Monitoring of Natural Pigments in Henna and Jagua Tattoos for Fake Detection

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Abstract: Temporary tattoos are a popular alternative to permanent ones. Some of them use natural pigments such as lawsone in the famous henna tattoos. Recently, jagua tattoos, whose main ingredients are genipin and geniposide, have emerged as an interesting option. This study was conducted to identify the presence and concentration of henna and jagua active ingredients (lawsone; genipin and geniposide, respectively) in commercial tattoo samples. Since natural pigments are often mixed with additives such as *p-phenylenediamine* (PPD) in the case of henna, PPD has been included in the study. Green and simple extraction methods based on vortex or ultrasound-assisted techniques have been tested. To determine the compounds of interest liquid chromatography (LC) with diode-array detection (DAD) has been applied; and PPD absence was confirmed by LC-QTOF (quadrupole-time of flight tandem mass spectrometry). This work demonstrated that only one out of 14 henna samples analyzed contained lawsone. For jaguas, genipin was found in all samples, while geniposide only in two. Therefore, quality control analysis on these semi-permanent tattoos is considered necessary to detect these ingredients in commercial mixtures, as well as to uncover possible fraud in products sold as natural henna.

Keywords: genipin; geniposide; henna; jagua; lawsone; temporary tattoos

1. Introduction

Tattoos have been used since ancient times, but in recent decades they have become more popular among people of different ages, backgrounds, and cultures. There are two types of "tattoos," permanent tattoos and temporary tattoos. Temporary tattoos are becoming a more common alternative to permanent ones, because of the risks associated with the inks used in permanent tattoos. Temporary tattoos like henna-based tattoos, use natural pigments such as lawsone, and recently, jagua-based tattoos, in which the main ingredients are genipin and geniposide, have emerged as an alternative in the market. They are usually sold on the Internet. From a regulatory point of view, while all types of tattoos can be included in the group of new format cosmetics or *borderline* products in the EU Regulation [1], temporary tattoos are a product with a diverse chemical composition and unclear legislation. They should be correctly labelled according to both the Cosmetics Regulation [2] and the Toys Directive [3] and they should also follow the guidelines of the Manual of Borderline Products. However, temporary tattoos do not meet these requirements. A recent study [4] showed that the labelling on stickers tattoos was either non-existent or had incorrect ingredients listed. Some natural pigments in temporary tattoos are mainly discussed throughout this article. The most well-known tattoos are henna- based tattoos, and in particular, the so-called black henna tattoos. This is where natural henna has been adulterated with p-phenylenediamine (PPD) in high concentrations [5,6]. Regulations on natural based pigments for temporary tattoos have not been yet issued and most commercial products of plant origin are

not labelled. While henna and its main active ingredient lawsone have been evaluated for safety in hair dye products [7], no such evaluation has been done yet for other similar products containing henna extracts such as temporary tattoos. Jagua-based tattoos have not even been considered from the regulatory point of view, even though there has already been evidence of its allergenic potential. The safety of these products and their potential to skin reactions is highly questionable. This situation is due to their recent appearance on the market, lack of regulation, and limited scientific research.

Natural henna (Lawsonia inermis, from the Lythraceae family) has as its active ingredient lawsone (2-hydroxy-1,4-naphthoquinone, HNQ), which is responsible for the typical reddish-brown coloring [8]. After drying and crushing the leaves and stems of this tropical plant, a brownish-green powder is obtained. After mixing it with water or essential oils, a paste is produced that is traditionally used as a dye to decorate nails, hands, and feet. However, different henna formulations can be found in the market that are sold as temporary natural henna tattoo dyes.

Lawsone and PPD are generally determined by high-performance liquid chromatography with a diode array detector (HPLC-DAD). Almeida et al. [9] have used this technique to quantify HNQ and PPD in 11 commercial henna products (9 had HNQ and 2 PPD) and in 3 preparations used by henna tattoo artists (finding PPD in all 3 but HNQ in only one of them). A few years ago, a qualitative and quantitative determination of HNQ and PPD in black henna tattoo samples was also proposed by HPLC-DAD [10]. The study was focused on products marketed in Turkey, where these tattoos are part of the popular culture. Lawsone was found in 21 of the 25 samples considered, while PPD was detected in all of them. In both papers, sample preparation was based on single dilution followed by sonication and final filtration before the analysis. Other techniques for analyzing PPD in henna powders or mixtures are discussed in a recent review [11]. However, there are no further papers determining lawsone in henna tattoos available globally through the Net; one of the main objectives of the present study.

