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Influence of bleaching, perming and coloring on cannabis content in hair.

By:

Nicolas Van Elsué

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Promotor(en): Dr. Michel Yegles

Copromotor: Pr. Dr. Hugo Neels

Coach: Dr. Michel Yegles

Adress of the lab

Laboratoire National de Santé

Rue Louis Rech 1, Dudelange, Luxembourg

Content

1. Summary (Dutch)

Haaranalyse kent de laatste jaren een enorme opmars voor het gemakkelijk en praktisch gebruik voor het bepalen van drugs in haar. Echter, de concentratie van drugs neemt af na cosmetische behandeling (bleken, een permanent laten zetten en kleuren) van haar. Tot nog toe bevestigde slechts 1 studie een afname in THC na bleken en kleuren van haar.
Een nieuw experiment werd uitgedacht om het effect van haarbehandelingen te testen op cannabinoïden in haar. Haarstalen van THC-positieve patiënten werden gesplitst in 2 haarlokken. De ene diende ter controle en de andere werd behandeld met de overeenkomstige haarbehandeling. Het doel van de studie was om concentraties van cannabinoïden (CBD, THC, CBN en THC-COOH) te vergelijken tussen behandelde en controlestalen en na te gaan wat het effect is van de behandeling alsook de invloed op de ratio van de cannabinoïden.
Haarstalen werden geïncubeerd met NaOH en nadien werd de IS toegevoegd. Stalen werden vervolgens gecentrifugeerd en Solid Phase Extractie werd uitgevoerd. De stalen werden gederivatiseerd met MSTFA alvorens ze te analyseren in de GC-MS² (met een EI-bron). Specifieke m/z-waarden werden geselecteerd, gebruikmakend van MRM. De procedure voor THC-COOH analyse werd aangepast voor een betere detectie. Een hogere concentratie NaOH werd na korte incubatie geneutraliseerd met azijnzuur en acetaat buffer werd gebruikt om de pH stabiel te houden. Ook werd een dubbele derivatisatie met PFP en PFPOH gebruikt alvorens de stalen te analyseren in de GC-MS², waar de EI-bron vervangen werd door een NCI-bron en een PTV-injectie voor verhoogde sensitiviteit.

Het bleken van haar had een duidelijke impact op de afname van cannabinoïd concentraties (THC, CBN en CBD waren meer getroffen dan THC-COOH). Incubatie met waterstof peroxide toonde een hoge reactiviteit aan met cannabinoïden. Een permanent zetten deed ook cannabinoïd concentraties dalen, maar het was vooral CBN welke minder beïnvloed werd in vergelijking tot bleken van haar. Tijdelijk kleuren van haar deed ook alle concentraties dalen, terwijl permanent kleuren van haar weinig tot geen effect had op cannabinoïden in haar.

Men kan dus concluderen dat cosmetische haarbehandelingen een effect hebben op cannabinoïd concentraties in haar. Producten welke hoge concentraties bevatten van reactieve producten zoals waterstof peroxide, hebben duidelijk een grotere impact op cannabinoïden. Speciale aandacht is vereist van bijkomstige effecten van uitspoeling tijdens de bepaalde haarbehandelingen welke drug concentraties kan doen dalen.

2. Abstract

The determination of drug of abuse in hair has been increasingly reported and is adapted frequently as routine analysis in the toxicological field. Cosmetic hair treatments could affect drug incorporation in hair. However, so far only 1 study found a decrease in THC content after colouring and bleaching.
A new experiment was set up to determine the effect of cosmetic hair treatments on cannabinoids in hair. Hair locks from THC-positive subjects were divided in 2 separate locks. Different applications were tested on one of the hair locks, such as bleaching, perming, temporary and permanent coloring. The aim of this paper was to compare the concentrations of cannabinoids of interest (CBD, THC, CBN and THC-COOH) in the treated samples and control samples, to determine any effect related to the treatment and to investigate the effect of the cosmetic hair treatment on cannabinoid ratios.
Hair was incubated with NaOH and spiked afterwards with IS. Samples were centrifuged and a solid phase extractions was performed after derivatisation with MSTFA, analysis was done by GC-MS^2 (with an EI-source) and specific m/z values were selected using MRM. For the detection of THC-COOH, the protocol was slightly altered: a higher concentration of NaOH was used for a shorter incubation period which was neutralized with acetic acid, a fresh made acetate buffer was used to maintain a stable pH. After a different solid phase extraction a double derivatization method with PFP and PFPOH was adopted for better detection in the GC-MS^2. The source was changed to a NCI-source and a PTV injection was done to increase sensitivity.

Bleaching had a clear impact on the decrease on all cannabinoids (THC, CBN and CBD were more affected then THC-COOH). Hydrogen peroxide showed to be highly reactive to cannabinoids. Perming also decreased all cannabinoids, but CBN concentrations were less altered in comparison to bleaching. Temporary coloring decreased cannabis content in hair while permanent coloring did not strongly affect the concentration of cannabinoids in hair.

