Chromium, nickel, and cobalt in cosmetic matrices: an integrated bioanalytical characterization through total content, bioaccessibility, and Cr(III)/Cr(VI) speciation

Analytical and bioanalytical chemistry 409 (29) 2017 : 6831-6841

DOI: 10.1007/s00216-017-0644-8

Chromium, Nickel and Cobalt in cosmetic matrices: an integrated bioanalytical characterization through total content, bioaccessibility and Cr(III)/Cr(VI) speciation

Maria Concetta Bruzzoniti[1]*, Ornella Abollino[1]*, Marco Pazzi[1], Luca Rivoira[1], Agnese Giacomino[2], Marco Vincenti[1]

[1] Department of Chemistry, University of Turin, Via P. Giuria 5, 10125 Turin (Italy)

[2] Department of Drug Science and Technology, University of Turin, Via P. Giuria 9, 10125 Turin (Italy)

*Corresponding authors:

Maria Concetta Bruzzoniti

Department of Chemistry, University of Turin, Via P. Giuria 5, 10125 Turin (Italy)

Email: mariaconcetta.bruzzoniti@unito.it

Orcid ID: 0000-0002-9144-9254

Ornella Abollino

Department of Chemistry, University of Turin, Via P. Giuria 5, 10125 Turin (Italy)

Email: ornella.abollino@unito.it

Orcid ID: 0000-0003-2350-4941

Abstract

The presence of certain metals naturally contained inside raw materials (e.g.: pigments) used to produce cosmetics for make-up may represent a serious concern for the final quality and safety of the product. The knowledge of the total concentration of metals is not sufficient to predict their reactivity and their toxicological profile. For these reasons, we set up a comprehensive approach to characterize the content of Co, Cr and Ni in two raw materials for cosmetic production, a *black iron oxide* and a *pearly pigment*, and in a finished product, *pearly powder eye shadow*. Namely, besides the total metal concentrations, the speciation of chromium and the bioaccessibility of the three metals were assessed. Since no standard method is so far available for hexavalent chromium extraction from cosmetic samples, three approaches were compared (EPA 3060A method, IRSA 16 method and a Na_3PO_4 extraction). Results show that Na_3PO_4 extraction is the most selective one. Cr(VI) was undetectable in *black iron oxide* and present at very low concentrations (about 0.3 mg/kg) in *pearly pigment* and in the *pearly powder eye shadow* samples. The extracted Cr(VI) concentrations are not related to the total Cr content in the samples. Bioaccessibility studies were performed by *in vitro* extractions with synthetic lacrimal fluids and sweat. Despite the wide range of metal concentrations in the samples, the amounts of bioaccessible elements were undetectable or very low (less than 0.4 mg/kg), thus suggesting that metals in the three samples are present in inert forms.

Keywords: cosmetics, metals, Cr speciation, bioaccessibility, simulated biological fluids

Introduction

Dyes and pigments are fundamental components in most cosmetics for make-up, such as eye-and face-powders and lipsticks. Many pigments authorized to be used contain or are made of metal oxides or minerals, e.g. iron oxide, chromium oxide, aluminium silicate and titanium oxide, which by their nature contain traces of heavy metals. So, it is important to accurately characterize the nature and the amount of the metal impurities in the pigments in order to assess the cosmetic products safety.

However, it must be pointed out that the knowledge of the total metal concentration in a solid sample is not sufficient to predict metal reactivity and effects on human health: indeed, metals

can be present in various forms (e.g. elementary, complexes, soluble or insoluble salts) with different chemical properties and consequently different potential toxicity for humans.

For chromium, the toxicity is strictly related to its oxidation states (III/VI). Cr(VI) is carcinogenic to humans (IARC group 1) [1] and its use as ingredient is prohibited by the European Community (EC/1223/2009) [2]. Nevertheless, the same regulation (article 17), declares that small quantities of prohibited substances, including Cr(VI), may be present in final products as technically unavoidable traces if these preparations are safe for human health. The safe levels of these technically unavoidable impurities are not defined in the regulation as it is under the responsibility of the producers of finished cosmetics to demonstrate that their unwanted presence in the product does not pose any health issue in normal condition of use. For the allergy concern and the risk of contact dermatitis, the German Federal Institute for Risk Assessment defines 1 mg/Kg chromium(VI) as the threshold value [3].

Speciation can be defined as an analytical approach aimed at gaining insight into the forms and the oxidation states of an element in a matrix. Unfortunately, for complex matrices, the distinction of the single forms is rather difficult, or even impossible. For this reason, one relies on fractionation techniques, in which the total amount of an element is partitioned into classes (fractions) of species with different chemical and/or physical properties, hence with different reactivity toward organisms. Fractionation methods often rely on single or sequential extractions with chemicals able to react with the matrix of interest with different mechanisms (e.g. saline media, acid solutions, reducing or oxidizing agents) [4]. Such methods have some drawbacks, mainly the nonselectivity of reagents, the possible readsorption and redistribution of extracted species, and the operationally defined nature of the results: however, they are extensively used to characterize solid matrices, since they provide valuable information on the behaviour and mobility of elements [5, 6]. An application of single and sequential extractions is the evaluation of bioaccessibility, which is defined by IUPAC as “the potential for a substance to come in contact with a living organism and then interact with it. This may lead to absorption” [7]. Most studies on this subject deal with oral bioaccessibility, i.e. the fraction of a compound that is released from its matrix in the gastrointestinal tract, and thus becomes available for intestinal absorption [8, 9], but also lung [10] and dermal [11] bioaccessibility have been investigated.

Bioaccessibility results are used to estimate bioavailability [8, 9]. Bioavailability is defined by IUPAC as “the extent of absorption of a substance by a living organism compared to a standard system [7].” It must be pointed out that the presence of an element in an extract does not provide

information on its actual absorption, and hence its entering the systemic circulation. As Semple et al. state, bioaccessibility encompasses what is actually bioavailable now plus what is potentially bioavailable [12]. Bioavailability should be determined *in vivo* on animals or humans after administration of an acute or chronic dose of the species of interest, but *in vivo* studies are expensive and ethically troublesome; furthermore, testing of finished cosmetic products and cosmetic ingredients on animals is forbidden in the European Union [2]. Finally, *in vitro* bioaccessibility tests are consistent with the Directive 2010/63/EU (revising Directive 86/609/EEC on the protection of animals used for scientific purposes) which is based on the "Three Rs" principle, i.e.: i) replacing the use of animals with alternative techniques, or avoid the use of animals altogether; ii) reducing the number of animals used to a minimum; iii) refining the way experiments are carried out, to make sure animals suffer as little as possible [13, 14].

At the best of our knowledge, no standardized protocols for total metal determination, extraction, speciation and fractionation are yet available in the field of cosmetics. In particular, many papers published on total element content in make-up products are mainly aimed at assessing exposure and potential health risks following the use of metal-containing cosmetics, and simply describe the analytical method adopted without discussing it [15, 16]. Within this frame, the aim of this work is the characterization of raw and cosmetic materials in terms of total metal content, speciation and bioaccessibility. We aim to propose analytical methods fully verified, discussing the possible sources of errors, such as the presence of interferences which are not always detectable by analysing reference materials, taking into account that no SRM for cosmetics are available.

Three metals, namely Cr, Co and Ni, were studied: Cr and Co are present in a wide variety of pigments, whereas the interest in Ni is mainly due to its allergenic properties.

For this purpose, we selected two raw materials (RMs) used in the cosmetic industry and an eye shadow sample expressly formulated for this research as being a formula with a high content of pigment, thus representing a "worst case" scenario in terms of Cr content. Of course, the selected samples are not representative of the whole range of cosmetic raw materials and finished goods in the market, but can be helpful in testing analytical procedures since they contain detectable amounts of the considered metals.

The total metal concentrations were determined by microwave oven dissolution prior to inductively coupled plasma optical emission spectroscopy (ICP-OES) detection, which was optimized in order to overcome the spectral interferences hampering the analysis.

As to Cr speciation, extraction of Cr (VI) from the cosmetics was addressed as crucial aspect of the whole analytical approach. The extraction method should solubilize all forms of Cr(VI) without Cr(III)-Cr(VI) interconversions. Regarding the determination of Cr(VI), literature methods available are based on spectrophotometry [17, 18], electroanalysis [19], ICP-OES or inductively coupled plasma-mass spectrometry (ICP-MS) detection [20] after chromatographic separation.

Under the rational to use, if possible, official methods already consolidated for other matrices, we compared the performance of different extraction approaches for Cr(VI). Namely, the US-EPA 3060A [21] and the Italy-IRSA 16 [22] methods were tested. They are recommended for extraction of Cr(VI) from waste (EPA and IRSA) and from soils, sludges, sediments (EPA) and claimed suitable for the extraction of both water-soluble and precipitated species of Cr(VI). Additionally, a sodium phosphate-based extraction proposed for leaching of Cr(VI) from soil [23] was here tested. After extraction, the analysis was performed by ion chromatography (IC) coupled with spectrophotometric detection after post-column reaction with 1,5-diphenyl carbazide (DPC) [18].

As for bioaccessibility assays, extractions were carried out with reagents simulating the effects of body fluids, such as artificial gastric and intestinal juices, sweat or lysosomal fluids [8-11]. This approach is commonly adopted in environmental science to estimate human exposure to pollutants present in soils [8], sediments [24] and atmospheric particulate matter [10] and in food science [8, 25]. Regarding cosmetics, artificial lacrimal fluid and sweat were used to assess bioaccessibillity in eye shadow [26, 27] and face powder [28] respectively; Gao et al. [29] adopted a different approach, and calculated the bioaccessibility in lipstick samples as the sum of the first three fractions (water soluble/exchangeable, reducible and oxidizable) obtained with a sequential extraction scheme (the BCR protocol) commonly used to study metal mobility in soils [4-6].

