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Synthesis and Application of ^{13}C-Labeled 2-Acetyl-4-((1*R*,2*S*,3*R*)-1,2,3,4-tetrahydroxybutyl)imidazole (THI), an Immunosuppressant Observed in Caramel Food Colorings

Paul W. Elsinghorst,*[,†,‡] Marion Raters,[§] Anna Dingel,[§] Jochen Fischer,[‡,∥] and Reinhard Matissek[§]

[†]Pharmaceutical Chemistry I, Pharmaceutical Institute, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany

[‡]ELFI Analytik GbR, Massenhausener Strasse 18a, D-85375 Neufahrn, Germany

[§]Food Chemistry Institute (LCI) of the Association of the German Confectionery Industry, Adamsstrasse 52-54, D-51063 Köln, Germany

[∥]Bioanalytics, Department of Nutrition and Food Sciences, University of Bonn, Endenicher Allee 11-13, D-53115 Bonn, Germany

ABSTRACT: 2-Acetyl-4-((1*R*,2*S*,3*R*)-1,2,3,4-tetrahydroxybutyl)imidazole (THI) is a minor toxic contaminant observed in caramel food colorings and was shown to exert immunosuppressant activity when fed to rodents. Because of this toxicity, maximum levels of THI in caramel food colorings have been defined by international and European authorities. Several reports of THI analysis using external standardization have been published for liquid foods such as beers and soft drinks. However, no suitable internal standard has yet been described allowing THI analysis in more complex samples. In this paper we describe the preparation of a labeled [$^{13}C_6$]THI analogue and its application for the successful validation of the first stable isotope dilution assay (SIDA) of THI in caramel food colorings. A brief survey of THI levels in commercially available caramel class III (E 150c) and IV (E 150d) food colorings is also included, corroborating that THI occurs only in caramel class III food colorings.

KEYWORDS: *isotopic labeling, ^{13}C, THI, immunosuppressant, caramel food colorings, SIDA*

INTRODUCTION

Browning, a welcome side effect of common food preparation processes, such as roasting or baking, has been known to mankind since ancient times. Today, modern science distinguishes between such nonenzymatic and, on the other hand, enzymatic browning reactions, which relate to the enzymatic darkening of cut or damaged fruits. Following a well-accepted etymology, the term "caramel" was established in the early 18th century, literally referring to "burnt sugar", i.e., a deliberately browned sugar preparation. Aside from various food preparations that take advantage of its pleasant taste, caramel has also been used as a food colorant. Following first commercial importance in the 18th century as an additive in brewery products and as a colorant for brandy, caramel colorings are nowadays used in all kinds of foods, e.g., in beverages and bakery or meat products. Today they account for more than 80% of all colorants added to foods, and annual global consumption exceeds 200 000 tons.[1,2]

Modern caramel colorings used as food additives are divided into four classes depending on their preparation and resulting properties. Of these, caramel of color classes III and IV is prepared by heating carbohydrates in the presence of ammonium compounds (III) and, additionally, sulfite (IV). During caramelization, these ammonium compounds serve as a source of nitrogen for a series of food contaminants, including a group of toxic imidazoles, e.g., 2-methylimidazole (2-MI) or 4-methylimidazole (4-MI) and 2-acetyl-4-((1*R*,2*S*,3*R*)-1,2,3,4-tetrahydroxybutyl)imidazole (THI). Mechanisms for imidazole formation during caramelization have been proposed for both methyl- and acetylimidazoles.[3,4] Each one relies on methylglyoxal as a product of carbohydrate pyrolysis, which subsequently condenses with concomitantly formed nitrogen synthons such as formamide or iminofructosamine to provide the imidazole heterocycle. In the case of THI, the stereochemistry of the side chain is inherited from the D-glucose or D-fructose precursor, a fact that has been synthetically exploited.[3,5−7]

