CARAMEL COLOURS

Prepared at the 74th JECFA (2011) and published in FAO JECFA Monographs 11 (2011), superseding specifications prepared at the 55th JECFA (2000), published in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). An ADI for Class I 'Not specified' was established at the 29th JECFA (1985), for Class II of 0-160 mg/kg bw was established at the 55th JECFA (2000) and an ADI for Class III of 0-200 mg/kg bw (0-150 mg/kg bw on solids basis) was established at the 29th JECFA (1985) and an ADI for Class IV of 0-200 mg/kg bw (0-150 mg/kg bw on solids basis) was established at the 29th JECFA (1985).

SYNONYMS

Caramel colours are divided into four classes. The synonyms for each class are:

Class I: Plain caramel; INS No.150a
Class II: Sulfite caramel; INS No.150b
Class III: Ammonia caramel; INS No.150c
Class IV: Sulfite ammonia caramel; INS No.150d

DEFINITION

Complex mixtures of compounds, some of which are in the form of colloidal aggregates, manufactured by heating carbohydrates either alone or in the presence of food-grade acids, alkalis or salts; classified according to the reactants used in their manufacture as follows:

Class I: Prepared by heating carbohydrates with or without acids or alkalis; no ammonium or sulfite compounds are used.
Class II: Prepared by heating carbohydrates with or without acids or alkalis in the presence of sulfite compounds; no ammonium compounds are used.
Class III: Prepared by heating carbohydrates with or without acids or alkalis in the presence of ammonium compounds; no sulfite compounds are used.
Class IV: Prepared by heating carbohydrates with or without acids or alkalis in the presence of both sulfite and ammonium compounds.

In all cases the carbohydrate raw materials are commercially available food-grade nutritive sweeteners consisting of glucose, fructose and/or polymers thereof. The acids and alkalis are food-grade sulfuric or citric acids and sodium, potassium or calcium hydroxides or mixtures thereof.

Where ammonium compounds are used they are one or any of the following: ammonium hydroxide, ammonium carbonate and ammonium hydrogen carbonate, ammonium phosphate, ammonium sulfate, ammonium sulfite and ammonium hydrogen sulfite.

Where sulfite compounds are used they are one or any of the following: sulfurous acid, potassium, sodium and ammonium sulfites and hydrogen sulfites.

Food-grade anti-foaming agents may be used as processing aids during manufacture.

DESCRIPTION

Dark brown to black liquids or solids having an odour of burnt sugar

FUNCTIONAL USES

Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Miscible with water

Identification of colouring matters (Vol. 4)

Passes test

Classification

Class I: Not more than 50% of the colour is bound by DEAE Cellulose and not more than 50% of the colour is bound by Phosphoryl Cellulose.
Class II: More than 50% of the colour is bound by DEAE Cellulose and it exhibits an Absorbance Ratio of more than 50.
Class III: Not more than 50% of the colour is bound by DEAE Cellulose and more than 50% of the colour is bound by Phosphoryl Cellulose.
Class IV: More than 50% of the colour is bound by DEAE Cellulose and it exhibits an Absorbance Ratio of not more than 50.
See description under TESTS

PURITY

Note: Arsenic and lead metals limits apply to all classes of caramel and are expressed on the basis of the product as is:
Other limits and ranges apply to the individual classes as indicated and, unless otherwise stated, are expressed on a solids basis.

Solid content

Class I: 62-77%
Class II: 65-72%
Class III: 53-83%
Class IV: 40-75%
See description under TESTS

Colour intensity

Class I: 0.01-0.12
Class II: 0.06-0.10
Class III: 0.08-0.36
Class IV: 0.10-0.60
See description under TESTS

Total nitrogen (Vol. 4)

Class I: max 0.1%
Class II: max 0.2%
Class III: 1.3 -6.8%
Class IV: 0.5-7.5%

Determine as directed under Nitrogen Determination (Kjeldahl Method) using Method II

Total sulfur	Class I: max 0.3% Class II: 1.3 -2.5% Class III: max 0.3% Class IV: 1.4-10.0% See description under TESTS
Sulfur dioxide	Class I: - Class II: max 0.2% Class III: - Class IV: max 0.5% See description under TESTS
Ammoniacal nitrogen	Class I: - Class II: - Class III: max 0.4% Class IV: max 2.8% See description under TESTS
4-Methylimidazole (MEI)	Class I: - Class II: - Class III: max 300 mg/kg & max 200 mg/kg on an equivalent colour basis Class IV: max 1000 mg/kg & max 250 mg/kg on an equivalent colour basis See description under TESTS
2-Acetyl-4-tetrahydroxy-butylimidazole (THI)	Class I: - Class II: - Class III: max 40 mg/kg & max 25 mg/kg on an equivalent colour basis. Class IV: - See description under TESTS
Arsenic (Vol.4)	Not more than 1 mg/kg (Method II)
Lead (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4, “Instrumental Methods”.

