

Chromium(VI) release from leather and metals can be detected with a diphenylcarbazide spot test

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Summary

Background. Along with chromium, nickel and cobalt are the clinically most important metal allergens. However, unlike for nickel and cobalt, there is no validated colorimetric spot test that detects chromium. Such a test could help both clinicians and their patients with chromium dermatitis to identify culprit exposures.

Objectives. To evaluate the use of diphenylcarbazide (DPC) as a spot test reagent for the identification of chromium(VI) release.

Methods. A colorimetric chromium(VI) spot test based on DPC was prepared and used on different items from small market surveys.

Results. The DPC spot test was able to identify chromium(VI) release at 0.5 ppm without interference from other pure metals, alloys, or leather. A market survey using the test showed no chromium(VI) release from work tools (0/100). However, chromium(VI) release from metal screws (7/60), one earring (1/50), leather shoes (4/100) and leather gloves (6/11) was observed. We found no false-positive test reactions. Confirmatory testing was performed with X-ray fluorescence (XRF) and spectrophotometrically on extraction fluids.

Conclusions. The use of DPC as a colorimetric spot test reagent appears to be a good and valid test method for detecting the release of chromium(VI) ions from leather and metal articles. The spot test has the potential to become a valuable screening tool.

Key words: allergic chromium dermatitis; chromium; chromium allergy; dermatitis; leather; metals; potassium dichromate; screening; spot test.

Chromium is a complex transition metal that has several different oxidation states, ranging from $-II$ to $+VI$. However, only chromium(III) and chromium(VI) are stable forms that can act as haptens inducing contact allergy, and chromium(VI) is recognized as the most potent allergen (1). Historically, occupational exposure to cement has been the primary cause of allergic chromium

dermatitis. However, a regulation on chromium in cement has changed the prevalence and epidemiology in Europe (2–5).

Today, leather articles are considered to constitute the leading cause of chromium contact allergy (6, 7). A new regulation, applying from May 2015 in all EU member states, on chromium(VI) release from leather articles is expected to change the epidemiology of chromium allergy, once again leading to a general decrease (8).

Along with chromium, nickel and cobalt are the clinically most important metal allergens. A colorimetric nickel spot test based on dimethylglyoxime (9) and a cobalt spot test based on disodium-1-nitroso-2-naphthol-3,6-disulfonate (10) are available

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and have proven to be valuable screening tools for the identification of excessive nickel and cobalt ion release (11–13).

The most frequently reported reagent used as a chromium(VI) indicator is diphenylcarbazide (DPC), which, when dissolved in a combination of acids and solvents, will turn red/violet. DPC is widely used as an indicator for chromium(VI) release. In 1958, Feigl (14) described the possibility of using the DPC reagent in a spot test. There have been many publications on the measurement of chromium(VI) in water and soil. However, to our knowledge, the DPC reagent has not yet been systematically evaluated as a potential colorimetric spot test. The DPC method is based on the reduction of chromium(VI) to chromium(III) in a reaction where 1,5-DPC is oxidized to 1,5-diphenylcarbazone (15). The DPC redox reaction is used in the ISO EN 17075 standard to determine the release of extracted chromium(VI) from leather samples (16), and the use of the DPC reagent has been previously reported (17–19).

Chromium(VI) is carcinogenic (20), and the detection of chromium(VI) in the environment has successfully been assessed with DPC as an indicator (21–23). Nevertheless, market surveys using the reagent as a spot test to screen for chromium(VI) ion release from items typically causing allergic chromium dermatitis have never been systematically evaluated. In this study, we evaluated the use and reliability of DPC as a spot test reagent for the identification of excessive chromium(VI) release and the estimation of chromium(VI) release from selected products.

Methods and Results

For statistical analysis, prevalence estimates were expressed with 95% confidence intervals (CIs), which were calculated with the Clopper–Pearson method.

Producing the DPC-based chromium(VI) spot test

A chromium(VI) test reagent based on DPC was produced by dissolving 0.4 g of 1,5-DPC (Merck KGaA®, Darmstadt, Germany) in a mixture of 20 ml of acetone (Merck KGaA®) and 20 ml of 96% ethanol (VWR BDH Prolabo, Fontenay-sous-Bois, France), and then adding 20 ml of 75% H₃PO₄ (VWR BDH Prolabo) and 20 ml of de-ionized water, in a borosilicate glass beaker (Pyrex®, SciLabware Limited, Staffordshire, UK).