In this study, two pathways of metal input into the human body were considered because deemed significant for the samples investigated: exposure to the metals leached by lacrimal fluid and sweat. Therefore, extraction with synthetic solutions of the above-mentioned fluids were performed, merging the approaches of previously published studies [26-28].

The novel integrated approach here provided meets specific needs of manufacturers of cosmetic products for internal control of the safety of raw materials used and of their finished products. This aspect is of paramount importance considering the number of cosmetic products used daily (about 20 for women and 10 for men), throughout the whole life and the worldwide cosmetics market, estimated at a total of 205 billion euros in 2016.

Materials and Methods

Samples

RMs and finished product were provided by the working group on heavy metals of the Technical Committee of *Cosmetica Italia* (the Italian association of personal care). The RMs are here indicated with the labels *black iron oxide* and *pearly pigment*; they are pigments in which the presence of technically unavoidable metals is well known. The pigments are not representative of the whole cosmetic raw materials.

The finished product is labelled as *pearly powder eyeshadow* and was expressly formulated by *Cosmetica Italia* as a case study with a very high amount of substances with a dark colour and probably represents the most extreme case among cosmetics containing heavy metals.

Chemicals

All chemicals used were of analytical grade or better; 65% HNO_3, 40% HF, 37% HCl and 30% H_2O_2 used for sample mineralization were purchased from Merck or Sigma Aldrich. Standard analyte solutions were prepared by dilution of 1000 mg/L concentrated stock solutions (Merck Titrisol or Sigma Aldrich). Na_2CrO_4 salt (Sigma-Aldrich) was used for the preparation of Cr(VI) standard solutions. Recovery of water-soluble and precipitated species of Cr(VI) by the extraction solutions was evaluated using Na_2CrO_4, and $PbCrO_4$ salts respectively.

H_2SO_4 (95-97%) and CH_3OH were purchased from Sigma Aldrich (Chemie, Steinheim, DE). $(NH_4)_2SO_4$ (Carlo Erba, Arese, Milano, Italy) and NH_4OH (Sigma Aldrich) were used for eluent preparation. The post-column reagent, 1,5-diphenyl carbazide (DPC), was from Carlo Erba. The chemicals present in the extracting solutions (see below) were purchased from Sigma Aldrich. High-purity water (HPW, 18.2 MΩ·cm resistivity at 25 °C), produced by an Elix-Milli Q Academic system (Millipore, Vimodrone, MI, Italy) was used for the preparation of eluent and standards.

Eluents and sample solutions were filtered through 0.45µm mixed cellulose ester membrane filters.

Instrumentation

Sample mineralization was carried out with a Milestone Ethos One (Milestone, Sorisole, Italy) microwave laboratory unit equipped with polytetrafluoroethylene (PTFE) bombs and temperature sensor. The total metal contents were determined by ICP-OES with a Perkin Elmer Optima 7000

(Perkin Elmer, Norwalk, Connecticut, USA) spectrometer by standard addition. The following emission wavelengths were selected: Co, 230.786 nm; Cr, 267.716 nm; Ni, 231.604 nm.

Chromatographic analysis was performed with a Dionex 4000i (Dionex ThermoFisher, Sunnyvale, CA, USA) ion chromatograph equipped with a spectrophotometric detector. The column used (Dionex) was an IonPac AS7 (250 x 4.0 mm) which was protected by a guard column IonPac AG7 (50 x 4.0 mm). The eluent, 250 mM $(NH_4)_2SO_4$ and 100 mM NH_4OH (pH 8.8), was delivered at 1 mL/min flow rate and the injection volume was 1000 µL. Spectrophotometric detection was performed at 530 nm after post-column derivatization (750 µL reaction coil) with a 0.5 M H_2SO_4 solution containing 2 mM DPC (10% CH_3OH). After optimization, the flow rate of post-column reagent was set at 0.33 mL/min. Chromatographic quantification of chromium in the extracts was obtained by the standard addition method.

Total chromium concentration in the extraction mixtures was evaluated by ICP-OES using matrix-matched calibration.

For Eh measurements, an Autolab PGSTAT12 from Metrohm was used. Counter and reference electrodes were Pt mesh and Ag/AgCl/KCl(1M), respectively. pH values were measured with an EA 920 Orion potentiometer provided with a combined glass-calomel ion selective electrode.

Field Emission Scanning Microscopy (FESEM) equipped with an EDS detector (Oxford Instruments) was used for determination of Fe content. For Fe(II) speciation, a Varian Cary 1E (Agilent, Cernusco sul Naviglio, Milan, Italy) spectrophotometer (510 nm) was used.

Metal concentrations in synthetic lacrimal fluid and sweat were determined with a Perkin Elmer Analyst 600 Graphite Furnace Atomic Absorption Spectrometer (GFAAS) equipped with an autosampler and Zeeman effect background correction using matrix-matched calibration. The following absorption wavelengths were selected: Co, 242.5 nm; Cr, 357.9 nm; Ni, 232.0 nm. All the sample extracts in simulated lacrimal fluids gave rise to a high background signal at the wavelength of Ni, which masked the analyte signal. This drawback was overcome after 1:1 dilution with water. The same dilution was carried out for the determination of Co only in the extracts of RMs in lacrimal fluid.

Sample treatments

Total concentrations

Aliquots of 0.3 g of sample were transferred into PTFE bombs and added with 4 ml of HNO_3, 2 ml of a HF/H_3BO_3 (6M) mixture and 2 ml of H_2O_2, unless otherwise stated. The bombs were heated in

the microwave oven according to the following scheme: from room temperature to 200 °C in 15 min, 15 min at 200 °C followed by a 5 min-ventilation step; maximum allowed T and power: 220 °C and 1500 W, respectively. After cooling, the digested samples were filtered through Whatman 5 filters, diluted to 50 ml with HPW and analyzed by ICP-OES.

Cr speciation

Three sample pre-treatments, namely EPA 3060 method, IRSA method and a Na_3PO_4 based extraction were evaluated in order to solubilize chromium species and to remove matrix interferences. Details are hereafter given.

Method 3060A-EPA: 2.5 g of solid sample were digested with 50 mL of a 0.28 M Na_2CO_3/0.5 M NaOH solution for 60 min at 90-95°C in order to dissolve Cr(VI) and stabilize it against reduction to Cr(III). After cooling, samples were filtered through a 0.45 µm membrane filter and analyzed after dilution 1:1 (v/v) with HPW.

Na_3PO_4 based extraction: 0.5 g of solid sample were added with 50 mL of a 0.01 M Na_3PO_4 solution (pH 11.7) and heated for 5 min at 100°C. The mixture was filtered through a 0.45 µm membrane filter and directly analyzed.

Method 16-IRSA: 0.5 g of solid sample were transferred into a 50 mL volumetric flask, added with 49.0 mL HPW and 1 mL H_2SO_4 (1:1, H_2SO_4 / H_2O, v/v) and magnetically stirred for 10 min. The supernatant was analyzed after filtration through a 0.45 µm membrane filter.

In the solutions deriving from each of the above-mentioned extraction protocols, the total chromium and the Cr(VI) content were measured by ICP-OES and IC, respectively.

Bioaccessibility

Synthetic sweat consisted of an aqueous solution of 0.1% (w/v) urea, 0.5 % NaCl and 0.1 % lactic acid. The pH was adjusted to 6.57 ± 0.01 with NH_3 [26].

Synthetic lacrimal fluid was prepared by dissolving NaCl (4.3 g), Na_2HPO_4-$12H_2O$ (8.7 g) and NaH_2PO_4 (0.59 g) in HPW and diluting to 1000 ml [27].

Aliquots of 2.0 g of sample were put into contact with 50 ml of each extractant in Falcon test tubes for 12 hours in a water bath at 36 °C. Then the suspensions were centrifuged at 3500 rpm, the supernatant was filtered and acidified with HNO_3. The extracts so obtained were analysed by GF-AAS.

Results and Discussion

Total concentration

Development of the digestion procedure

The sample digestion procedure was developed based on: i) the method developed by the U.S. Food and Drug Administration (FDA) for the determination of Pb in lipstick [30] and widely used by cosmetic companies for other make-up products; ii) the protocol recently proposed by the Italian National Institute of Health for the quantification of Cd, Co, Cr, Ni and Pb in face powder [31] and iii) two Milestone Application Notes for lipsticks and face powders [32]. The application of such methods is not straightforward, since the investigated matrices are different from those treated in the above-mentioned references. The conditions reported in "Sample treatments - Total concentrations" section were developed after some preliminary experiments with different amounts of reagents (HNO_3, H_2O_2, HF) and different thermal treatments. Two other reagent mixtures, namely HNO_3/H_2SO_4/HF (4/2/2 ml) and *aqua regia*/HF (6/2 ml), were tested to couple the oxidizing power of a sulfonitric mixture or *aqua regia* to the ability of HF to dissolve silicates, without observing any improvement with respect to the HNO_3/HF/H_2O_2 mixture (Table 1S, Electronic Supplementary Material). In no case, a complete solubilization of the samples was observed, since a small residue was left even in the presence of HF. It can be assumed that this refractory component has no relevance for the assessment of the product safety, since the metals possibly present in it would not be released. Besides the safety issues derived from the use of HF, the addition of boric acid to obtain fluoride volatilization makes digestions more complicated and gives rise to a more complex matrix, which may be inconvenient for the subsequent analysis. The fraction of metals that becomes extractable with HF is the one trapped in silicates that will not be solubilized in the real-life use of the raw material or finished products. The question of the balance between information provided / safety risk when using HF should be considered. In this study, we mineralized the samples with HF, since it can provide data closer to the actual metal content and hence more precautionary information.