TESTS

IDENTIFICATION TESTS

Classification/Colour bound by DEAE Cellulose	For the purposes of this specification, colour bound by DEAE cellulose is defined as the percentage of decrease in absorbance of a caramel colour solution at 560 nm after treatment with DEAE Cellulose. Special reagent DEAE (diethylaminoethyl) Cellulose of 0.7 meq/gram capacity, e.g. Cellex D from Bio-Rad or equivalent DEAE Celluloses of higher or lower capacities in proportionately higher or lower quantities. Procedure

Prepare a caramel colour solution of approximately 0.5 absorbance unit at 560 nm by transferring an appropriate amount of caramel colour into a 100-ml volumetric flask with the aid of 0.025 N hydrochloric acid. Dilute to volume with 0.025 N hydrochloric acid and centrifuge or filter, if solution is cloudy. Take a 20 ml aliquot of the caramel colour solution, add 200 mg of DEAE Cellulose, mix thoroughly for several min, centrifuge or filter, and collect the clear supernatant. Determine the absorbance of the caramel colour solution and the supernatant in a 1-cm cell at 560 nm, with a suitable spectrophotometer previously standardized using 0.025 N hydrochloric acid as reference. Calculate the percentage of Colour Bound by DEAE Cellulose by the formula:

$$\frac{(X_1 - X_2)}{X_1} \times 100$$

where

X_1 is the absorbance of the caramel colour solution at 560 nm; and
X_2 is the absorbance of the supernatant after DEAE Cellulose treatment at 560 nm.

Classification/Colour bound by phosphoryl cellulose

For the purposes of this specification colour bound phosphoryl cellulose is defined as the percentage of decrease in absorbance of a caramel colour solution at 560 nm after treatment with Phosphoryl Cellulose.

Special Reagent

Phosphoryl Cellulose of 0.85 meq/gram capacity, e.g. Cellex P from Bio-Rad or equivalent Phosphoryl Celluloses of higher or lower capacities in proportionately higher or lower quantities.

Procedure

Transfer 200-300 mg of caramel colour into a 100-ml volumetric flask, dilute to volume with 0.025 N hydrochloric acid, and centrifuge or filter, if solution is cloudy. Take a 40 ml aliquot of the caramel colour solution, add 2.0 g Phosphoryl Cellulose and mix thoroughly for several min. Centrifuge or filter, and collect the clear supernatant. Determine the absorbance of the caramel colour solution and the supernatant in a 1-cm cell at 560 nm, with a suitable spectrophotometer previously standardized using 0.025 N hydrochloric acid as reference. Calculate the percentage of Colour Bound by Phosphoryl Cellulose by the formula:

$$\frac{(X_1 - X_2)}{X_1} \times 100$$

where

X_1 is the absorbance of the caramel colour solution at 560 nm; and
X_2 is the absorbance of the supernatant after Phosphoryl Cellulose treatment at 560 nm.

Classification/ Absorbance ratio

For the purposes of this specification, Absorbance Ratio is defined as the absorbance of caramel colour at 280 nm divided by the absorbance of caramel colour at 560 nm.

Procedure

Transfer 100 mg of caramel colour into a 100-ml volumetric flask with the aid of water, dilute to volume, mix and centrifuge if solution is cloudy. Pipet a 5.0 ml portion of the clear solution into a 100-ml volumetric flask, dilute to volume with water, and mix. Determine the absorbance of the 0.1% solution in a 1-cm cell at 560 nm and that of the 1:20 diluted solution at 280 nm with a suitable spectrophotometer previously standardized using water as reference. (A suitable spectrophotometer is one equipped with a monochromator to provide a band width of 2 nm or less and of such quality that the stray-light characteristic is 0.5% or less.) Calculate the Absorbance Ratio of the caramel colour by dividing the absorbance units at 280 nm multiplied by 20 (dilution factor) by the absorbance units at 560 nm.