All reagents were measured with a volumetric bulb pipette (The Silberbrand Eterna; Brand®, Wertheim, Germany). By the use of a pH meter (PHM220; MeterLab®, Villeurbanne Cedex, France), the pH was determined

to be 0.41. The pH meter was calibrated with buffer solution (pH 10, pH 7 ± 0.02, and pH 4 ± 0.02) (VWR BDH Prolabo). The DPC powder was weighed on 0.3-mm polystyrene weight-boats (VWR BDH Prolabo).

The DPC spot test turns reddish-purple when a sample releases chromium(VI) ions

A white cotton stick was soaked in the DPC solution and rubbed against the sample for 30 seconds. If sufficient chromium(VI) ions are released, a characteristic reddish-purple colour on the cotton stick indicates the presence of chromium(VI) ions. Although an immediate colour reaction cannot always be seen, the colour will become darker and more apparent over time, as a result of reduction, and final readings should be performed no more than 2 min after rubbing (Fig. 1). As a result of the acidity, testing may cause destruction of the corrosive layer of metal objects, and discolour both metal and leather items. To prevent this destructive effect, the DPC test area should be rinsed with water after testing.

The DPC test is able to identify chromium(VI) release at 0.5 ppm

The threshold level of the DCP test was determined by applying 100 µl of DPC test reagent to 1 ml of a chromium(VI) standard solution (Specpure®, Alfa Aesar GmbH, Karlsruhe, Germany) diluted to different concentrations of chromium(VI) (0, 0.25, 0.50, 1.0, 2.5 and 5.0 ppm). A weak colour change to a light purple was visually detectable at 0.25 ppm, and a clear reddish-purple colour was visible at 0.5 ppm (Fig. 2). Under these conditions, we estimated that trained and untrained users of the chromium(VI) test may be able to detect a positive test reaction when the chromium(VI) ion concentration in a solution exceeds 0.5 ppm.

Performance of the DCP test is negatively affected by time and high temperatures

The DPC test was performed on a chromated steel specimen known to release chromium(VI) after the test reagent had been stored under different conditions. The shelf-life of the mixed DPC reagent was estimated to 4 hr at room temperature in daylight, and up to 14 days at 4°C; it could be extended up to 60 days if the reagent was stored at –18°C in a closed vessel (Fig. 3). Storage in a closed vessel at 60°C for 4 hr resulted in discolouration of the DPC reagent, whereby the colour of the test solution transformed from transparent to an orange–brown shade. Nevertheless, it could still detect chromium(VI) release from the chromated steel specimen.

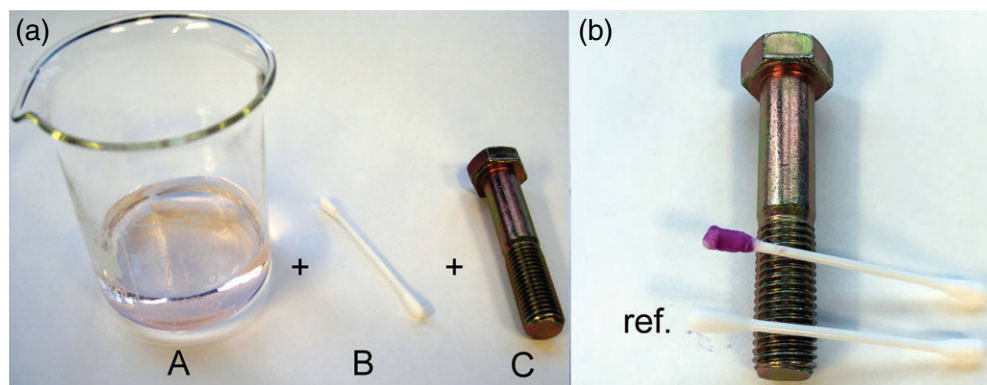


Fig. 1. A positive diphenylcarbazide spot test reaction on a chromated screw. A, diphenylcarbazide solution; B, cotton stick; C, chromated screw. (a) A cotton stick is soaked in the premade diphenylcarbazide solution and then rubbed firmly against the screw for 30 seconds. (b) In the presence of chromium(VI), oxidation of 1,5-diphenylcarbazide to 1,5-diphenylcarbazone will give a reddish-purple cotton stick, and the final reading should be performed after 2 min. A cotton stick immersed in diphenylcarbazide solution without colouration is shown as a reference (ref.).

