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Determination of 2,4,6-tribromoaniline in the color additives D&C Red Nos. 21 and 22 (Eosin Y) using solid-phase microextraction and gas chromatography-mass spectrometry

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Abstract

The present work demonstrates the presence of an impurity, 2,4,6-tribromoaniline (TBA), in the color additives D&C Red Nos. 21 and 21 lake (21L) and describes the determination of TBA in certified lots of D&C Red Nos. 21, 21L and 22 (Eosin Y). A method was developed using solid-phase microextraction with [$^{13}C_6$]TBA as an internal standard followed by gas chromatography-mass spectrometry analysis. Test portions from 23 lots of US-certified color additives D&C Red Nos. 21, 21L and 22 were analyzed for TBA using the new method. These lots represent domestic (four) and foreign (four) manufacturers that requested certification for the color additives during the past 2 years. Of the test portions analyzed, 12 (52.2%) contained TBA in amounts ranging from 19.9 to 638.9 ppm with an average value of ∼278.7 ppm. The remaining 11 (47.2%) test portions contained no detectable TBA or less than 0.01 ppm, which is the limit of quantification of the present method. The wide range of TBA levels found in lots submitted for certification suggest that the contamination with TBA may be avoided or significantly decreased through appropriate changes in the color-manufacturing process.

Keywords: D&C Red No. 21; D&C Red No. 22; Eosins; Aromatic amine; 2,4,6-Tribromoaniline; Color additives

1. Introduction

D&C Red No. 21 (R21, Colour Index 45380:2, mainly 2′,4′,5′,7′-tetrabromofluorescein, **1**; Fig. 1) and its disodium salt, D&C Red No. 22 (R22, Colour Index 45380, Eosin Y, mainly, **2**) as well as their lakes (R21 or R22 precipitated onto an insoluble substratum, e.g., alumina, at typically 10–20% total color content), are color additives used in the USA in drugs and cosmetics [1]. Their manufacture is schematically shown in Fig. 1 and involves several steps: condensing phthalic anhydride (or acid) with two equivalents of resorcinol in the presence of zinc chloride (or sulfuric acid); partially purifying the resulting fluorescein (F) and then brominating it to yield the main component of R21, **1**; and then hydrolyzing **1** with sodium hydroxide, resulting in the main component of R22, **2** (Fig. 1). R21, R22 and their Lakes are batch-certified by the US Food and Drug Administration (FDA) to ensure compliance with specifications described in the Code of Federal Regulations (CFR) [1]. The specifications limit the level of certain impurities in these color additives. Excess levels of specified or unspecified impurities may result in the failure of a batch of R21 or R22 to meet certification requirements established by the FDA. Sources of impurities may arise from unreacted starting materials (e.g., phthalic acid, specified at “not more than 1%”), reaction intermediates (e.g., fluorescein, specified at “not more than 0.2%”) or reaction byproducts (e.g., brominated resorcinol, specified at “not more than 0.4%”). Another source for the impurities in the color additives may be the components

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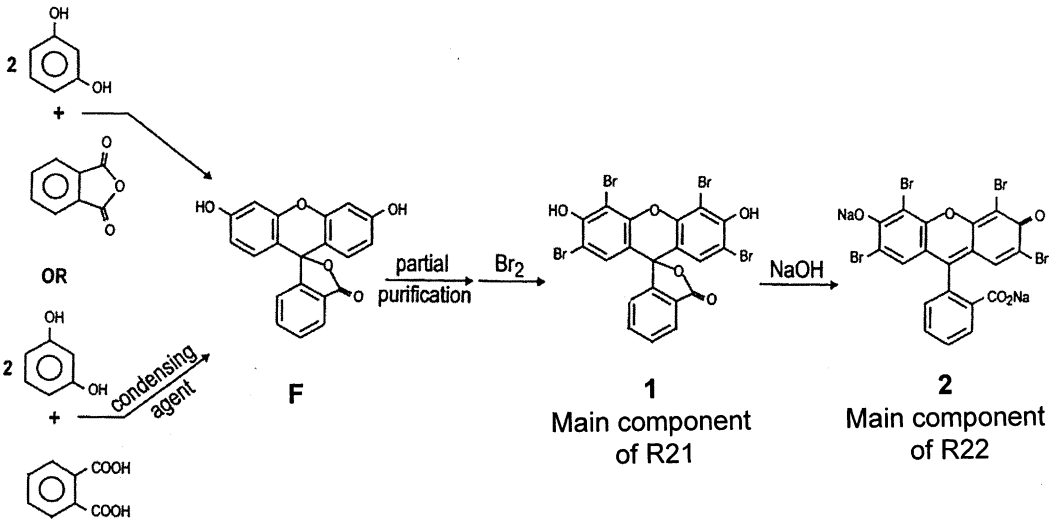