The current fashion for jagua tattoos is becoming more popular although they have been used in the past by certain populations. In jagua-based tattoos, the natural pigment is obtained from an Amazonian tropical fruit known as *Genipa americana* L., from the *Rubiaceae* family. The dye comes from the sap of an unripe fruit and it turns dark blue or blackish when it is applied to the body. Because of its coloring, it could be substitute for p-phenylenediamine in black henna tattoos to darken it. Jagua main ingredients are geniposide and its bioactive compound genipin. Genipin can be obtained by hydrolysis of the geniposide which is also present in other types of plants such as Gardenia jasminoides. Geniposide is a glycosylated iridoid and genipin is a colorless substance. However, when placed in contact with the skin, genipin reacts immediately to the skin's proteins to produce the pigment's color. Because the epidermis is constantly regenerating, it disappears from the skin in a few days. Certain studies suggest that jagua may become a potential new allergen in temporary tattoos [12,13]. A recent case of allergic contact dermatitis caused by temporary tattoos named Earth Jagua® and bought on the Internet has been described [14]. The list of ingredients for this brand of temporary tattoo which caused the allergic reaction mentioned above was incomplete. However, after extraction and LC-UV analysis, the presence of genipin and geniposide was confirmed, ruling out the possibility of a PPD allergy. Genipin was identified as the compound causing the allergic reaction. This is probably due to its high affinity for proteins, making it a possible contact allergen candidate. Although some methods have been reported for the quantification of geniposide in pharmacokinetic works [15–17], no studies have been conducted on analytical determinations in cosmetic samples. All those methods used HPLC. Nathia-Neves et al. [18] determined both genipin and geniposide directly in the unripe fruits of *Genipa* in their research. While other surveys have been carried out [19–22], there are no studies about the determinations of these two ingredients in temporary jagua tattoos samples. Hence, up to now there are a very few studies published about both compounds found in jagua-based tattoo samples, and most of them deal with allergy cases. Hence, analytical methodologies for the detection and determination of genipin and geniposide in products intended for use as tattoos are required.

Thus, this study was mainly conducted to verify the natural origin of 19 commercial tattoo samples based on henna and jagua, using their active pigments as quality markers. Following the desired trend toward simplifying and standardizing the analytical methodology, simple sample preparation procedures are applied. It is also interesting to develop selective and reliable methods for the quantitation of these compounds. To meet these objectives, liquid chromatography with diode-array detection was used for the simultaneous quantification of all the markers involved (lawsone in henna; genipin and geniposide in jagua). In addition, LC with quadrupole time-of-flight (QTOF) mass spectrometry detection was applied to check for the presence or absence of the recognized allergen PPD. These approaches are useful in two ways: first, for the quality control of these semi-permanent tattoos, and second, to detect potential fraud in beauty products containing these ingredients in their formulations and sold as natural when they are not. As a result, a quantitative analysis of the bioactive compounds in jagua natural tattoos has been proposed in this study, as currently the analytical methodology published is scarce or non-existent. To the best of our knowledge, this is the first methodological approach that can help to determine fraud jointly in both henna and jagua temporary tattoos.

2. Materials and Methods

2.1. Chemicals and Reagents

All solvents and reagents were of analytical grade. MS-grade methanol and acetonitrile were provided by Sigma-Aldrich Chemie GmbH (Steinheim, Germany), ultrapure water MS-grade by Scharlab (Barcelona, Spain), and formic acid was obtained from Merck (Darmstadt, Germany). The analyzed compounds, their chemical names, structures, Chemical Abstract Services (CAS) numbers, and purity of the standards are shown in Table 1. Genipin, geniposide, and PPD are white powders while lawsone is a greenish powder. Genipin was purchased from Biosynth Carbosynth (Berkshire, United Kingdom), geniposide from Sigma Aldrich (Steinheim, Germany), lawsone was supplied by Alfa Aesar (Karlsruhe, Germany), and PPD by Tokyo Chemical Industry (TCI) Europe (Zwijndrecht, Belgium). Individual stock solutions of each compound were prepared in methanol as well as further dilutions and mixtures. All stock solutions were stored in glass vials protected from light and kept in a freezer at −20 °C.

Table 1. Target compounds: chemical names and structures, purity, and Chemical Abstract Services (CAS).

