Determination of 2-Acetyl-4(5)-(tetrahydroxybutyl)imidazole in Ammonia Caramel Color by HPLC

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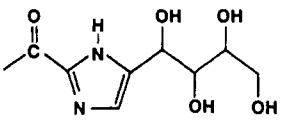
A new approach for the determination of 2-acetyl-4(5)-(tetrahydroxybutyl)imidazole (THI) in ammonia caramel color has been developed. THI was extracted from samples by cation-exchange solid-phase extraction (SPE). After the cation exchange column extraction, a phenyl boronate agarose (PBA-30) affinity column was used for further cleanup. In the boronate extractor, a cyclic boronate ester was formed between the tetrahedral boronate anion and the *cis*-diol groups of THI. THI was isolated from most remaining matrix interferences and was recovered by cleaving the formed ester with acetic acid. The collected samples were then determined by HPLC–UV, using a diol bonded phase column. The optimum HPLC conditions for the separation of THI from ammonia caramel color matrices were also investigated. The minimum detection limit of THI by this approach was at the 1 ppm level in ammonia caramel color.

2-Acetyl-4(5)-(tetrahydroxybutyl)imidazole (THI) (Figure 1) is a compound that may be formed during the production of ammonia caramel color. Previous methods for determining THI have employed cation-exchange solid-phase extraction followed by reversed-phase HPLC with UV detection (Kroplien, 1986; Kroplien et al., 1985; Lawrence, 1987). These methods suffered from incomplete chromatographic resolution of THI from caramel matrix compounds. Thus, there exists a need to develop improved methods for the determination of THI.

We have now developed a new and improved approach for the determination of THI in ammonia caramel color samples. The keys to this method are the use of boronic acid solid-phase extraction after a cation-exchange solid-phase extraction step and the use of a diol bonded phase column for the HPLC analysis. These two factors rely on the polyhydroxy structure of THI. More specifically, the boronate extraction is based upon the fact that THI has several *cis*-diol functionalities which can form a cyclic boronate ester. The entire procedure was automated using a Supelco vacuum manifold for sample preparation and applied to single blind, spiked samples, as well as to actual ammonia caramel samples.



2-Acetyl-4-(1, 2, 3, 4-tetrahydroxybutyl)-imidazole

Figure 1. Structural formula of THI.

EXPERIMENTAL PROCEDURES

Chemicals and Experimental Conditions. *Chemicals.* Carboxymethylcellulose (CMC) CM-52, a preswollen microgranular cation exchanger, was obtained from Whatman BioSystems, Ltd. (Maidstone, Kent, U.K.). Sulfonic acid type strong cation exchange resin, AG 50 W-X8, -400 mesh, was from Bio-Rad Laboratories (Rockville Centre, NY). Phenyl boronate agarose (PBA-30), *m*-aminophenylboronic acid covalently bound with a boron content of 48.8 mmol/mL, preswollen gel, was obtained from Amicon Corp. (Amicon Division of W. R. Grace and Co., Danvers, MA). THI was obtained from the International Technical Caramel Association (ITCA) (Washington, DC). THI stock solution was prepared by dissolving 10 mg of THI in 100 mL of deionized water. The THI solution was stored in the refrigerator at 4.0 °C and later diluted to the desired concentrations for standard addition experiments. HPLC solvents were obtained from EM Science, Inc. (Gibbstown, NJ), as their Omnisolv HPLC brand/grade. THI samples stored in a dissolved solution of mobile phase at room temperature were stable throughout the duration of this project, which was completed over 2 years. All HPLC solvents were used after filtration through a 0.45-μm solvent filter (GVWP; Millipore Corp., Bedford, MA) and degassed under vacuum with stirring.

Apparatus. A Waters 501 HPLC pump (Millipore Corp., Waters Chromatography Division, Milford, MA), a syringe loading injector, Rheodyne Model 7125 (Rheodyne, Cotati, CA) with 20-μL loop, a variable-wavelength UV detector (Knauer, GmbH, Berlin, FRG) at 285 nm, and a recording integrator HP 3394 (Hewlett-Packard, Avondale, PA) were used. The HPLC column was a Lichrospher 100 diol bonded phase (E. Merck, GmbH, EM Science, Inc., Gibbstown, NJ), 10 μm, 250 × 4 mm, with a guard column of the same Lichrospher Diol, 5 μm; mobile phase was 5% MeOH and 0.5% acetic acid in deionized water, delivered at 0.6 mL/min.

Sample Preparation. *SPE Column Packing.* All of the SPE columns were slurry packed with packing materials in empty SPE cartridges on a vacuum manifold (Supelco, Bellefonte, PA). For the cation-exchange columns, the bed height was 4 cm in a 3-mL SPE tube, two of which were used in series (Figure 2). The upper column was CMC, and the lower column was AG 50 W-X8. For the PBA-30 column, the bed height was 3.5 cm in a 1-mL SPE tube, and two of them were used in series.