The toxicity of THI was first recognized as a lymphopenic effect, i.e., a reduction in circulating lymphocyte counts, when fed at high concentrations to rats receiving a diet deficient or marginal in vitamin B_6.[8−11] Inhibition of pyridoxal kinase has been suggested as a possible mechanism but has not been further evaluated. Additional research revealed that THI also impairs the immunity of rats and mice by modifying splenocyte function.[12−15] Mice fed with THI showed less $CD4^+$ and $CD8^+$ T-cells, and THI probably prevents the recruitment of $CD4^+$ T-cells in draining lymph nodes.[16,17] These and further reports of immunotoxicity have been evaluated by international food safety authorities, and maximum levels of THI in caramel class III food colorings have been defined.[18−21] While the Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommends a maximum amount of THI below 40 or 25 mg/kg on an equivalent color basis, the European Commission sets the maximum at 10 mg/kg on an equivalent color basis.[22] No maximum levels have been defined for caramel class IV food colorings, which are considered free of THI to date.[19]

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Several chromatographic methods have been published for the quantitation of THI, some of them simultaneously determining 2- and/or 4-MI.[3,23−32] Most of these investigate liquid samples, e.g., beers, soft drinks, or food coloring preparations, usually applying external standardization and no sample pretreatment except for dilution. However, quantitation results in these cases may be flawed by matrix interferences, a problem that can be avoided by the application of internal standards, especially of stable-labeled isotopomers in combination with hyphenated LC−MS technology (SIDA).[33,34] Solid samples obviously require a suitable workup to make the analytes of interest accessible to chromatography. Solid-phase extraction (SPE) of spiked ground coffee gave incomplete recovery of THI, a drawback that was overcome by the application of supercritical fluid extraction (SFE).[24,29] Instrumentation for SFE, however, is less common, whereas LC−MS is more widely available. Such LC−MS analyses of complex samples involving preparation techniques such as SPE require a suitable internal standard to account for analyte extraction losses, e.g., a stable-labeled isotopomer.

A deuterated analogue of 4-MI is commercially available and has been applied in simultaneous 4-MI/THI analysis of beverages.[32] Nevertheless, in this and other studies, THI was quantitated by external standardization. It remains questionable if 4-MI-d_3 is a suitable internal standard to reflect matrix interferences affecting THI. Not only polarity and thus extraction recoveries but also chromatographic behavior and ionization characteristics of 4-MI and THI differ considerably. Therefore, a stable-labeled THI internal standard was prepared, and a stable isotope dilution assay for THI in caramel food colorings was developed and fully validated. The assay was successfully applied to a small set of commercially available caramel class III and IV food colorings.

MATERIALS AND METHODS

Chemicals. [$^{13}C_6$]Glucose was purchased from Omicron Biochemials (South Bend, IN). Trimethylsilyl cyanide, tin(II) chloride, bromoacetaldehyde diethyl acetal, and diethylamine were from ABCR (Karlsruhe, Germany). All other reagents were provided by Sigma-Aldrich (Steinheim, Germany). Analytical grade solvents and gases were obtained from Sigma-Aldrich, Acros Organics (Geel, Belgium), or Linde (Pullach, Germany) and used without further purification.

Instrumentation. Melting points were determined using a capillary tube apparatus (Apotec, Wepa, Hillscheid, Germany) and are uncorrected. LC−MS analysis for compound characterization was performed on an API 2000 mass spectrometer (ESI; AB Sciex, Darmstadt, Germany), coupled with an Agilent 1100 HPLC system (Agilent Technologies, Böblingen, Germany) using a C_{18} column (Phenomenex Luna, 3 μm, 50 × 2 mm; Aschaffenburg, Germany) and a gradient of methanol (MeOH)/H_2O. GC−MS analysis was carried out using a Varian GC-450, equipped with a Varian VF-5 ms capillary column (30 m × 0.25 mm × 0.25 μm, flow 1.0 mL/min; temperature program, start 50 °C, rate 10 °C/min, end 280 °C), coupled to a Varian MS-240 ion trap (CI (MeOH), split 1:20, scan range 50−300 m/z; Varian, Darmstadt, Germany). ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) spectra were recorded on an Avance DRX 500 spectrometer (Bruker BioSpin, Rheinstetten, Germany), and chemical shifts δ are given in parts per million referring to the signal center using the solvent peaks for reference ($CDCl_3$, 7.26/77.0 ppm; DMSO-d_6, 2.49/39.7 ppm). To characterize the spin multiplicity, the following abbreviations are used: s, singlet; br s, broad singlet; d, doublet; br d, broad doublet; dd, doublet of doublets; ddd, doublet of doublets of doublets; t, triplet; q, quartet; m, multiplet. Elemental composition was determined by combustion analysis using a PerkinElmer 2400 series II CHNS/O elemental analyzer (PerkinElmer, Rodgau, Germany).