Fig. 1. Preparation of D&C Red Nos. 21 and 22 by condensation of phthalic anhydride/acid with resorcinol followed by bromination of the condensation product fluorescein (F).

present in a low-grade starting material, which can be carried over to the final product during the manufacturing process [2]. When impurities not specified in the CFR are found in color additives [2–4], their toxicity is assessed by the FDA and specifications limiting their levels may be added to the CFR.

In a prior study in our lab, 2,4,6-tribromoaniline (TBA), a previously unknown impurity, was found to be present in batches of R22 [5]. Halogenated aromatic amines are generally toxic and carcinogenic [6,7]. In order to establish limiting specifications, information was needed on the presence of TBA in related color additives. The present work describes the determination of TBA in certified lots of the color additives R21, R22 and their lakes. A method was developed using solid-phase microextraction (SPME) for the sample preparation, followed by gas chromatographic separation and mass spectrometric detection (SPME-GC-MS). The SPME technique introduced by Pawliszyn and co-workers 15 years ago [8,9] is "a powerful sample preparation tool" [10] that now has many applications. It is based on an equilibrium process in which the organic analytes partition between a fiber coated with a thin polymeric film and an aqueous media or gas media. In using the SPME-GC-MS technique, the adsorbed organic analytes are subsequently thermally desorbed into the injection port of a gas chromatograph, separated and analyzed using a mass spectrometer. The SPME theory and method development was described in detail [11,12] and its combination with mass spectrometric analysis was recently reviewed [10].

Aromatic amines have been analyzed by SPME-GC-MS in environmental water samples [13–15], biological fluids [16,17], color additives [3] and colored textiles and leather [18]. R22 is water soluble and R21 is soluble in basic aqueous solutions, and therefore, the TBA content of both color additives can be analyzed by SPME-GC-MS.

2. Experimental

2.1. Materials and instrumentation

Most of the materials, instrumentation and procedures used were described previously [3]. The SPME holder, the SPME fiber [100 μm polydimethylsiloxane (PDMS), 65 μm carbowax-divinylbenzene and 65 μm PDMS-DVB (StableFlex), coatings] assembly, silanized SPME injection port sleeves (0.75 mm i.d. for Hewlett–Packard and Agilent gas chromatographs) and the glassware surface deactivating reagent (Sylon-CT, 5% dimethyldichlorosilane in toluene) were purchased from Supelco (Bellefonte, PA, USA). The 2-ml silanized vials (Target DP) capped with TEF/SIL septum screw caps were from National Scientific (Atlanta, GA, USA). Custom pyrex coated stir bars (7 mm × 2 mm) were ordered from Spectrum Chemicals & Laboratory Products (New Brunswick, NJ, USA). The 2,4,6-tribromoaniline [^{12}C]TBA purchased from Aldrich (Milwaukee, WI, USA) was purified by flash chromatography [CH_2Cl_2/EtOAc (1:1)] prior to use as a standard (white cottony material, mp 110 °C). The [$^{13}C_6$]TBA purchased from Isotech (Miamisburg, OH, USA, minimum 99 at.% ^{13}C) was used as internal standard as received. Prior to use, the water had been deionized with a Milli-Q water system from Millipore (Bedford, MA, USA). Methylene chloride, ammonium hydroxide (28–30% NH_3 in water) and sodium hydroxide (99%) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sodium chloride (≥99.5%) was from Fluka. The analyzed samples of R21, R21 lakes (R21L) and R22 had been submitted to the FDA for batch certification during the past 2 years.