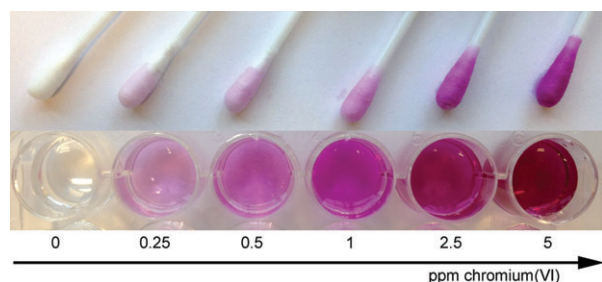


Fig. 2. The threshold level of the diphenylcarbazide spot test was evaluated. Specifically, the threshold level for the reddish-purple colour change in a mix of 1 ml of potassium dichromate standard solution and 100 µl of diphenylcarbazide test reagent was estimated visually by the investigators. The colour change was estimated to be clear and visible at a chromium(VI) concentration of 0.5 ppm.

No interference was observed when pure metals, alloys and leather were tested

Interference was defined as discolouration of the spot test that could be interpreted as false-positive findings. We used pure metals and alloys known not to contain chromium to further evaluate the performance of the DPC test. Thus, solid cylindrical samples, with a diameter of 10 mm and a height of 10 mm, of pure metals (Department of Mechanical Engineering, Technical University of Denmark) made of massive Cu, Ni, Ag, Al, Sn and Ti SAE 304 stainless steel and cast iron were used (Fig. 4).

We tested with liquid serial dilutions (0, 0.5, 1.0, 2.0, 5.0 and 10.0 ppm total ions) of Ni (PerkinElmer[®], Shelton, CT, USA), Zn (PerkinElmer[®]), Pb (Merck KGaA[®]), Ag (PerkinElmer[®]), Cd (PerkinElmer[®]), Cr³⁺ (Merck



Fig. 3. Development of the diphenylcarbazide reagent over time under different conditions: the left glass beaker was stored at room temperature (22–23°C) without sunlight protection; the right glass beaker was stored at –18°C in darkness. (a) After 0 hr. (b) After 4 hr. (c) After 1 day. (d) After 60 days.

KGaA[®]), and Cr⁶⁺ (Specpure[®]; Alfa Aesar GmbH). Similarly, liquid serial dilutions (0, 0.5, 1.0, 2.0, 5.0 and 10.0 ppm total ions) were performed on a multi-standard solution (PerkinElmer[®]): 500 ppm Al; 250 ppm V; 100 ppm As, Be, Cr, Co, Fe, Mn, Ni, Pb, and Zn; 25 ppm Cd and Se; and 5 ppm Hg. Single non-dilutions samples were made on 10 ppm Cr³⁺; 10 ppm Cr³⁺ + (NH₄)₂S₂O₈. Negative control chambers contained 1 ml of purified water (Milli-Q[®]; Merck KGaA[®]). Positive control chambers contained 10 ppm Cr⁶⁺ and 10 ppm Cr⁶⁺ + (NH₄)₂S₂O₈.