PURITY TESTS

Solids content

The solids content of Caramel Colour is determined by drying a sample upon a carrier composed of pure quartz sand that passes a No. 40 but not a No. 60 sieve and has been prepared by digestion with hydrochloric acid, washed acid-free, dried and ignited. Mix 30.0 g of prepared sand accurately weighed with 1.5-2.0 g Caramel Colour accurately weighed and dry to constant weight at 60^{o} under reduced pressure 50 mm/Hg (6.7 kPa). Record the final weight of the sand plus caramel. Calculate the % solids as follows:

$$\% \text{ solids} = \frac{(w_F - w_S)}{w_C} \times 100$$

where
w_F is the final weight of sand plus caramel;
w_S is the weight of sand; and
w_C is the weight of caramel initially added.

Calculation on a solids basis

The contents of Total Nitrogen, Total sulfur, Ammoniacal nitrogen, sulfur dioxide, 4-MEI and THI are expressed on a solids basis. The concentration (C_I) of each impurity is determined on an "as is" basis; the concentration (C_S) on a solid basis is then calculated using the formula:

$$C_S = \frac{C_i \times 100}{\% \text{ solids}}$$

Colour Intensity

For the purpose of this specification, Colour Intensity is defined as the absorbance of a 0.1% (w/v) solution of Caramel Colour solids in water in a 1 cm cell at 610 nm.

Procedure

Transfer 100 mg of Caramel Colour into a 100 ml volumetric flask, dilute to volume with water, mix and centrifuge if the solution is cloudy. Determine the absorbance (A_{610}) of the clear solution in a 1 cm cell at 610 nm with a suitable spectrophotometer previously standardized using water as a reference. Calculate the Colour Intensity of the Caramel Colour as follows:

$$\text{Colour intensity} = \frac{A_{610} \times 100}{\%\ \text{solids}}$$

Determine % solids as described under Solids content.
Calculation on an equivalent colour basis: Where additional limits for 4-MEI and THI are expressed on an equivalent colour basis the concentrations are first calculated on a solids basis as directed under "Calculations on a solids basis", and then expressed on an equivalent colour basis according to the formula:

$$\text{Equivalent colour basis} = \frac{C_S}{\text{Colour intensity}} \times 0.1$$

where
C_S is the concentration on a solids basis.

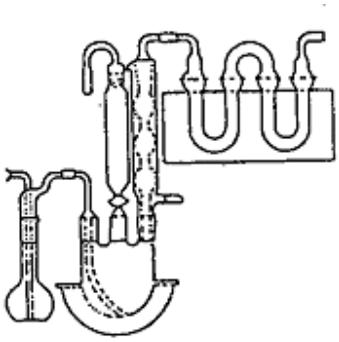
This gives content expressed in terms of a product having a Colour Intensity of 0.1 absorbance units.

Total sulfur

In the largest available casserole that fits in an electric muffle furnace, place 1-3 g MgO or equivalent quantity of $Mg(NO_3)_2 \cdot 6H_2O$ (6.4 - 19.2 g), 1 g powdered sucrose, and 50 ml HNO_3. Add 5-10 g caramel colour. Place same quantities of reagents in another casserole for blank. Evaporate on steam bath to paste. Place casserole in cold electric muffle (25º) and gradually heat until all NO_2 fumes are driven off. Cool, dissolve and neutralize with HCl (1+2.5), adding excess of 5 ml. Filter, heat to boiling, and add 5 ml 10% $BaCl_2 \cdot 2H_2O$ solution dropwise. Evaporate to 100 ml, let stand overnight, filter, wash, ignite, and weigh the $BaSO_4$. Correct result for $BaSO_4$ obtained in blank and report as mg S/100 g. Commercial instruments that analyse for total sulfur such as, the Leco-Combustion/Titration procedure can also be used and are recommended for sample amounts of about 200 mg.

Sulfur dioxide

Apparatus
Use a modified Monier-Williams apparatus (available from 5GA Scientific, Inc., Bloomfield, N.J., USA) for the determination of sulfurous acid, or construct the apparatus as shown in the figure. The assembly consists of a 1000-ml three-neck round-bottom distillation flask having 24/40 standard-taper ground-glass joints. A 30-cm Allihn condenser is attached in the reflux position to an outer neck of the flask, and the other end of the condenser is connected with 1/4-inch. Tygon or silicon tubing (preboiled with 1 in 20 hydrochloric acid solution and rinsed with water) to the absorption tube assembly (having 35/20 ball joints or the equivalent). Connect the centre neck of the flask with a 125-ml cylindrical separator, and attach a piece of tubing to a short U-tube inserted through a rubber stopper in the neck of the separator. Attach a curved glass inlet tube, reaching nearly to the bottom of the flask, to the other outer neck of the flask, and connect the inlet tube to a 250-ml gas-washing bottle with a piece of the tubing. The gas-washing bottle, in turn, is connected by tubing to a nitrogen cylinder.