We hypothesize that the mixture of reagents adopted in this study would be applicable, at least in principle, also to other kinds of RMs or cosmetic products: HF would be suitable to dissolve silicate-based components, whereas HNO_3 and H_2O_2 would ensure the oxidation of the organic components. Indeed, it is advisable to optimize the ratios among the three reagents and the duration and temperature of the digestion for each matrix.

Identification and correction of spectral interferences

A preliminary analysis was done in order to identify the concentration range of the metals in the matrices and to select the instrumental conditions for the measurement.

The wavelength usually adopted for the determination of Co by ICP-OES (228.616 nm) is not suitable for the analysis of the *pearly pigment* and the *pearly powder eye shadow*, because the peak of the metal is overlapped with another peak, already present in the blank (Figure 1S, Electronic Supplementary Material). This interference is negligible for *black iron oxide*, in which a higher concentration of Co is present. The identification of the interfering peak was attempted: elements with emission lines closest to 228.616 nm are Ba (228.611 nm) and W (228.590 nm), but their presence was ruled out because it can be presumed that they are not contained in the blank at detectable levels; Fe (whose presence in a blank might be likely) has a weak emission line at 228.615 nm, but the height of the interfering peak does not increase upon addition of a relatively high Fe concentration (40 mg/l). In conclusion, it was not possible to ascertain the source of the spectral interference.

In order to find the most suitable wavelength for Co determination, the spectra of 0.050 mg/l solutions of Co in dilute nitric acid and in the digested blank were recorded around the main emission lines of this element: 228.616 nm, 230.786 nm, 231.160 nm, 236.380 nm and 238.892 nm. As Figure 1 shows, at 231.160 nm a peak in the digested blank is overlapped to the signal of Co, and at 236.380 nm a shoulder makes it difficult to integrate the peak of Co correctly. The addition of Fe (not shown) gives rise to a peak close to 231.160 and 238.892 nm, which would cause a spectral interference in the analysis of *black iron oxide*. The emission line at 230.786 nm is well differentiated from the signal of the digested blank and is not influenced by the presence of iron, so it was chosen for sample analysis.

Analysis of cosmetic matrices

Table 1 shows the concentrations found in the two RMs and in the finished product. Since no certified reference materials for cosmetics are available, the accuracy of the procedure was checked by analysing fortified samples. The recoveries of the added spikes, reported in Table 1, are included within 79% and 95%. The recoveries obtained were significantly higher than those obtained by Bocca et al. [31] for face powders for Cr, but they were lower for Co and Ni. These

values overall comply with the requisites of the Commission Decision 2002/657/EC [33] concerning the performance of analytical methods. Regarding precision, relative standard deviations of the results reported in Table 1 range from 1.6 to 16.2%, with most values being below 10%. The median precision is 7.4%.

As to the composition of the samples, the *black iron oxide* has the highest concentrations of cobalt and especially of nickel. These elements are not intentionally added by the producers of the RMs, but represent impurities of the main component. The *pearly powder eye shadow* was found to have a very high content of chromium due to the presence of chromium oxide green C77288, which is an allowed pigment according to Annex IV of European Regulation. From the point of view of the samples, the concentrations increase in the order: *pearly pigment* < *pearly powder eye shadow* < *black iron oxide* for Co and Ni; *black iron oxide* < *pearly pigment* << *pearly powder eye shadow* for Cr.

Determination of other elements

The content of other elements in the RMs was also determined. The concentrations of Ba were 85 mg/kg in the *pearly pigment* and 11 mg/kg in the *black iron oxide*, whereas Zn content was 23 and 42 mg/kg in the two products respectively. Pb was undetectable even by GFAAS (< 0.3 mg/kg).

Chromium speciation

Cr(VI) determination

For this step, it is necessary to use, as much as possible, highly sensitive techniques capable of detecting low concentrations of Cr(VI).

Unlike the colorimetric techniques that may be affected by positive interferences from other metals present in cosmetics, the chromatographic techniques allow a correct identification of Cr(VI).

In this work, the analysis of the extracts (see below) was performed by ion chromatography with post-column derivatization and spectrophotometric detection. For this technique, detection and quantitation limits, calculated according to the ICH guidelines [34], were respectively 0.05 and 0.15 µg/L for Cr(VI) in water. Relative standard deviation, RSD, (n=7) was 10.1%. Linearity was verified within two orders of magnitude. For peak area, intraday precision was 6.75% (n=5), while interday precision was 9.2% (n=13, 3 days). Detection and quantitation limits evaluated in 0.01 M Na_3PO_4 extractant solution remained constant within the RSD of the method. Detection and

quantitation limits evaluated in the 0.18 M H_2SO_4 extraction solution were 0.15 and 0.45 µg/L, respectively. Detection limits are much lower than those achievable by colorimetric detection (0.5 mg/L) and slightly better than those obtained by ICP-OES (1 µg/L) for total Cr. As an example, a typical chromatogram for 0.05 µg/L Cr(VI) in water is shown in figure 2.

Cr(VI) extraction

The Cr contents determined by the different extraction procedures are summarized in Table 2 for the two RMs. Concentration of total chromium in the extracts was measured by ICP-OES, whereas the concentration of Cr(VI) was measured by IC. Cr(III) is calculated as the difference between total Cr and Cr(VI). Results obtained are hereafter given and discussed.

Method 3060A-EPA

For both the samples, it was impossible to quantify Cr(VI) following the EPA extraction procedure [21] since a strong interference affected the spectrophotometric detection. This interference was ascribed to CO_2 evolution originated during the post column reaction with acidic DPC visualized by bubble formation. The pH adjustment of the extracted solution to match the pH value of the eluent did not anyway allow the determination of Cr(VI).

However, the total Cr content in the extract, 0.267 mg/Kg for the *pearly pigment*, allows us, on a precautionary basis, to hypothesize a similar or even lower concentration value for Cr(VI). Differently, since no Cr was detected in the extract of *black iron oxide* by ICP-OES, we can affirm that hexavalent chromium is not present.

Na_3PO_4 based extraction

This procedure was selected since claimed suitable for the extraction of insoluble Cr(VI) species [23], considering the relevant content of metals in the raw matrices. In fact, if Cr is present as $BaCrO_4$, the Na_3PO_4 extraction should be effective to dissolve it, according to the solubility product constants (K_{PS}) reported in Table 3 (compare $BaCrO_4$ and $Ba_3(PO_4)_2$ K_{PS} values).

The recovery yield for soluble and insoluble compounds was evaluated using Na_2CrO_4 and $PbCrO_4$ (as models of soluble and water insoluble salts, respectively). Recovery of Cr(VI) was assessed both from pure salts and from the fortified RMs. The results obtained showed a recovery of Cr(VI) respectively of 100% from Na_2CrO_4 and 21% from $PbCrO_4$ for both the above-mentioned approaches, supporting the absence of a matrix effect. The low recovery obtained from $PbCrO_4$ is

unexpected, speculating merely on the K_{PS} values of the salts (K_{PS} $Pb_3(PO_4)_2$ =$1.6 \cdot 10^{-32}$ vs K_{PS} $PbCrO_4$ =$2.8 \cdot 10^{-13}$). It should be highlighted that, differently from this work, the method proposed by Mandiwana [23] did not study the recovery of insoluble Cr(VI).

As far as Cr(III) is concerned, it must be underlined that at the pH value (11.7) of the extraction procedure, Cr is present as $Cr(OH)_3$, suggesting its precipitation. The similarity of the concentration values found for Cr(VI) (0.245 mg/kg) and total Cr (0.30 mg/kg) in the extract for *pearly pigment* confirms that Cr(III) is present at very low concentration, in agreement with the above-mentioned hypothesis. Typical chromatograms obtained for Cr(VI) determination in the *pearly pigment* are shown in Figure 3.

This extraction protocol applied to the *pearly powder eye shadow* provided a Cr(VI) concentration of 0.31 mg/kg, despite the significant total Cr content (about 2600 mg/kg), highlighting the importance of an integrated analytical approach that includes speciation besides total metal concentration.

Method 16-IRSA

Data obtained applying the IRSA method [22] show a value for Cr(VI) in agreement with Na_3PO_4 extraction, but a higher efficiency for total chromium in respect to the other two extraction approaches (Table 2). This result is due to the high acidity of the extractant (pH 0.7).

This procedure provided recovery yields of Cr(VI) from Na_2CrO_4 and $PbCrO_4$ of 100% and 8% respectively, showing a lower dissolution ability than the Na_3PO_4 extraction towards insoluble species.

Due to the oxidative conditions measured for the extracted *pearly pigment* sample (Eh= 799 mV), the stability of the chromium species in this extract was measured at different times. Results showed that, after 1 hour, the Cr(VI) concentration was comparable to the one measured just after extraction, whereas, after 24 hours, all the Cr was reduced to Cr(III). This was attributed to the presence of a reducing agent [35] in the sample such as Fe(II), which can be usually present in different kinds of mica [36]. For the *pearly pigment* sample, Fe(II) concentration was evaluated by extraction with acetate buffer [37] and by addition of 1,10-phenantroline. The Fe(II) content was 36.6 mg/Kg which corresponds to about 100 times the amount of Cr(VI) extracted (Table 2), supporting the hypothesis of a redox reaction proceeding with time. Total concentration of Fe in the *pearly pigment* determined after microwave digestion by ICP-OES was 14600 mg/kg, in agreement with FESEM-EDX measurements on solid *pearly pigment* (1.3%).

For *black iron oxide* sample, the total Cr concentration extracted was mainly due to Cr(III) with a Cr(VI) concentration lower than detection limits.