The GC-MS analyses were performed with an Agilent 6890N Network GC system interfaced with an Agilent 5973 mass-selective detector (Agilent Technologies, Wilmington, DE, USA). The gas chromatograph was equipped with a HP-

5 MS (cross-linked 5% phenyl-methylsilicone) fused-silica capillary column, 30 m × 0.25 mm i.d. with 0.25 μm film thickness.

2.2. Glassware treatment

All the glassware used for the analysis of TBA in R21 and R22 was sylanized prior to use, as described previously [19]. Silanized vials were commercially available (see Section 2.1). The volumetric flasks were filled with 5% solution of dimethyldichlorosilane in toluene (Sylon-CT). After a waiting period of 5–10 min, they were rinsed twice with toluene and twice with methanol followed by oven drying at 135 °C for 30 min.

2.3. Preparation of solutions to be analyzed

2.3.1. Preparation of the stock solutions

The stock solutions of [^{12}C]TBA [(A) 1.0552 ± 0.001 mg/ml and (B) 0.061 ± 0.001 mg/ml] were prepared by dissolving [^{12}C]TBA in methylene chloride [(A) 5.276 mg in 5 ml, (B) 0.606 mg in 10 ml]. The stock solution of [$^{13}C_6$]TBA (0.1596 ± 0.001 mg/ml) was prepared by dissolving [$^{13}C_6$]TBA (1.596 mg) in methylene chloride (10 ml). These solutions were stored in a refrigerator at ∼8 °C.

2.3.2. Preparation of solutions for calibration curves

For the [^{12}C]TBA calibration curve, approximately 50 mg of R21 (a batch of dye that was found to be free of TBA by SPME-GC-MS, sample 1 in Table 1) was placed in each of six 5-ml volumetric flasks. [^{12}C]TBA stock solution (A) or (B) was added to the dry dye to eventually yield standard solutions containing 0.012, 0.121, 0.528, 1.055, 3.166 and 7.386 ppm [^{12}C]TBA, respectively. A constant amount, 10 μl, of [$^{13}C_6$]TBA stock solution (0.1596 μg/μl) was added to the dry dye in each of the six volumetric flasks. The dye was dissolved (agitation) and brought to volume with a solution of 10% NaCl in aqueous ammonia (∼3% NH_3) (pH ∼ 11.2). For the calibration curve, 1.5 ml of each of the six standard solutions was analyzed by SPME-GC-MS as described in Sections 2.4 and 2.5

2.3.3. Preparation of dye solutions for SPME analyses

For the preparation of each of the 23 solutions of R21, R21L and R22, a test portion (approximately 50 mg dye) was placed in a 5-ml volumetric flask and 10 μl of [$^{13}C_6$]TBA stock solution (0.1596 μg/μl) was added to the dry dye as an internal standard. (Note: prior to addition as an internal standard, the refrigerated stock solution was warmed to room temperature and brought to the last recorded mass by adding methylene chloride). Approximately 2 ml of 10% NaCl in aqueous ammonia (∼3% NH_3) was then added to each volumetric flask. The resulting solutions were agitated until all the dye (R21 and R22) dissolved. (For R21L, alumina remained as a fine powder on the bottom of the flask). The solutions were diluted to volume with 10% NaCl in aqueous ammonia (∼3% NH_3).

2.4. Extraction procedure

A 2-ml silanized vial containing a custom-made pyrex coated magnetic stir bar (see Section 2.1) was filled with 1.5 ml of the [$^{13}C_6$]TBA spiked dye solution (see Section 2.3.3). The metallic needle of the SPME holder was inserted into the vial through the septum, and the SPME fiber was submerged in the sample while the sample was vigorously stirred at room temperature (25 ± 1 °C). A Model S7805, Thermolyne magnetic stirrer (Barnstead, Dubuque, IA, USA) was used with the speed controller set at 8 (∼800 rpm). Only the fiber (not the metallic needle itself) was in contact with the sample solution. After 25 min of exposure (adsorption step), the fiber was retracted into the SPME needle and immediately inserted into the heated GC injector (desorption step) for GC-MS analysis.