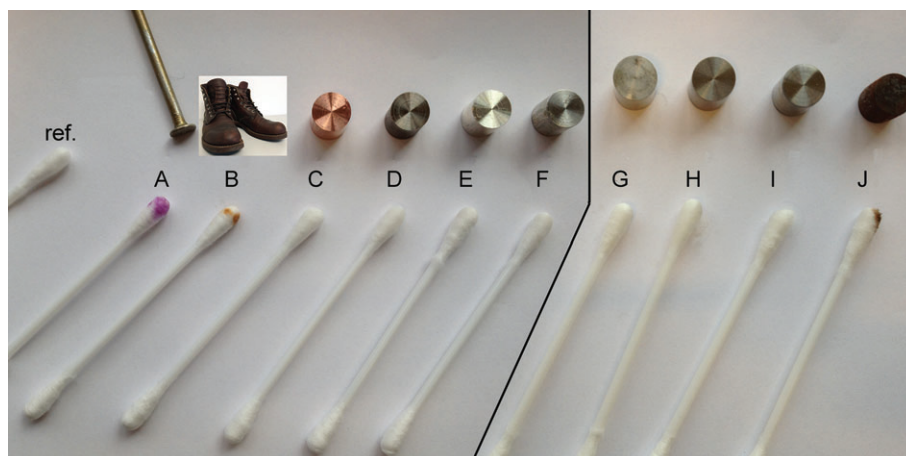


Fig. 4. No interference with the diphenylcarbazide test was observed when pure metals, alloys and leather were tested. However, discolouration was observed from sample B (leather) and sample J (cast iron). ref., reference; A, Cr(VI); B, leather; C, Cu; D, Ni; E, Ag; F, Al; G, Sn; H, Ti; I, 304 stainless steel; J, cast iron.

These tests showed no interference by the following metals and alloys: Al, V, As, Be, Cr, Co, Fe, Mn, Ni, Pb, Zn, Cd, Se, Hg, Cu, Ag, Pb, Sn, Ti, stainless steel, and cast iron (Fig. 4).

DPC spot test screening of leather, screws and earrings showed release of chromium(VI)

The DPC test was used to screen for chromium(VI) release from various items found in retail stores (shoes, gloves, tools, and screws) for this study. The earrings derived from a study aimed at identifying excessive nickel release from various earrings for sale in San Francisco in October 2007 (24). Among 277 earrings, the majority were later used for destructive analyses when the specificity and sensitivity of the nickel spot test was evaluated (9). Hence, for the present study, a random, and probably non-representative, sample of the remaining earrings was used. Notably, all remaining spot test screens in the present study were conducted on-site in the retail stores in 2014. Here, DPC test-positive items were purchased for further analysis. We only tested parts of the items that could potentially come into prolonged or repeated contact with the skin during normal usage, for example the vamp or toe box of shoes and the inner part of the gloves. The examined products were categorized as metal and leather groups.

The leather samples consisted of 100 pairs of footwear representing 20 brands, and 11 pairs of leather work-gloves representing four brands. All leather samples came from Danish retail stores. Of 100 pieces of footwear, four pairs were DPC test-positive (4%, 95%CI: 0.1–9.9%). Of 11 pairs of work-gloves, 6 were DPC test-positive (55%, 95%CI: 23.4–83.3%). Thus, a total of 10 DPC spot test-positive leather samples were identified.

The metal samples consisted of work tools, screws, and jewellery. A total of 100 hand-held non-professional work

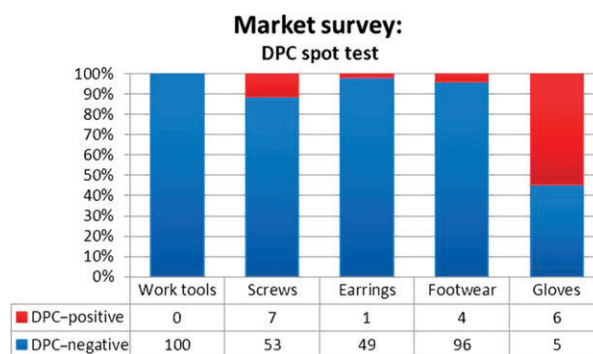


Fig. 5. The market survey results for chromium(VI) with the diphenylcarbazide (DPC) test. All samples came from Danish retail stores except for the earrings, which were North American.

tools were available at local retail stores for analysis. The tools came from 17 different brands. No work tools (0 of 100 items) gave a positive test reaction with the DPC spot test (0%, 95%CI: 0–3.6%). A total of 60 screws from the same retail stores were analysed, and 11.7% (7 of 60 items) gave a positive test reaction with the DPC spot test (11.7%, 95%CI: 4.8–22.6%). As stated, the earrings had been purchased in North America in relation to a previous study on nickel (24). A total of 50 earrings were analysed, and one gave a positive test reaction with the DPC test (2%, 95%CI: 0.1–10.6%). Thus, a total of eight DPC test-positive metal samples were identified (Fig. 5).