Bioaccessibility of Co, Cr and Ni in cosmetic matrices

The bioaccessibility of Co, Cr and Ni both from RMs and from the finished product was assessed *in vitro* using extraction solutions which simulate the *in-vivo* conditions. Differently from Cr(VI)/Cr(III) speciation studies, the aim of the treatments is not to obtain a complete extraction of the analyte, and the extractability is expected to be different in samples of different nature, depending on how strongly metals are retained to the matrix. The objective is to simulate the effect of sweat and tears, biological fluids potentially in contact with the cosmetics. Extraction conditions (12 hours at 36 °C) were chosen to match those of a daily use at medium-high climate temperatures.

The results were expressed considering a precautionary quantitation limit for GFAAS of 5 µg/L for each analyte, corresponding to 0.125 or 0.250 mg/kg in the solid sample, depending on the dilution performed on the samples before analysis (Table 4). Relative standard deviation ranges were 4.9 – 8.0% and 7.1 – 13.6% for extraction into sweat and lacrimal fluid respectively. Spike (5 µg/L) recoveries were 98 ± 11% for sweat and 85 ± 8% for lacrimal fluids. As an example, Figure 4 shows the time-resolved absorbance for the extracts of the *Pearly Pigment* as such and after spiking with 5 µg/L of each analyte at the three wavelengths chosen for the analysis. Figures 2S-4S (Electronic Supplementary Material) collect the time-resolved absorbance plots for all samples and both simulating fluids. Interestingly, Co and Ni were not extracted at detectable levels from any sample. The concentrations of Cr released into the two simulating fluids were undetectable for *black iron oxide* and very low for the other two samples. Despite the significant Cr content (about 2600 mg/kg) in the *pearly powder eye shadow* sample, only less than 0.4 mg/kg was extracted by the two fluids. Low metal bioaccessibility was also observed in other studies [26, 28] on powder-based cosmetics.

Conclusions

Reliable analytical approaches to be routinely applied are needed to ensure the availability of essential information in order to assess the safety of cosmetic products and to provide data to confirm their compliance with the legal requirements.

Sample digestion for the determination of the total concentrations of Co, Cr and Ni requires drastic conditions including the use of strong acids and high pressure and temperature. In

addition, careful selection of detection wavelength in the subsequent ICP-OES analysis must be performed, in order to avoid interferences. In this study, a spectral interference for Co was identified and corrected.

As regards Cr speciation, extraction by Na_3PO_4 coupled to IC determination with post-column derivatization ensures proper identification of Cr(VI), as confirmed by the recovery yields obtained. Na_3PO_4 method is preferred towards the IRSA method since this last approach is affected by possible interconversion of Cr species. In fact, due to the acidity of the IRSA extracting solution, underestimation can occur if easily oxidable metal species are present in the matrix and co-extracted. The speciation study reveals that Cr(VI) was not present in *black iron oxide* and present at very low concentrations in *pearly pigment* and in the *pearly powder eye shadow* samples, despite the significant total Cr content in the latter sample.

The results from bioaccessibility study show that the constituent materials have low solubility in biological fluids that may come more likely into contact with cosmetic products. In particular, the amounts extracted from the investigated samples were undetectable or very low even in the "worst case" sample (*pearly powder eye shadow*). This finding suggests that the choice of measuring the amount of bioaccessible metals in the samples can provide a most realistic and appropriate set of data for a correct overall assessment of the safety of a cosmetic product.

The three-step integrated approach (determination of total metal content, speciation, bioaccessibility) adopted in this study can be used as a suitable procedure to characterize cosmetic products and their raw materials in a more comprehensive way in comparison to the determination of total metal concentration only.

Acknowledgements

The authors would like to thank *Cosmetica Italia* for the supply of the samples and for fruitful discussion. Financial support from MIUR (Ministero dell'Università e della Ricerca, Italy) is also gratefully acknowledged.

Conflict of interest

The authors declare that they have no competing interests.

Table 1. Element concentrations (expressed in mg/kg) in the investigated samples (n=2). Spike recoveries (%) are shown in parenthesis*.

	Co	**Cr**	**Ni**
Pearly pigment	3.4 ± 0.04 (80.1 ± 0.9)	33.0 ± 4.0 (91.6 ± 11.2)	11.7 ± 1.0 (95.2 ± 8.5)
Black iron oxide	52.9 ± 3.9 (79.8 ± 5.9)	17.1 ± 1.2 (91.0 ± 6.7)	157 ± 1 (78.9 ± 0.5)
Pearly powder eye shadow	8.4 ± 1.4 (80.5 ± 13.0)	2597 ± 42 (91.2 ± 1.5)	20.8 ± 2.1 (80.7 ± 8.2)

*Spiking concentrations (mg/kg) for Co, Cr and Ni respectively: *Pearly pigment*: 8.2; 25.0; 8.3. *Black iron oxide*: 16.3; 10.0; 117. *Pearly powder eye shadow*: 8.2; 2500; 8.3.

Table 2. Analysis of the extracts for the determination of total Cr, Cr(VI) and Cr(III) in the RMs. Cr(III) was determined by difference between total extracted Cr and Cr(VI). n.d.=not detected (lower than the detection limits). Relative standard deviations are below 10% for all measurements for n=3 repeated extractions.

Extraction procedure	**Pearly pigment**			**Black iron oxide**		
	Cr(VI)	*Cr(III)*	*total Cr*	*Cr(VI)*	*Cr(III)*	*total Cr*
	mg/kg			*mg/kg*		
Method 3060A-EPA			0.267			n.d.
Na_3PO_4 extraction	0.245	0.050	0.30	n.d.	-	n.d.
Method 16-IRSA	0.225	0.945	1.17	n.d.	1.54	1.56

Table 3. Metal ions present in RMs and solubility product constants (K_{PS}) with anions involved in the extraction procedures.

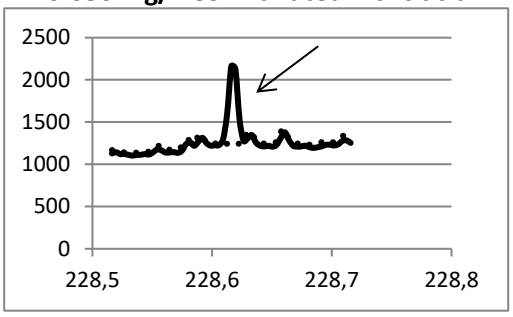
Metal	K_{ps}			
	OH^-	CO_3^{2-}	PO_4^{3-}	CrO_4^{2-}
Co	$1.6\ 10^{-44}$	$1\ 10^{-10}$		
Ba	$5\ 10^{-3}$	$1\ 10^{-8.3}$	$1.3\ 10^{-29}$	$2.4\ 10^{-10}$
Zn	$1.2\ 10^{-17}$	$1\ 10^{-10}$		
Ni	$2\ 10^{-15}$	$1\ 10^{-6.9}$		

Table 4. Bioaccessibility of Co, Cr, Ni evaluated by solutions simulating biological fluids (n=2). Extraction percentages are calculated with respect to total concentrations.

	Co		**Cr**		**Ni**	
Synthetic sweat	*mg/kg*	*%*	*mg/kg*	*%*	*mg/kg*	*%*
Pearly pigment	n.d.	<3.7	0.324 ± 0.016	1.0	n.d.	<1.1
Black iron oxide	n.d.	<0.2	n.d.	<0.4	n.d.	<0.1
Pearly powder eye shadow	n.d.	<1.5	0.200 ± 0.016	$8 \cdot 10^{-3}$	n.d.	<0.6
Synthetic lacrimal fluid	*mg/kg*	*%*	*mg/kg*	*%*	*mg/kg*	*%*
Pearly pigment	n.d.	<7.4	0.326 ± 0.023	1.0	n.d.	<2.1
Black iron oxide	n.d.	<0.5	n.d.	<0.7	n.d.	<0.2
Pearly powder eye shadow	n.d.	<1.5	0.368 ± 0.05	0.01	n.d.	<1.2

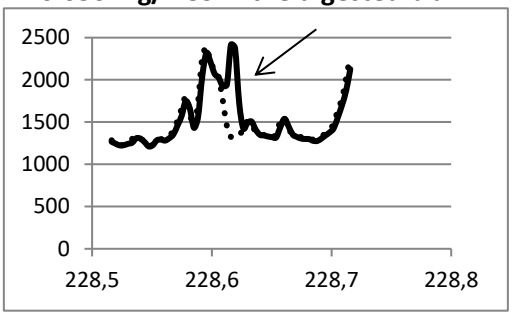
n.d. = not detected, i.e. <0.125 mg/kg in all samples except for synthetic lacrimal fluid for Ni in all samples and Co in RMs (<0.250 mg/kg)