2.5. GC-MS method, analysis and quantification

The desorption step was achieved by exposing the SPME fiber to the hot (280 °C) GC injector for 1 min with the GC injector purge flow turned off. The injector purge was then turned on and the fiber was left in the injection port for an additional 3 min to remove any residual analytes from the fiber. The fiber was then retracted into the SPME holder, and removed from the injector, ready for the next extraction. The GC conditions were as follows: initially, the oven temperature was maintained at 60 °C for 2 min; then it was increased to 165 °C at a rate of 15 °C/min; then to 190 °C at 2 °C/min; and finally, to 300 °C at 40 °C/min, where it was held for 1 min. Helium was used as the carrier gas at a flow-rate of 40 cm/s. The temperature of the MS transfer line was 280 °C. The MS source temperature was 230 °C. The MS quadrupole temperature was 150 °C. The MS operating conditions were as follows: ionization was performed by electron ionization at 70 eV; the mass spectrometer was scanned over the range *m/z* 80–380; the threshold was set at 150; the solvent delay was set to 8 min. The total time required for the GC-MS analysis of each sample was ∼25 min. A deionized water blank was analyzed prior to the first dye sample of the day to verify that the SPME fiber and the GC column had no detectable amounts of TBA.

The TBA present in R21, R21L and R22 was quantified by using the ratio of the integrated peak areas from the extracted mass chromatogram of the ions *m/z* 329 ([^{12}C]TBA) and *m/z* 335 ([$^{13}C_6$]TBA). The amount of [^{12}C]TBA was calculated using the following equation [20]:

$$Q_{[^{12}\mathrm{C}]\mathrm{TBA}} = \frac{A_{[^{12}\mathrm{C}]\mathrm{TBA}}\, Q_{\text{internal standard}}}{A_{[^{13}\mathrm{C}_6]\mathrm{TBA}} \times \mathrm{RRF}} \quad (1)$$