In conclusion, hair treatments affect cannabinoid concentrations in hair. Products containing higher concentrations of reactive products such as hydrogen peroxide have clearly a stronger impact on cannabinoids. One must also be aware of additional leaching-out effects during the application of certain products.

3. Abbreviations

- 11-OH THC - 11-hydroxy-D9-tetrahydrocannabinol
- CBD - Cannabidiol
- CBG - Cannabigerol
- CBN - Cannabinol
- CYP2C9/450 - Cytochrome P450 Oxidase
- D^3-mix - Deuterated drug mixture
- EI - Electron Impact
- EtG - Ethyl Glucuronide
- GC-MS^2 - Gas Chromatography – Mass Spectrometer – Mass Spectrometer
- H-mix - Hydrogen (proton + neutron) drug mixture
- IS - Internal Standard
- LLOQ - Lowest Limit Of Quantification
- LOD - Lowest Limit of Detection
- MeOH - Methanol/Methyl Hydroxide
- MSTFA - N-methyl-N-(trimethylsilyl) Trifluoroacetamide
- NaOH - Natrium Hydroxide
- NCI - Negative Chemical Ionization
- PFP - Pentafluoropropionyl
- PFPOH - Pentafluoropropanol
- THC - D-9-tetrahydrocannabinol
- THC-A - Tetrahydrocannabinolic acid
- THC-COOH - 11-nor-D9-tetrahydrocannabinol-9-carboxylic acid

4. Introduction

A project called “Driving Under the Influence of Drugs, Alcohol and Medicines (DRUID)” that was co-funded by the European Commission in the early 2010s stated that 4 - 14% of drivers (depending on the country) involved in road accidents tested positive for cannabis [1]. The fraction of drivers under the influence of cannabis, who were not involved in car accidents, remains poorly estimated [2]. Cannabis is known to have an impairment on driving performance (estimating distance, staying in lane, etc.), cognitive functions and psychomotor abilities, but it does also strongly affects the brain [2, 3]. Concentrations of a few ng/ml found in blood are correlated with elevated crash risk [4]. The plant from which cannabis is derive - *Cannabis Sativa L.* - contains the main psychoactive compound D-9-tetrahydrocannabinol (THC) as well as other non-psychoactive congeners including the biogenetic precursor Cannabidiol (CBD), the oxidized form Cannabinol (CBN) and Cannabigerol (CBG) [3, 5-7].

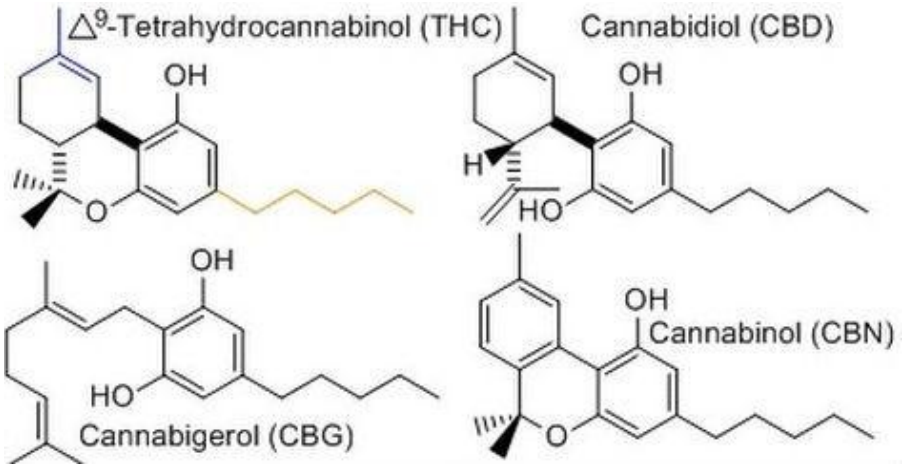


Figure 1. Chemical structures of THC, CBD, CBG and CBN.

D-9-tetrahydrocannabinolic acid A (THC-A), an alternative form of THC is also present in the plant [2, 7]. THC is converted by hepatic enzymes (CYP450) to 11-hydroxy-D9-tetrahydrocannabinol (11-OH-THC) which will be further metabolized to a non-active form called 11-nor-D9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) as shown in figure 2 [5, 8, 9]. Due to fast metabolization, concentrations of 11-OH-THC in the body are extremely low, thus reducing its importance as a useful biomarker.

