Determination of Polycyclic Aromatic Hydrocarbons in Lipstick by Gas-Chromatography coupled to Mass Spectrometry: a case history

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Highlights

Development of an extraction protocol for the determination of PAHs in cosmetic products

Optimization and validation of a GC/MS method for the analysis of PAHs

Assessment of the potential risk for consumer health

Abstract

A suitable extraction protocol based on an liquid-liquid extraction with hexane/dimethyl sulfoxide and a GC/MS method were developed and validated to determine the concentration of six prohibited Polycyclic Aromatic Hydrocarbons (PAHs; benzo[a]pyrene; dibenz[a,h]anthracene; benz[a]anthracene; benzo[j]fluoranthene; benzo[k]fluoranthene; chrysene) in lipsticks commissioned by a cosmetic company to a manufacturer. The lipsticks were produced in four different colors. Analyses confirmed the presence of benz[a]anthracene and chrysene only in two colors in a concentration of 9.3-9.4 ng/g. The concentration of PAHs was 250 times lower than what is considered a toxic level on the basis of what reported in the litaraure and guidances for cosmetic ingredients; therefore we could assume that the risk for consumer health was negligeble.

Keywords Polycyclic Aromatic Hydrocarbons (PAHs); Cosmetic Products; GC/MS; Liquid-liquid Extraction

1. Introduction

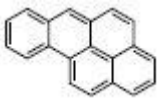
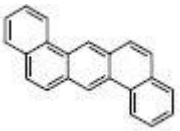
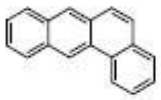
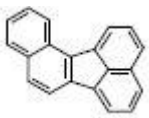
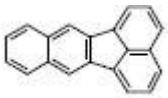
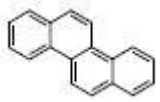
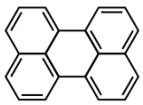
Polycyclic aromatic hydrocarbons (PAHs) are a large group of more than two hundred compounds consisting of two or more fused aromatic rings. They are formed during incomplete pyrolysis of organic materials and are diffused environmental pollutants. They are introduced or emitted into the environment, and contaminate and accumulate in such plants and animals which are part of the food chain. [1,2]; in fact, for non-smokers the main way of assumption is food, contribution of smoking being significant. Food can be contaminated from environmental sources or way of cooking (i.e. roasting and baking). Many studies have been reported on the determination of PAHs in environmental and food samples. Examples of the latter include tea, coffee, fruits, vegetables, oils, milk, cheese, roasted meat and fish [3-9].

As regards as cosmetic and personal care products, to the best of our knowledge, very few scientific reports are present on the presence of PAHs in these preparations [10] and no data are available in lipsticks. The manufacturing process is designed to exclude substances with carcinogenic potential like polycyclic hydrocarbons (PAH) in cosmetic ingredients but mineral oil and waxes, the main components of the lipsticks, could be contaminated deriving form petroleum. Moreover even pigments or dyes could be PAH-contaminated materials.

Toxicity of PAHs has been evaluated by several organizations [11-16]; they have been found to be carcinogenic in experimental animals after inhalation or intratracheal ingestion. The United States Environmental Protection Agency (EPA) monitors 16 priority PAHs in air due to health concerns: naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, chrysene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, B[a]P, indeno[1,2,3-c,d]pyrene, benzo[g,h,i]-perylene, and dibenz[a,h]anthracene [11]. International

Agency for Research on Cancer (IARC) classified benzo[a]pyrene as a human carcinogen (Group1); dibenz[a,h]anthracene as carcinogen (Group 2A), whereas chrysene, benz[a]anthracene, benzo[j]fluoranthene, benzo[k]fluoranthene are possible human carcinogens (Group 2B) as reported in Table 1 [1].