Grind 4.5 g of pyrogallol (pyrogallic acid) with 5 ml of water in a small mortar, and transfer the slurry to the gas-washing bottle. Grind the residue again, and transfer it quantitatively to the bottle with two 5-ml portions of water. Pass nitrogen from the cylinder to the bottle to flush out air, and then add to the bottle, through a long-stem funnel, a cooled solution of 65 g of potassium hydroxide in about 85 ml of water. Place the head of the bottle in position, and bubble nitrogen through it to remove air from the headspace. Clamp off the tubing on both sides of the bottle, and connect it to the glass inlet tube of the distillation flask. The gas-washing bottle must be prepared with fresh pyrogallol solution as described on the day of use.

To each U-tube of the absorption tube assembly add the following: two pieces of 8-mm glass rod about 25 mm in length, 10 ml of 3-mm glass beads at the exit side, 10.0 ml of 3% hydrogen peroxide solution, and 1 drop of methyl red TS.

Assemble all pieces of the apparatus, and check for leaks by blowing gently into the tubing attached to the neck of the separator. While blowing, close the stopcock of the separator. Let stand for a few min; if the liquid levels in the U-tubes equalize, reseal all connections and test again. If the system is airtight proceed as directed below.

Procedure

Disperse about 25 g of the sample, accurately weighed, in 300 ml of recently boiled and cooled water, and transfer the slurry to the flask with the aid of water, using a large-bore funnel. Dilute to about 400 ml with water, and reseal the separator. Add 90 ml of 4 N hydrochloric acid to the separator, and force the acid into the flask by blowing gently into the tube in the neck of the separator. Close the stopcock of the separator. Unclamp the tubing on both sides of the gas-washing bottle, and start the nitrogen flow at a steady stream of bubbles. Heat the distilling flask with a heating mantle to cause refluxing in approximately 20 min. When steady refluxing is reached, apply the line voltage to the mantle and reflux for 1.75 h. Turn off the water in the condenser, and continue heating until the inlet joint of the first U-tube shows condensation and slight warming. Remove the separator and turn off the heat.

When the joint at the top of the condenser cools, remove the connecting assembly and rinse it into the second U-tube, leaving the crossover tube attached to the exit joint of the first U-tube but disconnected from the entrance of the second U-tube. Rotate the crossover tube until the free end almost touches the entrance of the first U-tube. Add 1 drop of methyl

red TS to the first U-tube, and titrate with 0.1 N sodium hydroxide just to a clear yellow colour, mixing with a gentle rocking motion. After titrating the first U-tube, remove the crossover tube, attach it to the second U-tube exit, and titrate similarly. Record the sum of the two titers as S, in ml.

Perform a blank determination, and record the volume of 0.1 N sodium hydroxide required as B. Calculate the percentage of sulfur dioxide in the sample by the formula:

$$SO_2\ \% = \frac{(S - B) \times 0.0032 \times 100}{W}$$

where

W is the weight of the sample taken, in g.

Ammonium nitrogen

Add 25 ml of 0.1 N sulfuric acid to a 500-ml receiving flask, and connect it to a distillation apparatus consisting of a Kjeldahl connecting bulb and a condenser such that the condenser delivery tube is immersed beneath the surface of the acid solution in the receiving flask. Transfer about 2 g of caramel colour, accurately weighed, into an 800-ml long-neck Kjeldahl digestion flask, and to the flask add 2 g of magnesium oxide (carbonate-free), 200 ml of water, and several boiling chips. Swirl the digestion flask to mix the contents, and quickly connect it to the distillation apparatus. Heat the digestion flask to boiling, and collect about 100 ml of distillate in the receiving flask. Wash the tip of the delivery tube with a few ml of water, collecting the washings in the receiving flask, then add 4 or 5 drops of methyl red indicator (500 mg of methyl red in 100 ml of alcohol), and titrate with 0.1 N sodium hydroxide, recording the volume, in ml, required as S. Conduct a blank determination, and record the volume, in ml, of 0.1 N sodium hydroxide required to neutralize as B. Calculate the percentage of ammoniacal nitrogen in the sample by the formula:

$$\text{Ammoniacal nitrogen} = \frac{(B - S) \times 0.0014 \times 100}{W}$$

where

W is the weight of caramel colour taken, in g.

