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Note

Determination of 2-acetyl-4(5)-tetrahydroxybutylimidazole in beers by high-performance liquid chromatography with confirmation by chemical derivatization

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2-Acetyl-4(5)-tetrahydroxybutylimidazole (THI) is a compound formed during the production of ammonia type caramel which is used as a colouring agent for beers. The substance has been found to cause lymphocyte depression in rats fed vitamin B_6-deficient diets[1]. As a result, international specifications now include a maximum concentration of 40 μg/g of THI in ammonia caramels (based on total solids content of the sample).

Two publications on the determination of THI in caramel have appeared in the past three years[2,3]. Both of these employ high-performance liquid chromatography (HPLC) either directly[2] or after derivatization of THI with 2,4-dinitrophenylhydrazine[3]. These methods were concerned with mainly caramel itself and not with determination in beers. Since caramel colour can be diluted by up to 1000 times in beer, methodology should be capable of quantitating 40 ng/ml THI in commercial samples. The present work includes method development for THI in beers that enables the detection of as low as 10 ng/ml THI with confirmation by chemical derivatization followed by analysis of the product under different HPLC conditions.

EXPERIMENTAL

Reagents

THI was dissolved in deionized water and diluted to yield a concentration of 10 μg/ml. Aliquots of this were used for direct HPLC analysis, for sample spiking and for derivatization studies. Pyridine "plus" (Applied Science Labs., State College, PA, U.S.A.) and acetic anhydride (J. T. Baker, Phillipsburgh, NJ, U.S.A.) were used as received. All other solvents and chemicals were analytical reagent-grade materials.

Cation-exchange columns

About 100 g each of Amberlite CG-50(H) type 1 (BDH, Toronto, Canada) and Dowex 50W-X8 (100–200 mesh) (Bio-Rad, Richmond, CA, U.S.A.) were allowed to soak in deionized water overnight. Each batch was rinsed several times with water, then a slurry poured to a bed height of 10 cm into separate 1 cm I.D. glass columns

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containing a glass wool plug. Each column was rinsed with at least 20 ml of water before addition of the samples.

HPLC

Separations were carried out employing a Beckman Model 114 pump, a Model 210 sample injection port (20 μl or 50 μl loop size) and a Micromeritics Model 788 variable-wavelength detector at 287 nm or 300 nm and 0.005 a.u.f.s. A Supelcosil LC-18 (5 μm) column (Supelco, Bellefonte, PA, U.S.A.) (15 cm × 4.6 mm I.D.) was used for the separations. For direct analysis of THI, two mobile phases were evaluated. These were; 0.005 *M* KH_2PO_4 (adjusted to pH 4.5 with 10% H_3PO_4) used for routine analysis and 0.02 *M* KH_2PO_4 (pH 4.0) used for confirmation. All mobile phases were filtered (0.45 μm) and degassed before use. The flow-rate was 0.7 ml/min. THI eluted around 6 min. For quantitation, peak height measurements were used.

A step gradient to 5% acetonitrile in mobile phase was initiated at 3 min and appeared in the chromatograms at 7 min. The step gradient was continued for 7 min to remove late eluting peaks before the next injection.

For determination of acetylated THI, the same HPLC system was used with a change in the mobile phase composition and the absorbance wavelength set to 287 nm. Three mobile phases were evaluated for the derivative. They all consisted of 0.02 *M* KH_2PO_4 (pH 6.0) containing either 12% (v/v) tetrahydrofuran, 32% methanol or 20% acetonitrile. The flow-rate was 1.0 ml/min. No step gradient was employed for the derivative.

Sample extraction

A 50-ml volume of degassed beer was passed through the Amberlite weak cation-exchange column and the eluate collected. The column was washed with 75 ml water and the eluate collected. The total eluate was then added to the Dowex strong cation-exchange column. The eluate was discarded. The column was then washed with 100 ml water which was also discarded. The THI was eluted with 100 ml 0.3 *M* hydrochloric acid and the eluate collected in a 500-ml round bottom flask. The solution was evaporated completely to dryness at 45°C for 30 min. The residue was dissolved in 5 or 10 ml water for HPLC analysis.