Table 1. PAHs considered in the study

PAH	Molecular weight	Structure	IARC Classification [12]	Log P	Ref. number [17]	CAS Number
Benzo[a]pyrene	252.32		1	6.0	612	00050-32-08
Dibenz[a,h]anthracene	278.35		2A	6.5	637	000053-70-3
Benz[a]anthracene	228.28		2B	5.8	638	000056-55-3
Benzo[j]fluoranthene	252.32		2B	6.4	640	000205-82-3
Benzo[k]fluoranthene	252.32		2B	6.8	642	000207-08-9
Chrysene	228.28		2B	5.7	643	000218-01-9
Perylene	252.32		3	5.7	Internal standard	00050-32-08

The exposure to PAHs is a significant health problem. Their presence in cosmetic products is regulated by the Regulation (EC) No 1223/2009 of the European Parliament and of the Council of

30 November 2009 [17]. In this regulation, the six PAHs reported in Table 1 (benzo[a]pyrene; dibenz[a,h]anthracene; benz[a]anthracene; benzo[j]fluoranthene; benzo[k]fluoranthene; chrysene, perylene was used as internal standard, IS) are prohibited. On the other hand, the preamble 37 of the regulation cites "In order to ensure product safety, prohibited substances should be acceptable at trace levels only if they are technologically inevitable with correct manufacturing processes and provided that the product is safe" [17].

In this frame, a cosmetic company, that had commissioned some batches of lipsticks to a manufacturer, contested the production because of the presence of PAHs included in the list of the substances prohibited in cosmetics [17]. The manufacturer stated that the presence of PAHs was inevitable because they were present in the raw materials used for the production of the lipsticks, and that their presence was not dangerous for human health.

To settle the matter, the Court gave us the task to determine the concentration of the six prohibited PAHs reported in Table 1 in the lipsticks, and to assess if their possible presence could be risk for consumer health.

Among the analytical methods reported in the literature [18-21] we chose GC/MS, as it was indicated as the most suitable method for PAH determination [22-25]. As regard as sample preparation many extraction methods from different matrices have been proposed such as liquid-liquid extraction (LLE) [26] or solid phase extraction (SPE) [27]. The application of these extraction methods on lipsticks resulted quite complicated, due to the hydrophobicity and complexity of the matrix. So that we carried out an extensive study on the extraction method and on the analytical conditions in order to optimize PAH recovery and detection. The optimized analytical method was then validated and applied for the determination of PAHs in the lipsticks.

2. Materials and methods

2.1 Seized materials

Five lipsticks for each different color (honey, coral, flamingo and toffee) were delivered to our laboratory for the analysis.

2.2 Reagents and standards

Hexane (>97%), cyclohexane (>99%), chloroform (>99%); dimethyl sulfoxide (>99%); anhydrous sodium sulfate; sodium chloride; benzo[a]pyrene (>96,7%) ; dibenz[a,h]anthracene (>99%); benz[a]anthracene (>99,8%); benzo[j]fluoranthene (>99,7%); benzo[k]fluoranthene (>99,7%); chrysene (>99,5%); perylene (IS, >98%) were purchased form Sigma Aldrich (Milan, Italy); water (18.2 $\Omega \cdot cm^{-1}$) was prepared by a Milli-Q System (Millipore, Darmstadt, Germany).

Two standard mixtures (Std mix1; Std mix 2) were prepared in cyclohexane to ensure a comparable response of the different PAHs: Std mix 1: benz[a]anthracene, 10 µg/mL; chrysene, 10 µg/mL, benzo[j]fluoranthene, 4.0 µg/mL; benzo[k]fluoranthene, 4.0 µg/mL; benzo[a]pyrene, 4.0 µg/mL; dibenz[a,h]anthracene, 20 µg/mL. Std mix 2 was obtained diluting 100 fold Std mix 1: benz[a]anthracene, 0.10 µg/mL; chrysene, 0.10 µg/mL, benzo[j]fluoranthene, 0.04 µg/mL; benzo[k]fluoranthene, 0.04 µg/mL; benzo[a]pyrene, 0.04 µg/mL; dibenz[a,h]anthracene, 0.20 µg/mL.

