Journal of Chromatography, 362 (1986) 286–290
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 687

Note

Quantitative analysis of 2-acetyl-4(5)-tetrahydroxybutylimidazole

U. KRÖPLIEN
Coca-Cola GmbH, Max-Keith-Strasse 66, D-4300 Essen 1 (F.R.G.)
(Received March 26th, 1986)

Of the four classes of caramel colours[1], some caramel colours III (also known as ammonia caramels and often called "beer caramels") have been found to be capable of decreasing the number of circulating lymphocytes in rats fed on a diet deficient in vitamin B_6. None of the other caramel classes provoked such a response. The substance responsible for this effect could be identified as 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI)[2].

Separation of THI from other caramel constituents of similar basicity and polarity is achieved by a two-step procedure. Sequential treatment of caramel colours III (AC) with weakly and strongly acidic cation-exchange resins removes neutral, acidic and more basic substances. Weak bases, among them THI, are recovered with hydrochloric acid from the strong cation-exchanger. Reaction with the carbonyl reagent 2,4-dinitrophenylhydrazine converts THI into a coloured derivative. The latter is separated from excess reagent and interfering substances on a reversed-phase high-performance liquid chromatography (HPLC) column, and quantified spectrophotometrically.

EXPERIMENTAL

Solvents and other chemicals

These were of reagent-grade quality from Merck (Darmstadt, F.R.G.), Baker (Gross-Gerau, F.R.G.) and Fluka (Buchs, Switzerland).

Purification of dimethoxyethane

Dimethoxyethane was distilled from dinitrophenylhydrazine (DNPH), then from sodium hydroxide, stored over sodium sulphate, and percolated through alkaline alumina immediately prior to use.

2,4-DNPH · HCl

Reagent-grade 2,4-DNPH (Fluka)*, recrystallized from ethyl acetate, was suspended in water, and hydrochloric acid (32%) was added until the yellow hydro-

* Use of Fluka DNPH is recommended because DNPH from several other manufacturers remained impure even after recrystallization, leading to spurious peaks in HPLC.

0021-9673/86/$03.50

chloride was formed completely and the reddish-orange free base was no longer detectable. The crystals were dried under suction, washed repeatedly with dimethoxyethane, then with diethyl ether, and carefully dried.

Strong cation-exchanger

Dowex 50 AG × 8, 100–200 mesh, H^+ (Bio-Rad, Munich, F.R.G.) was used.

Weak cation-exchanger

Amberlite CG AG 50 I, 100–200 mesh, H^+ (Serva, Heidelberg, F.R.G.) was used. The resin must be sedimented repeatedly to remove fines.

Methanol, carbonyl-free

This was prepared[3] by treatment with Girard P reagent.

THI–2,4-DNPH

THI–HCl (133 mg) was dissolved in 95% ethanol (10 ml) and 32% hydrochloric acid (0.2 ml) was added, followed by 10 ml of a saturated solution of 2,4-dinitrophenylhydrazine hydrochloride in dimethoxyethane. After standing for 1 h, the solution was taken to dryness under vacuum. The residue was treated with 95% ethanol (2 ml), then dimethoxyethane (5 ml) was added. The yellow crystals were dried under suction, washed with dimethoxyethane, then with diethyl ether, and dried. Yield, 205 mg (88%); m.p., 151°C. Calculated for $C_{15}H_{18}N_6O_9 \cdot H_2O \cdot HCl$: C = 38.75%; H = 4.52%; N = 18.08%; Cl = 7.64%. Found: C = 38.71%; H = 4.59%; N = 16.97%; Cl = 7.33%.

Combination columns (clean-up device)

A lower column (175 mm × 10 mm I.D., with a PTFE stopcock and capillary outlet), an upper column (150 mm × 12.5 mm I.D., with a capillary outlet of 1 mm I.D.) and a 100-ml dropping funnel with a PTFE stopcock[4] were used. All parts were linked by standard ground-glass joints (14.5 mm).

High-performance liquid chromatographic equipment

A double-piston pump 6000 A (Waters, Eschborn, F.R.G.), an injector U6K (Waters, Eschborn, F.R.G.), a variable-wavelength detector 87.00 (Knauer, Bad Homburg, F.R.G.) and a recording integrator HP 3390 (Hewlett-Packard, Düsseldorf, F.R.G.) were used. Commercial* LiChrosorb RP-8, 10 μm, 250 × 4 mm I.D. "Vertex" columns (Knauer) were used. The HPLC mobile phase was methanol–0.02–0.05 *M* phosphoric acid (50:50, v/v); the concentration of phosphoric acid depended on the column performance.

Reacti-Vials

These were of 1-ml volume from Pierce/Karl (Geisenheim-Johannisberg, F.R.G.) with Tuf-Bond Teflon-Silicone septa.

---

* Other RP-8 materials such as "LiChrosorb" manufactured in America or "Nucleosil" were unsuitable. They were found not to retain THI–DNPH, which is eluted in the void volume together with non-carbonylic contaminants.

Caramel colours III and IV

These were provided by the International Technical Caramel Association (Washington D.C., U.S.A.).