Acetylation

A 1.0-ml aliquot of the sample extract was passed through a C_{18} disposable cartridge (Sep-Pak, Waters Assoc., Milford, MA, U.S.A.) and the eluate collected. The cartridge was then washed with 3 ml water which was combined with the first eluate. This was diluted to exactly 4.0 ml then a 1-ml aliquot was transferred to a 3-ml Reactivial (Pierce, Rockford, IL, U.S.A.) and evaporated to dryness at 50°C under a stream of nitrogen. To the residue were added 10 μl of pyridine and 150 μl of acetic anhydride. The vial was capped and the contents were mixed and heated for 10 min at 90°C. After the reaction, the contents were evaporated just to dryness at room temperature under a stream of nitrogen. The evaporation was continued for an additional 5 min after dryness. The residue was dissolved in 1.0 ml water. Then, 1.0 ml chloroform was added and the mixture shaken gently. After separation, most of the chloroform was removed to a clean Reactivial and the extraction repeated with two 0.5-ml volumes of chloroform. The combined chloroform extracts were dried with

a small quantity of anhydrous sodium sulfate and then transferred to a clean vial and evaporated just to dryness at room temperature under nitrogen. The final residue was dissolved in 1 ml of HPLC mobile phase for analysis. This required allowing the solution to sit at room temperature for several hours or overnight.

RESULTS AND DISCUSSION

Direct analysis of THI in beer

The original method we employed for the direct determination of THI in caramel[2] was not suitable for routine determination of the compound in beers, due to the presence of too many coextractives. As a result, the two-column ion-exchange cleanup described by Kröplien[3] for caramel colours was employed with a reduced volume of beer (50 ml instead of 200 ml). The elution scheme was chosen after a number of optimization studies were carried out to obtain the best recovery with the least amount of interfering coextractives. Fig. 1 shows typical chromatograms for blank and spiked beer samples using 0.005 *M* KH_2PO_4 (pH 4.5) as the mobile phase and 300 nm as the absorption wavelength. At 7 min, a step gradient to 5% acetonitrile in mobile phase was incorporated to remove late eluting peaks. This shortened analysis time appreciably since otherwise some coextractives would still elute after 40 min.

The detection limit for most beers was estimated to be about 10 ng/ml although in some cases as low as 2 ng/ml could be detected when more sample was injected. Any sample extracts showing values above 10 ng/ml were reanalysed with a slightly different mobile phase (0.02 *M* KH_2PO_4, pH 4.0). It was found that this mobile phase

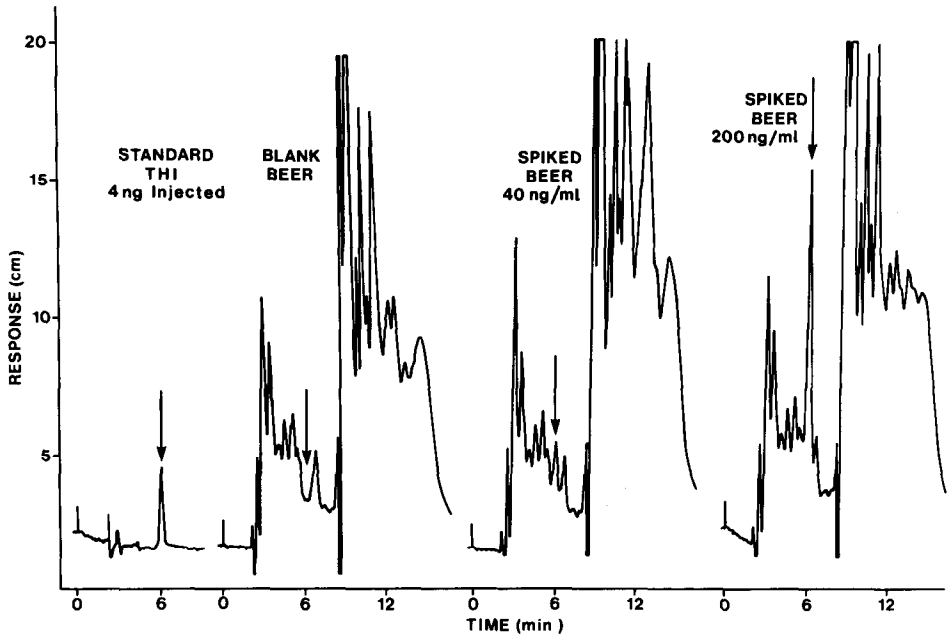

Fig. 1. Chromatograms of a pale beer spiked to contain 40 and 200 ng/ml THI. Mobile phase, 0.005 *M* KH_2PO_4 (pH 4.5). Wavelength, 300 nm. Sample extract dissolved in 10 ml mobile phase.