4-Methylimidazole

NB: Information on an improved method is sought.
The following materials and reagents are required (the reagents should be ACS grade or equivalent where applicable).

Materials

Pyrex glasswool, 22 x 300 mm chromatography column with PTFE stopcock (e.g. Kimax 17800); 150 ml polypropylene beaker (e.g. Nalge 1201); 250 ml round-bottom flask (e.g., Pyrex 4320); 75 mm powder funnel; 5 cm spatula; rotary vacuum evaporator; hot plate; pan for water bath; disposable Pasteur pipets; 5 ml volumetric flask.

Reagents

Acetone; Celite 545; methylene chloride; sodium hydroxide; and tetrahydrofuran.

Procedure

After thoroughly mixing the caramel colour sample by shaking or stirring, weigh a 10.00 g aliquot into a 150 ml polypropylene beaker. Polypropylene is considered superior to glass because of its hydrophobic surface which facilitates quantitative sample transfer. A 5.0 g portion of 3.0 N NaOH is added and thoroughly mixed to ensure that the pH of the entire sample exceeds 12. A 20 g portion of Celite 545 is added to the beaker, and the contents are mixed until a semi-dry mixture is obtained. This normally requires approximately 2 to 3 min. With samples of unusually high water content, the resultant caramel colour-Celite 545 mixture may be overly wet. In such cases, a 5.00 g aliquot of caramel colour may be mixed with 2.5 g of 3.0 N NaOH and 15 g of Celite 545 and carried through the remainder of the analysis.

A plug of Pyrex glasswool is placed in the bottom of a 22 x 300 mm chromatographic column with PTFE stopcock. The caramel colour-Celite 545 mixture is placed in the column with the aid of a 75 mm powder funnel. The column contents are settled by repeatedly allowing the column to fall vertically about 10 cm to a padded surface. When properly settled, the caramel colour-Celite 545 mixture should occupy approximately the lower 250 mm of the column. Care should be exercised at this point to avoid a column bed which is either too loosely or too tightly packed. Loose packing will allow too rapid elution of the methylene chloride and possibly incomplete extraction. A too tightly packed column, e.g., the result of tamping down the column contents, can result in regions of the bed which are relatively inaccessible to the extraction solvent. This can also result in incomplete extraction.

With the stopcock open, the column is filled with methylene chloride poured from the sample beaker. When the solvent reaches the glasswool plug, the stopcock is closed and the solvent is allowed to stand in contact with the bed for 5 min. The stopcock is then opened and the column is further eluted with methylene chloride until 200 ml have been collected in a 250 ml round-bottom flask. A 1.00 ml aliquot of 2 MEI internal standard solution (50.0 mg of 2 MEI/50.0 ml of methylene chloride) is added to the collected eluate. The 2 MEI is well separated from the 4 MEI under the GLC conditions employed and has not been found in caramel colour.

The bulk of the solvent is then removed from the eluate on a rotary vacuum evaporator operated at 45-50 kPa and with the round-bottom flask maintained at 35° in a water bath. The extracted residue is transferred quantitatively to a 5 ml volumetric flask with a disposable Pasteur pipet, by rinsing the round-bottom flask several times with small (ca. 0.75 ml) portions of either tetrahydrofuran or acetone. Both solvents have been used with equal success. After mixing the contents thoroughly by several inversions of the flask, the extract is ready for GLC analysis. The extracts should be analysed as soon as possible after their preparation, because stability problems have occasionally been encountered with extracts more than 1 day old.

The GLC analysis is carried out using a gas chromatograph equipped with a hydrogen flame detector. The column is glass, 1 mm x 6 mm o.d. x 4 mm i.d., filled with 7.5% Carbowax 20M + 2% K0H on 90/100 mesh Anakrom

ABS. The GLC parameters are as follows: carrier, nitrogen, 50 ml/min; hydrogen, 50 ml/min; oxygen, 80 ml/min; injection port, 200°; column isothermal, 180°; detector, 250°; sample size, 5 µl. All quantitation is done by using the internal standard technique.