0.050 mg/L Co in diluted nitric acid

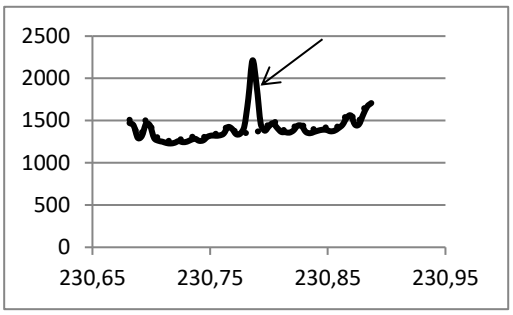


λ=228.616 nm

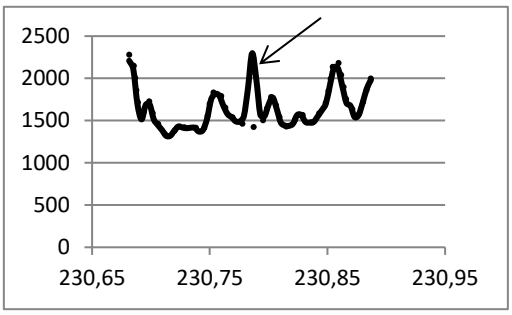
0.050 mg/L Co in the digested blank



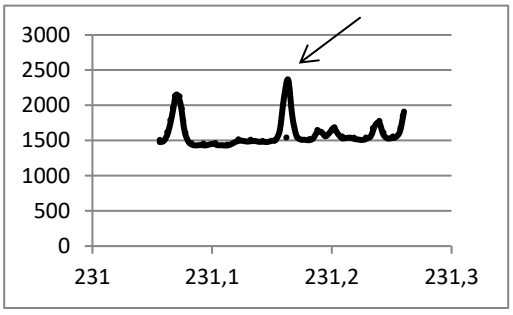
λ=228.616 nm



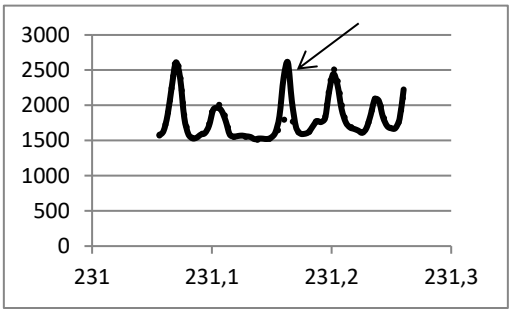
λ=230.786 nm



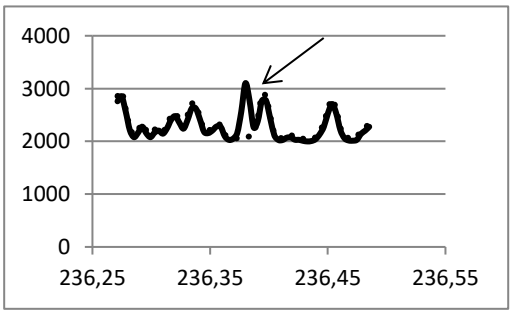
λ=230.786 nm



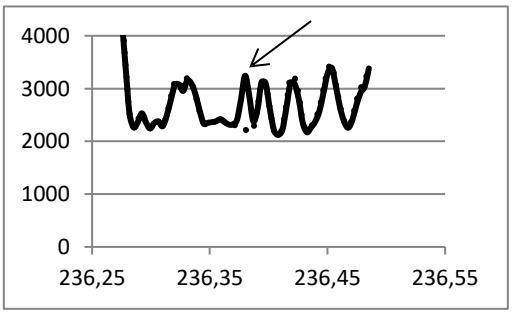
λ=231.160 nm



λ=231.160 nm



λ=236.380 nm



λ=236.380 nm

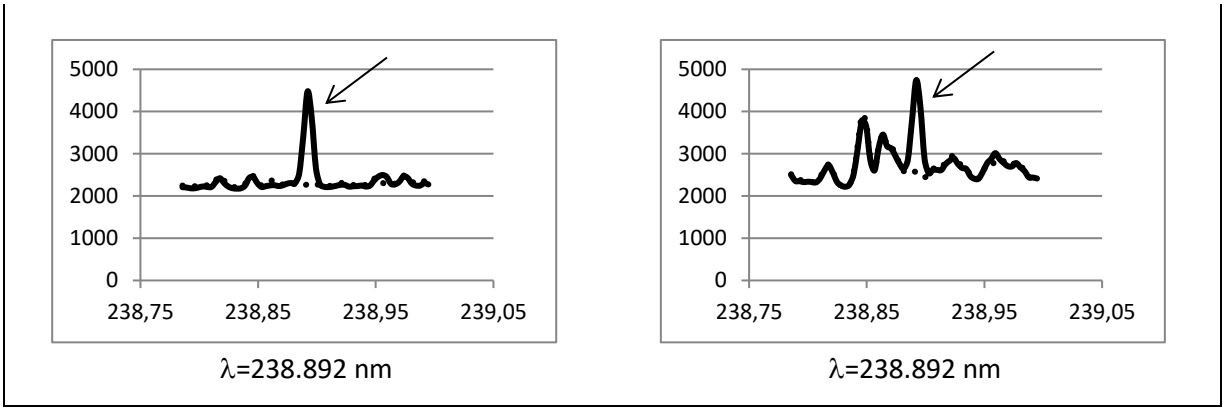


Figure 1. Emission spectra of 0.050 mg/l Co in diluted nitric acid and in the digested blank at the following wavelengths (nm): 228.616; 230.786; 231,160; 236.380; 238.892. Dotted lines represent the corresponding blanks. Arrows indicate Co emission peak. Abscissae: wavelength (nm). Ordinates: emission power (arbitrary units).

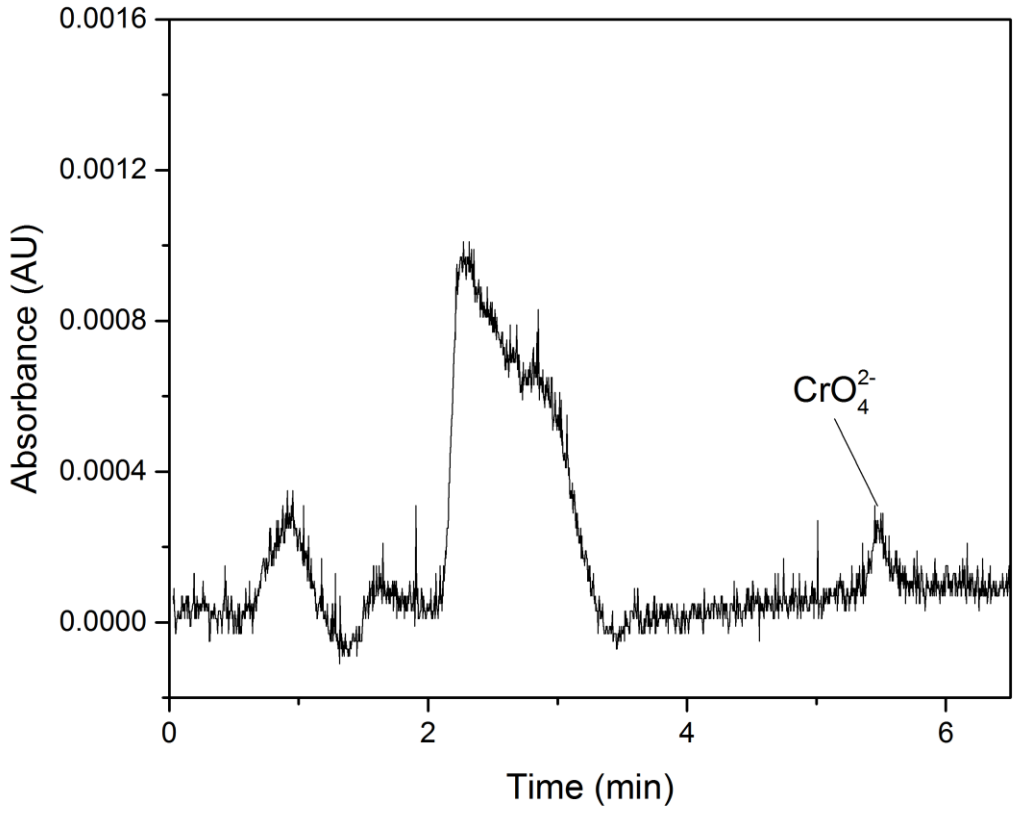


Figure 2. Typical chromatogram obtained for 0.05 µg/L Cr(VI). Chromatographic conditions as follows. Column: IonPac AG7 (50 x 4.0 mm) and IonPac AS7 (250 x 4.0 mm). Eluent: 250 mM $(NH_4)_2SO_4$, 100 mM NH_4OH (pH 8.8), 1 mL/min flow rate. Injection volume: 1000 µL. Detection: spectrophotometric (530 nm) after post-column derivatization (750 µl reaction coil) with 0.5 M H_2SO_4, 2 mM DPC (10% CH_3OH).

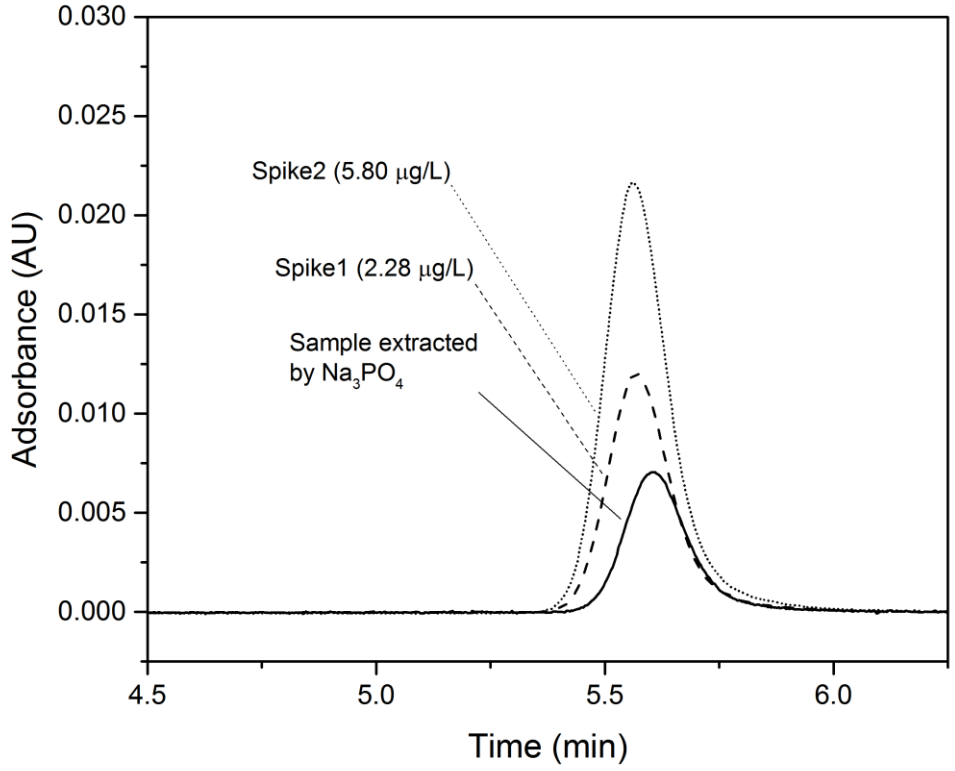


Figure 3. Determination of Cr(VI) in the pearly pigment after extraction with Na_3PO_4 and anion-exchange chromatography with post-column detection. Overlay of sample solution extract with some of the spikes used for Cr(VI) quantification with standard addition method. For chromatographic conditions, see figure 2.

