, it is observed that the main fractions of beer protein have molecular weight <100000. In all cases (Tables I and II) clarification of beer by the media employed decreased the content of all forms of nitrogen.

In Table II, columns a, b, c, d were respectively non-filtered beer; a control beer, filtered through a controlled filtering mass; a test beer, filtered through a test filtering mass; and a test beer, which was filtered twice through a test filtering mass.

Filtration techniques, using the experimental filtering mass as filtered bed and double filtration, appeared to be most effective in removing β-globulin, amino acids, albumin, coagulable, $MgSO_4$-precipitable, organic, salt-soluble, amino, formaldehyde-titratable, and Lundin fraction A nitrogen (Table II).

From the results in Table II it can be seen that not all the nitrogenous compounds were soluble.

The following observations regarding the distribution of the various forms of nitrogen-containing compounds can be made (see Table II): of the total nitrogen, 45–50% is in soluble form, and of this, 40% is coagulable. Also that 40% of coagulable nitrogen was possibly β-globulin, which during the fermentation process forms complex molecules of high molecular weight, which were insoluble. The coagulable nitrogen, perhaps, was hordein, whose presence together with anthocyanogens was identified by chill haze. The coagulable nitrogen is present in smaller amount than the Lundin fraction A nitrogen, and formaldehyde titratable nitrogen is present in smaller amount than the amino acid Lundin C fraction. The salt-soluble fraction which is assumed to contain the β-globulin present was in greater amount that the β-globulin found by electrophoretic treatment of the total protein.

Electrophoretic fractionation of proteins divides them into two groups: the albumins-glutelins and the globu-

Table II. Nitrogenous Composition (mg/100 mL) of Samples by Different Means of Clarification

Different forms of nitrogen	Non-filtered (col. a)	Control[a] (col. b)	Test[b] (col. c)	Test (double filtration)[b] (col. d)	Bentonite	Stabifix	Diatomite	Test and diatomite
Total								
Kjeldahl	56.18	53.40	46.24	43.97	48.24	50.14	50.00	44.15
Diffusion	56.37	53.90	46.90	43.90	48.70	51.02	50.07	44.24
Duma	56.23	53.49	46.33	44.02	48.94	50.90	49.17	44.80
Organic	47.85	45.37	33.92	32.47	34.48	34.00	34.14	32.87
Lundin fractions								
A	19.42	18.32	12.00	10.96	12.94	12.00	12.00	11.32
B	9.52	9.40	9.28	9.18	9.41	9.34	9.40	9.30
C	26.38	25.40	23.14	22.18	24.37	23.64	24.18	22.75
Total protein	44.80	43.25	32.84	32.21	42.15	36.15	40.12	32.37
Formaldehyde titratable	16.23	15.94	12.95	12.00	14.92	13.83	14.60	12.75
Amine	8.14	8.00	7.47	7.15	7.94	7.65	7.40	7.47
β-Globulin electrophoresis	8.23	8.03	6.38	6.00	7.49	6.14	7.0	6.18
Hordein electrophoresis	9.24	9.06	8.21	6.98	7.94	7.15	7.40	7.35
Amino acids	23.18	22.15	18.24	17.26	21.14	20.15	21.08	17.93
Soluble	26.41	24.99	20.24	19.73	23.75	23.00	23.40	20.89
Coagulable	13.43	17.80	9.15	8.40	11.15	10.93	11.00	8.85
$MgSO_4$-precipitable	12.35	12.00	10.04	9.18	11.90	9.00	10.12	9.98
Water-soluble (albumin)	13.24	12.80	12.15	11.50	12.00	11.90	12.0	12.00
Salt-soluble (globulin)	9.13	9.00	7.16	7.00	8.12	7.94	8.04	7.10
Alkaline-soluble (glutelin)	15.25	15.00	14.70	14.00	15.00	14.85	15.0	14.90
Alcohol-soluble (hordein)	11.14	10.94	10.00	9.40	11.80	10.80	11.40	9.70
Limit of precipitation (mL of ammonium sulfate/100 mL)		8.0	11.0	12.0	10.0	11.0	11.0	12.0

[a] Clarified with filter mass 34 nephelos. [b] Clarified with filter mass 55 nephelos.

lins-hordeins. The banding patterns of the extracted proteins in the precipitate after clarification by all adsorbents are qualitatively the same and identical with α-hordein and β-globulin. From the data compiled in Table I, it was possible to establish the degree of adsorption of proteins in the β-globulin and α-hordein electrophoretic fractions to the various adsorbents employed according to the following ascending order: aluminum oxide > silica gel > perlite > molecular sieve and molselect G25 > polyacrylamide > silicic acid > acrylex P30 > polyamide resin "Perlon" > Florisil > bentonite > diatomite > stabifix (Table I). These data can be applied to the clarification of beer, leading to higher chill haze stability of the product.