Compound	Chemical Name	Chemical Structure	Purity (%)	CAS No.
Genipin	methyl (1*R*,4*aS*,7*aS*)-1-hydroxy-7-(hydroxymethyl)-1,4*a*,5,7*a*-tetrahydrocyclopenta[c]pyran-4-carboxylate		≥98%	6902-77-8
Geniposide	methyl (1*S*,4*aS*,7*aS*)-7-(hydroxymethyl)-1-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-1,4*a*,5,7*a*-tetrahydrocyclopenta[c]pyran-4-carboxylate		98%	24512-63-8
Lawsone	2-Hydroxy-1,4-naphthoquinone		>98%	83-72-7
PPD	p-Phenylenediamine; benzene-1,4-diamine		>98%	106-50-3

2.2. Tattoo Samples

Tattoo samples (12 henna tattoos and 4 jagua tattoos) were obtained via the Internet from a well-known site available to everyone. These hennas samples were provided by two different sellers. Two additional henna samples were collected from a local source in Morocco, labelled as from Pakistan origin. There were a total of 14 hennas and as many jagua samples as we could make available at the time of acquisition. Additionally, other plant origin sample (labelled as herbaceous plant tattoo), whose composition and origin species are unknown, was also purchased online. All these samples are described in detail in Table 2. Until their analysis, samples were kept in their original containers at room temperature and protected from light.

Table 2. Characteristics of the samples. HNT: henna natural tattoo; JNT: Jagua natural tattoo; HPT: herbaceous plant tattoo.

Sample Code	Color	Sample Type	Labelled Ingredients	Other Comments on the Label
HNT-1	Black			
HNT-2	Red			
HNT-3	White			
HNT-4	Orange			100% VEG, India origin, does not contain PPD, clinically tested
HNT-5	Pink	Paste	None	
HNT-6	Reddish			
HNT-7	Green			
HNT-8	Blue			
HNT-9	Violet			
HNT-10	Black			
HNT-11	Brown	Paste	None	100% VEG, India origin
HNT-12	Red			
HNT-13	Black	Paste	None	Pakistan origin
HNT-14	Red	Paste	None	Pakistan origin
JNT-1	Black	Paste	*Genipa americana* fruit juice, xanthan gum, citric acid, potassium sorbate, *Lavandula angustifolia* flower oil, limonene, linalool	Non tested on animals, non-toxic, PPD free, latex free
JNT-2	Black	Paste	Water, alcohol denat, *Genipa americana,* xanthan gum, citric acid, potassium sorbate	100% Natural, for external use only
JNT-3	Black	Paste	*Genipa americana* fruit extract, xanthan gum, citric acid, *Lavandula angustifolia* herb oil, potassium sorbate	Dermatologically tested, vegan
JNT-4	Black	Solid	Genipa Americana, sugar, xanthan gum, citric acid	Safe and non-toxic, not for use by children 12 years and under, adult supervision advised, non-permanent, 100% natural
HPT	Black	Liquid-paste	None	Herbaceous plant tattoo fluid. vegetable dye, skin retention time varies between individuals because skin metabolism is slightly different

2.3. Sample Preparation

For each sample, 0.02–0.03 g of raw material and 7.5 g of methanol were exactly weighted into a 10 mL glass vial. All solutions were colorful. The complete solubility of the samples was assured by shaking them in an ultrasonic bath Raypa® model UCI 150 (Barcelona, Spain) at 35 kHz of ultrasound frequency for 5 min. The dilution factor was modified according to the concentration of the analytes in the samples. Samples were injected directly. The sample solutions were stored in glass vials at −20 °C. Prior to the chromatographic analysis, solutions were filtered through 0.22 µm polytetrafluoroethylene (PTFE) syringe filter. Figure 1 below illustrates the process described.
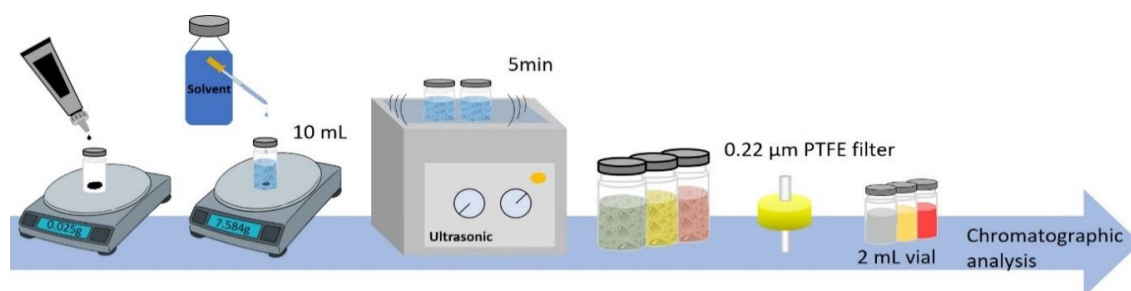
Figure 1. Sample preparation procedure.