Stable Isotope Dilution Assay. Samples for stable isotope dilution analysis were commercially available caramel food colorings produced in the European Union for the German market. For analysis of caramel food colorings, samples (0.5 g) were spiked with [$^{13}C_6$]THI (150 μL, c = 100 μg/mL in MeOH/H_2O, 50:50, v/v), incubated for 30 min, diluted with MeOH/H_2O (50 mL; 50:50, v/v), and clarified by the method of Carrez.[35] Subsequent LC−MS analysis was accomplished on an API 4500 mass spectrometer (ESI; AB Sciex, Darmstadt, Germany), coupled with an Eksigent ekspert microLC 200 microflow UHPLC system (AB Sciex) using a C_{30} reversed-phase column (Phenomenex Develosil, 3 μm, 50 × 1 mm; Aschaffenburg, Germany), a gradient mobile phase of A (100%, 0−2 min), 0.1% aqueous formic acid, and B (100%, 2−3 min), 10% methanol, 90% aqueous ammonia (0.05%), with a flow of 30 μL/min at 20 °C. Mass spectrometric detection (ESI$^+$) used selected reaction monitoring transitions specific to THI (m/z 231 → 153 (target), 231 → 195 (qualifier)) and [$^{13}C_6$]THI (m/z 237 → 159 (target), 237 → 201 (qualifier)). A set of calibration levels (0.01, 0.05, 0.1, 0.5, 1, 10, and 50 mg/kg) were prepared in analyte-free matrix and analyzed. Assay sensitivity was determined by repeated dilution until a signal/noise ratio of 3 (limit of detection, LOD) or 9 (limit of quantitation, LOQ) was obtained. Intraday accuracy was evaluated after multiple analyses of spiked analyte-free matrix (n = 4, c = 2.14−2.88 mg/kg), while intra- and interday precision was determined after multiple analyses (n = 12) of a single sample.

2-Ethoxyacrylonitrile (1). A stirred suspension of tin(II) chloride (1.0 mmol, 0.19 g) in bromoacetaldehyde diethyl acetal (180 mmol, 35.6 g) was ice-cooled, and trimethylsilyl cyanide (180 mmol, 17.9 g) was added dropwise over a period of 30 min. The reaction mixture was allowed to warm to room temperature and stirred overnight. Subsequently, the reaction mixture was diluted with methyl *tert*-butyl ether (85 mL), and diethylamine (270 mmol, 19.7 g) was added dropwise while the reaction mixture was cooled. After 30 min methyl *tert*-butyl ether (25 mL) was added to the resulting thick slurry, which was stirred overnight. Afterward, the solids were filtered off and washed with methyl *tert*-butyl ether (2 × 50 mL), and the filtrate was evaporated in vacuo. Vacuum distillation provided **1** as a colorless liquid (11.5 g, 66%), bp 45−49 °C, 25 mbar. ^{1}H NMR ($CDCl_3$): δ 1.32 (t, 3H, J = 6.9 Hz, CH_3), 3.82 (q, 2H, J = 6.9 Hz, CH_2), 4.83 (d, 1H, J = 3.5 Hz, CH_AH_B), 4.93 (d, 1H, J = 3.2 Hz, CH_AH_B). ^{13}C NMR ($CDCl_3$): δ 14.00 (CH_3), 65.10 (CH_2), 100.69 (CH_AH_B), 114.79 (CN), 136.15 (C). GC−MS: purity 99%. MS (CI): m/z calcd for C_5H_7NO 97.1, found 98.1 [M + H]$^+$.