The presence of chromium was confirmed with X-ray fluorescence (XRF)

An X-Strata 980 GMF Maxi bench top XRF-analyser (Oxford Instruments®, Shanghai, China) was used on all DPC test-positive items to confirm the presence of chromium. Measurements were performed at 45.0 kV

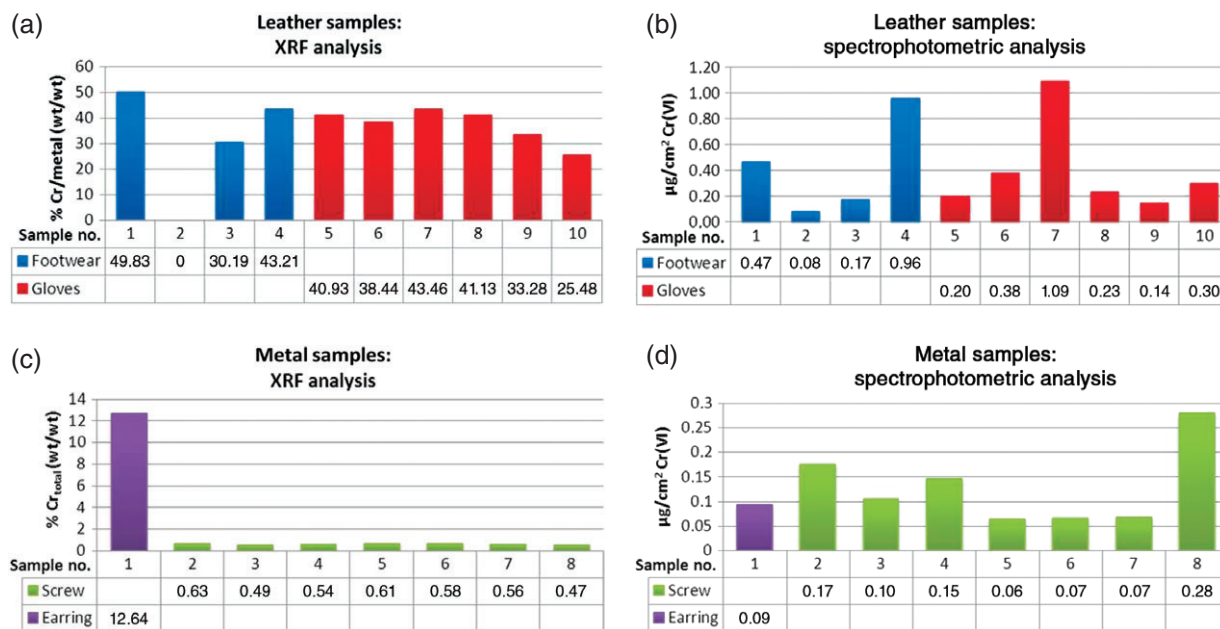


Fig. 6. The market survey findings with the diphenylcarbazide test are correlated with positive X-ray fluorescence (XRF) and spectrophotometric analysis results. (a, b) Leather samples. (c, d) Metal samples. The content given as wt% Cr per total detectable metal in (a) can only be used as qualitative indication of the presence of Cr, and is not an exact quantitative measure.

and 0.053 mA. The instrument was calibrated on a validated metal disc before and after screening (results not shown). The level of detection was estimated to be 0.01%, and the XRF unit performed analysis of each sample for 15 seconds. The XRF analysis gives percentages of elemental metal content, and is recorded for each element, indicating the weight proportions of the complete metal mass. It is ideally used for characterization of solid metal components; for leather, XRF can only be used as a semi-quantitative screening tool. The XRF analysis showed the presence of chromium in three of four footwear samples and in six of six gloves (Fig. 6a). All of the DPC test-positive metal samples contained chromium according to the XRF analysis (Fig. 6c).

Quantitative determination of chromium(VI) release from DPC test-positive samples was performed with spectrophotometry

A UV-2600 ultraviolet (UV)–visible spectrophotometer (Shimadzu®, Kyoto, Japan) was used for the quantitative determination of chromium(VI) content. The spectrophotometric analysis was performed in accordance with a previous study (17) and in accordance with ISO 17075 (16); the concentration of chromium(VI) was determined according to the oxidation of 1,5-DPC to 1,5-diphenylcarbazone, which gives a red–violet

complex with chromium that can be quantified spectrophotometrically at 540 nm. Blank extraction solution and known concentrations of chromium(VI) (Specpure®; Alfa Aesar GmbH) were used as calibration samples. As in the ISO 17075 standard test, 70% phosphoric acid and DPC solution (1.0 g of 1,5-DPC in 100 ml of acetone, acidified with one drop of glacial acetic acid) were used, and all samples had the same volume ratio: 2.5 ml of sample (96%), 0.05 ml of phosphoric acid (2%), and 0.05 ml of DPC solution (2%). The calibration standards were prepared at concentrations of 0, 125, 250, 500 and 1000 µg/l chromium(VI); retesting of the calibration standards was performed after 2 hr, and no deviance was observed. All calibration curves were linear (correlation coefficient for calibration curves: phosphate buffer, $r^2 = 0.99610$; artificial sweat, $r^2 = 0.99895$).