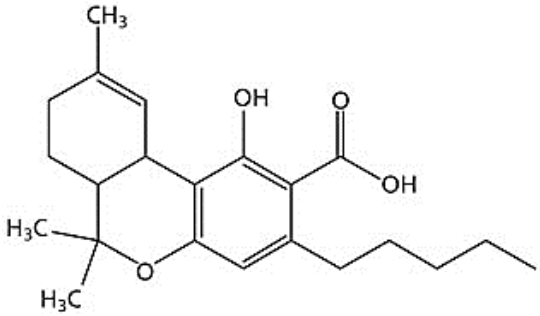


Figure 2. Chemical structure of THC-COOH.

Metabolites are being glucuronidated to excrete them via the urine. THC has a distribution volume of 9 - 11 L/kg, meaning that after binding to lipoproteins it will be distributed rapidly to fat and muscle tissue resulting in lower plasma concentrations. The bioavailability of THC ranges from 10 - 27 % approximately, the half-life expands from 19 to 96 hours and has a terminal half-life of 3 - 13 days [2, 4, 9-11].

Blood and urine are good matrices for measuring acute usage of cannabis. However they do not suffice when tests are being carried out after a period of abstinence to determine true renouncement of the drug. Drug testing in hair has gained popularity due to its easy accessibility and advantages over blood and urine. However, confirmation with urine samples may often be required as concentrations measured in urine are higher compared to concentrations in hair [6, 8, 9, 12, 13].

The core of the hair is called the medulla which contains mainly lipids and proteins [1]. The medulla is small in size and is not always present. Studies show that the medulla has no significant importance for the hair [14-18]. The medulla is covered by a thick layer, the cortex. It contributes the most to the hair size and its properties. These cells are aligned longitudinally and contain intermediate filaments, keratin-associated proteins and in between the cells, melanosomes can also be found [1, 18, 19]. The outer layer is called the cuticle and consists mainly of protein. The cuticle can be subdivided into the epi-, exo- and endocuticle which can be characterized depending on their cysteine content [18]. Matrix cells in the hair follicle multiply and differentiate to form the different hair cells. Cannabinoids are delivered to the matrix cells at the basement membrane of the hair follicle by blood vessels that surround the hair shaft (active or passive diffusion) or by sebum and sweat that is secreted (in low concentration) as shown in figure 3 [6, 7, 9, 11, 20].

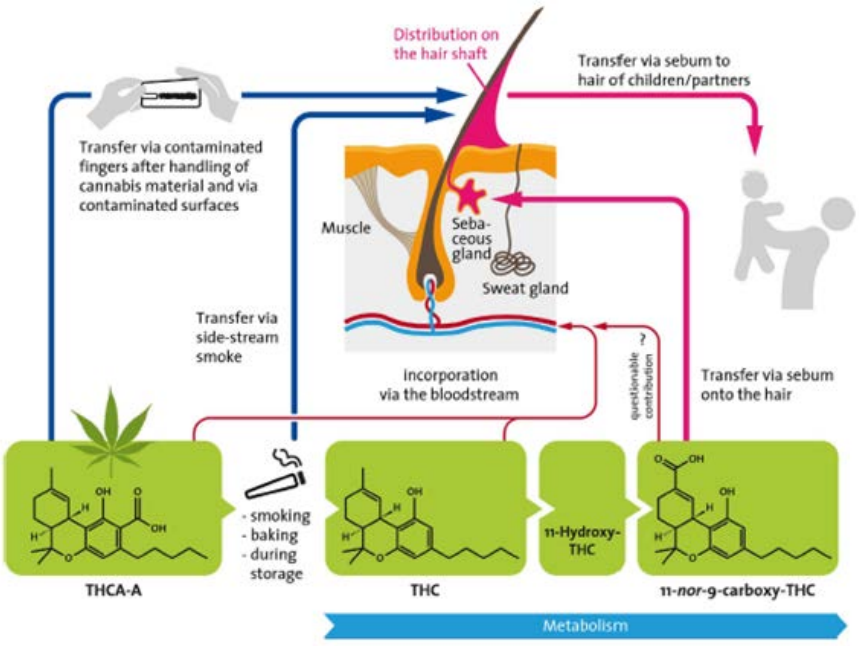


Figure 3. Overview of distribution pathways of cannabinoids and metabolites into hair. This can occur by direct contact or smoke with the hair, by the blood so substances get incorporated into the hair and by sweat or sebum that comes in direct contact with the hair.

Different research groups have shown that cannabinoids have a very low incorporation rate in hair. However, the hypothesis on why this is the case, differs between scientists. On one hand, acidic cannabinoids like THC-COOH have a much lower incorporation in hair as the hair matrix is more acidic compared to blood (pH = 7.4). In contrast, the larger Area Under the Curve (AUC) of THC-COOH in blood suggests an increase of the likelihood of incorporation [7, 8, 10]. Also the presence of melanin is thought to play a role in the incorporation of cannabinoids in hair. Melanin causes pigmentation of hair and does not favor the embodiment of THC and its metabolites in hair [5, 6, 9-11, 21, 22]. Other genetic parameters like sex, age and general health condition could alter drug deposition in the inner space of hair [11].
Finally hair samples for analysis can be contaminated by external THC that originates from the smoke produced when a joint is lit [7, 9, 11, 12, 20, 23, 24]. Many researchers advice to wash the hair samples thoroughly to remove any external trace of THC. According to others, this difficult procedures can be circumvented if metabolites (especially THC-COOH) are being studied. Bioconversion with CYP-enzymes is needed in order to find these metabolites, so external contamination (i.e. smoke, direct contact, etc.) can be excluded [9, 12, 25].

