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Rapid quantification of hexachlorobenzene in the color additives D&C Red Nos. 27 and 28 (phloxine B) using solid-phase microextraction and gas chromatography–mass spectrometry

Denis Andrzejewski[a], Adrian Weisz[b,*]

[a]*Office of Scientific Analysis and Support, US Food and Drug Administration, Washington, DC 20204, USA*
[b]*Office of Cosmetics and Colors, US Food and Drug Administration, Washington, DC 20204, USA*

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Abstract

The present paper describes the development of a method for the quantification of hexachlorobenzene (HCB) in the color additives D&C Red Nos. 27 and 28 (phloxine B) using solid-phase microextraction followed by gas chromatography–mass spectrometry (GC–MS) analysis. The method is simple and fast (1 h for each analysis), generates little solvent waste, and does not involve a solid matrix, thus permitting a more efficient extraction than does a previously developed Soxhlet extraction–GC–MS method. Test portions from 30 batches of US-certified color additives D&C Red Nos. 27 and 28 were analyzed for HCB using the new method. Those batches represent domestic (five) and foreign (one) manufacturers that requested certification for the colors during the past four years. All the samples contained HCB, ranging from 0.2 ppm to 244.3 ppm. The analyses revealed significant differences in the levels of HCB across batches from the same manufacturer as well as among different manufacturers. The range of HCB levels found in the analyzed batches (0.2–244.3 ppm) suggest that the contamination with HCB may be decreased by avoiding use of starting material (tetrachlorophthalic anhydride) heavily contaminated with HCB. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: D&C Red No. 27; D&C Red No. 28; Phloxines; Hexachlorobenzenes; Color additives

1. Introduction

D&C Red No. 27 (R27, Colour Index 45410:1, mainly 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein, **1**; Fig. 1) and its disodium salt, D&C Red No. 28 (R28, Colour Index 45410, phloxine B, mainly **2**) as well as their lakes (R27 or R28 precipitated onto an insoluble substratum, e.g., alumina, at typically 10–40% total color content), are US-certified color additives listed for use in drugs and cosmetics. They are batch-certified by the US Food and Drug Administration (FDA) to ensure compliance with the specifications described in the Code of Federal Regulations (CFR) [1]. The first step in the manufacture of these colors involves the condensation of tetrachlorophthalic anhydride with resorcinol (Fig. 1).

In a previous study [2], it was found that hexachlorobenzene (HCB), which is present in tetrachlorophthalic anhydride as a contaminant, is carried over into the color additives during the manufacturing process. HCB has been reported to be toxic to humans, affecting their neurological [3,4], metabolic [5], and immune [6] systems. HCB was found to be

*Corresponding author. Fax: +1-202-205-5098.

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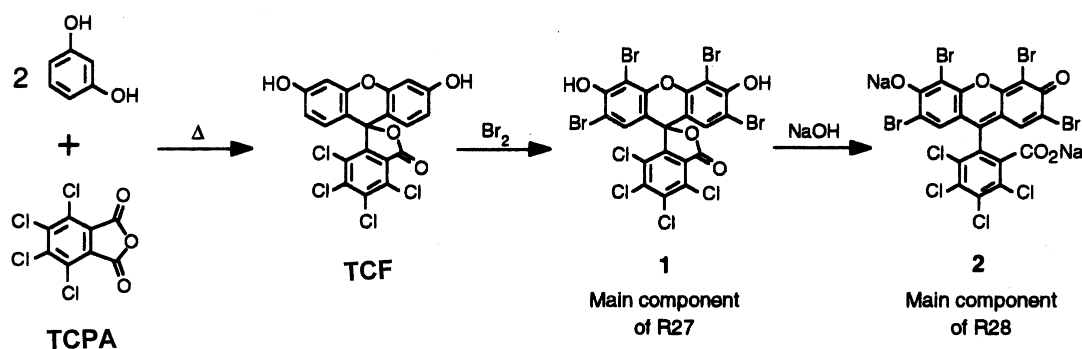

Fig. 1. Preparation of D&C Red Nos. 27 and 28 through the intermediate 4,5,6,7-tetrachlorofluorescein (TCF) by condensing resorcinol with tetrachlorophthalic anhydride (TCPA).

carcinogenic in experimental animals [7,8] and the International Agency for Research on Cancer (IARC) has also assessed its possible carcinogenic risk to humans [9]. It was therefore of interest to determine the extent and level of HCB contamination in certified lots of D&C Red Nos. 27 and 28. In the previous study [2], each color test portion (30–60 g) was Soxhlet-extracted with chloroform (350 ml) for approximately 50 h. The solvent was eliminated by rotary evaporation and an aliquot of the redissolved residue was analyzed by gas chromatography–mass spectrometry (GC–MS). While that method was reproducible, it required considerable time, generated a large volume of solvent waste, and yielded low HCB recoveries.