Duplicate samples of leather for extraction ($n = 10$) were exposed to a phosphate buffer (initial pH 8.0; composed of 11.8 g/l $K_2HPO_4 \cdot 3H_2O$, adjusted to $pH 8.0 \pm 0.1$ with phosphoric acid, and used non-deaerated). This is the extraction solution used in ISO 17075. The samples (~ 1 g, size $3.5 \times 3.5 \times 0.2$ cm³) were immersed in 50 ml of phosphate buffer for 3 hr at room temperature (22–23°C) in darkness.

Duplicate samples of metal for extraction ($n = 8$) were immersed in artificial sweat (initial pH 6.5) consisting of

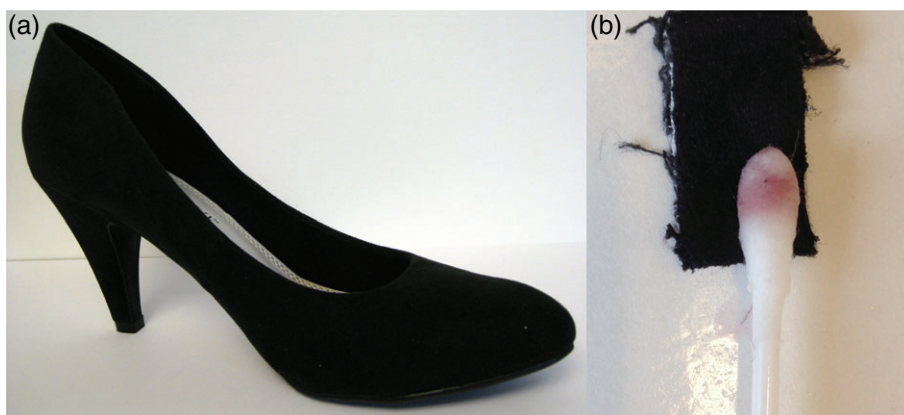


Fig. 7. A difficult positive finding, owing to simultaneous discolouration from the leather dye. The sample result with X-ray fluorescence was 0.0% total chromium, and spectrophotometry gave $0.08 \mu\text{g}/\text{cm}^2$ chromium(VI) in the extract from the leather sample.

5.0 g/l NaCl, 1.0 g/l lactic acid, and 1.0 g/l urea, adjusted to $\text{pH } 6.5 \pm 0.05$ with NaOH. This is the extraction solution used in EN 1811, and each sample was immersed at room temperature ($22\text{--}23^\circ\text{C}$) for 48 hr in darkness in a volume determined individually with regard to the size of the item.

Chromium(VI) was released from all leather samples in amounts between 0.08 and $1.09 \mu\text{g}/\text{cm}^2$ (Fig. 6b). Chromium(VI) was released from all metal samples in amounts between 0.06 and $0.28 \mu\text{g}/\text{cm}^2$ (Fig. 6d).

Discussion

We set out to determine whether the DPC test can potentially work as a rapid and inexpensive tool to detect excessive chromium(VI) release from items that chromium-allergic patients are exposed to. On the basis of these early results, the DPC test appeared to rapidly and reliably detect chromium(VI) release.

A key finding was that the DPC test could identify chromium(VI) ions in a chromium(VI) standard solution at 0.5 ppm. Also, DPC test-positive reactions were detected when both leather articles and metallic items were analysed. The 0.5 ppm threshold level is below the level set by the upcoming European regulation on leather, which will come into force from May 2015 (<3 ppm). The lower threshold level has the potential to help chromium-allergic patients to better identify the presence of chromium(VI) in products that comply with the regulation but could cause morbidity. The specific dose of chromium(VI) that can elicit dermatitis has been evaluated in several dose–response studies (25–34). When these studies were reviewed and the minimal eliciting threshold (MET) in 10% of the chromium-sensitive patients was calculated, it was found that the MET10% for chromium(VI) ranged from 7 ppm (32, 34) to 45 ppm (28). Notably, in a subsequent study, the MET10% was judged to decrease to 1 ppm and the MET50% to 5 ppm

(27). These results imply that regulations might not have sufficient cut-off levels to protect all chromium-allergic patients, but will probably protect the majority against sensitization.