2.3 GC/MS analysis

GC/MS analyses were carried out on a 6890 Series Plus gas chromatograph equipped with an Agilent 7683 autosampler and coupled to a 5973N mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Data were analysed with MSD ChemStation D.03.00 software (Agilent Technologies). Chromatographic separation was carried out on a J&W DB- 5MS UI capillary

column (30 m × 0.25 mm I.D., thickness 0.25 µm; Agilent Technologies) and pulsed splitless injection mode (pressure 18 psi for 1 min) split ratio (36:1) was used. The GC/MS system was operated under the following conditions: injection temperature: 300°C; interface transfer line: 280°C; ion source: 230°C; initial column temperature: 70°C. The temperature was subsequently increased to 180°C at a rate of 40°C min^{-1}, then to 300°C at a rate of 10°C min^{-1} and held at this temperature for 7.25 min. Helium was used as carrier gas at a flow rate of 1.1 mL/ min. MS analysis was performed in scanning mode (40-550 *m*/*z*) and selected ion monitoring mode (SIM) with a quadrupole mass detector operated in electron ionization mode, with beam energy of 70 eV. The ions selected for SIM mode acquisition and the retention times are reported in Table 2. In Figure 1 the chromatogram obtained for a standard mixture 100 µg/mL of PAHs and IS is shown. In these conditions the most dangerous PAHs, i.e. benzo[a]pyrene and dibenz[a,h]anthracene were well separated and detected in a reasonable lapse of time; on the other hand the two isomers benzo[j]fluoranthene and benzo[k]fluoranthene, were not separated. To separate these two peaks it would have been necessary too long analysis time or a dedicated GC column. This was not considered a problem because benzo[j]fluoranthene and benzo[k]fluoranthene were not present in the lipstick samples considered. Therefore we decided to measure their sum.

Table 2. Retention times and fragmentation ions of PAHs: in bold the quantifier ions.

PAHs	**T_R**	**m/z**
benz[a]anthracene	11.574 ± 0.1 min	114, **228**
chrysene	11.650 ± 0.1 min	114, **228**
benzo[j]fluoranthene	13.903 ± 0.1 min	126, 224, **252**
benzo[k]fluoranthene	13.956 ± 0.1 min	126, 224, **252**
benzo[a]pyrene	14.543 ± 0.1 min	126, **252**
IS	14.725 ± 0.1 min	126, **252**
dibenz[a,h]anthracene	17.115 ± 0.1 min	139, 250, **278**

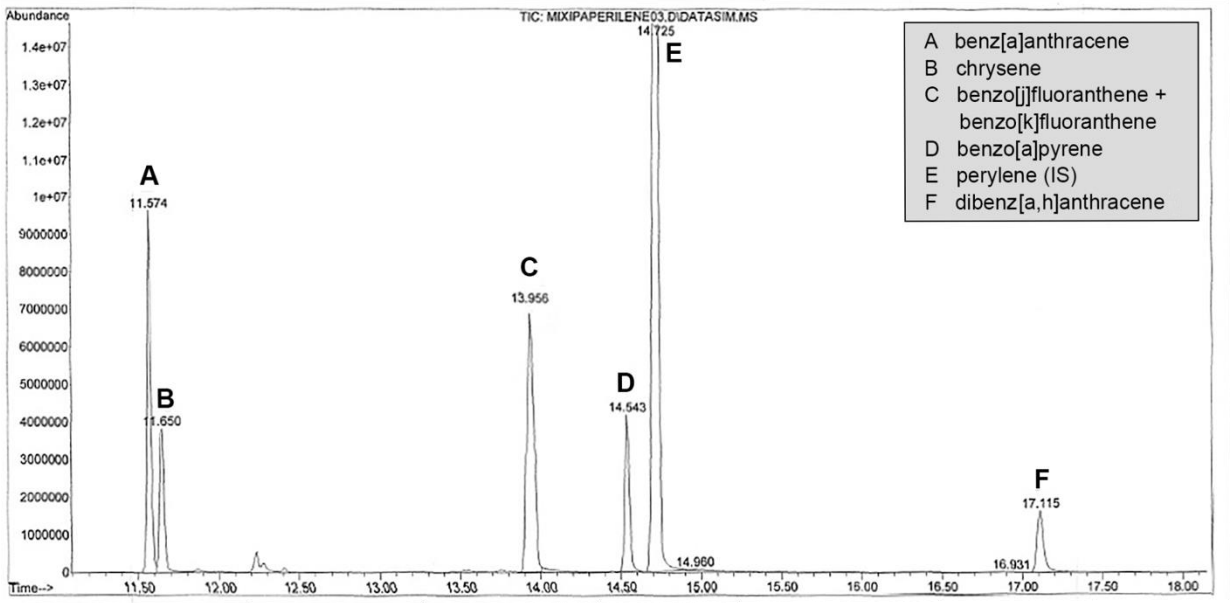


Figure 1. Standard mixture 100 µg/mL of PAHs and IS.