[$^{13}C_6$]-1-Deoxy-1-(*N*,*N*-dibenzylamino)-D-fructose (2). To a mixture of [$^{13}C_6$]-D-glucose (27.1 mmol, 5.04 g) and dibenzylamine (27.4 mmol, 5.40 g) in absolute ethanol (100 mL) was added glacial acetic acid (1.6 mL), and the mixture was refluxed under a gentle stream of nitrogen for 4 h. The reaction mixture temporarily turned clear before forming a precipitate that was recovered by suction filtration. The precipitate was washed with absolute ethanol (20 mL), followed by petroleum ether (2 × 20 mL), and dried to obtain **2** (8.96 g, 92%) as white crystals, mp 163 °C, lit.[36,37] mp 162−163 °C. LC−MS: purity >99%. MS (ESI$^+$): m/z calcd for $^{12}C_{14}{}^{13}C_6H_{25}NO_5$ 365.2, found 366.2 [M + H]$^+$.

[$^{13}C_6$]-1-Amino-1-deoxy-D-fructose Hydroacetate (3). A suspension of **2** (16.4 mmol, 6.0 g) and Pd/C (10% Pd, 50% H_2O, 1.2 g) in a mixture of absolute ethanol (80 mL) and glacial acetic acid (40 mL) was hydrogenated at ambient pressure for 12 h. The suspension was filtered off, and the solids were extracted with methanol (10 × 50 mL) to separate all of the product from the catalyst. Evaporation of the combined filtrate to approximately 80 mL provided a white precipitate, which was recovered by suction filtration, washed with petroleum ether (25 mL), and dried to obtain **3** (2.52 g, 63%) as a white powder, mp 141 °C, lit.[36] mp 140−141 °C. LC−MS: purity >99%. MS (ESI$^+$): m/z calcd for $^{13}C_6H_{13}NO_5$ 185.1, found 186.3 [M + H]$^+$.

[$^{13}C_6$]-1-(4-((1*R*,2*S*,3*R*)-1,2,3,4-Tetrahydroxybutyl)-1*H*-imidazol-2-yl)ethanone ([$^{13}C_6$]THI, 4). A mixture of **1** (10.3 mmol, 1.0 g) and sodium methoxide (6.2 mmol, 1.41 mL, 25%) in anhydrous methanol (20 mL) was stirred for 1 h at room temperature. **3** (10.0 mmol, 2.45 g) was added, and the reaction mixture was left stirring.