Current US regulations for D&C Red Nos. 27 and 28 do not include a limit for HCB, although, the FDA is assessing the need to limit the HCB levels in these color additives. For batch certification purposes, the development of a simple, rapid, sensitive and reliable method for the quantification of HCB in these colors is necessary. This paper reports the development of such a method by using solid-phase microextraction (SPME). SPME was relatively recently developed by Pawliszyn and co-workers [10,11], as a simple technique for the quantitative analysis of organic compounds in aqueous (solid or gaseous) media. The principle of the technique has been previously described in detail [12,13], and it is based on the equilibrium process in which the analytes partition between a fiber coated with a polymeric film and the aqueous media. The adsorbed analytes are subsequently either thermally desorbed into the injection port of a gas chromatograph, separated and analyzed (SPME–GC or SPME–GC–MS) or solvent-desorbed in a special desorption chamber prior to liquid chromatography (LC) (SPME–HPLC and SPME–LC–MS) [14–16]. A recent book by Pawliszyn describes the details of SPME method development [17]. Two reviews summarize the many applications of SPME [18,19], which include the analysis of polyhalogenated aromatic compounds in aqueous media [10,20–23]. D&C Red No. 28 dissolves in water and D&C Red No. 27 can be dissolved in a basic aqueous solution, and therefore, the HCB content of both color additives can be analyzed by SPME–GC–MS.

2. Experimental

2.1. Materials and instrumentation

The SPME holder, the SPME fiber [100 μm poly(dimethylsiloxane) (PDMS) coating] assembly, silanized SPME injection port sleeves (0.75 mm I.D. for Hewlett-Packard 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 and non-silanized vials (Target DP) capped with TEF/SIL septum screw caps were from National Scientific (Atlanta, GA, USA). The micro stir bars (7 mm length×2 mm diameter) coated with PTFE were purchased from Cole-Parmer Instrument Company (Vernon Hills, IL, USA). The hexachlorobenzene [^{12}C]HCB obtained from Aldrich (Mil-

waukee, WI, USA) was recrystallized from 2-propanol prior to use as a standard. Prior to use, the water had been deionized with a Milli-Q water system from Millipore (Bedford, MA, USA). The tetrabromotetrachlorofluorescein used as a matrix for the calibration curve had been purified by pH-zone-refining counter-current chromatography [24] and was free of HCB (by SPME–GC–MS). Alternatively, a portion of D&C Red No. 28 dissolved in water and extracted several times with methylene chloride can be used for the calibration curve as a matrix free of HCB. The analyzed samples of D&C Red Nos. 27 and 28 had been submitted to the FDA for batch certification during the past four years. Methylene chloride (J.T. Baker, Phillipsburg, NJ, USA), ammonium hydroxide (28–30% NH_3 in water, Fluka, Buchs, Switzerland), [$^{13}C_6$]HCB (99%, Cambridge Isotope Labs., Woburn, MA, USA) were used as received.

The GC–MS analyses were performed with an HP-5890 Series II gas chromatograph interfaced with an HP-5971 mass-selective detector (Hewlett-Packard, Wilmington, DE, USA). The gas chromatograph was equipped with an 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 HCB in the color additives was silanized prior to use, as described previously [20]. The volumetric flasks and the vials used were filled with 5% solution of dimethyldichlorosilane in toluene (Sylon-CT). After a 12–16 h waiting period, they were rinsed once with toluene and once with methanol followed by oven drying at 150°C for 1 h. Alternatively, silanized vials may be purchased (see Section 2.1).

2.3. Preparation of standard solutions

2.3.1. Preparation of the stock solutions

The stock solutions of [^{12}C]HCB [e.g., (A) 0.051 μg/μl, (B) 1.5 μg/μl] were prepared by dissolving [^{12}C]HCB [e.g., (B) 15.0 mg], recrystallized from 2-propanol, in methylene chloride (10 ml) in a 10-ml volumetric flask. The stock solution of [$^{13}C_6$]HCB (e.g., 0.15453 μg/μl) was prepared by dissolving [$^{13}C_6$]HCB (e.g., 1.5453 mg) in methylene chloride (10 ml) in a 10-ml volumetric flask. These solutions were stored in the freezer (~−14°C).