CMC and AG 50 W-X8 Cation Exchange Column Sample Cleanup. (1) Add 4 mL of pH 5.0 (0.1 M) acetate buffer to wash the columns, in series. (2) Apply 0.1 g of ammonia caramel color sample in pH 5.0 buffer solution to the top of the first column above. (3) Spike THI in the ammonia caramel color sample before addition of samples to the SPE procedure for standard addition. (4) After the sample passes through both columns, wash both columns with 20 mL of pH 5.0 acetate buffer and discard the eluent. (5) Remove the upper CMC column and wash only the lower column with 5 mL of distilled water. (6) Wash the lower column with 16 mL of 0.5 M HCl. Discard the first 4 mL of eluent and collect the next 12 mL of eluent. (7) To this 12 mL

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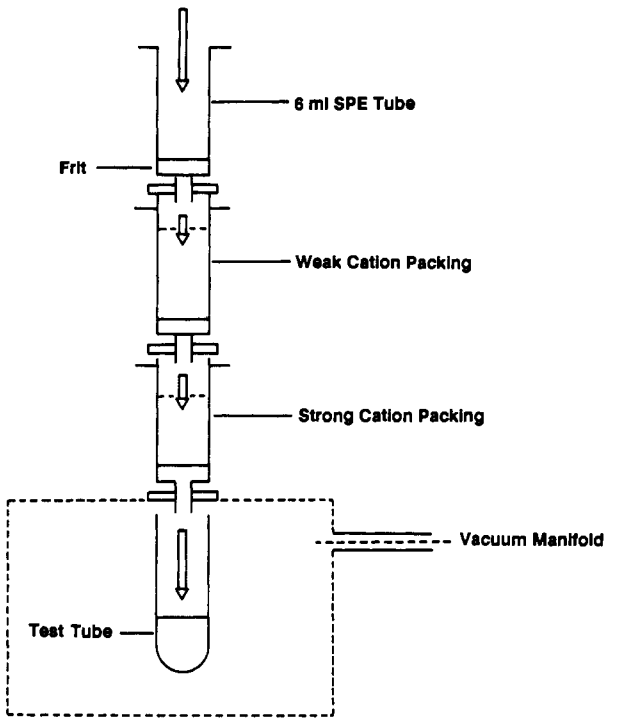

Figure 2. Weak cation exchanger and strong cation exchanger cleanup procedure, automated on a vacuum manifold.

of eluent, add about 2 mL of 5.0 M ammonium hydroxide to adjust the pH of the solution to a basic condition of about pH 9.0.

PBA Column Sample Cleanup. (1) Apply 6 mL of a pH 8.8 (50 mM) ammonium acetate buffer to wash the columns and discard the eluent. (2) Apply the basic caramel solution on top of the PBA column. (3) Wash with 4 mL of pH 8.8 (50 mM) ammonium acetate buffer. (4) Wash column with 3 mL of 0.5 M acetic acid and collect the eluent (3.0 mL) or note eluent volume for HPLC injection and final analysis.

RESULTS AND DISCUSSION

THI is a weakly basic compound with an imidazole ring and four hydroxyl groups. Obviously, the ammonia caramel color matrices are composed of many very polar components, many of them having physical and chemical properties similar to those of THI. Therefore, it was very difficult to extract and separate trace amounts of THI from other compounds in the matrices. In view of this, combined approaches were required. The first step was similar to Kroplien's work (Kroplien et al., 1985; Kroplien, 1986) but was improved by using a weak acid cation exchange SPE column (CMC) and a strong acid cation exchange SPE column (AG-50 W) with a vacuum manifold to extract THI from matrices (Kroplien et al., 1985; Kroplien, 1986; Lawrence and Charbonneau, 1987). The weak cation exchange column was used to remove high molecular weight impurities from the caramel matrix, and no attempt was made to recover any THI that may have been bound to this column. The optimization of volume of eluent for recovery of THI from the strong cation exchange column, AG-50 W-X8, using 0.5 M HCl as eluent, is shown in Figure 3. On the basis of the data in Figure 3, it was decided to first wash the strong cation exchange column with 4.0 mL of 0.5 M HCl to wash off any weakly bound impurities and then elute the THI with 12.0 mL of 0.5 M HCl. This 12.0 mL of eluent recovered most of the THI but not quite all. The implications of this will be discussed below.