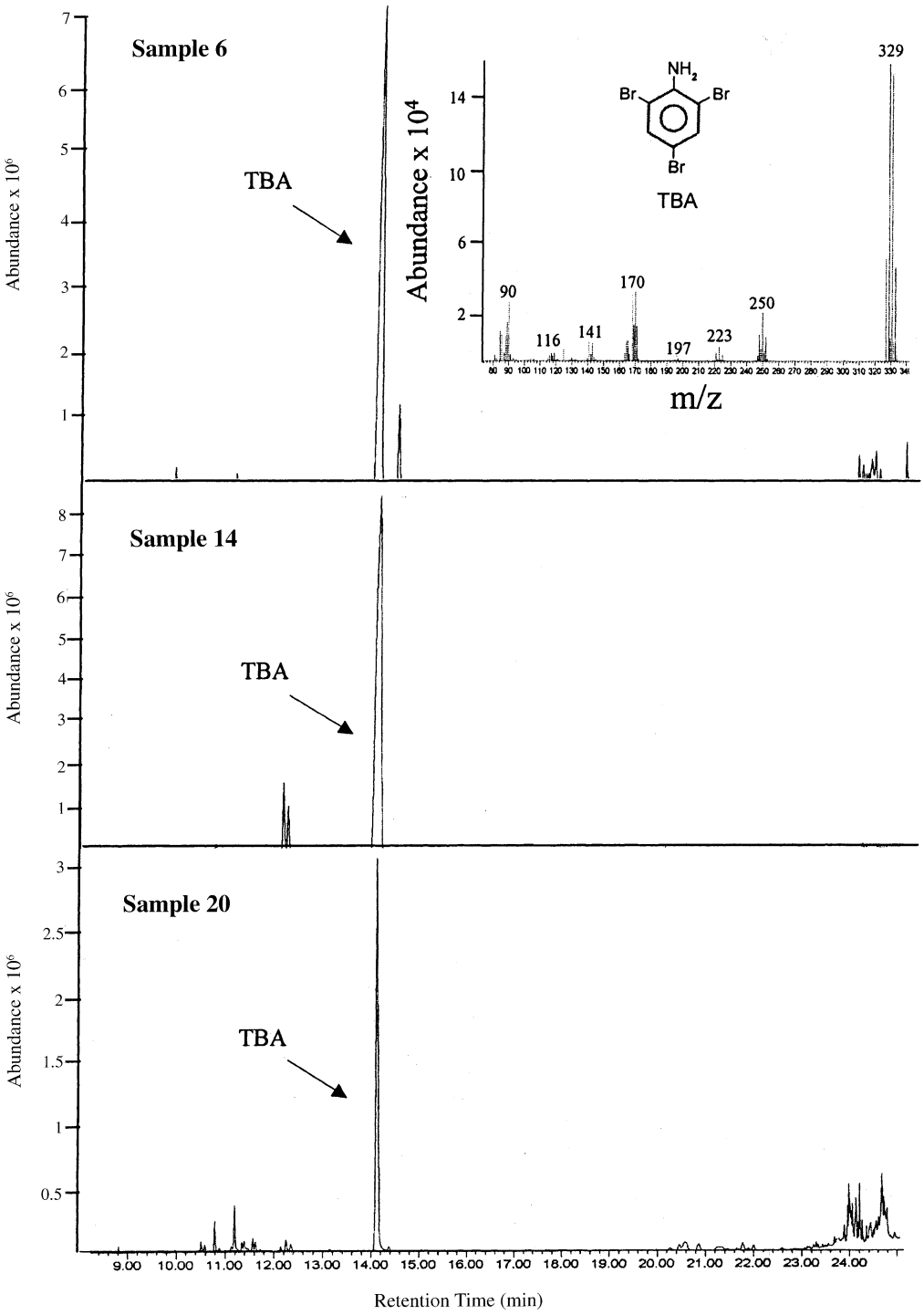

Fig. 2. Total ion chromatograms obtained by SPME-GC-MS analyses of batches of D&C Red Nos. 21 and 21L (samples 6, 14 and 20 in Table 1) and TBA mass spectrum.

where Q [^{12}C]TBA is the quantity of [^{12}C]TBA in μg; $Q_{\text{internal standard}}$ the quantity, in μg, of [$^{13}C_6$]TBA internal standard added to the 1.5 ml sample before extraction; A [^{12}C]TBA the integrated area of m/z 329 obtained from GC/MS; A [$^{13}C_6$]TBA the integrated area of m/z 335 obtained from GC-MS; RRF the relative response factor of [^{12}C]TBA/[$^{13}C_6$]TBA (the RRF was determined for four readings in the concentration range of 0.121–3.166 ppm TBA. The average value obtained was 1.05).

3. Results and discussion

The SPME method for the extraction of TBA from the color additives R21 and R22 was based on the method de-

veloped previously for the analysis of hexachlorobenzene [2] and a similar polyhalogenated aromatic amine [3] in closely-related dyes. The dye samples were prepared in a basic aqueous solution (∼3% NH_3; pH ∼ 11.2). The main components of R21 and R22 are dissociated in the aqueous solution at that pH and thus do not interfere with the extraction of TBA into the fiber coating. As in the previous studies, a fiber coated with PDMS (100 μm film thickness) and an extraction time of 25 min were found to be satisfactory for the extraction of TBA. A shorter extraction time (i.e., 15 min) was not sufficient for TBA to reach equilibrium between the aqueous phase and the PDMS fiber coating. A longer extraction time (i.e., 35 min) did not increase the amount of TBA adsorbed onto the PDMS coating. To enhance the extraction of TBA, NaCl (10%) was added to the aqueous ammonium hydroxide solution (∼3% NH_3). The availability of an isotopically-labeled internal standard ([$^{13}C_6$]TBA) that has the same chemical and physical properties as the analyte ([^{12}C]TBA), eliminated the necessity for further optimization of extraction conditions. To obtain reproducible results, as reported previously [2], the internal standard ([$^{13}C_6$]TBA dissolved in methylene chloride) was added directly onto the dry dye in a volumetric flask. To avoid contamination from commercially available PTFE-coated magnetic stir bars, which were shown to possibly retain small amounts of polychlorinated aromatic compounds after use [21,2] and leach the plasticizer dibutyl phthalate [3], custom-made pyrex coated stir bars (7 mm × 2 mm, Spectrum) were used.Fig. 2 shows total ion chromatograms (TIC) obtained using SPME-GC-MS analyses of samples of R21 and R21L (samples 6, 14 and 20, respectively in Table 1). In these TIC, TBA is the most abundant component. Its mass spectrum is shown in the attached box on the upper TIC in Fig. 2. Fig. 3a shows the TIC obtained from the analysis of a sample of R22 (sample 22 in Table 1) to which [$^{13}C_6$]TBA was added as internal standard for quantification purposes. The internal standard ([$^{13}C_6$]TBA) and