Sample clean-up

The lower part of the combination column was filled with strong cation-exchanger to a bed-height of 60 mm. Then the upper column was connected and filled with weak cation-exchanger to a bed-height of 80–90 mm. After passage of *ca.* 20 ml of water through the columns, the device was operational. Caramel (200–250 mg, weighed accurately) was dissolved in water (*ca.* 3 ml) and the solution was transferred quantitatively to the combination column (preferably with a Pasteur pipet). The dropping funnel was adjusted and the column system eluted exhaustively with water (80–100 ml). The upper column was then disconnected and the lower column eluted with 0.5 *M* hydrochloric acid (45 ml). The first 10.0 ml were discarded, and the remaining 35 ml were collected.

Preparation of derivative

The solution was evaporated to dryness under vacuum at 40–45°C. The glassy or syrupy yellow or light brown residue is dissolved in carbonyl-free methanol (250 µl), and a saturated solution of 2,4-DNPH · HCl in dimethoxyethane (250 µl)[5] is added by syringe. The reaction mixture is transferred rapidly, to avoid solvent losses, to a septum-capped vial.

High-performance liquid chromatography

The derivative solution was kept for 5 h at room temperature. Volumes between 1 and 25 µl (usually 5 or 10 µl) were injected onto the HPLC column, which was eluted isocratically with the mobile phase at a flow-rate of 2.0 ml/min. THI–2,4-DNPH was detected and measured at 385 nm and 0.16 a.u.f.s., using peak-height determination because of the occasional occurrence of a slightly later eluting, scarcely resolved peak.

For calibration, THI dinitrophenylhydrazone (*ca.* 100 mg) was dissolved in absolute, carbonyl-free methanol. Stored in a refrigerator, the solution is stable for at least twelve months if kept in alkali-free glassware.

RESULTS

A typical chromatogram is shown in Fig. 1. The largest peak represents excess reagent.

Calibration

THI–DNPH was dissolved in methanol. Volumes from 1 to 25 µl were injected onto the HPLC column. A linear response was found for the whole range of 5–1200 ng of THI. Above 25 ng, relative standard deviations (R.S.D.) of 3.65% (peak area mode) and 2.25% (peak height mode) were found. R.S.D. values increased to 13% with 10 ng, and to 23% with 5 ng of THI, with corresponding averages of 106% and 146% of theoretical amounts being measured.

Responses and errors did not vary significantly with time. However, for peak

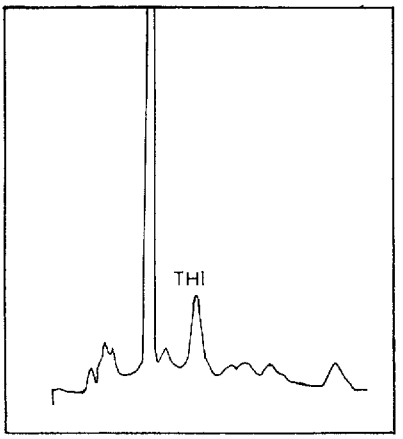

Fig. 1. Chromatographic separation of THI–DNPH on LiChrosorb RP-8 (10 μ). Mobile phase, methanol–0.02 *M* phosphoric acid (50:50, v/v); detector wavelength, 385 nm.

height mode determinations, one or two injections for recalibration are indispensable owing to slight changes of column efficiencies with time.

Limit of detection

A signal with less than 2000 peak height impulses cannot be distinguished clearly from noise, corresponding to a detection limit of *ca.* 5 ng of THI. This value corresponds to 1 mg/kg (1 ppm) in caramels under standard conditions (sample weight, 250 mg; derivative volume, 500 μl, injection volume, 10 μl). Matrix effects may raise this limit to up to 5 ppm, depending on the nature of the caramel sample.

Recovery

THI was processed through the clean-up and derivatization steps, or was previously added to caramel colours IV, also known as sulphite–ammonia caramels (THI-free) or caramel colours III (ammonia caramels). The amounts corresponded to 45–1130 ppm in the caramels. Recoveries ranged from 95.7% to 103.4%, averaging 98.6%. They were not matrix- or concentration-dependent.

THI concentrations determined in a number of commercial caramel colours III (AC) are listed in Table I.

TABLE I

CONCENTRATIONS OF THI DETERMINED IN COMMERCIAL CARAMEL COLORS III (AC)

AC	*THI (ppm)*	*AC*	*THI (ppm)*
1	41	7	61
2	373	8	173
3	68	9	8
4	196	10	44
5	162	11	2
6	11	12	3

CONCLUSIONS

The recently detected constituent of some caramel colours III, 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI), can be determined by HPLC with high accuracy. THI contents of caramel colours III manufactured between 1979 and 1983 cover a range from virtually nil to 400 ppm. Further work will show how these levels are affected by manufacturing conditions.

ACKNOWLEDGEMENTS

The author is grateful to The Coca-Cola Company for permission to publish his results. Thanks are due to Mr. J. Rosdorfer for his skillful technical assistance, and to Drs. D. V. Myers and T. Radford for their help in preparing the manuscript.

REFERENCES

1 *Specifications for Caramel Color*, International Technical Caramel Association, Washington, DC, Dec. 1984, amended May 1985.
2 U. Kröplien, J. Rosdorfer, J. van der Greef, R. C. Long, Jr. and J. H. Goldstein, *J. Org. Chem.*, 50 (1985) 1131.
3 Y. Peleg and C. H. Mannheim, *J. Agric. Food Chem.*, 18 (1970) 176.
4 U. Kröplien, *J. Agric. Food Chem.*, 22 (1974) 110.
5 S. Honda and H. Kakehi, *J. Chromatogr.*, 152 (1978) 405.