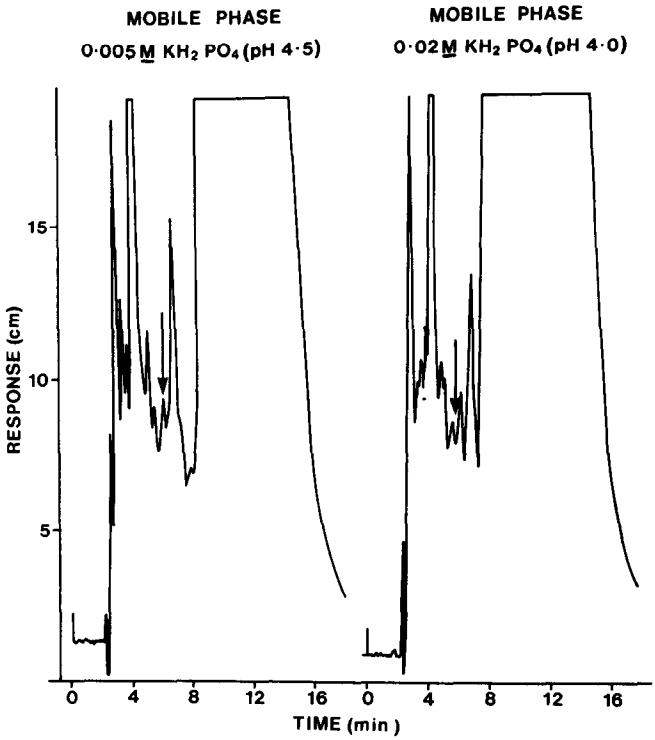

Fig. 2. A comparison of results obtained for a stout extract using the two different mobile phases studied. Wavelength, 300 nm. Sample extract dissolved in 5 ml mobile phase.

provided enough selectivity difference to act as a confirmation. In several instances values above 15 ng/ml were found to be less than 10 ng/ml with the second mobile phase. The lower value was always taken as being more accurate. Fig. 2 compares an extract of a stout with the two mobile phases. As can be seen, in one system a peak is observed corresponding to 9 ng/ml while in the second system no peak is observed with a retention time equivalent to THI although two peaks are close. With some other beers the opposite situation was observed.

We found that although 287 nm was the absorbance maximum for THI, 300 nm was chosen because of the increased selectivity obtained. The result was improved chromatograms in spite of the slightly reduced sensitivity to THI.

Recovery of THI added to different beer samples ranged from 57–70% at spiking levels of 20, 40, 60 and 200 ng/ml. The repeatability coefficient of variation of triplicate determinations each at 12 and 40 ng/ml were 12% and 18%, respectively.

In a limited survey of 18 domestic and 15 imported beers only two dark beers showed levels consistently above 10 ng/ml with the two mobile phases employed. Both of these samples produced extracts which contained much more coextractive material than the others including even stout, and thus had to be diluted in order to have the chromatograms appear on-scale.