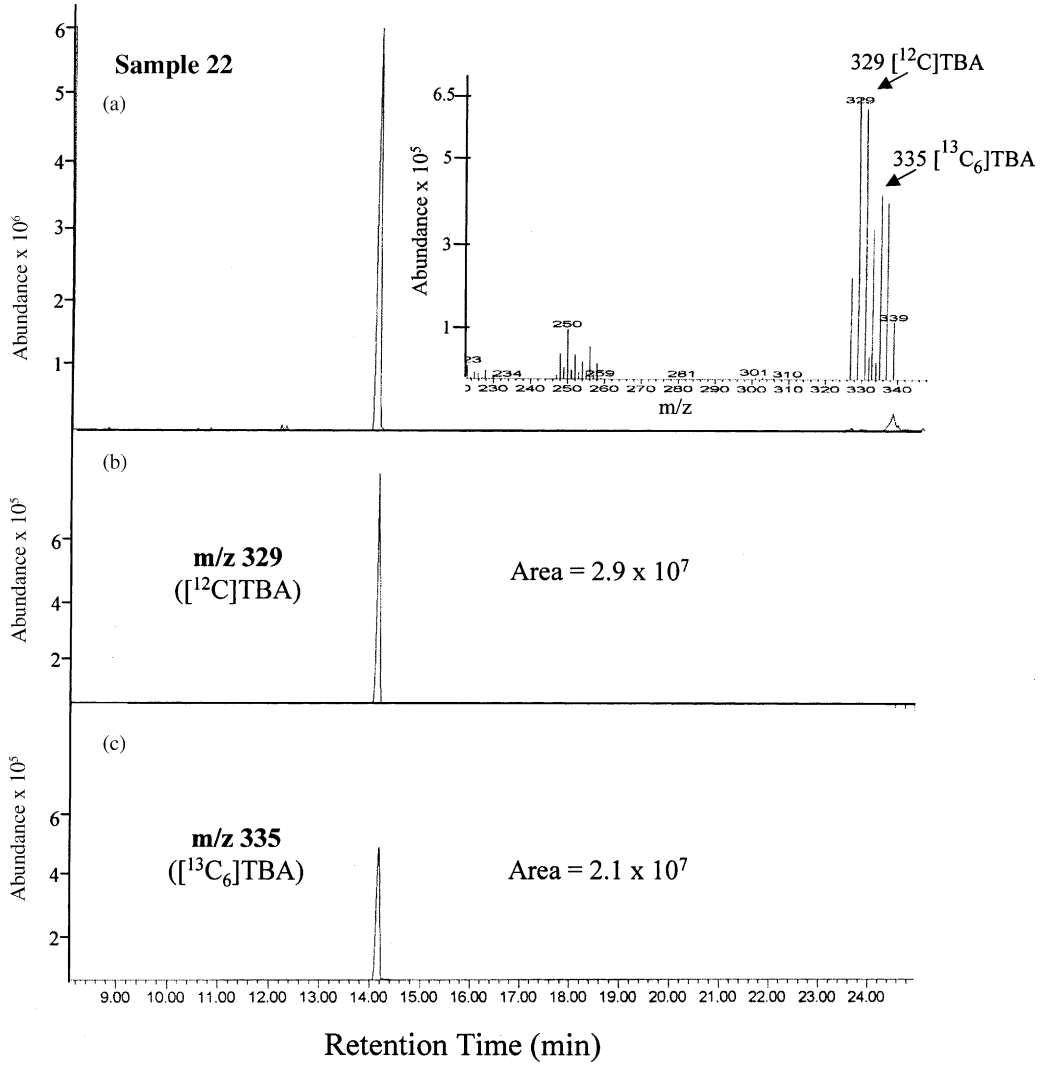

Fig. 3. SPME-GC-MS determination of the TBA present in a batch of D&C Red No. 22 (sample 22 in Table 1). (a) Total ion chromatogram of the [$^{13}C_6$]TBA spiked sample 22, (b) extracted mass chromatogram for ion *m/z* 329 of TBA present in sample 22, (c) extracted mass chromatogram for ion *m/z* 335 of [$^{13}C_6$]TBA added as an internal standard.