2.4 Matrix

A typical matrix was prepared in order to validate the method (extraction efficiency). Percentage of the single ingredients (castor oil, octildodecanol, microcristalline wax, candelilla wax, multi wax, ozokerite, kaolin) were establisehd on the basis of the composition reported in the label of the products and is not reported for reasons of secrecy. As shown in Figure 2, the reconstitued matrix was free of PAHs.

The compostion of the matrix and the dyes used were not reported for reasons of company confidentiality.

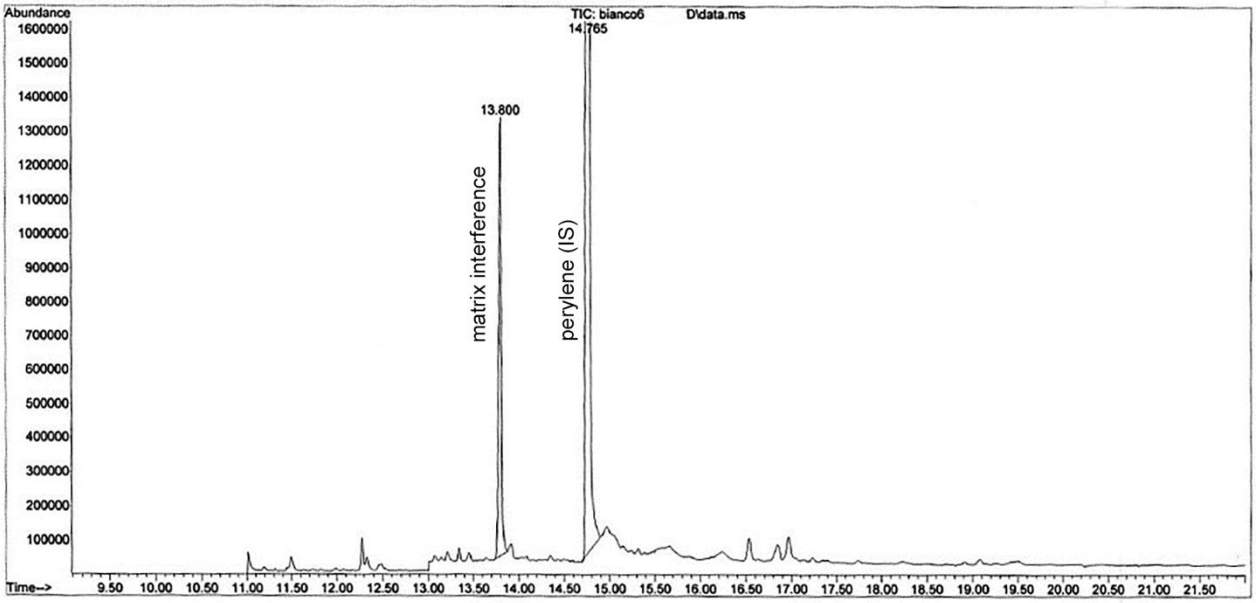


Figure 2. SIM mode chromatogram of the reconstituted matrix.