2.3.2. Preparation of the solutions for the calibration curve

For the [^{12}C]HCB calibration curve, approximately 50 mg of tetrabromotetrachlorofluorescein free of HCB was placed in each of six 5-ml silanized volumetric flasks. [^{12}C]HCB stock solution (A) or (B) was added to the dry dye to eventually yield standard solutions containing 0.01, 0.05, 0.1, 0.6, 1.05 and 3.0 ppm [^{12}C]HCB, respectively. A constant amount, 15 μl, of [$^{13}C_6$]HCB stock solution (1.5453 mg/10 ml) was added to the dry dye in each of the six volumetric flasks. Approximately 2 ml of aqueous ammonia (≈3% NH_3) was then added to each volumetric flask. The resulting solutions were sonicated and vigorously agitated until all the dye dissolved. The solutions were diluted to volume with aqueous ammonia (≈3% NH_3). For the calibration curve, 1.5 ml of each of the above standard solutions were analyzed as described in Section 2.4 below.

2.3.3. Preparation of the dye solutions for direct SPME analyses

For the preparation of each solution of R27 and R28, a test portion (approximately 50 mg) was placed in a 5-ml silanized volumetric flask and 15 μl of [$^{13}C_6$]HCB stock solution (1.5453 mg/10 ml) was added onto the dry dye as an internal standard. (Note: prior to addition as an internal standard, the freezer kept stock solution was warmed to room temperature and brought to the last recorded mass by adding methylene chloride). Approximately 2 ml of aqueous ammonia (≈3% NH_3) was then added to the volumetric flask. The resulting solution was sonicated and mixed until all the dye dissolved. The solution was diluted to volume with aqueous ammonia (≈3% NH_3).

2.3.4. Preparation of the Soxhlet chloroform extracts for SPME analyses

The present analyses involved use of previously-obtained [2] extracts of test portions of D&C Red Nos. 27 and 28. In that earlier work, each test

portion (30–60 g) was Soxhlet-extracted with chloroform for approximately 50 h. The solvent was eliminated by rotary evaporation and the residue obtained was analyzed for HCB by GC–MS. In the present study, the residues obtained earlier were re-analyzed for HCB by SPME–GC–MS as described below. Each residue was re-dissolved in 5 ml of methylene chloride, 1 ml of the resulting solution was placed into a massed silanized 5-ml volumetric flask and the solvent was evaporated at room temperature. The flask was then massed again. The difference represents the chloroform residue obtained from the extraction of 1/5th of the original dye. For the SPME extraction, 10 μl of [$^{13}C_6$]HCB stock solution (1.5453 mg/10 ml) was added to the dry residue as an internal standard and the mixture was dissolved and diluted to volume with aqueous ammonia (≈3% NH_3). SPME extraction of 1.5 ml of this solution was then conducted as described in Section 2.4.

2.3.5. Preparation of the Soxhlet-extracted dyes (thimble residues) for SPME analyses

The dye that remained in the Soxhlet thimble after the chloroform extractions described above (Section 2.3.4) was analyzed for HCB by SPME–GC–MS. The first step of this process was thorough mixing of the dry dye contained in the Soxhlet thimble that had undergone Soxhlet extraction with chloroform. Approximately 50 mg of the dye from the thimble was placed in a 5-ml silanized volumetric flask and 15 μl of [$^{13}C_6$]HCB stock solution (1.5453 mg/10 ml) was added to the dry dye as an internal standard. The dye was dissolved and diluted to volume with aqueous ammonia (≈3% NH_3) as described above (Section 2.3.3).