Table 1
Tribromoaniline (TBA) found in certified batches of color additives D&C Red Nos. 21, 21L and 22 using SPME-GC-MS

Sample number	Manufacturer	Color additive[a]	TBA found[b] (ppm)
1	AA4533[c]	R21	ND[d]
2	A	R21	ND
3	A	R22	ND
4	A	R22	ND
5	B	R21	315.6
6	B	R21	307.2
7	B	R21L[e]	26.7
8	B	R21L	40.9
9	C	R21	ND
10	C	R21	ND
11	C	R21	ND
12	D	R21	464.6
13	D	R21	448.6
14	D	R21	638.9
15	E	R22	ND
16	E	R22	ND
17	E	R22	ND
18	F	R21L	19.9
19	F	R21L	151.1
20	F	R21L	261.9
21	G	R21	539.8
22	G	R22	128.8
23	H	R22	ND

[a] Color batches certified during 2002 and 2003.
[b] Average of duplicate analyses.
[c] From toxicology test batch.
[d] Not detected (<0.01 ppm).
[e] Lake, R21 precipitated onto an insoluble substratum, e.g., alumina, at typically 10–20% total color content.

the analyte ([^{12}C]TBA) co-elute from the GC system (retention time ~14 min). Quantification of the analyte is obtained by extracting the mass chromatogram of ion *m*/*z* 328.7 for the analyte (Fig. 3b) and ion *m*/*z* 334.7 for the internal standard (Fig. 3c) and comparing their areas (see Eq. (1) in Section 2.5). Fig. 4 shows a calibration curve obtained by plotting the ratio between the peak area of [^{12}C]TBA and the peak area of [$^{13}C_6$]TBA versus the [^{12}C]TBA concentration. The

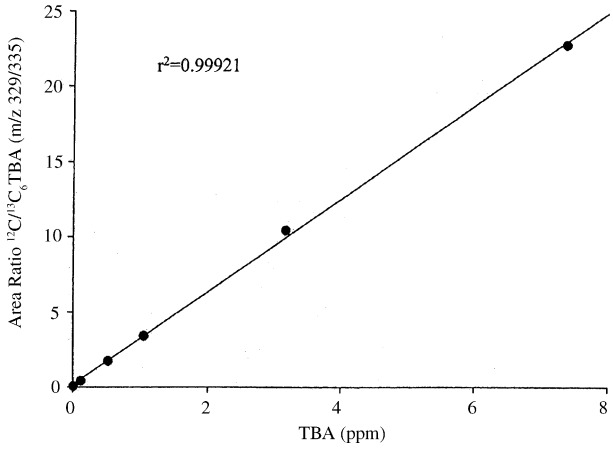

Fig. 4. A standard calibration curve for quantitative determination of TBA in the color additives D&C Red Nos. 21, 21L and 22 using SPME-GC-MS.

data points ranged from 0.01 to 7.39 ppm. Over this range, the SPME-GC-MS method shows excellent linearity, with a correlation coefficient for the linear regression of 0.999. Each determination was made in duplicate (two different aliquots for each calibration point). The precision of the method in terms of relative standard deviation (R.S.D.), measured using a 40 ppm concentration of TBA, is 0.61% as determined by analyzing three vials of the same sample.

Test portions from 23 certified lots of D&C Red Nos. 21, 21L and 22 were analyzed for TBA using the SPME-GC-MS method. The lots were submitted for certification during the past 2 years by both domestic companies (Table 1, A, C, E and F) and foreign companies [Table 1, France (B), UK (D) and India (G and H)]. The study also included one lot of R21 (Table 1, sample 1 "toxicology test batch") that was used in the animal feeding studies upon which the FDA based its safety evaluation of R21 and R22. Of the test portions analyzed, 12 (52.2%) contained TBA in amounts ranging from 19.9 to 638.9 ppm with an average value of ~278.7 ppm. The remaining 11 (47.2%) test portions contained no detectable TBA (<0.01 ppm, limit of quantification). Notably, six samples had over 300 ppm of TBA. Samples from one manufacturer (Table 1, D) had over 400 ppm of TBA in all three lots of color additive analyzed. Those six samples had a substantially higher level of TBA than that found in the "toxicology test batch", which contained no detectable TBA or <0.01 ppm (sample 1 in Table 1).