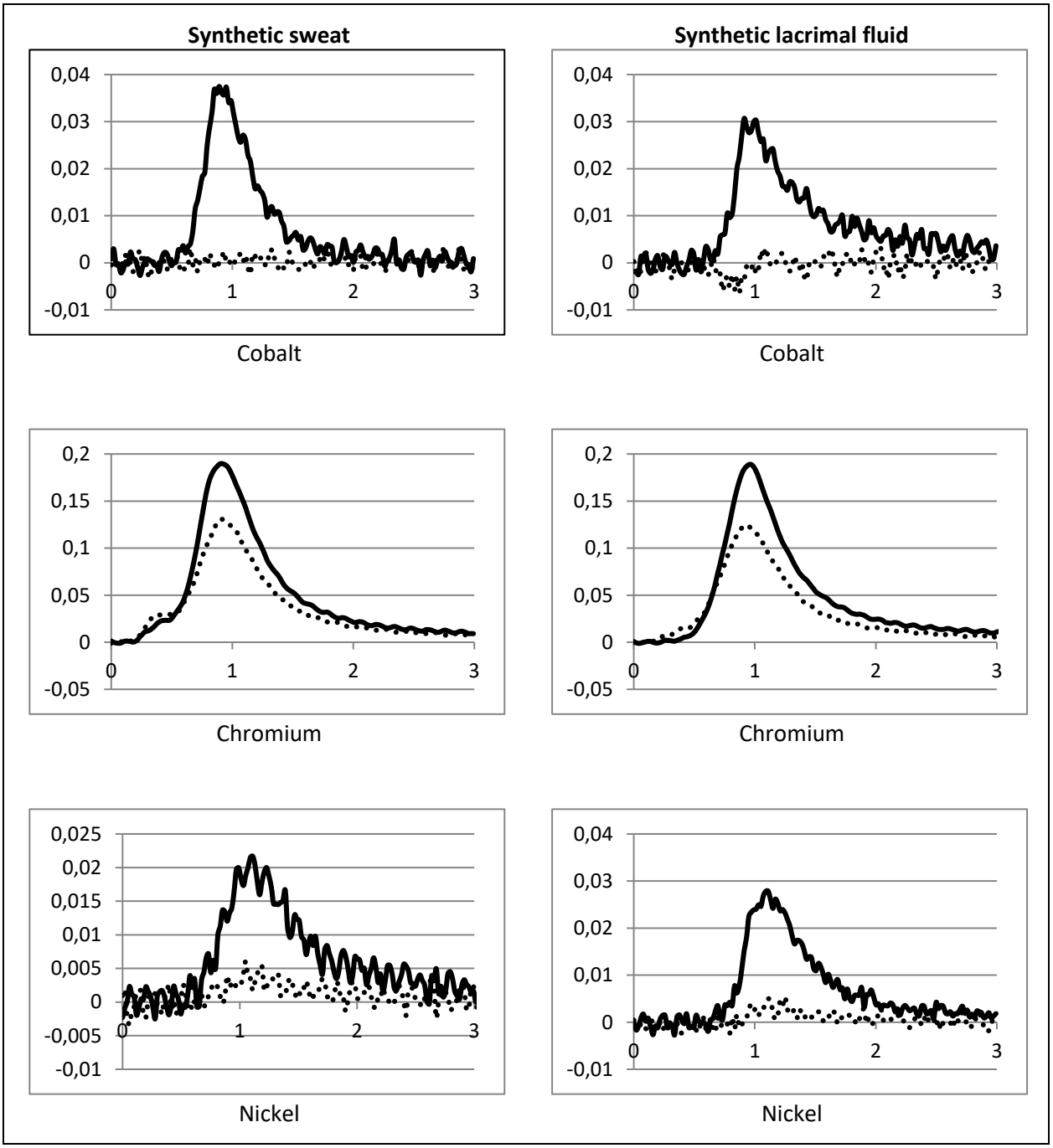


Figure 4. Time-resolved absorbance of pearly pigment extracts in simulated sweat and lacrimal fluid at the absorption lines of Co (242.5 nm), Cr (357.9 nm) and Ni (232.0 nm). Dotted lines: unspiked extracts; Solid lines: extracts spiked with 5 µg/L of each element. Abscissae: time (s). Ordinates: absorbance.

References

1. Straif K, Benbrahim-Tallaa L, Baan R, Grosse Y, Secretan B, El Ghissassi F et al. A review of human carcinogens--part C: metals, arsenic, dusts, and fibres. Lancet Oncol. 2009;10(5):453-4.
2. European Commission. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetics products. 2009. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:342:0059:0209:en:PDF. Accessed 07 Jun 2017.
3. Bundesinstitut fur Risikobewertung. BfR empfiehlt, Allergie auslösendes Chrom (VI) in Lederprodukten streng zu begrenzen 2007. http://www.bfr.bund.de/cm/343/bfr_empfiehlt_allergie_ausloesendes_chrom_in_lederprodukten_streng_zu_begrenzen.pdf. Accessed 07 Jun 2017.
4. Methodologies for Soil and Sediment Fractionation Studies Edited by Ph. Quevauviller (European Commission, DG Research, Brussels). Cambridge: Royal Society of Chemistry; 2002.
5. Bacon JR, Davidson CM. Is there a future for sequential chemical extraction? Analyst. 2008;133(1):25-46.
6. Abollino O, Malandrino M, Giacomino A, Mentasti E. Investigation of metal pollution in soils by single and sequential extraction procedures. In: Contaminated Soils: Environmental Impact, Disposal and Treatment. New York: Nova Science Publishers; 2011. p. 140-80.
7. Environmental Health &Toxicology. IUPAC Glossary. https://sis.nlm.nih.gov/enviro/iupacglossary/glossaryb.html. Accessed 07 Jun 2017.
8. Intawongse M, Dean JR. In-vitro testing for assessing oral bioaccessibility of trace metals in soil and food samples. TrAC - Trend Anal. Chem. 2006;25(9):876-86.
9. Giacomino A, Abollino O, Casanova C, La Gioia C, Magi E, Malandrino M. Determination of the total and bioaccessible contents of essential and potentially toxic elements in ayurvedic formulations purchased from different commercial channels. Microchem. J. 2015;120:6-17.
10. Guney M, Chapuis RP, Zagury GJ. Lung bioaccessibility of contaminants in particulate matter of geological origin. Environ. Sci. Pollut. R. 2016;23(24):24422-34.
11. Mazinanian N, Hedberg Y, Odnevall Wallinder I. Nickel release and surface characteristics of fine powders of nickel metal and nickel oxide in media of relevance for inhalation and dermal contact. Regul. Toxicol. Pharm. 2013;65(1):135-46.
12. Semple KT, Doick KJ, Jones KC, Burauel P, Craven A, Harms H. Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated. Environ. Sci. Technol. 2004;38(12):228a-31a.
13. European Commission. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. 2010. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF. Accessed 07 Jun 2017.
14. Russell WMS, Burch RL. The principles of humane experimental technique. 1st ed. London: Methuen; 1959.
15. Al-Saleh I, Al-Enazi S, Shinwari N. Assessment of lead in cosmetic products. Regul. Toxicol. Pharm. 2009;54(2):105-13.
16. Oh J, Lee H, Choi Y, Choi H, Byun J. Metal allergy in eyelid dermatitis and the evaluation of metal contents in eye shadows. J Eur. Acad. Dermatol. Venereol. 2016;30(9):1518-21.
17. US EPA. Chromium Hexavalent (colorimetric), Method 7196A. 1992. https://www.epa.gov/sites/production/files/2015-12/documents/7196a.pdf. Accessed 07 Jun 2017.
18. US EPA. Determination of Hexavalent Chromium in drinking water, groundwater and industrial wastewater effluents by ion chromatography. Method 7199. 1996. https://www.epa.gov/sites/production/files/2015-12/documents/7199.pdf. Accessed 07 Jun 2017.
19. Grabarczyk M. Protocol for Extraction and Determination of Cr(VI) in Solid Materials with a High Cr(III)/Cr(VI) Ratio Using EDDS as a Leaching Agent for Cr(VI) and a Masking Agent for Cr(III). Electroanal. 2008;20(17):1857-62.
20. Byrdy FA, Olson LK, Vela NP, Caruso JA. Chromium speciation by anion-exchange high-performance liquid chromatography with both inductively coupled plasma atomic emission spectroscopic and inductively coupled plasma mass spectrometric detection. J. Chromatogr. A. 1995;712(2):311-20.