2.4. Extraction procedure

A 2-ml silanized vial fitted with an unused PTFE-coated micro magnetic stir bar was filled with 1.5 ml of the [$^{13}C_6$]HCB spiked dye solution (see Sections 2.3.3–2.3.5). A previously unused stir bar was necessary for each extraction because it was observed that the PTFE coating retained a small amount of HCB. The metallic needle of the SPME holder was inserted into the vial through the septum, and the SPME fiber was submerged in the sample solution while the sample was vigorously stirred at room temperature (26±2°C). A Model 4805, Micro-V magnetic stirrer (Cole-Parmer, Chicago, IL, USA) was used with the speed controller set at MAX (approximately 1100 rpm). Care was taken to ensure that only the fiber (not the metallic needle itself) was in contact with the sample solution. After exactly 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

To achieve complete desorption, the SPME fiber was exposed to the hot (280°C) GC injector for 3 min with the GC injector purge flow 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 contaminants from the fiber. After that period of time, the fiber was retracted into the SPME holder which was removed from the injector and it was ready for the next extraction. The GC operating conditions were as follows: initially, the oven temperature was maintained at 70°C for 4 min, then it was increased to 150°C at a rate of 15°C/min, then to 180°C at 4°C/min, and finally, to 300°C at 20°C/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 operating conditions were as follows: ionization was performed by electron impact at 70 eV. The mass spectrometer was scanned over the range m/z 220–320. The threshold was set at 150. The solvent delay was set to 13 min. The total time required for the GC–MS analysis of each sample was approximately 23 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 HCB.

The [^{12}C]HCB present in R27 and R28 was quantified by using the ratio of the integrated peak areas from the extracted mass chromatogram of the ions m/z 286 ([^{12}C]HCB) and m/z 292 ([$^{13}C_6$]HCB). The amount of [^{12}C]HCB was calculated using the following equation [25]:

$$Q_{[^{12}C]HCB} = \frac{A_{[^{12}C]HCB} \cdot Q_{\text{internal standard}}}{A_{[^{13}C_6]HCB} \cdot \text{RRF}} \quad (1)$$

where $Q_{[^{12}C]HCB}$ = quantity of $[^{12}C]$HCB in μg, $Q_{internal\ standard}$ = quantity, in μg, of $[^{13}C_6]$HCB internal standard added to the 1.5-ml sample before extraction, $A_{[^{12}C]HCB}$ = integrated area of m/z 286 obtained from GC–MS, $A_{[^{13}C_6]HCB}$ = integrated area of m/z 292 obtained from GC–MS, and RRF = relative response factor of $[^{12}C]$HCB/$[^{13}C_6]$HCB (the RRF was determined for seven readings in the concentration range of 0.01–5 ppm HCB. The average value obtained for this experiment was 1.03).