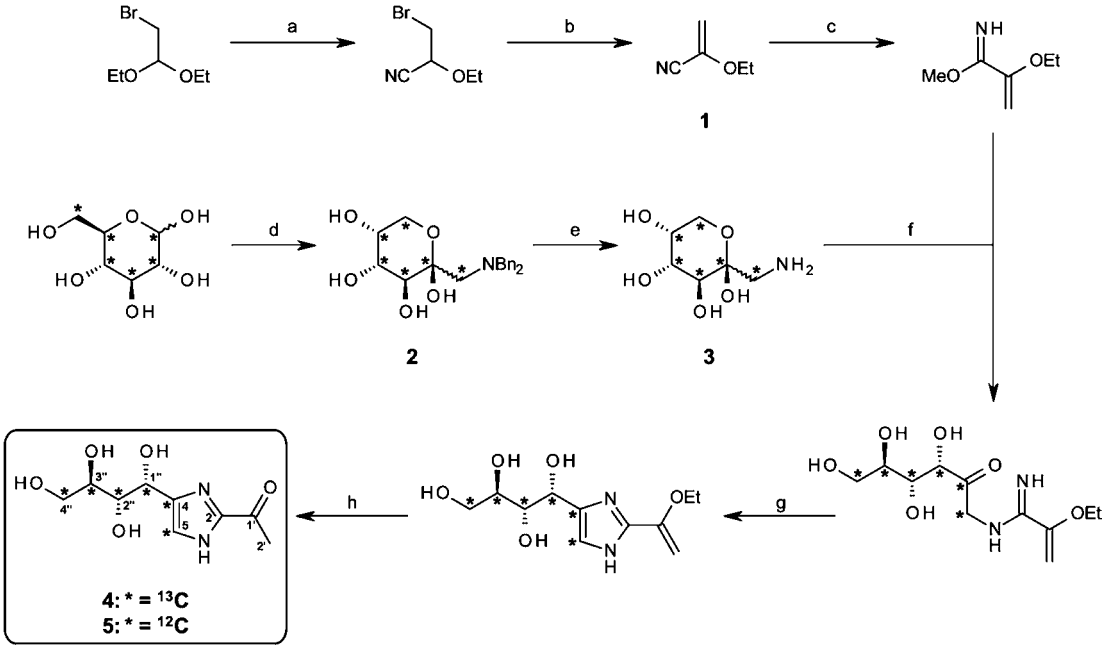

Figure 1. Synthesis of [$^{13}C_6$]THI. Reagents and conditions: (a) Me_3SiCN, $SnCl_2$, 10 °C, 12 h; (b) Et_2NH, TBME, rt, 24 h; (c) NaOMe, MeOH, rt, 1 h; (d) Bn_2NH, AcOH, EtOH, reflux, 4 h; (e) H_2, Pd/C, AcOH, EtOH, rt, 12 h; (f) MeOH, rt, 6 h; (g) NaOMe, MeOH, rt, 16 h; (h) AcOH, H_2O, 60 °C, 1 h.

After 6 h a second portion of sodium methoxide (5.2 mmol, 1.18 mL, 25%) was added, and the reaction mixture was stirred for a further 16 h. Subsequently, water (20 mL) and glacial acetic acid (20.6 mmol, 1.18 mL) were added, and the resulting solution was stirred at 60 °C for 1 h. Evaporation to approximately 15 mL followed by cooling to 0 °C for 1 h led to a precipitate that was recovered by suction filtration. The precipitate was resuspended in water (10 mL), heated to reflux for 5 min, and then cooled again to 0 °C for 1 h. Final suction filtration and drying in vacuo provided **4** (1.42 g, 60%) as an off-white powder, mp 233 °C, lit.[5] mp 234–236 °C. ^{1}H NMR (DMSO-d_6): (tautomer A (minor)) δ 2.47 (br s, 3H, 2′-H), 3.30–3.70 (m, 4H, 2″/3″/4″-H), 4.29 (br s, 1H, 4″-OH), 4.53 (br s, 1H, 3″-OH), 4.58 (br s, 1H, 2″-OH), 4.85 (br d, 1H, J = 185 Hz, 1″-H), 4.98 (br s, 1H, 1″-OH), 7.04 (dd, 1H, J = 13.9, 188 Hz, 5-H), 12.71 (br s, 1H, NH); (tautomer B (major)) δ 2.47 (br s, 3H, 2′-H), 3.30–3.70 (m, 4H, 2″/3″/4″-H), 4.28 (br s, 1H, 4″-OH), 4.29 (br s, 1H, 2″-OH), 4.53 (br s, 1H, 3″-OH), 4.75 (br s, 1H, 1″-OH), 4.76 (br d, 1H, J = 185 Hz, 1″-H), 7.20 (dd, 1H, J = 8.5, 190 Hz, 5-H), 12.96 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6): (tautomer A (minor)) δ 25.3 (C-2′), 63.5 (d, J = 40 Hz, C-4″), 64.9 (dd, J = 42, 54 Hz, C-1″), 71.4 (dd, J = 42 Hz, C-3″), 73.9 (dd, J = 43 Hz, C-2″), 128.1 (d, J = 63 Hz, C-5), 139.4 (dd, J = 61 Hz, C-4), 144.2 (d, J = 61 Hz, C-2), 188.5 (C-1′); (tautomer B (major)) δ 25.5 (C-2′), 63.7 (d, J = 41 Hz, C-4″), 67.3 (ddd, J = 3.5, 40, 60 Hz, C-1″), 71.4 (dd, J = 42 Hz, C-3″), 73.9 (dd, J = 43 Hz, C-2″), 119.2 (dd, J = 4.5, 65 Hz, C-5), 144.2 (d, J = 61 Hz, C-2), 147.0 (dd, J = 63 Hz, C-4), 188.5 (C-1′). LC–MS: purity >99%. MS (ESI$^+$): m/z calcd for $^{12}C_3{}^{13}C_6H_{14}N_2O_5$ 236.1, found 237.2 [M + H]$^+$, 259.4 [M + Na]$^+$. Anal. Calcd for $^{12}C_3{}^{13}C_6H_{14}N_2O_5$: C, 48.3; H, 6.0; N, 11.9. Found: C, 48.6; H, 6.1; N, 11.8.