After the cation exchange columns, a phenyl boronate agarose (PBA-30) affinity column was used for further

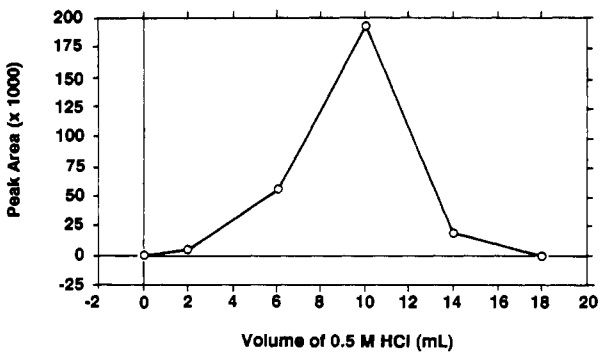

Figure 3. Effect of the elution volume of 0.5 M HCl on the recovery of standard THI from the AG 50 W-X8 strong cation exchange column.

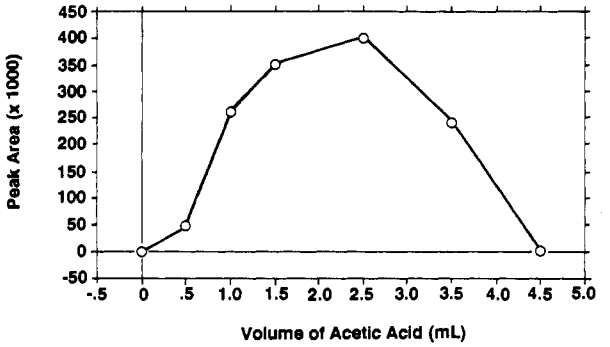

Figure 4. Effect of the elution volume of 1% acetic acid on the recovery of standard THI from the PBA-30 boronate affinity column.

extraction of THI from other compounds (Mazzeo and Krull, 1989). At basic conditions (pH around 9.0), hydroxylation of the boronate occurs, converting the boronate from the plane trigonal neutral form to its negatively charged, tetrahedral form, where it can interact with the *cis*-diols of THI to form a five-membered ring. THI was retained on the PBA column and isolated from other remaining matrix interferences. Since the PBA affinity column binding was weak, it was very sensitive to the pH of the eluent. The optimum concentration and volume of acetic acid for recovery of THI from the PBA-30 column have also been investigated. Figure 4 shows the optimization of the volume of 1% acetic acid for the elution of THI from the boronate affinity solid-phase extraction column. In the actual sample cleanup procedure, the THI was eluted from the PBA-30 column with 3.0 mL of 1% acetic acid. As was the case with the strong cation exchange column, this washing recovered most of the THI but not quite all.

When the entire solid-phase extraction procedure was employed, including both cation exchange columns as well as the boronate affinity column, it was determined that the percent recovery of standard THI was 70.0%. The less than quantitative recovery can be explained by considering Figures 3 and 4 and the actual amount of elution solvent used for both the strong cation exchange column and the boronate affinity column. We did not use enough eluent to recover all of the bound THI. However, there was a compromise between the maximum percent recovery and minimum interferences in the resulting chromatograms. To reduce interferences in the later HPLC determination, some sacrifice of sample recovery had to be made. Hence, the elution conditions used led to the best compromise between maximum recovery and cleanest chromatograms.

It was also found that the recovery of THI in the cleanup procedures depended on the ammonia caramel color

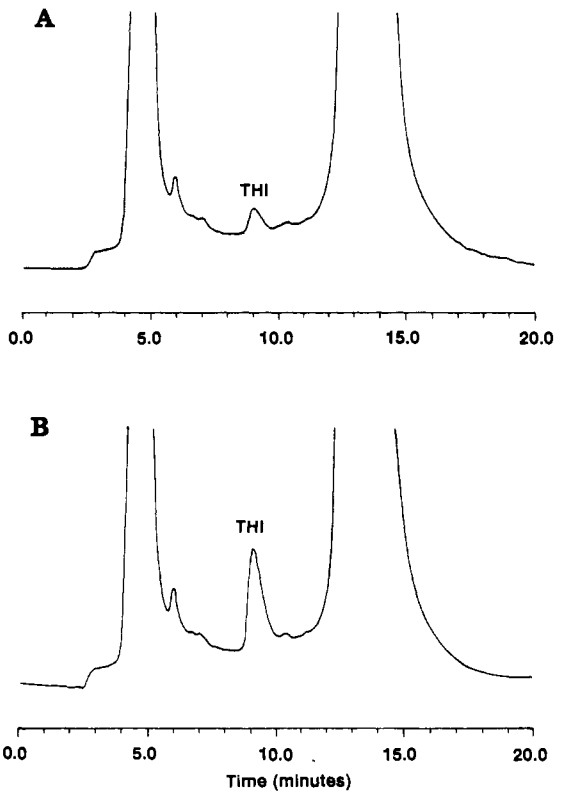

Figure 5. (A) Chromatogram of ammonia caramel color at incurred level, ca. 10 μg/g of THI. (B) Chromatogram of ammonia caramel color sample spiked with 80 μg/g of THI. HPLC conditions: column, Lichrosorb diol 100 (5 μm, 350 × 4 mm); detector, UV at 285 nm, 0.1 AUFS, chart speed 0.5 cm/min; 20-μL injection; mobile phase, 5% methanol, 0.5% acetic acid in water; flow rate, 0.6 mL/min.