2-Acetyl-4-tetrahydroxy-butylimidazole (THI)

NB: Information on an improved method is sought.
THI is converted into its 2,4-dinitrophenylhydrazone (THI-DNPH). This derivative is separated from excess reagent and carbonylic contaminants by HPLC on RP-8, then determined by its absorbance at 385 nm.

Procedure

Caramel colour (200-250 mg) is weighed accurately, then dissolved in water (3 ml). The solution is transferred quantitatively to the upper part of a Combination Column. Elution with water is started, and a total of about 100 ml of water is passed through the columns.

The upper column is then disconnected. The lower column is eluted with 0.5 N HCl. The first 10.0 ml of eluate are discarded, then a volume of 35 ml is collected.

The solution is concentrated to dryness at 40^{o} and 15 torr. The syrup residue is dissolved in carbonyl-free methanol (250 µl) and the 2,4-dinitrophenylhydrazine reagent (250 µl) is added. The reaction mixture is transferred to a septum-capped vial and stored for 5 h at room temperature.
A volume of 5 µl (but also from 1 to 25 µl) is injected onto a LiChrosorb RP-8 (10 µm) HPLC column. The mobile phase is MeOH/0.1 M H_3PO_4 50/50 (v/v). Adjustments in mobile phase composition may be needed as column characteristics vary, depending upon the manufacturer. (Use of LiChrosorb RP-8, 10 µm, 250 x 4 mm "Vertex" column manufactured by Knauer, Bad Homburg, F.R.G. is strongly recommended). At a mobile phase flow rate of 2 ml/min and column dimensions of 250 x 4.6 mm, THI-DNPH is eluted at about 6.3±0.1 min. It is detected at 385 nm and the peak height is measured. The amount is calculated from a calibration curve prepared with THI-DNPH in methanol.

Materials

- 2,4,-Dinitrophenylhydrazine hydrochloride reagent: Commercial 2,4-dinitrophenylhydrazine (5 g) is added to concentrated hydrochloric acid (10 ml) in a 100-ml Erlenmeyer flask, and the latter is gently shaken until the free base (red) is converted to the hydrochloride (yellow). Ethanol (100 ml) is added and the mixture is heated on a steam bath until all the solid has dissolved. After crystallization at room temperature, the hydrochloride is filtered off, washed with ether, dried at room temperature and stored in a desiccator. On storage the hydrochloride is slowly converted to the free base. The latter can be removed by washing with dimethoxyethane. The reagent is prepared by mixing 0.5 g of 2,4-dinitrophenylhydrazine hydrochloride in 15 ml of 5% methanol in dimethoxyethane for 30 min. It should be stored in the refrigerator and be checked periodically by HPLC.

- Cation-exchange resin (strong): Dowex 50 AG x 8, H^+, 100-200 mesh.
- Cation-exchange resin (weak): Amberlite CG AG 50 I, H^+, (100-200 mesh). (Sediment two or three times prior to use).

- Methanol, carbonyl-free: Methanol is prepared after Y. Peleg and C.H. Mannheim, J. Agr. Fd. Chem, 18 (1970) 176, by treatment with Girard P reagent.
- Dimethoxyethane: If impure, dimethoxyethane is purified by distillation from 2,4-dinitrophenylhydrazine in the presence of acid and redistilled from sodium hydroxide. Immediately prior to use it is passed through a column of neutral aluminium to remove peroxides.

Instrumental

Combination Columns: Similar to the set-up described in J. Agr. Fd. Chem. 22 (1974) 110. The upper column (150 x 12.5 mm, filling height max. 9 cm, or 200 x 10 mm, filling height max. 14 cm, with capillary outlet of 1 mm i.d.) is filled with weakly acidic cation-exchanger; bed height, approx. 50-60, or 80-90 mm, respectively. The lower column (total length 175 mm, i.d. 10 mm, with capillary outlet and Teflon stopcock) is filled with strongly acidic cation-exchanger to a bed-height of 60 mm. As a solvent reservoir, a dropping funnel (100 ml) with Teflon stopcock is used. All parts are connected by standard ground-glass joints (14.5 mm).

HPLC: With the column specified above and an ultraviolet detector capable of reading at 385 nm.

Calibration: THI-DNPH is dissolved in absolute, carbonyl-free methanol (about 100 mg/l; calculated concentration of THI: 47.58 ng/µl). A portion of this solution is diluted tenfold with methanol (4.7 ng THI/µl). THI-DNPH standard solutions are stable for at least twenty weeks when stored in the refrigerator.