1-(4-((1*R*,2*S*,3*R*)-1,2,3,4-Tetrahydroxybutyl)-1*H*-imidazol-2-yl)ethanone (THI, 5). Unlabeled THI (**5**) (1.01 g, 44%) was obtained as an off-white powder following the steps outlined above but using [$^{12}C_6$]glucose instead of [$^{13}C_6$]glucose, mp 234 °C, lit.[5] mp 234–236 °C. ^{1}H NMR (DMSO-d_6): (tautomer A (minor)) δ 2.46 (s, 3H, 2′-H), 3.35–3.63 (m, 4H, 2″/3″/4″-H), 4.32 (t, 1H, J = 5.7 Hz, 4″-OH), 4.53 (d, 1H, J = 5.4 Hz, 3″-OH), 4.59 (d, 1H, J = 6.3 Hz, 2″-OH), 4.90 (dd, 1H, J = 1.6, 7.6 Hz, 1″-H), 4.98 (d, 1H, J = 7.3 Hz, 1″-OH), 7.04 (s, 1H, 5-H), 12.70 (br s, 1H, NH); (tautomer B (major)) δ 2.47 (s, 3H, 2′-H), 3.35–3.63 (m, 4H, 2″/3″/4″-H), 4.28 (t, 1H, J = 5.7 Hz, 4″-OH), 4.29 (d, 1H, J = 6.3 Hz, 2″-OH), 4.53 (d, 1H, J = 6.3 Hz, 3″-OH), 4.75 (d, 1H, J = 6.6 Hz, 1″-OH), 4.81 (dd, 1H, J = 0.7, 7.3 Hz, 1″-H), 7.21 (s, 1H, 5-H), 12.96 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6): (tautomer A (minor)) δ 25.3 (C-2′), 63.5 (C-4″), 64.9 (C-1″), 71.4 (C-3″), 73.9 (C-2″), 128.1 (C-5), 139.4 (C-4), 143.9 (C-2), 188.5 (C-1′); (tautomer B (major)) δ 25.5 (C-2′), 63.7 (C-4″), 67.3 (C-1″), 71.4 (C-3″), 73.9 (C-2″), 119.2 (C-5), 143.9 (C-2), 147.0 (C-4), 188.5 (C-1′). LC–MS: purity 99%. MS (ESI$^+$): m/z calcd for $C_9H_{14}N_2O_5$ 230.1, found 231.4 [M + H]$^+$, 253.0 [M + Na]$^+$. Anal. Calcd for $C_9H_{14}N_2O_5$: C, 47.0; H, 6.1; N, 12.2. Found: C, 46.7; H, 6.2; N, 12.1.

RESULTS AND DISCUSSION

Published procedures for the synthesis of THI may be divided into two groups. The first group considers synthetic strategies aiming solely at THI itself and comprises those works using a D-glucose- or D-fructose-derived precursor to obtain the correct stereochemistry of the THI side chain.[3,5−7] The second group of reactions also considers the synthesis of possible analogues, e.g., with different side chain substitutions, with different imidazole substitutents, or with thiazole replacing the imidazole moiety. These procedures involve the use of sophisticated protecting groups to allow for coupling of the substituted imidazole/thiazole moiety to suitable side chain synthons by Stille coupling or organolithium-facilitated reactions.[38−44]

For the stable-labeled THI internal standard, labeling with carbon-13 was preferred over a deuterated analogue as the latter still shows significantly different physicochemical properties.[33,45] [$^{13}C_6$]THI was prepared by a multistep convergent synthesis adapting reported procedures as outlined in Figure 1.[5,7] 2-Ethoxyacrylonitrile (**1**) was prepared in a two-step sequence starting from commercially available bromoacetaldehyde diethyl acetal. The tin(II)-catalyzed reaction with trimethylsilyl cyanide provided intermediate 3-bromo-2-ethoxypropanenitrile,[46] which was subsequently dehydrobrominated using diethylamine to obtain **1** in an overall yield of 66%. Treatment of **1** with alkoxides has been shown to give the corresponding vinylimidates, which in turn are known synthons