21. US EPA. Method 3060A, Alkaline digestion for hexavalent chromium. 1996. https://www.epa.gov/sites/production/files/2015-12/documents/3060a.pdf. Accessed 07 Jun 2017.
22. Istituto di Ricerca Sulle Acque IRSA. Cromo esavalente. 1968. http://ctntes.arpa.piemonte.it/Raccolta%20Metodi%202003/pdf/Metodi%20IRSA/16cromoesavalente.pdf. Accessed 07 Jun 2017.
23. Mandiwana KL. Rapid leaching of Cr(VI) in soil with Na_3PO_4 in the determination of hexavalent chromium by electrothermal atomic absorption spectrometry. Talanta. 2008;74(4):736-40.
24. Turner A, Hambling J. Bioaccessibility of trace metals in sediment, macroalga and antifouling paint to the wild mute swan, Cygnus olor. Water Air Soil Pollut. 2012;223(5):2503-9.
25. Verhoeckx K, Cotter P, López-Expósito I, Kleiveland C, Lea T, Mackie A, Requena T, Swiatecka D, Wichers H, editors. The Impact of Food Bioactives on Health in vitro and ex vivo models. Cham (CH): Springer International Publishing; 2015.
26. Contado C, Pagnoni A. A new strategy for pressed powder eye shadow analysis: Allergenic metal ion content and particle size distribution. Sci. Total Environ. 2012;432:173-9.
27. Wurster S, Kratz E, Lachenmeier DW, Mildau G. Spectrophotometric Quantification of Toxicologically Relevant Concentrations of Chromium(VI) in Cosmetic Pigments and Eyeshadow Using Synthetic Lachrymal Fluid Extraction. Int. J. Spectrosc. 2012;2012.
28. Capelli C, Foppiano D, Venturelli G, Carlini E, Magi E, Ianni C. Determination of Arsenic, Cadmium, Cobalt, Chromium, Nickel, and Lead in Cosmetic Face-Powders: Optimization of Extraction and Validation. Anal. Lett. 2014;47(7):1201-9.
29. Gao P, Liu S, Zhang Z, Meng P, Lin N, Lu B et al. Health impact of bioaccessible metal in lip cosmetics to female college students and career women, northeast of China. Environ. Pollut. 2015;197:214-20.
30. Hepp N, Mindak W, Cheng J. Determination of total lead in lipstick: development and validation of a microwave-assisted digestion, inductively coupled plasma–mass spectrometric method. J. Cosmetic Sci. 2009;60(4):405-14.
31. Bocca B, Forte G, Pino A, Alimonti A. Heavy metals in powder-based cosmetics quantified by ICP-MS: an approach for estimating measurement uncertainty. Anal. Methods. 2013;5(2):402-8.
32. Milestone. SK-10 High Pressure Rotor. Application Book. Methods HPR-CA-16 and HPR-CA-18.
33. European Commission. 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results 2002. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32002D0657. Accessed 07 Jun 2017.
34. International Council for Harmonisation, ICH Expert Working Group, editor. Validation of analytical procedures: text and methodology Q2 (R1). Proceedings of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2005.
35. Rai D, Eary L, Zachara J. Environmental chemistry of chromium. Sci. Total Environ. 1989;86(1-2):15-23.
36. Hudson Institute of Mineralogy, Mindat.org. http://www.mindat.org/min-6728.html. Accessed 07 Jun 2017.
37. Kumada K, Asami T. A new method for determining ferrous iron in paddy soils. Soil Sci. Plant Nutr. 1957;3(1):187-93.

SUPPLEMENTARY MATERIAL

Chromium, Nickel and Cobalt in cosmetic matrices: an integrated bioanalytical characterization through total content, bioaccessibility and Cr(III)/Cr(VI) speciation

Maria Concetta Bruzzoniti[1]*, Ornella Abollino[1]*, Marco Pazzi[1], Luca Rivoira[1], Agnese Giacomino[2], Marco Vincenti[1]

[1] Department of Chemistry, University of Turin, Via P. Giuria 5, 10125 Turin (Italy)
[2] Department of Drug Science and Technology, University of Turin, Via P. Giuria 9, 10125 Turin (Italy)

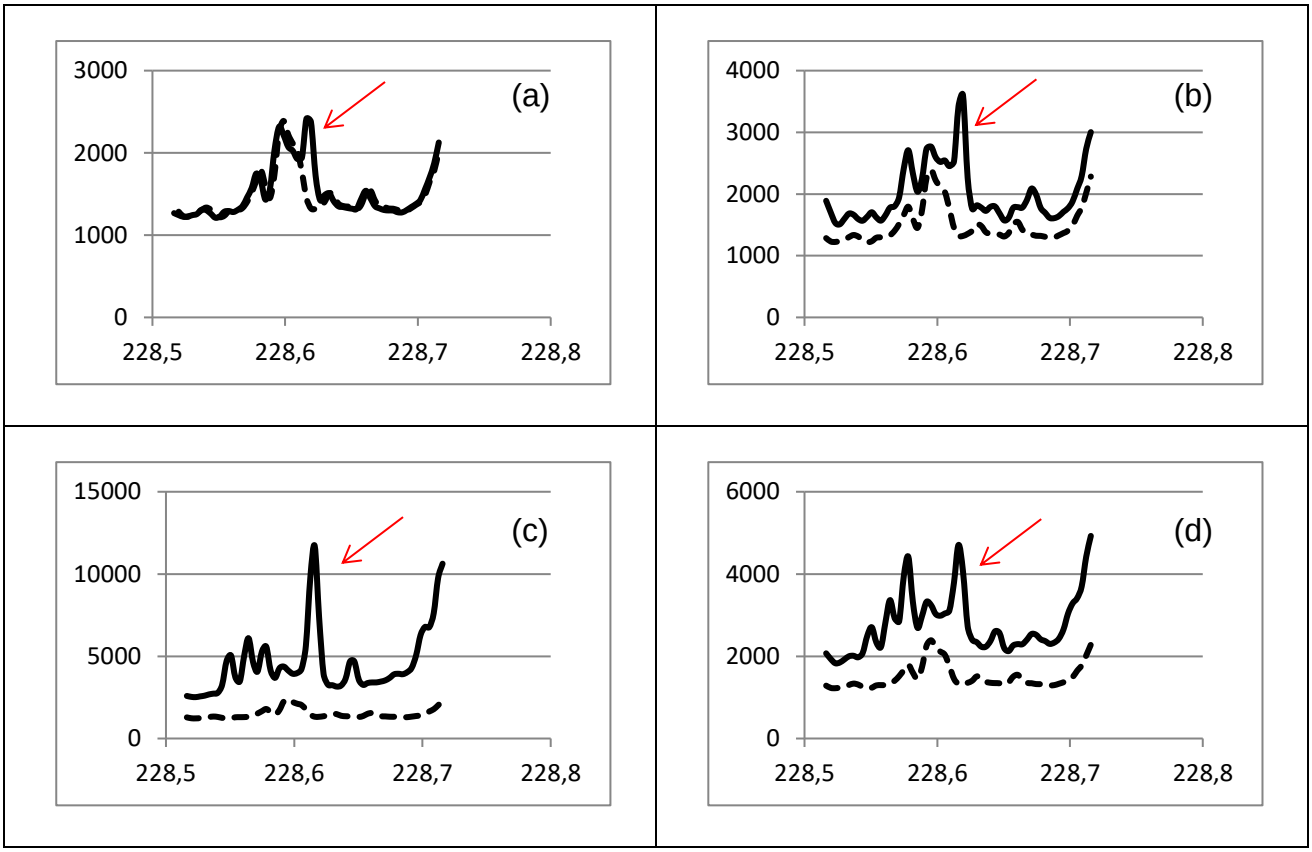


Figure 1S. Emission spectra of: digested blank spiked with 0.050 mg/l Co (a); unspiked digested solutions of pearly pigment (b), black iron oxide; (c) and pearly powder eye shadow (d) around 228.616 nm. Dotted lines represent the blank (same blank for all samples). Arrows indicate Co emission peak. Abscissae: wavelength (nm). Ordinates: emission power (arbitrary units).