3. Results and discussion

For the development of the present SPME method,

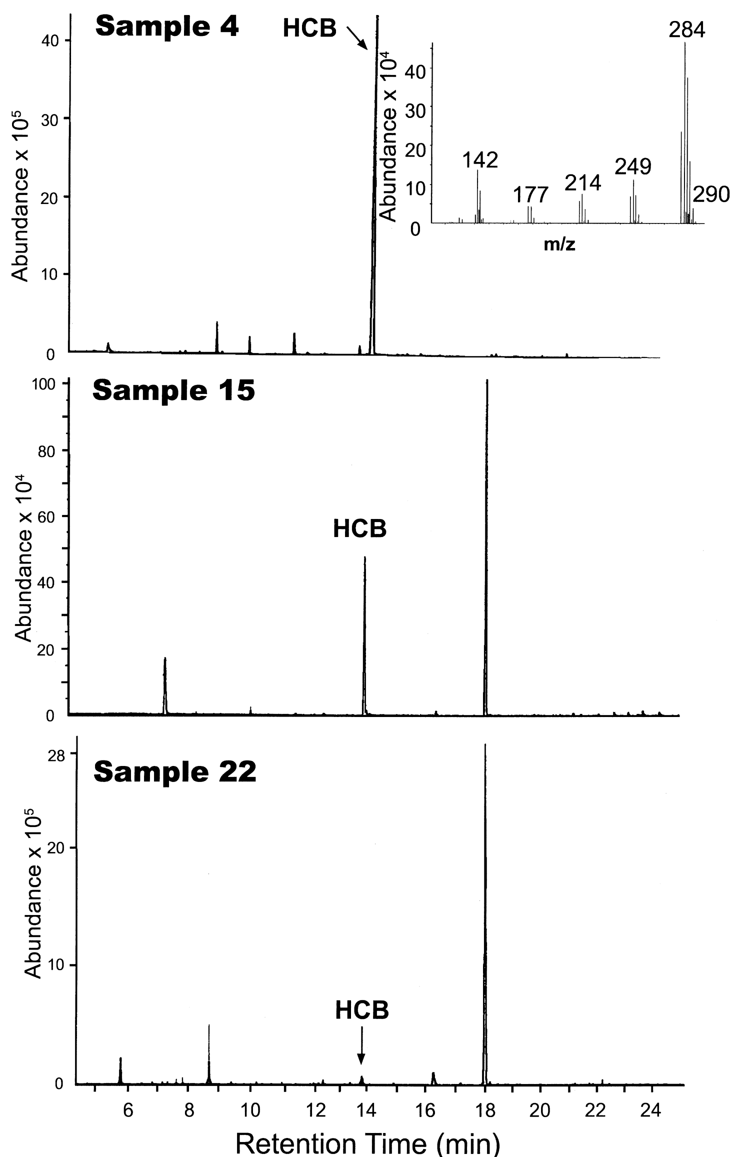

Fig. 2. Total ion chromatograms obtained by direct SPM–GC–MS analysis of batches of D&C Red Nos. 27 and 28 (samples 4, 15 and 22 in Table 1) and HCB mass spectrum.

we followed the guidelines outlined by Pawliszyn in his recently published book [17]. The commonly-used fiber coated with PDMS of 100 μm film thickness and a 25-min extraction time were found to be suitable for the extraction of HCB from dyes. Experiments with other coatings [i.e., poly(acrylate)] and film thicknesses showed no observable advantages. The availability of an isotopically-labeled internal standard ([$^{13}C_6$]HCB) that has the same chemical and physical properties as the analyte ([^{12}C]HCB), eliminated in this case the necessity for the optimization of extraction conditions. The dye samples were prepared in a basic aqueous solution (pH approximately 11.4). At that pH level, the main components of D&C Red Nos. 27 and 28 are dissociated in the aqueous solution and thus do not interfere with the extraction of HCB by the fiber's coating. In an attempt to enhance the extraction of HCB, salt was added to the aqueous solution. When an NaCl saturated ammonium hydroxide solution (~3% NH_3) was used to dissolve the sample, the dye precipitated out of the solution and the extraction of HCB was poor. When a 1% NaCl solution of ammonium hydroxide (~3% NH_3) was used to