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for imidazole synthesis.[5] The isotopically labeled precursor was obtained starting from commercially available [$^{13}C_6$]glucose. Condensation with dibenzylamine under mild acidic conditions promoted an Amadori rearrangement to provide dibenzyl-substituted fructosamine (**2**),[7] which was hydrogenated in ethanol/acetic acid under ambient conditions to afford [$^{13}C_6$]fructosamine (**3**) as its hydroacetate in an overall 58% yield. Both compounds crystallize in pyranose form,[47,48] which is also the predominant form in solution, and the compounds are therefore depicted as such in Figure 1. Following an established procedure,[5] reaction of **1** with sodium methoxide, followed by addition of **3**, produced an intermediate imidamide that was subjected to a base-promoted cyclization to provide the imidazole heterocycle. The 2-acetyl substituent of the imidazole ring was liberated from the enol ether by addition of acetic acid, and the product was isolated by precipitation. Purification from hot water provided analytically pure [$^{13}C_6$]THI (**4**) in 60% yield, which is an overall yield of 35% starting from [$^{13}C_6$]glucose.

Because of the six ^{13}C nuclei in [$^{13}C_6$]THI and the resulting complex $^{1}H-^{13}C$ coupling patterns, the acquired proton and carbon NMR spectra of **4** provided little information on first view to structurally assign the observed signals. Therefore, a ^{12}C-isotopomer of THI, i.e., compound **5**, was prepared following the synthetic pathway outlined before. NMR analysis of **5** allowed full assignment of all signals and was in accordance with the anticipated structure and literature values. On the basis of this information, the complex signals in the proton and carbon NMR spectra of **4** were successfully assigned and corroborated its identity. However, only one literature report described NMR spectra of the free THI base, i.e., not dissolved in D_2O/DCl but in DMSO-d_6.[6] These recordings showed the two annular tautomers of the imidazole, which however were not quantitated. Our observation that approximately 2/3 accounts for the 1*H*-tautomer and only 1/3 for the 3*H*-tautomer agrees well with the predicted distribution.[49] Furthermore, comparison of **4** and **5** with respect to their melting points, elemental composition, and chromatographic and mass spectrometric behavior provided a reliable basis to ensure the identity of **4**.

Excellent suitability of **5** for analysis of THI was observed as an LC−MS procedure was developed for its quantitation in caramel food colorings. Chromatographic separation of THI from the remaining matrix interferences was achieved by adapting the reported conditions to the available instrumentation.[32] While early reports of THI analysis applied acidic or neutral conditions using C_{18} or diol-substituted reversed phases,[23,26−28] recent techniques tend to use neutral or basic eluents under hydrophilic interaction liquid chromatography (HILIC) or reversed-phase conditions.[24,25,29−32] As shown in Figure 2 selected reaction monitoring (SRM) was carried out for mass spectrometric detection (ESI^+) of THI and [$^{13}C_6$]THI using specific fragmentation patterns. A neutral loss of two water molecules being liberated from the side chain gives an ion with m/z 195 ([$^{13}C_6$]THI, m/z 201), which subsequently eliminates ketene from the acetyl substituent to provide the second SRM ion of m/z 153 ([$^{13}C_6$]THI, m/z 159).[29] Monitoring of these transitions allows for selective detection of THI and [$^{13}C_6$]THI in caramel food colorings after simple dilution.

Table 1 summarizes the validation parameters obtained when the developed methodology is applied. A linear calibration range from 0.01 to 50 mg/kg (r^2 > 0.999) was established,