2.4. Liquid Chromatography (LC) with Diode-Array Detector (DAD)

High-performance liquid chromatography (HPLC-DAD) was performed in a Jasco LC Net II, equipped with the PU-4180 quaternary pump, the AS-4150 auto sampler and the MD-4010 diode detector. The system was controlled with the JASCO ChromNAV Version 2.01.00 (JASCO International Co., Ltd., Tokyo, Japan). The separations were carried out using a Kinetex chromatographic column 5 µm C18-100Å (150 mm × 4.6 mm, 2.6 µm) supplied by Phenomenex, (Torrance, CA, USA). The mobile phase consists of water (A) and methanol (B) both acidified with 1% formic acid, with the following gradient program: 0 min, 70% A; 10 min, 70% A; 15 min, 0% A; 18 min, 70% A and 23 min, 70% A. The temperature and flow rate that allowed the best chromatographic performance were 30 °C and 1.0 mL/min, respectively, resulting a total run time of 23 min, including column clean-up and re-equilibration. Re-equilibration time is necessary in gradient HPLC to ensure that the column environment has returned to the initial stable conditions. Five microliter of the sample was injected in duplicate. The UV-Vis absorption spectra of standards and samples were acquired in the range of 200 to 600 nm to determine the absorption maxima of the three target compounds: lawsone, genipin, and geniposide. The listed compounds were identified in the real samples by comparison of their retention times and UV-Vis spectra to those of pure standards. Quantification was performed by external standard calibration. In the particular case of the PPD, several analyses were carried out using the column mentioned above. In addition, a Kinetex chromatographic column HILIC-100Å (150 mm × 2.1 mm, 2.6 µm) supplied by Phenomenex, (Torrance, CA, USA) was also used.

2.5. Ultra High Performance Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-QOF-MS)

Rapid analysis of PPD was carried out in an Elute UHPLC 1300 coupled to a quadrupole time-of-flight mass spectrometry (QTOF) Compact Instrument (Bruker Daltonics, Bremen, Germany). Separation was carried out on an Intensity Solo HPLC column C18 (100 mm × 2.1 mm, 2.0 µm; Bruker Daltonics, Bremen, Germany) which was kept at a constant temperature of 40 °C. The mobile phase consists of 0.1% formic acid in both water (A) and methanol (B) and the flow rate was 0.25 mL/min. The gradient method was in 95% A for 0.4 min, then was from 5% B to 35% for 0.1 min, and to 100% B for 7 min, and then held for 5 min, thus returning to the initial conditions until reaching the 15 min of total running time. Two µL of the sample were injected in triplicate.

The mass spectrometer was operated in the electrospray ionization (ESI) in positive mode, detecting mainly pseudo molecular ions $[M + H]^+$. The MS method used was a broadband collision-induced dissociation (bbCID) approach, which allows the exhaustive recording of all detectable precursor and products ions, independently of precursor intensity. The voltage ramp applied was from 10 to 105 eV, with spectra rate of 8 Hz and mass filtering from 20 to 1000 m/z, with a total cycle time range equal to 1 s. All acquisitions were obtained using the Compass HyStar software and quantification was performed using the TASQ Version 2.1 (Build 201.2.4019) software.

3. Results and Discussion

3.1. Solubility Studies

The solubility of the studied compounds was evaluated in both water and methanol. Slusarewicz et al. [23] have already explored the aqueous stability of genipin. In their work, they concluded that genipin is decomposed in aqueous solution, drastically influencing the pH of that solution in the degradation. In addition, the poor solubility of lawsone in water (1 mg·mL^{-1} [24]) could be a problem. Based on these evidences and the fact that the compounds are properly soluble in methanol, it was decided to discard the use of water in the preparation of individual standards, mixtures or samples, with the aim of using a common solvent for all markers.