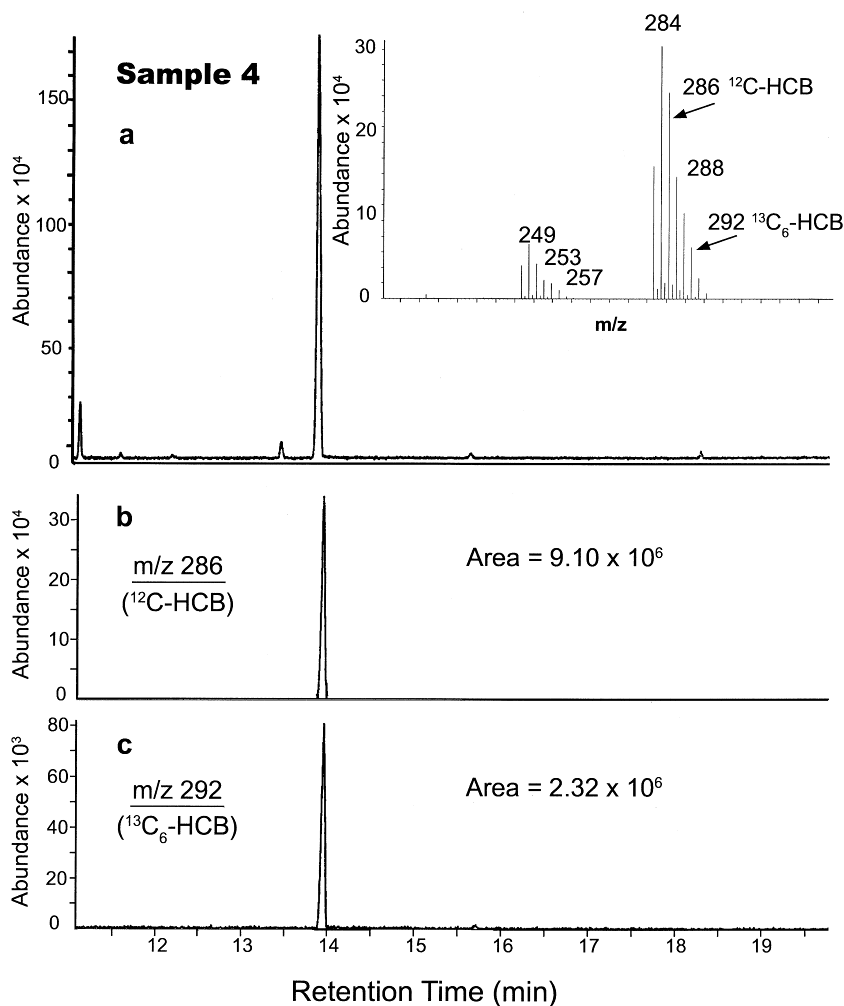

Fig. 3. SPME–GC–MS determination of the HCB present in a batch of D&C Red No. 28 (sample 4 in Table 1). (a) Total ion chromatogram of the [$^{13}C_6$]HCB spiked sample 4, (b) extracted mass chromatogram for ion m/z 286 of HCB present in sample 4, (c) extracted mass chromatogram for ion m/z 292 of [$^{13}C_6$]HCB added as internal standard.

dissolve the sample, the extraction of the HCB by the fiber was enhanced by a factor of approximately 2, and the ratio of the extracted [^{12}C]HCB to [$^{13}C_6$HCB] remained very close to the ratio obtained when the extraction was performed in the un-salted aqueous solution. The 1% NaCl ammonium hydroxide solution may be used for the SPME–GC–MS method when a lower detection limit is needed for HCB. The limit of detection using the un-salted aqueous solution was determined by spiking a portion of HCB-free dye with known quantities of [^{12}C]- and [$^{13}C_6$]HCB standards and analyzed by the SPME–GC–MS method. Using a signal-to-noise ratio of 3:1, the limit of detection was found to be 5 ppb of HCB. The results described in the present paper were obtained using for SPME the un-salted aqueous ammonium hydroxide solution.

To obtain reproducible results, the internal standard ([$^{13}C_6$]HCB dissolved in methylene chloride) must be added directly onto the dry dye in the volumetric flask. Adding it to the aqueous dye solution causes the methylene chloride to settle at the bottom resulting in the release of unequal amounts of the internal standard from one analysis to the next. During the development of the method, it was observed that the PTFE-coated magnetic stir bars retained a small quantity of HCB that could not be removed even by prolonged sonication in methanol. Because of this contamination, a new stir bar was used for each analysis. A similar contamination of the PTFE-coated stir bars was observed during SPME analyses of polychlorinated biphenyls (PCBs) in water samples [26].

Fig. 2 shows a typical total ion chromatogram (TIC) obtained from the SPME–GC–MS analysis of a sample of D&C Red No. 28. In this specific case, the HCB response is the most abundant component of the TIC. Its mass spectrum is shown in the attached box. Fig. 3a shows the TIC obtained from the analysis of the same dye sample to which internal standard was added for quantification purposes. The internal standard ([$^{13}C_6$]HCB) and the analyte

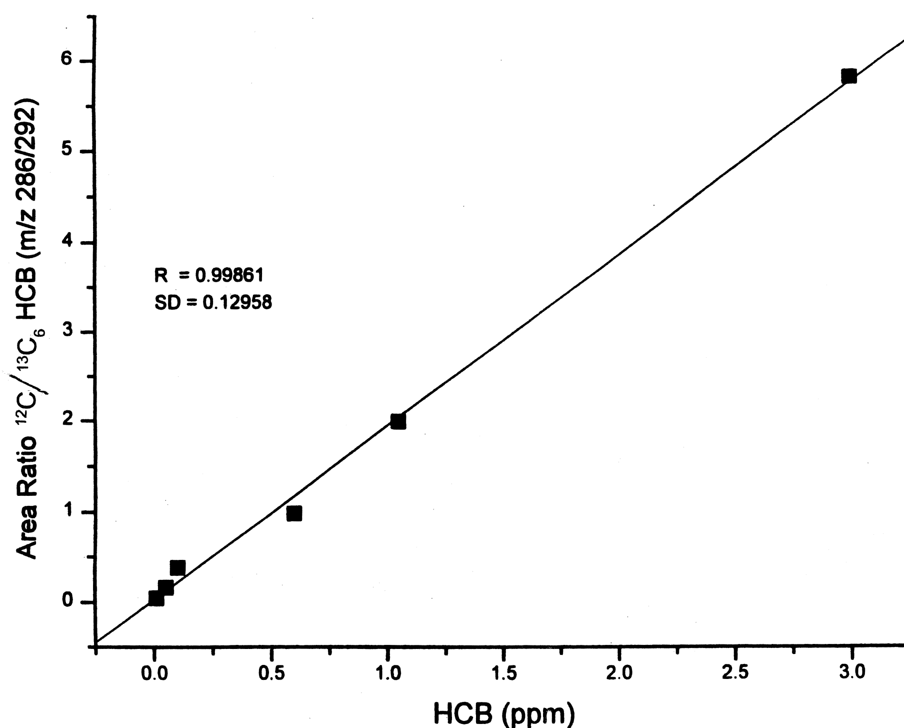

Fig. 4. A standard calibration curve for quantitative determination of HCB in the color additives D&C Red Nos. 27 and 28 using SPME–GC–MS.