The analyses of the hydrolyzed protein precipitate of the sample after clarification with different adsorbents were thus: (a) amino acid chromatographic analyses show the qualitative content of amino acids: aspartic, threonine, serine, glutamic, proline, glycine, alanine, valine, methionine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine; (b) IR ν max (KBr) 3400 (C=NH), 3300 (broad OH, NH), 1715 (ketone), 1660 (C=O), 1650 (amide), and 1580–1500 cm^{-1} (C=C aromatic); (c) UV_{max} (MeOH), λ_{max} 225 and 275 nm; (d) ^1H NMR (60 MH_z, D_2O) δ_{TSP} 8.65, 7.3, 6.7. The peaks obtained in IR, UV, and NMR spectra have the same chemical shifts both for control and test samples.

The amino acid composition which was determined in the chill haze is the same as was found in beer (Gorinstein, 1974c). Perhaps, in chill haze formation it was an effect of sulfur-containing amino acid such as methionine.

The chemical shifts of the IR, UV, and NMR produced by the chill haze show that this contained nitrogenous compounds (also aromatic), which gave under hydrolytic dissociation the following groups: sulfur, imine, carbonyl, carboxyl, and hydroxyl.

The chill haze stability (see Table II, the last line) is found to be in inverse proportion to the different forms of organic nitrogen. The greatest amount of nitrogen is in the salt-soluble fraction which has the lowest chill haze stability and the least nitrogen is in the soluble fraction (albumin). A decreased amount of coagulable protein thus results in a higher quality of beer.

The greatest colloidal stability (limit of precipitation) was attained by double filtration through filtering masses and also by clarification through both filtering mass and diatomite, good adsorbents were also stabifix and bentonite.

ACKNOWLEDGMENT

I am very greatful to Professor S. Sarel for his interest in this work and R. Victor for her help in the organization of this manuscript.

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Received for review December 4, 1975. Accepted June 3, 1977.

Effect of Ethanol on Optical Rotation, Velocity of Mutarotation, and Equilibrium Constant of Lactose

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The effects of ethanol on polarimetric readings of lactose were studied. The specific rotation of both α- and β-lactose was less in ethanol solutions than in water. Also, as the percentage of ethanol increased, the specific rotation of lactose decreased. The relation was linear between the percentage of ethanol and the final specific rotation of lactose. The equilibrium constant of lactose was less in ethanol than in water, decreasing proportionately with the percentage of ethanol, $K = 1.68651 - 0.00415(\%\ \text{EtOH})$. Therefore, there was a direct relation between percentage of ethanol and percentage of α-lactose at equilibrium. The mutarotation of lactose was 2.3 times as fast in water as in 50% (v/v) ethanol. Both k_1 and k_2 were less in ethanol solution than in water, but k_1 decreased more (to 41%) than k_2 (to 46.5%) in 50% (v/v) ethanol compared to their values in water. This is responsible for the lower equilibrium constant of lactose in ethanol solutions than that in water.

Mutarotation, a phenomenon characteristic of lactose, is influenced by many factors (Nickerson, 1974). It is accelerated by increased temperature, becoming almost instantaneous at about 75 °C, and also by bases and acids (with the former more effective). Mutarotation rate is minimal at about pH 5.0, whereas at pH 9 equilibrium is established within a few minutes (Troy and Sharp, 1930). The action of salts is variable. Many salts can accelerate the mutarotation of lactose; for example, Haase and Nickerson (1966) have shown that whey–salt solutions increased the rate of interchange. Other substances can retard mutarotation. For example, mutarotation of lactose slows in the presence of sucrose (Patel and Nickerson, 1970), alcohols, and acetone (Herrington, 1934a; Richards et al., 1927). Various workers are not in complete agreement on the value of the mutarotation constant.

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The ratio of β to α at equilibrium, which defines the equilibrium constant ($K = \beta/\alpha = k_1/k_2$), is influenced by various factors. Temperature has a slight effect; with rising temperature the equilibrium constant decreases (Gillis, 1920; Nickerson, 1956; Parisi, 1930). Also influencing this value is the nature of the solvent. Bleyer and Schmidt (1923) stated that the equilibrium was displaced toward the formation of α-lactose in very strong acid solutions, and toward the formation of β-lactose in strong bases. Values for K, k_1 (the velocity constant of the α to β change), and k_2 (the velocity constant of the β to α change) have been calculated at different temperatures and pH by various workers (Hudson, 1904; Parisi, 1930).

The specific rotation of lactose varies with temperature and solvent. The specific rotation of lactose is increased in glycerol solutions and decreased in solutions of ethyl or methyl alcohol or acetone (Herrington, 1934b; Hudson and Yanovsky, 1917).

Previously (Majd and Nickerson, 1976) it was shown that alcohols decrease lactose solubility and that the

0021-8561/78/1426-0207$01.00/0