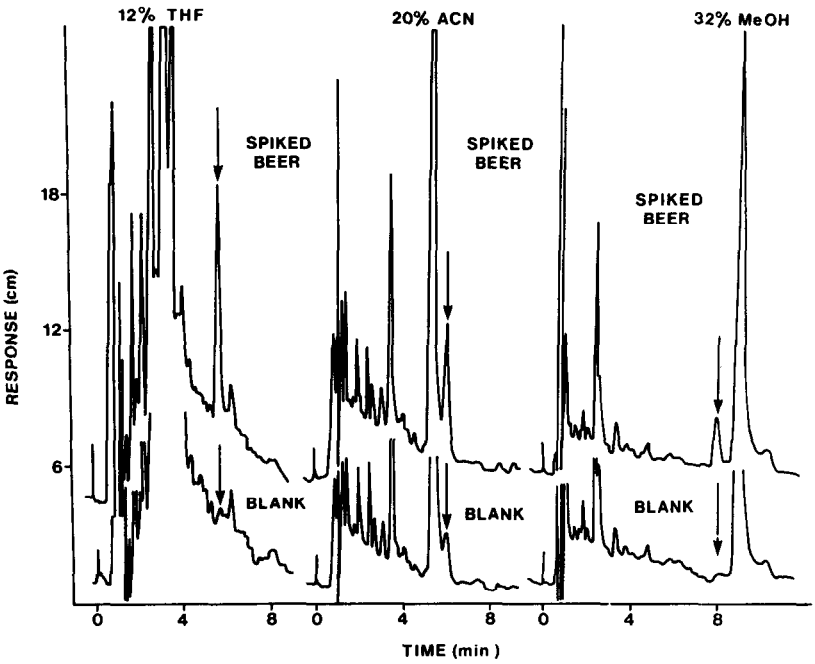

Fig. 3. Comparison of chromatograms obtained with three different mobile phases for the same sample extracts (blank and spike, 200 ng/ml) after acetylation. Reaction and chromatography conditions described in the text. Wavelength, 287 nm. Injection volumes, 20 μl for acetonitrile (ACN) and methanol (MeOH) and 50 μl for the tetrahydrofuran (THF) mobile phase.

Confirmation by acetylation

In order to confirm positive THI results obtained by direct HPLC we decided to attempt to acetylate the –OH and –NH moieties using acetic anhydride and pyridine as has been reported for sugars[4]. After a number of studies where quantities of reagents, temperature and time were varied, we obtained the reaction conditions described in the experimental section. Under those conditions a single peak was produced that was much less polar than THI but exhibited a very similar UV spectrum and the same sensitivity at the absorbance maximum of 287 nm. These characteristics would be expected if only the addition of acetyl groups were involved. Mass spectrometry (probe, electron impact) of the derivatives did not yield an unequivocal structure but it appears that three acetyl groups are added to THI as a result of the derivatization.

The Sep-Pak C_{18} cleanup was required to improve the yield of the reaction when applied to real samples. It removed coloured (pale yellow) material that turned brown during the reaction and resulted in low recoveries and interferences. After the reaction, the mixture was evaporated to dryness and then shaken with water and chloroform. This was found necessary to remove traces of pyridine which eluted as a broad tailing peak causing some difficulty in quantitating the derivative. It was observed that after the final chloroform evaporation, the THI derivative was difficult to re-dissolve in mobile phase for HPLC analysis. It required continuous vortex stirring for 15 min in order to have recoveries greater than 80%. Also, we found that just allowing the

solution to sit overnight was adequate to ensure dissolution of the derivative. After this time, the solution was stable for at least two weeks.

Three mobile phases were evaluated for the analysis of acetylated THI in the beer samples. Fig. 3 compares chromatograms obtained with each for a derivatized beer (porter) extract spiked with 200 ng/ml THI. At this spiking level the THI is clearly observed although the chromatographic patterns are quite different. The mobile phase containing tetrahydrofuran was chosen for routine use. The others were also useful, but occasional interfering peaks appeared with some samples that were not observed or were smaller with the tetrahydrofuran mobile phase (compare blanks for example, in Fig. 3). The lower detector response to THI with the methanol containing mobile phase appears to be the result of peak broadening due to increased retention time as well as perhaps other chromatographic effects. This was consistent for all samples and standards analysed with that system.

The detection limit using the acetylation was estimated to be about 10 ng/ml in the beers studied. Lower levels could be detected depending upon the particular sample and the quantity injected. Yields of derivative from reactions carried out in triplicate at 50 and 200 ng/ml THI in beer were 81 and 88% respectively compared to a pure standard carried through the same reaction. The corresponding coefficients of variation were 24% at 50 ng/ml and 5% at 200 ng/ml THI. The linear range for the acetylation reaction extended from the detection limit to at least 200 ng/ml THI. Higher concentrations were not evaluated.

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