2.4 Extraction method

Standard preparation: to 3.3 g of blank matrix were added with 25 μL IS (perylene, 1 mg/mL) and different μL of Std mix 2 (from 500 μL to 5000 μL) depending on desired PAHs concentrations. For seized lipstick samples: to 3.3 g of lipstick were added with 25 μL IS (perylene, 1 mg/mL). The sample preparation would be the same regardless of the type of sample (calibration standard in matrix or seized lipstick): 25 mL hexane were added, the mixture was stirred for 15 min and divided into two falcon tubes. 12.5 mL dimethyl sulfoxide were added in each falcon tube and the mixtures were centrifuged for 30 min (6708 x g). The latter operation was repeated another time for a complete extraction. The phases below were withdrawn, added with 120 mL water in which sodium chloride (6 g) was dissolved. The mixture was transferred in separation funnel with 50 mL cyclohexane. The organic phase (above) was separated and the residue extracted twice with 50 mL cyclohexane. The combined organic phases were concentrated using a rotavap until a volume of 5 mL was reached, dried with anhydrous sodium sulfate and concentrated again to 1 mL.

The concentrated organic phase was purified by solid phase extraction (SPE) on 6 mL Superclean™ LC-Florisil® Tubes (Supelco, Bellefonte, Pennsylvania, US) by 9 mL of chloroform as elution solvent. The eluate was concentrated under a gentle stream of nitrogen until a volume of 1 mL was reached and then purified again by SPE in the same conditions. The eluate (9 mL) was concentrated until a volume of 1 mL was reached and analyzed by GC/MS.

In Figure 3 a GC/MS chromatogram of the extracted reconstituted matrix containing 100 ng_{tot} of Std mix 2 is reported.

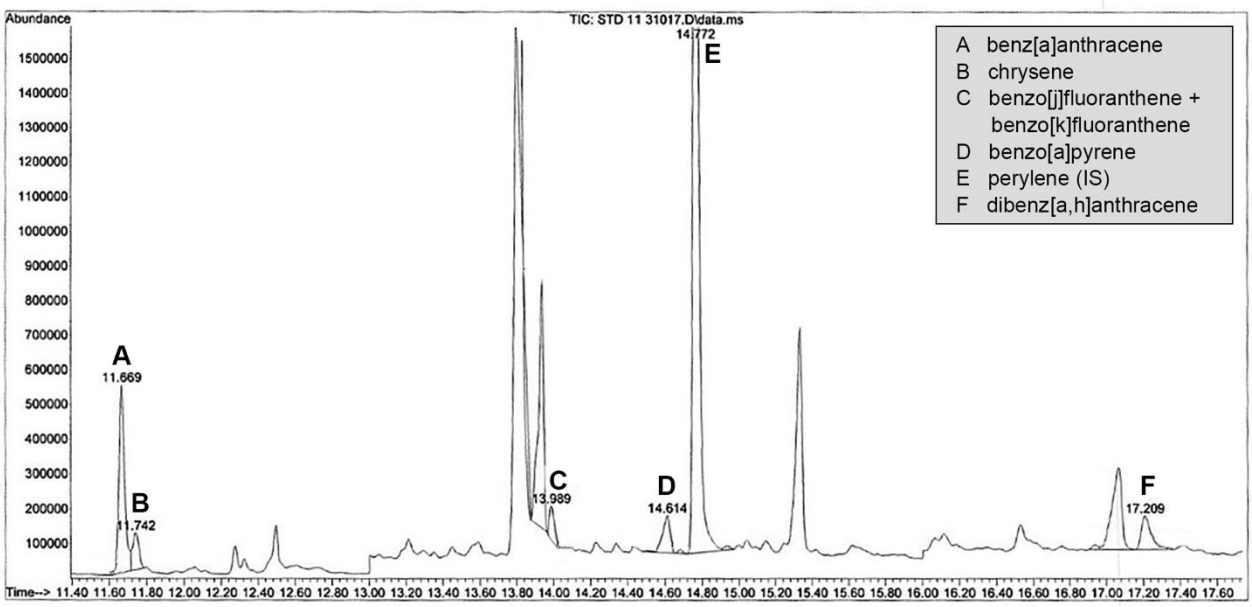


Figure 3. GC/MS chromatogram of the extracted reconstituted matrix containing 100 ng_{tot} of benz[a]anthracene, 100 ng_{tot} of chrysene, 40 ng_{tot} of benzo(j)fluorantene, 40 ng_{tot} of benzo(k)fluorantene, 40 ng_{tot} of benzopirene, 200 ng_{tot} of dibenzo(a,h)antracene

As it is possible to note from the magnification of the chromatographic peaks of benz[a]anthracene and chrysene in Figure 4, in the presence of the reconstituted matrix, the two analytes were not baseline separated and then quantitative determinations were carried out considering the sum the areas of the two chromatographic peaks.