3.2. Chromatographic Analysis

The chromatographic conditions were optimized to achieve an efficient separation of the three target compounds used in natural pigments-based tattoos.

Retention times (RT) were 3.50, 5.35, and 11.04 min, for geniposide, genipin, and lawsone, respectively. The elution order of geniposide and genipin was the same observed by other authors [19–22]. The HPLC-DAD method was validated in terms of linearity and precision, limits of detection (LODs), and limits of quantification (LOQs). The results are summarized in Table 3. Calibration curves were obtained employing standard solutions prepared in methanol covering a concentration range from 1 to 100 µg·mL^{-1} (geniposide, genipin and lawsone), with six concentration levels and three replicates per level. For the quantitative analysis absorption, 250 nm was selected. Figure 2 shows a section of the chromatogram obtained for an intermediate calibration level, as well as the UV-spectra of the three compounds. It should be noted that, although the first two spectra are identical because the differential moiety of the geniposide does not absorb in UV, both compounds are clearly identified by their different chromatographic retention as shown below. The method showed a good linearity, with coefficients of determination (R^2) higher than 0.9990. The instrumental precision was evaluated within a day (n = 3) for all concentration levels showing mean relative standard deviation (RSD) values about 2%. The LODs and LOQs were calculated as the compound concentration giving a signal-to-noise ratio of three (S/N = 3) and ten (S/N = 10), respectively.

Table 3. High-performance liquid chromatography with a diode array detector (HPLC-DAD) method performance. Linearity, precision, limits of detection (LODs) and limits of quantification (LOQs).

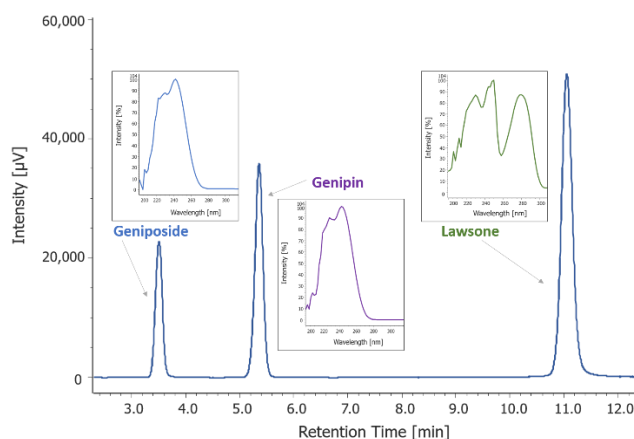| Compound	Linearity	Precision RSD (%)	LODs (µg·mL^{-1})	LOQs (µg·mL^{-1})
	R^2	Intra-Day [a]		
Genipin	0.9994	0.9	0.1	0.3
Geniposide	0.9991	1.4	0.1	0.4
Lawsone	0.9990	1.3	0.2	0.6

[a] 10 µg·mL^{-1}.

Figure 2. Chromatogram (λ = 250 nm) and UV-spectra comparison of a mixture of the standards obtained by HPLC–DAD.

The same chromatographic conditions were applied in the case of PPD. However, because of its high polarity, it was not detected. Alternatively, a HILIC (hydrophilic interaction liquid chromatography) column was chosen owing to its different selectivity and efficiency in the separation of polar compounds. Several tests were carried out with different elution gradients (now mixing water and acetonitrile as mobile phase). However, no results were achieved, hence, it was decided to perform the PPD analysis using UHPLC-ESI-QTOF-MS. Direct infusion of the PPD pattern was performed to search for the mass transitions that were subsequently selected. Figure 3 shows the mass spectrum of PPD. The peak of the $[M^+ + 1]$ ion (m/z = 109.08, $C_6H_9N_2$) was accurately determined. The theoretical exact masses of the protonated compound $[M+H]^+$ were calculated in the TASQ software based on the molecular formula resulting in an accurate mass. The retention time for the PPD was 2.23 min.
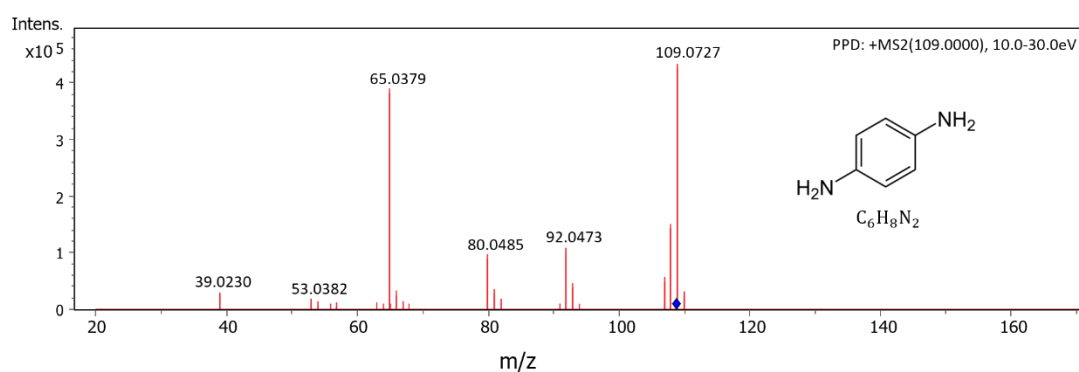