([^{12}C]HCB) co-elute from the GC system (retention time 14 min). Quantification of the analyte is obtained by extracting the mass chromatograms of ion m/z 285.8 for the analyte (Fig. 3b) and ion m/z 291.8 for the internal standard (Fig. 3c) and comparing their area (see Eq. (1) in Section 2.5). A calibration curve obtained by plotting the peak area ratios between [^{12}C]HCB and [$^{13}C_6$]HCB against the [^{12}C]HCB concentration is shown in Fig. 4. The data points ranged from 0.01 ppm to 3 ppm [^{12}C]HCB. Over this range, the SPME–GC–MS method shows very good linearity. The correlation coefficient for the linear regression was 0.998. The precision of the method in terms of relative standard deviation (RSD), determined by analyzing six vials of the same sample, is 0.33%.

Test portions from 30 certified lots of D&C Red Nos. 27 and 28 were analyzed for HCB using the SPME–GC–MS method. The manufacturers represented in those lots include both domestic (A, B, C, G in Table 1) and foreign companies (H – Japan, I – France) and all have requested certification for the colors during the past four years. The study includes two lots of D&C Red No. 27 (‘‘toxicology test batches’’, samples 1 and 2 in Table 1) that were used in the animal feeding studies upon which the FDA based its safety evaluation of D&C Red Nos. 27 and 28. All the samples analyzed were found to contain HCB, at levels ranging from 0.2 ppm to 244.3 ppm. Notably, one manufacturer (A in Table 1) had over 100 ppm of HCB in seven of the eight lots of color additive analyzed. This is a substantially higher level than that found in the batches that were the subject of animal testing, and which contained 11.7 and 14.5 ppm, respectively (Table 1).

Table 1
Hexachlorobenzene found in certified batches of color additives D&C Red Nos. 27 and 28 by direct SPME–GC–MS

Sample No.	Manufacturer	Color additive[a]	HCB found[b] (ppm)
1	AA-4623[c]	R27	11.7[d]
2	AA-8945[c]	R27	14.5[d]
3	A	R27	67.8
4	A	R28	191.6
5	A	R28	115.9
6	A	R28	244.3
7	A	R28	167.6
8	A	R28	108.0
9	A	R28	155.2
10	A	R28	120.1
11	B	R28	3.3
12	B	R28	7.3
13	B	R28	2.4
14	B	R28	2.4
15	C	R28	11.5
16	C	R28	0.2
17	C	R28	0.6
18	C	R28	49.5
19	C	R28	3.1
20	C	R27	0.2
21	C	R27	31.5
22	G	R27	0.8
23	G	R27	8.6
24	G	R27	2.1
25	H	R28	4.1
26	H	R27	2.4
27	H	R27	1.8
28	H	R28	1.7
29	H	R28	1.2
30	I	R27	0.5
31	I	R27	1.1
32	I	R28	1.4

[a] Color batches certified between 1994 and 1998.
[b] Average of duplicate analyses.
[c] From toxicology test batch.
[d] Average of triplicate analyses.

In a previous study [2], test portions (30–60 g) of D&C Red Nos. 27 and 28 were Soxhlet-extracted with chloroform (350 ml) for approximately 50 h. The solvent was eliminated by rotary evaporation and an aliquot of the redissolved residue was analyzed for HCB by GC–MS. For validation purposes, several colors from the above study [2] were reanalyzed by direct SPME–GC–MS. The previously-analyzed chloroform extracts were re-analyzed for HCB by SPME–GC–MS. The results obtained for this comparative study are shown in Table 2. The HCB levels obtained by SPME–GC–MS analyses of the chloroform extracts were similar to those obtained previously by GC–MS (columns 4 and 5 in Table 2). The slightly lower levels obtained by SPME–GC–MS can be attributed to the depletion of the analyte during the earlier GC–MS analyses. In contrast, a much higher quantity of HCB (between 6- to 39-times more) was found by direct SPME–GC–MS analyses of the colors than was reported previously by the Soxhlet–GC–MS method (compare columns 7 and 4 in Table 2). The reason for the discrepancy was therefore investigated. SPME–GC–MS analyses of the dye remaining in the thimble