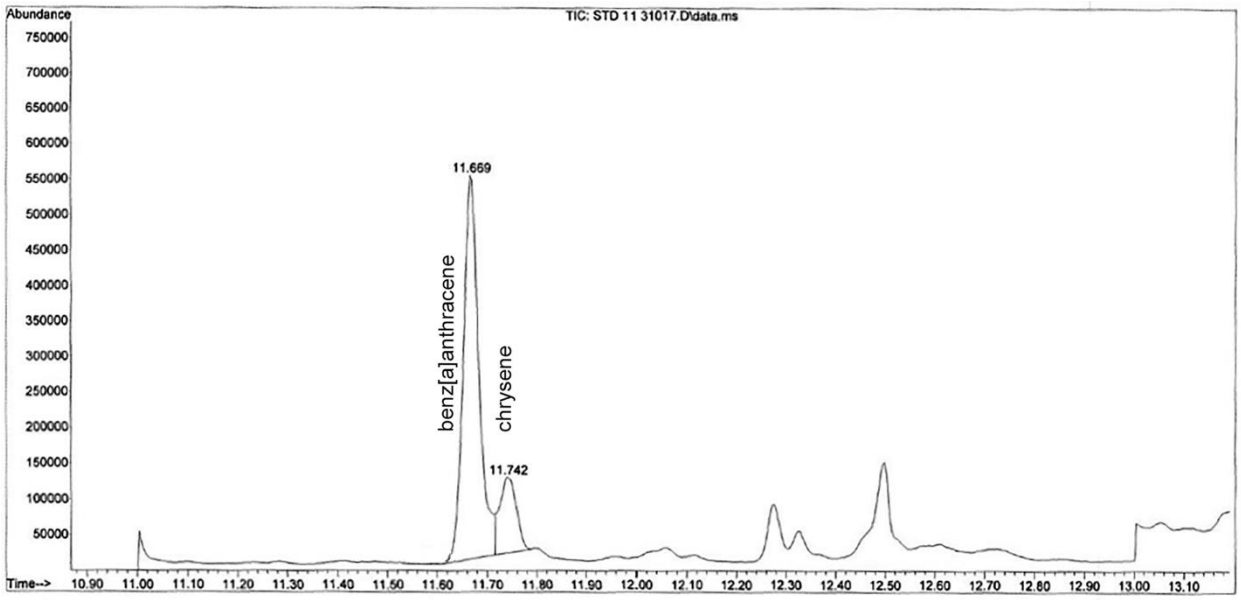


Figure 4. Magnification of benz[a]anthracene and chrysene chromatographic peaks.

2.5 Validation

Prior to application to real samples, the method was tested in a validation protocol scheme following ICH guidelines [28]. Validation protocol applied in the present study included specificity, precision, accuracy, linearity and limits of detection (LOD), quantification (LOQ) and percentage recovery. Standard samples containing different PAH amounts were prepared by adding suitable amounts of Std mix 2 solution to 3.3 g of reconstituted matrix. The amount of reconstituted matrix was chosen taking into account that the weight of a lipstick is 3.3 g. The standard samples were then treated as reported in the "extraction method" section.

The specificity was assessed by extracting control blank samples of reconstituted matrix in each validation run. The lack of interfering peaks at the same analyte retention times was considered as an acceptable selectivity.

Validation parameters for precision and accuracy were calculated using different replicates of samples in different working days. Accuracy was expressed as the percent mean error ($\%ER_m$)

between the theoretical and the experimental value, while precision was measured as coefficient of variation (CV%). A CV% below 10% was considered suitable.

Calibration curves were calculated by plotting peak area PAHs / area IS versus the total amount (ng_{tot}) of PAHs added to 3.3 g of blank reconstituted matrix. Linearity was evaluated in the interval 100-1000 (100, 200, 300, 400, 600, 1000) ng_{tot} for benz[a]anthracene + chrysene and dibenz[a,h]anthracene, 40-400 (40, 80, 120, 160, 240, 400) ng_{tot} for benzo[j]fluoranthene + benzo[k]fluoranthene and 20-200 (20, 40, 60, 80, 120, 200) ng_{tot} for benzo[a]pyrene.