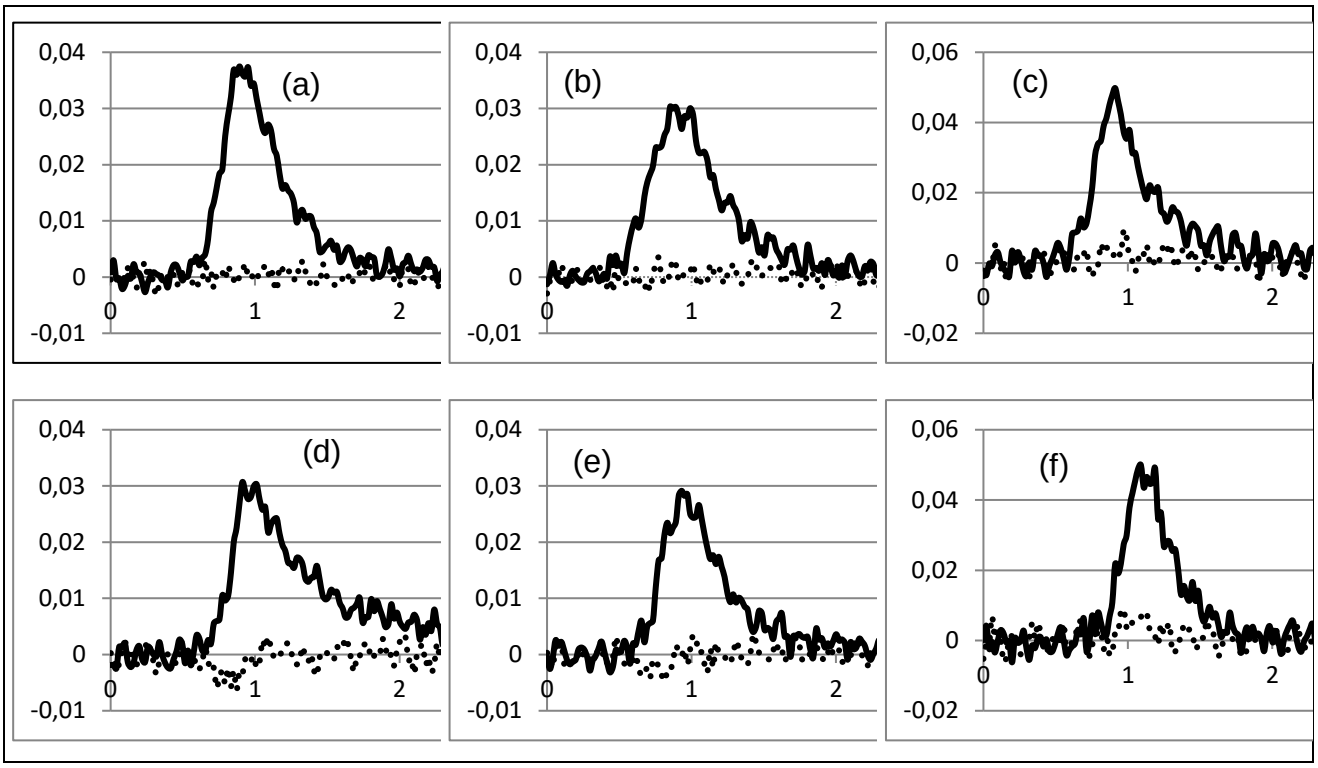


Figure 2S. Time-resolved absorbance of sample extracts in simulated sweat (plots a-c) and lacrimal fluid (plots d-f) at the absorption line of Co (242,5 nm). (a) and (d): pearly pigment; (b) and (e): black iron oxide; (c) and (f): pearly powder eye shadow. Dotted lines: unspiked extracts; Solid lines: extracts spiked with 5 µg/L Co. Abscissae: time (s). Ordinates: absorbance.

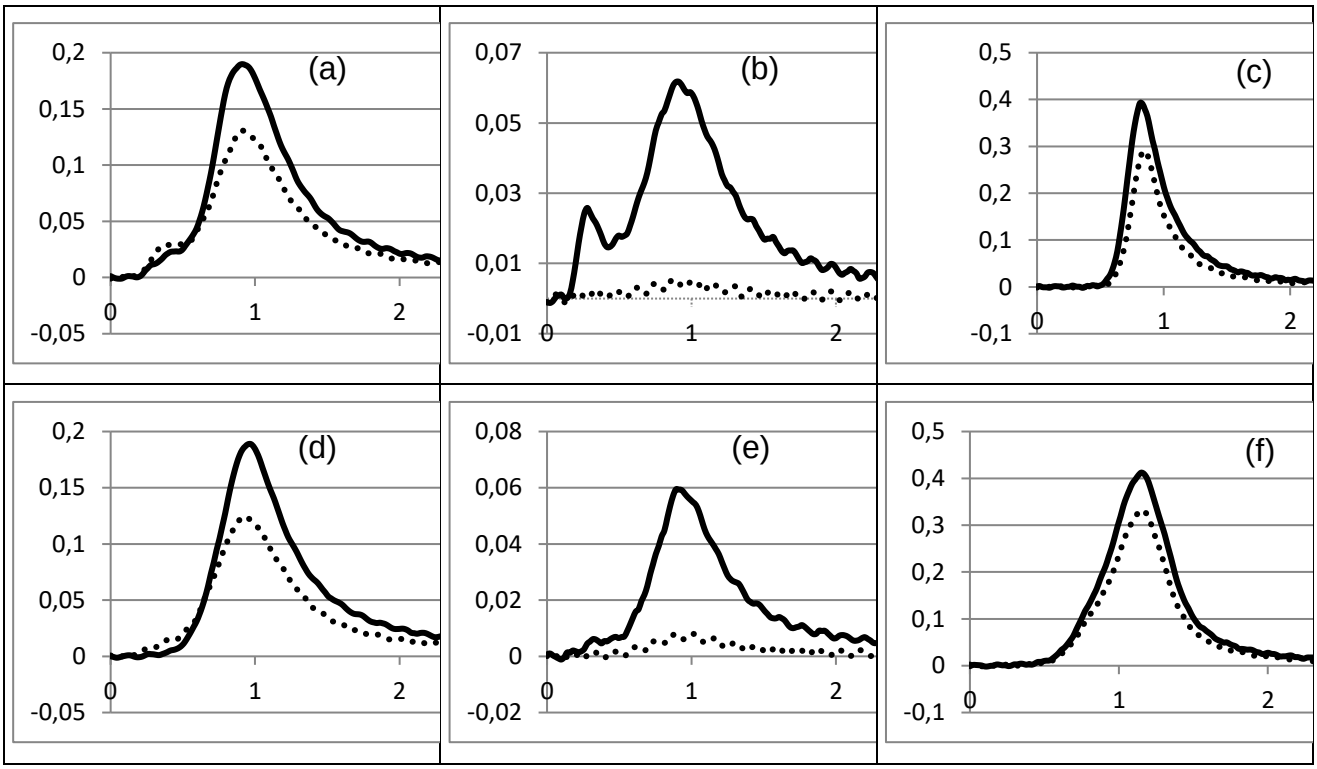


Figure 3S. Time-resolved absorbance of sample extracts in simulated sweat (plots a-c) and lacrimal fluid (plots d-f) at the absorption line of Cr (357.9 nm). (a) and (d): pearly pigment; (b) and (e): black iron oxide; (c) and (f): pearly powder eye shadow. Dotted lines: unspiked extracts; Solid lines: extracts spiked with 5 µg/L Cr. Abscissae: time (s). Ordinates: absorbance.

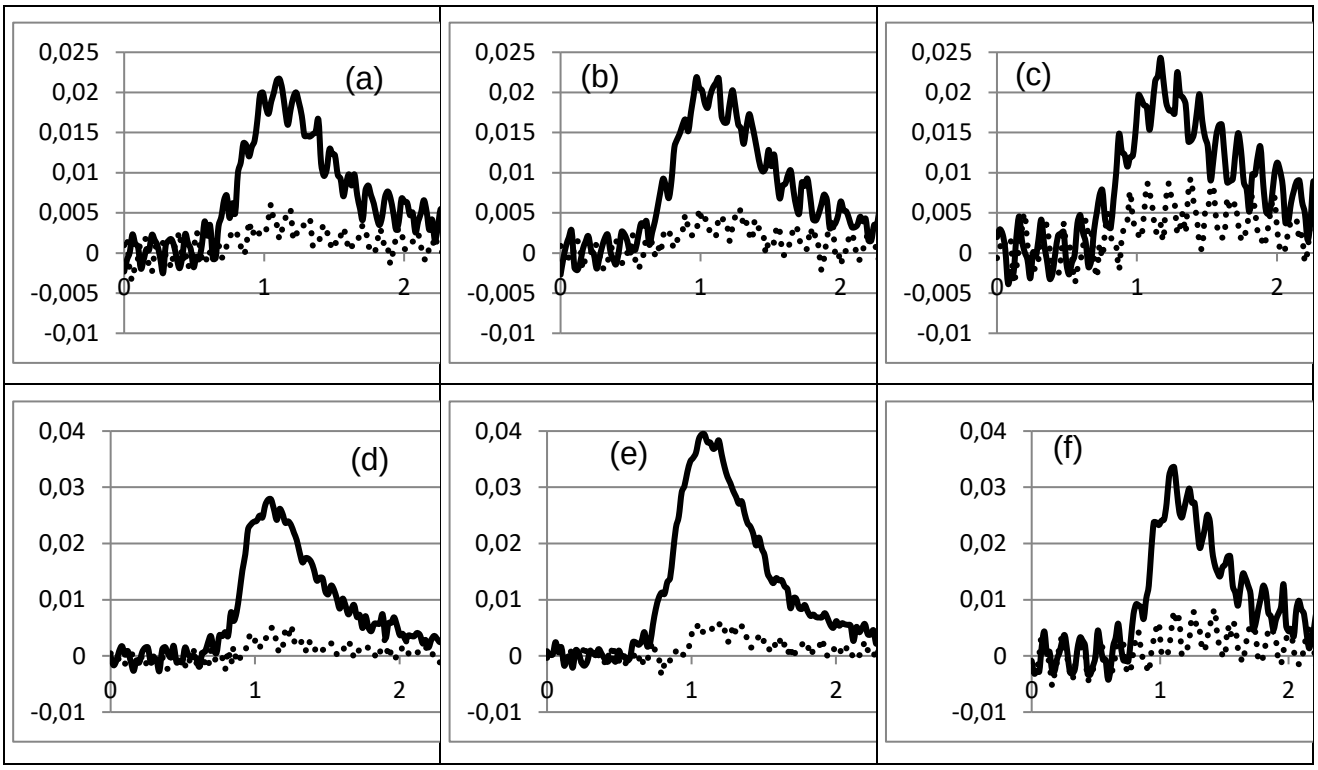


Figure 4S. Time-resolved absorbance of sample extracts in simulated sweat (plots a-c) and lacrimal fluid (plots d-f) at the absorption line of Ni (232.0 nm). (a) and (d): pearly pigment; (b) and (e): black iron oxide; (c) and (f): pearly powder eye shadow. Dotted lines: unspiked extracts; Solid lines: extracts spiked with 5 µg/L Cr. Abscissae: time (s). Ordinates: absorbance.

Table 1S. Element concentrations (expressed in mg/kg) in the investigated samples after digestion with two different acid mixtures.

	Co	**Cr**	**Ni**
HNO_3/H_2SO_4/HF (4/2/2 ml)			
Pearly pigment	-	33.7	9.5
Black iron oxide	50.3	14.2	144
Pearly powder eye shadow	n.d.	n.d.	n.d.
Aqua regia/HF (6/2 ml)			
Pearly pigment	2.3	22.4	11.8
Black iron oxide	45.2	12.1	128
Pearly powder eye shadow	15.1	394	20.0

n.d. = not determined