Table I. Recoveries of THI in Caramel Color Samples

sample	recovery, w/w %	SD[a]
VT-1	50.0	5.3
VT-2	64.2	6.0
VT-3	48.5	2.8
VT-4	48.9	1.0
VT-5	65.4	1.8
VT-6	66.5	8.1
VT-8	38.0	2.0
VT-9	61.9	4.5
VT-10	86.4	4.9
VT-11	78.0	4.4
VT-12	52.0	3.7
VT-13	41.0	6.8

[a] Standard deviation, $n = 3$.

matrix. The results of the recovery experiments of THI from various ammonia caramel color samples are summarized in Table I. This study was performed using ammonia caramel color samples that had been previously determined to contain amounts of THI below the minimum detectable level. THI was then spiked in by one analyst and the sample measured by another. The percent recoveries are seen to range from as low as 38% to a high of 86.4%.

The variation of percent recovery of THI with caramel matrix can easily be understood by considering the vast number of compounds in a typical caramel matrix. Some of these compounds may compete with THI for binding sites on the solid-phase extraction column, thereby influencing the percent recovery for THI. The number and amount of compounds that may compete with THI will be matrix specific and not easily predicted.

Since the recovery of THI varied with the caramel matrix, it was necessary to use a standard addition method to account for this variation. First, a caramel sample was worked through the solid-phase extraction procedure and analyzed for THI by HPLC, with the amount present after the extraction estimated by using an external standard calibration curve. The estimated amount and twice the estimated amount were then spiked into two different samples of the caramel matrix, and these two samples were then worked through the solid-phase extraction procedure as well as the native, incurred sample. Standard addition plots were then generated, plotting spiked level vs THI peak area, using the negative value of the x intercept as the native level. Standard addition plots were linear with correlation coefficients of 0.99 or better, suggesting that the percent recovery of THI did not vary with concentration in a given matrix.

Table II. Results of the Determination of Single Blind Spiked Caramel Color Samples

sample	spiked THI level, μg/g	determined	SD[a]
VT-1	19.1	18.8	2.0
VT-2	13.0	15.0	1.4
VT-3	16.8	15.6	0.9
VT-4	2.6	1.0	0.0
VT-5	24.1	25.7	0.7
VT-6	1.7	3.3	0.4
VT-8	22.6	24.3	1.3
VT-9	28.4	27.6	2.0
VT-10	15.5	12.4	0.7
VT-11	4.6	5.3	0.3
VT-12	28.1	32.0	2.3
VT-13	9.5	9.1	1.5

[a] SD, standard deviation, $n = 3$.

Table III. Summary of Caramel Color Samples Analyzed for THI Using the Analytical Methods Developed at Northeastern University

sample	supplier	level, μg/g	SD[a]
1	A	18.3	2.9
3	B	13.5	1.3
6	C	9.5	2.0
8	D	7.1	0.3
18	E	9.7	1.1
20	F	6.8	1.0

[a] SD, standard deviation, $n = 3$.

For HPLC determinations, a diol column was used instead of the popular C-18 column. Because of the selective interaction of the diol groups of the packing material with the hydroxyl groups of THI, the chromatographic behavior was much better, with THI sufficiently resolved from any matrix peaks. Dual-wavelength absorbance ratioing was used to confirm purity across the THI peak. Furthermore, on-line linear diode array spectroscopy was taken of the THI peak from a caramel sample and compared to that of standard THI, with the two exactly matching. It was shown that under our final conditions the diol column had been used for more than 6 months, presenting both repeatable and reliable results. A typical chromatogram is shown in Figure 5, of both native level THI and spiked level THI.

The overall approach was validated by the determination of single blind spiked caramel color samples. As with the recovery study, caramel samples that were determined to have levels of THI below the detection limit were spiked with THI by one analyst and determined by another. The results are summarized in Table II. Good agreement between spiked and determined levels was achieved at levels of 5 μg/g and above. However, as the spiked level approached the lower limit of detection, which was about 1 μg/g, the agreement was poor. Thus, the minimum quantifiable level was about 5 μg/g.

Having validated the method for samples above 5 μg/g of THI, we next analyzed ammonia caramel samples from different manufacturers around the world. These results are presented in Table III. A good level of precision was obtained for these samples.

CONCLUSIONS

By appropriate method validation techniques, it was shown that our newly developed and improved approach for the determination of THI in ammonia caramel colors is reliable and repeatable. It has been routinely used in our laboratory for the determination of ammonia caramel color samples from different manufacturers around the world.

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