The LOD, defined as the lowest concentration of analyte that can be clearly detected, was estimated as three times the signal to noise ratio. LOQ was considered as the lowest concentration that met a signal-to-noise ratio of at least 10. Percent recovery was evaluated as the percent ratio between the amount of the analyte after the extraction and the amount of analyte added to the blank reconstituted matrix. This parameter was assessed at two different concentrations 100 and 500 ng_{tot} for each analyte.

3. Results

To optimize and validate the extraction and analytical method it was necessary to obtain a matrix as similar as possible to that of the lipsticks. To this end the manufacturer gave us a reconstituted matrix made up with the main components reported in the label of the lipsticks. The compostion of the matrix and the dyes used were not reported for reasons of secrecy. The matrix was free of PAHS as reported in Figure 2. To this reconstituted matrix suitable amounts of the six PAHs were added in order to optimize the extraction protocol and to validate the analytical method.

Different extraction protocols (see Supplemetary Material) were evaluated in order to optimize the recovery of the analytes and to eliminate the interferences from the hydrophobic matrix components.

The first step of the best extraction protocol was the solubilization of the matrix containing the analytes, to this end hexane had proved to be the best solvent. Then an extraction with dimtehyl sulfoxide was carried out followed by a second extraction with cyclohexane. The concentrated organic phases were then purified by two subsequent SPE. All these steps were necessary to separate the hydrophobic analytes form the matrix, which is also highly hydrophobic. Nevertheless, as it is evident form the chromatogram obtained for the extracted reconstituted matrix containing the analytes (Fig. 3), it was not possible to avoid the presence of peaks related to the matrix. However, working in SIM mode, it was possible to identify the analytes without interferences.

Different chromatographic conditions (see Table 1S) were tested in order to enhance sensitivity. To this end the application of the pulsed splitless injection mode was crucial because with this technique pressure pulse contains sample expansion and transfers analytes to the column faster, in this way it is possible to obtain a more efficient sample transfer leading to sharper peaks and increasing sensitivity. The choice of the internal standard was also very important. Initially *tert*-butylanthraquinone was used, but it did not show suitable features to the quantitative purposes. On the other hand, perylene (Table 1) which is structurally related to the analyte but well separated resulted as the optimal choice.

The optimized analytical method was validated following ICH guidelines [28]. The results are reported in Table 3.

Table 3. Validation parameters

		benz[a]anthracene +chrysene		benzo[j]fluoranthene +benzo[k]fluoranthene		benzo[a]pyrene		dibenz[a,h]anthracene	
LOD ng/g		1		1		1		3	
LOQ ng/g		5		4		3		10	
Linearity		y=0,00005x-0.0024 100-1000 ng_{tot}		y=0,0003x+0.0131 40-400 ng_{tot}		y=0,00005x+0.004 20-200 ng_{tot}		y=0,0004x-0.0039 100-1000 ng_{tot}	
R^2		0.9899		0.9960		0.9877		0.9923	
% Recovery	100 ng_{tot}	66.3		64.9		66.2		77.6	
	500 ng_{tot}	68.4		65.9		67.6		81.1	
Precision (n=3)		Conc (ng_{tot})	%CV	Conc (ng_{tot})	%CV	Conc (ng_{tot})	%CV	Conc (ng_{tot})	%CV
		100	7.3	40	0.9	20	6.7	100	3.6
		200	1.6	80	3.9	40	2.8	200	2.5
		300	2.6	120	3.8	60	0.7	300	2.5
		400	1.8	160	7.2	80	2.6	400	3.9
		600	2.0	240	1.1	120	2.9	600	4.6
		1000	2.0	400	1.7	200	1.1	1000	2.9
Accuracy (n=6)		Conc (ng_{tot})	$\%ER_m$	Conc (ng_{tot})	$\%ER_m$	Conc (ng_{tot})	$\%ER_m$	Conc (ng_{tot})	$\%ER_m$
		400	7.2	160	8.2	80	7.6	400	10.6
		600	4.1	240	6.8	120	7.2	600	9.3