We used XRF and spectrophotometric analysis of the DPC test-positive samples to confirm the findings. All were verified with both XRF and spectrophotometric analysis. The use of XRF on leather is assumed to acceptably assess whether the specified metal is present or not, and the content given as wt% in Fig. 6a can only be used as qualitative indication of the presence of Cr. Although XRF is not intended for the analysis of leather, we have had good experience with XRF when analysing cobalt in leather (manuscript in preparation). Results from XRF analyses will differ according to various factors, including, but not limited to, the sample size, thickness, area, and surface flatness, equipment parameters, and matrix effects (e.g. plastic, rubber, metal, glass, ceramic, and leather). It is a semi-quantitative screening method that can determine whether or not the element in question is present, but not the ion form or whether it is released from the item. The XRF-negative sample was a leather shoe that also was the one with the lowest chromium(VI) release of all tested samples, which could indicate that the amount of total chromium was below the XRF machine's detection limit. Sometimes, testing samples of tanned leather can result in discolouration of the cotton stick, owing to contamination from the leather dye or polish. We retested the shoe several times, and concluded that the shoe was indeed DPC test-positive as a result of chromium(VI) release, and not false-positive as a result of discolouration (Fig. 7).

We found that the DPC test reagent had a shelf-life of ~ 4 hr before the solution began to discolour (Fig. 3). This limited shelf-life could be extended to >60 days (end of study period) by storage in a freezer at approximately -18°C . One should be aware that the DPC test should be handled with care, as it will stain if spilled. Use of

the test on leather products was sometimes followed by discolouration of the product; on metals, the corrosive outer layer was oxidized, but this could be limited by rinsing with water after testing. Thus, the DPC test should be regarded as destructive.

To our knowledge, the DPC test is the only screening tool that can be used to measure chromium(VI) ion release outside a laboratory. During our screening sessions, we found no false-positive test reactions, indicating relatively high sensitivity. However, we emphasize that we did not determine the sensitivity and specificity in this study. The literature has shown interference in specific oxidation steps of several elements (35). Even though the DPC reaction is considered to be nearly specific, there have been reports of interference during extraction by thiosulfate, Mo(VI), vanadium, iron and mercury salts in specific settings (36). In our study, no interference was found in the tested items. Nevertheless, false-negative findings cannot be completely excluded, as the presence of underlying iron may reduce the chromium(VI) to chromium(III), and thereby lead to false-negative test results. This phenomenon was described when DPC reagent used directly on the subject gave strong effervescence (19), but similar reactions were not observed with the cotton stick DPC test. Further exploration is necessary to make the test easily available in clinical settings.

Ideally, a spot test will be developed that shows positivity for both chromium(III) and chromium(VI) release with different colours. However, chromium(III) remains a version of the chromium allergen that, for years, has been neglected, but that does seem to play an important but as yet not fully understood role in chromium allergy and dermatitis.

Use of the DPC test for screening purposes resulted in the identification of chromium(VI) release from leather, screws, and earrings. In a former study, we screened 63 alloy parts from 52 failed hip implant patients, and found no positive test reactions with the DPC test (37). The high rate of positive findings when leather articles were screened was unexpected, but the DPC test appeared to reliably detect chromium(VI) release from leather. The DPC-positive metallic items evaluated in our study are likely to have a surface coating causing the release of chromium(VI). Chromium(VI) is typically used to give an anticorrosive property to metal alloys.

Conclusion

This study showed that the DPC test was able to identify chromium(VI) in a solution at ~0.5 ppm, a limit that is below the current European legislation limits regarding cement and the upcoming regulation on leather articles. The DPC spot test showed consistency when findings were validated with XRF and spectrophotometric tests. We found that leather, screw and earring samples released chromium(VI). The DPC test has the potential to become a valuable screening tool for identifying chromium(VI) release from articles that may cause chromium allergy and dermatitis.

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