4. Conclusions

This study demonstrates the presence of TBA, a halogenated aromatic amine, in lots of D&C Red Nos. 21, 21L and 22 submitted for FDA certification. The wide range of TBA levels found in lots submitted for certification (Table 1 and Fig. 2) suggest that the presence of TBA may be avoided or significantly decreased through appropriate changes in the manufacturing process. Current regulations for D&C Red Nos. 21, 21L and 22 do not specify a limit for TBA. FDA may consider limiting the TBA levels in these color additives through specifications in the CFR. The SPME-GC-MS method presented here could be used to enforce specifications in the batch certification of these colors.

References

[1] Code of Federal Regulations, Titles 74.1321 and 74.1322, US Government Printing Office, Washington, DC, 2003.
[2] D. Andrzejewski, A. Weisz, J. Chromatogr. A 863 (1999) 37.
[3] A. Weisz, D. Andrzejewski, J. Chromatogr. A 1005 (2003) 143.
[4] A. Weisz, Dyes Pigments 35 (1997) 101.
[5] A. Weisz, D. Andrzejewski, in: Proceedings of the 41st ASMS Conference on Mass Spectrometry and Allied Topics, San Francisco, CA, 31 May to 4 June 1993, p. 137a.
[6] Y.T. Woo, D.Y. Lai, Aromatic amino and nitro-amino compounds and their halogenated derivatives, in: E. Bingham, B. Cohrssen, C.H. Powell (Eds.), Patty's Toxicology, Wiley, New York, 2001, pp. 969–1105.

[7] R.J. Lewis, Sax's Dangerous Properties of Industrial Materials, 8th ed., Van Nostrand Reinhold, New York, 1992.
[8] R.G. Belardi, J. Pawliszyn, Water Pollut. Res. J. Can. 24 (1989) 179.
[9] C.L. Arthur, J. Pawliszyn, Anal. Chem. 62 (1990) 2145.
[10] G. Vas, K. Vekey, J. Mass Spectrom. 39 (2004) 233.
[11] J. Pawliszyn, Solid Phase Microextraction—Theory and Practice, Wiley-VCH, 1997.
[12] Z. Penton, Solid Phase Microextraction—A Practical Guide, Dekker, 1999.
[13] L. Muller, E. Fattore, E. Benfenati, J. Chromatogr. A 791 (1997) 221.
[14] Z. Zeng, W.J. Qiu, M. Yang, X. Wei, Z. Huang, F. Li, J. Chromatogr. A 934 (2001) 51.
[15] T. Zimmermann, W.J. Ensinger, T.C. Schmidt, Anal. Chem. 76 (2001) 1028.
[16] L.S. DeBruin, P.D. Josephy, J.B. Pawliszyn, Anal. Chem. 70 (1998) 1986.
[17] L.S. DeBruin, J.B. Pawliszyn, P.D. Josephy, Chem. Res. Toxicol. 12 (1999) 78.
[18] F. Cioni, G. Bartolucci, G. Pieraccini, S. Meloni, G. Moneti, Rapid Commun. Mass Spectrom. 13 (1999) 1833.
[19] C.L. Arthur, K. Pratt, S. Motlag, J. Pawliszyn, J. High Resolut. Chromatogr. 15 (1992) 741.
[20] Y. Tondeur, W.F. Beckert, Method 8290: Analytical Procedures and Quality Assurance for Multimedia Analysis of Polychlorinated Dibenzo-p-Dioxins and Dibenzofurans by High-Resolution Gas Chromatography-High-Resolution Mass Spectrometry, U.S. Environmental Protection Agency, EMSL-Las Vegas, NV, 1987.
[21] Y. Yang, D.J. Miller, S.B. Hawthorne, J. Chromatogr. A 800 (1998) 257.