The results obtained from the validation study fulfilled the expectations. Initially, blank reconstituted matrix was subjected to the optimized extraction method and no interfering peaks appeared at the retention time of the analytes and IS molecule. The linearity was proven according to the regression line by the method of least squares and expressed by the coefficient of determination (R^2). Six-point matrix-matched calibration curves were evaluated by spiking increasing amounts of the analyte in blank reconstituted matrix samples. Calibration curves were obtained by plotting the ratio between the peak area of the quantifier ion of the analyte and the peak area of the quantifier ion of the internal standard versus the corresponding concentrations of the analytes in concentration ranges. We observed linearity in the whole range. The values of the correlation factors R^2 of the calibration curves were satisfactory. The LOD and

LOQ values obtained were suitable for the purposes of the work (Table 3). The precision and accuracy values resulted acceptable. The recovery was not very high due to the complicated extraction procedure, however quantitative data were not compromised because they were calculated on the basis of extracted standard sample.

The validated method was applied for the determination of PAHs in the lipsticks. Three samples were analyzed for each color in triplicate. Only two analytes were found (benz[a]anthracene + chrysene) only in two colors (honey kiss and soft toffee).

As PAHs were detected only in two colors and the matrix of the lipsticks was the same in all colors, by exclusion we could assume that the presence of PAHs could be related to the dyes. On the other hand the presence of PAHs could be due to different batches of mineral oil and waxes, which are derived from petroleum.

The concentration of the two analytes was evaluated as sum, due to the fact that in the presence of the matrix the two chromatographic peaks were not baseline separated. The concentration of benz[a]anthracene + chrysene was 9.3 ± 0.06 ng/g in the honey kiss lipsticks and 9.4 ± 0.3 ng/g.

4. Discussion

Benz[a]anthracene + chrysene were detected only in two kinds of colors, this was probably related to the different pigments employed. The judge asked us to establish if the limits imposed by the UE 1223/2009 Regulation [17] were respected. As stated in the Introduction, the six PAHs reported in Table 1 (benzo[a]pyrene; dibenz[a,h]anthracene; benz[a]anthracene; benzo[j]fluoranthene; benzo[k]fluoranthene; chrysene) are prohibited. On the other hand, the preamble 37 of the regulation says that if traces of the prohibited substances are not technically evitable, because they are present in the raw materials and the finished products are not toxic, they are allowed. It is worth noting that in the Regulation no definition of the meaning of “traces” is reported and no quantitative

limits are indicated. In this frame, it is not possible to assess if the amounts of PAHs present in the lipsticks is acceptable or not, only the safety assessor of the company can give an indication. However, we can refer to the current regulations in the food sector [29]. The maximum levels allowed for benzo[a]pyrene is 2.0 ppb, while for the sum of benzo[a]pyrene, benz[a]anthracene, benzo[b]fluoranthene, and chrysene is 10.0 ppb. In this frame, we can say that levels of PAHs found in the lipsticks follow below these limits.

As regard as health safety we can make some considerations: on the basis of what reported in the Table 2 of the 9th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation" (SCCS/1564/15) [30], the amount of lipstick applied daily is 0.057 g. Considering that the concentration of PAHs found in the lipsticks is 9.4 ng/g the consumer is exposed daily to an amount of PAHs of 0.538 ng. On basis of what stated in a study on carcinogenic substances [31] in cosmetic products an acceptable level of genotoxic or carcinogenic substances is considered 150 ng/die. The levels of PAHs in the lipsticks is more than 250 times lower. In this frame we can say that the health safety risk related to the lipsticks is negligeble.

5. Conclusions

In conclusion appropriate extraction protocol and anlytical conditions for the detection of PAHs in lipsticks were studied to evaluate if their concentration could be dangerous for human health. The concentration of PAHs was 250 times lower than what is considered a toxic level. Therefore on the basis of what reported in the litaraure and guidances for cosmetic ingredients we can say that the risk for consumer health is negligeble.

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