Figure 3. Mass spectrum of PPD.

3.3. Application to Real Samples

The previously described methodology was applied to the analysis of 19 natural tattoo samples, which present a wide variety of tonalities in the case of henna products. Analyses of the samples showed the presence of some of the target analytes. Figure 4 shows overlaid chromatograms of some selected samples and standards. Lawsone was only found in one (HNT-11) out of the 14 henna samples (Figure 4a). The analysis by HPLC-DAD showed an amount of 8736.47 μg·g^{-1} lawsone. For the other colored hennas, their active ingredient was not detected in any of the cases (Figure 4b). The absences of lawsone in right henna samples have already been reported [9,10]. However, the ratio of samples with HNQ was around 70–80% while here it scarcely reaches 10% of the total. It is also important to highlight that samples reported were black, brown, or red, but in this study less typical color samples were considered like pink, green, or blue, among others, which led to the perception that these colored pigments are not based on natural henna.
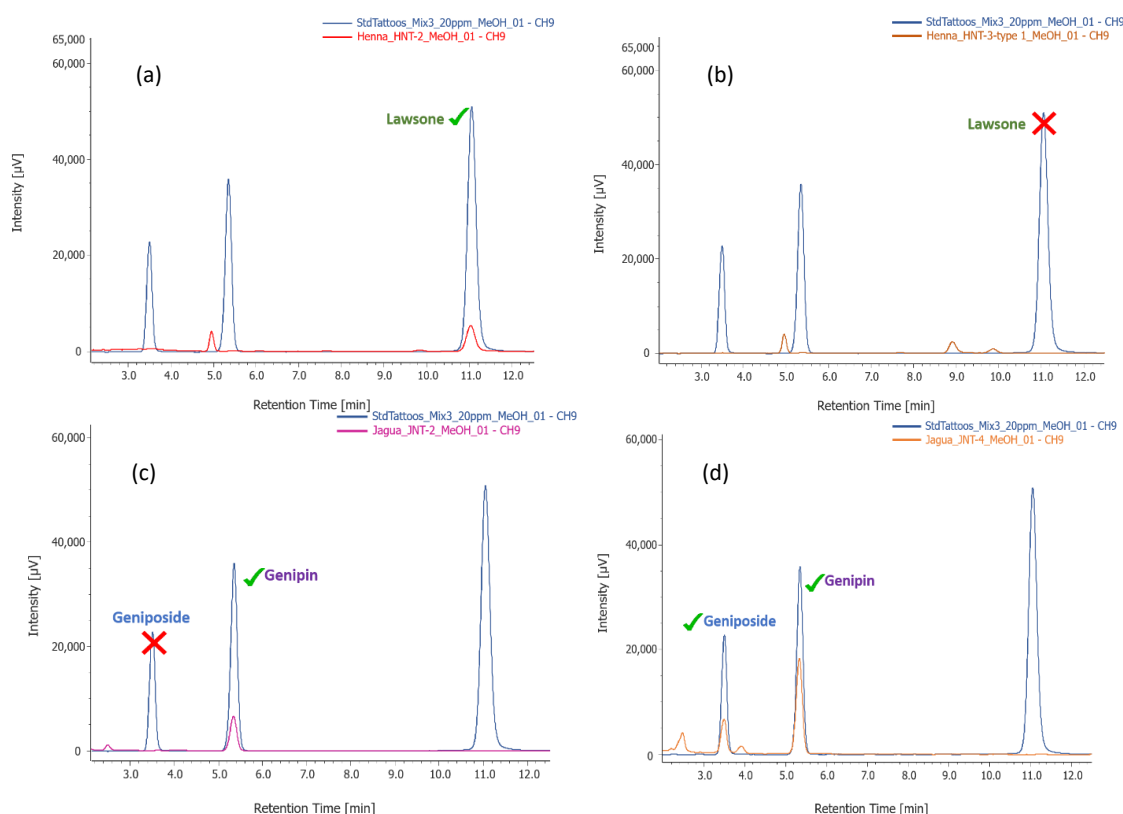
Figure 4. HPLC-DAD profiles of henna and jagua sample. (**a**) Henna right sample. (**b**) Henna fake sample. (**c**) Jagua sample only with genipin. (**d**) Jagua sample with the two targeted compounds.