Table 2
Hexachlorobenzene found in certified batches of color additive D&C Red No. 28 by Soxhlet extraction–GC–MS as compared to Soxhlet extraction–SPME–GC–MS and direct SPME–GC–MS

Sample No.	Manufacturer	Color additive[a]	HCB found (ppm)			
			Soxhlet–GC–MS (chloroform extract)[b]	Soxhlet–SPME–GC–MS (chloroform extract)	Soxhlet–SPME–GC–MS (thimble residue)	SPME–GC–MS
1	A	R28	0.20	0.16	1.43	1.67
2	B	R28	0.03	0.01	0.92	0.82
3	C	R28	0.20	0.08	1.56	1.26
4	C	R28	0.30	0.03	2.94	2.89
5	C	R28	1.90	2.01	13.60	11.50
6	D	R28	18.90	6.76	227.00	270.90
7	E	R28	0.30	0.36	2.18	2.79
8	F	R28	0.08	0.02	3.54	3.10
9	F	R28	0.20	0.09	2.26	2.25

[a] Color batches used in Ref. [2].
[b] From Ref. [2].

after Soxhlet extraction demonstrated that the solid dye matrix prevented quantitative extraction of HCB. Thus, the sum of the HCB found in the Soxhlet extract (column 4 in Table 2) and the HCB remaining in the dye after extraction (column 6 in Table 2) was in agreement with the quantity of HCB found by direct SPME–GC–MS analysis (column 7 in Table 2) of the respective dye.

As was mentioned previously [2], the major source for the HCB present in D&C Red Nos. 27 and 28 is the starting material tetrachlorophthalic anhydride (TCPA). SPME–GC–MS analyses of commercial batches of TCPA obtained from three different suppliers, confirmed the earlier results (Fig. 5). Each of the analyzed batches of TCPA contained HCB as a contaminant.

4. Conclusions

The results obtained demonstrate that the SPME–GC–MS method described here for the determination of HCB in the color additives D&C Red Nos. 27 and 28 is faster and more quantitative than the previously developed Soxhlet–GC–MS method [2]. It is also simpler to implement, significantly reduces analysis time (1 h for each analysis), and generates much less solvent waste. The range of HCB levels found in batches submitted for certification (e.g., Table 1 and Fig. 2) suggest that the contamination with HCB may be decreased by avoiding use of starting material (TCPA) heavily contaminated with HCB [27]. Current regulations for D&C Red Nos. 27 and 28 do not specify a limit for HCB. FDA is considering the

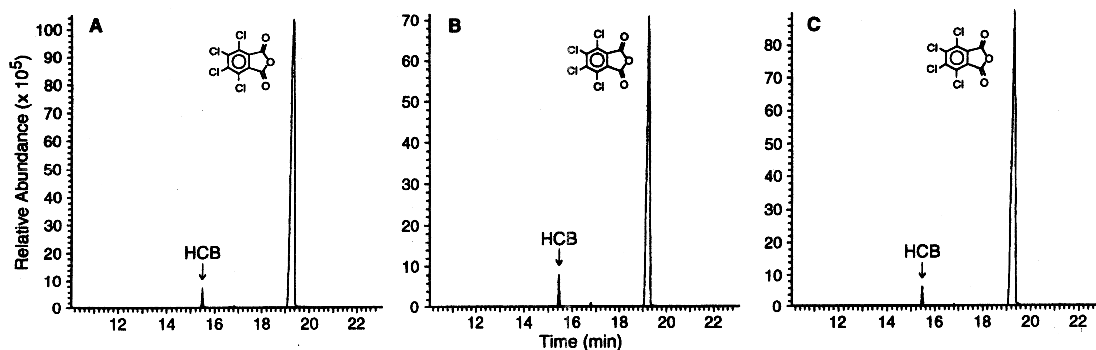

Fig. 5. Total ion chromatograms obtained by direct SPME–GC–MS analyses of tetrachlorophthalic anhydride from three different sources.

need to limit the HCB levels in these color additives. The technique of SPME–GC–MS appears applicable to the enforcement of future HCB specifications in the routine batch-certification analyses of these colors.

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