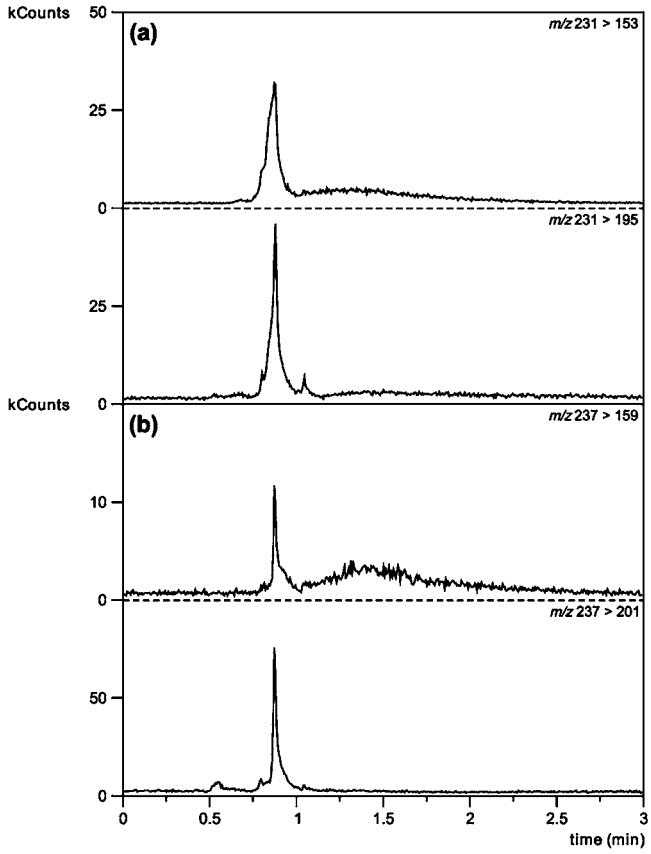

Figure 2. LC−MS analysis of a caramel class III food coloring sample (c = 0.44 mg/kg THI, approximately 2 × LOQ) showing SRM traces of THI (a) and the added internal standard [$^{13}C_6$]THI (b).

Table 1. Validation Parameters Obtained for Stable-Isotope Dilution Analysis of THI in Caramel Food Colorings

parameter	range	parameter	range
calibration range (mg/kg)	0.01−50 (r^2 > 0.999)	intraday accuracy (%, n = 4)	95.3−108.0
LOD (mg/kg)	0.08	intraday precision (%, n = 12)	9.1
LOQ (mg/kg)	0.23	interday precision (%, n = 12)	10.6

covering the expected levels in caramel food colorings. Comparison of the signals obtained in the matrix and eluent did not indicate any matrix interference. The LOQ reached in caramel food colorings was 80 μg/kg, which is equivalent to a concentration of 0.8 μg/L in solution. In addition, acceptable intraday accuracy (95.3−108.0%, mean 103.1%) and precision (intraday 9.1%, interday 10.6%) values were obtained. Overall, these parameters provide a sound proof of the excellent performance of the established stable isotope dilution assay for THI analysis.

THI levels of a set of commercially available caramel class III and IV food colorings obtained with the new assay are given in Table 2. THI levels in caramel class III food colorings were observed within the expected range (<50 mg/kg). Other findings that caramel class III food colorings may be free of THI may be biased because of sample selection or higher quantitation limits of the applied method.[31] No levels of THI in caramel class IV food colorings above the LOQ of 80 μg/kg were observed, corroborating the current position that no

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Table 2. THI Levels of Seven Commercially Available Caramel Class III (E 150c) and IV (E 150d) Food Colorings

sample	caramel class	THI[a] (mg/kg)	sample	caramel class	THI[a] (mg/kg)
1	III	20.6	5	IV	nd
2	III	20.9	6	IV	nd
3	III	47.7	7	IV	nd
4	III	1.5			

[a]Not color normalized. nd = not detectable (<80 μg/kg).

significant levels of THI are present in caramel class IV food colorings. Furthermore, investigation of THI in more complex samples giving matrix-rich extracts will be very susceptible to matrix interferences. In this respect the developed stable isotope dilution assay will be a valuable contribution to the field. Extension of this application to foods colored using caramel class III and IV food colorings, e.g., confectionery and bakery or meat products, as well as soft drinks, will be reported in due course.

AUTHOR INFORMATION

Corresponding Author

*(P.W.E.) Mailing address: Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstrasse 11, D-80937 München, Germany. Phone: ++49-89-3168-2305. Fax: ++49-89-3168-2333. E-mail: paul.elsinghorst@uni-bonn.de.

Notes

The authors declare no competing financial interest.

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