The situation was quite different for jagua samples. The average results for jagua tattoos are summarized in Table 4. Genipin was found in the all samples, its concentration in the first three samples (JNT-1 to JNT-3) being different from that of the fourth sample, where the genipin concentration is much higher. Geniposide was identified in two jagua samples (Figure 4c,d). As for genipin, the highest concentration was found in the JNT-4 sample in both cases, being very low in the other tattoo preparations. The reason for the higher amounts of both compounds in the JNT-4 sample may be related to its physical state, since it is the only solid sample. In fact, this commercial product was not dissolved in solvents or essential oils, so it could be considered as pure jagua. Additionally, this sample label claims to be 100% natural. If the two compounds are compared, concentrations of the main active ingredient genipin are higher than those of geniposide.

Table 4. Analysis of target compounds in jagua samples.

	HPLC-DAD Analysis			
	Genipin		Geniposide	
Sample Code	($\mu g \cdot mL^{-1}$)	($\mu g \cdot g^{-1}$)	($\mu g \cdot mL^{-1}$)	($\mu g \cdot g^{-1}$)
JNT-1	4.44	2051.10	0.40	184.92
JNT-2	8.43	3060.32	–	–
JNT-3	0.32	133.61	–	–
JNT-4	25.86	11,423.48	13.09	5783.10
HPT	–	–	–	–

Up to now, few works about both analytes have been reported in natural tattoo samples, and only one of them mentions the genipin detection. In any case, no previous analytical approaches have been reported for the simultaneous quantification of these target compounds. Lastly, with regard to the sample considered as an herbaceous plant tattoo (HPT), neither genipin nor geniposide was detected. This implies that its origin is not *Lawsonia inermis* or *Genipa americana* L.

In summary, the proposed analytical method is suitable for identification and determination of the natural coloring agents considered. In addition, although the main objective of this work was the detection and determination of active compounds in henna and jagua semi-permanent tattoos, since PPD is a very popular additive in hennas and some of the jagua samples were labelled as PPD-free, all samples were analyzed by LC-QTOF. PPD was not found in any of the 19 samples studied, indicating that the considered samples are been adulterated with PPD; however, in the case of most hennas, they did not contain the active ingredient either.

4. Conclusions and Future Trends

In this work a method based on HPLC-DAD has been proposed to simultaneously evaluate the presence of the active ingredients in plant pigments-based tattoos formulations. It is worthy to mention that the proposed sample preparation procedure is quite simple, rapid, and easy to handle. The method performance study showed that HPLC-DAD was appropriate, allowing a rapid recognition of a sample as natural or fake, depending on the presence or lack of its expected active ingredients. Only one out of 14 henna samples analyzed contained lawsone. For jaguas, genipin was found in all samples, while geniposide only in two. Then, the determination of the marker compounds was performed by a simple chromatographic method that can be easily applied in laboratory. However, if the presence of PPD is suspected, it is necessary to reconfirm it using more sophisticated equipment. In this work, LC-QTOF was applied for the rapid testing of PPD adulteration in the samples.

The growing use of temporary tattoos because of the global availability on the Internet and the current regulatory situation, can cause an increase in allergic reactions in the near future. Another issue relates to the lack of information about concentrations added to the incorrect or non-existent labelling. In parallel to the necessity of improvement in these issues, the development of analytical methods to control pigments, impurities, as well as potential undesirable additives, is highly important. It is probable that commercial products may contain prohibited or restricted compounds in cosmetics, as well as dyes to obtain such attractive colors. In addition, in further experiments, untargeted approaches should be included to better characterize the composition of these beauty products.

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