Additionally, the incorporation of cannabinoids in hair can also be influenced by external factors. This opposes problems when results need to be interpreted. Cannabinoids are photo-chemically not stable, so concentrations found in hair can decrease when exposed to sunlight and UV-light or when the hair is physically damaged [10, 20, 24]. Different cosmetic treatments including the use of shampoo, thermal straightening of hair, permanent waving, coloring and bleaching could have an influence on cannabinoids in hair [10, 11, 21]. For different drugs, research was already performed: Shampooing does not alter drug concentrations [26, 27], but special solutions like Ultra Clean® have been proven to affect drugs like cannabis and cocaine [28]. Pötsch and Skopp showed that permanent waving decreases opiates in hair and bleaching has been proven to also affect various drugs such as opiates and cocaine [27, 29-32].
A study from Jurado et al. (1997) observed an effect of dyeing and bleaching on drug concentrations in hair [29]. Few studies after the initial work of Jurado et al. exist that investigated the effect of bleaching and perming. In particular, a study on ethyl glucuronide (EtG), a metabolite of alcohol, was carried out by the same research group where this master dissertation was written [33-35]. They concluded that EtG was decreased after hair treatment, indicating that a similar effect might take place on cannabinoids in hair.

As briefly mentioned, there are different cosmetic hair treatments that are frequently adapted. Each of them has an impact on hair structure and appearance.
Firstly, bleaching is causing a lot of stress on hair due to oxidative damaging, degradation of melanin granules (splitting disulphide bonds) and decoloration of the solubilized pigment [36, 37]. Damage on the hair caused by bleaching alters proteins in the hair matrix, especially on cysteine residues.
Secondly, perming is a process that consists of a physical and a chemical component. The physical changes to induce the specific shape are achieved by rolling hair into curls. Afterwards, hair is treated with various chemicals, including Ammonium thioglycolate and hydrogen peroxide [18, 19]. Ammonium thioglycolate has 2 main effects on the hair: the Ammonium (alkaline reductive agent) opens the cuticle by lifting up the scales so thioglycolate (reducing agent) can diffuse into the cortex where it breaks disulphide bonds to separate sulfhydryl groups, which are mostly present in the cortex layer [18, 19, 38]. The hydrogen peroxide (oxidizing agent) is applied afterwards to

allow reformation of the disulphide bonds so the hair gains its typical curl, but does simultaneously increase surface damage and decrease hair stiffness [39]. One study found that protein and lipid content changed after perming [40]. Certain drugs with a more hydrophilic nature are thought to be lost for this reason after such hair treatment.
Thirdly, dyeing or coloring of hair can be done in a permanent or a more temporary fashion. Permanent dyeing is achieved by using alkali solutions to "open" the hair, allowing hydrogen peroxide to enter and damage the pigments. Colorless dye precursors enter the hair afterwards and react with hydrogen peroxide to express the desired color which is maintained by using a conditioner to close the cuticle layer. Semi-permanent coloring also uses alkali solutions for dye molecules to enter the cuticle layer, but the hydrogen peroxide concentration is very low so less damage is caused to the hair. Shampooing the hair will allow the dyes to be washed out. Temporary dyes, the last type of coloring, can be applied easily and do not require any additional chemical treatment of hair. Since the cuticle layer isn't opened and the product is simply applied on the hair, it can be washed off easily [41].
One additional study mentioned the effect of coloring on incorporated xenobiotics in hair, but additional research would be needed to confirm this [34]. From what these few papers suggested, an effect on cannabinoid content in hair due to cosmetic hair treatments like bleaching, perming and coloring was expected. However, there is little to none research yet published to support this theory. The question on what the relation of hair treatments was on cannabis content in hair remained unanswered.

Therefore, a new experiment will be developed which will be adopted for a master dissertation and will mainly focus on the effect of bleaching, perming and coloring on cannabinoid content in hair. The cannabinoids of interest that will be studied contain CBD, THC, CBN and THC-COOH. Additionally the ratios between these cannabinoids will be evaluated to possibly retrospectively indicate which treatment has been performed.

5. Material and methods

Hair samples were stored in aluminum foil and kept at room temperature (20 °C). The serial code attached to each sample was run through a database to determine if the sample originated from a person who tested positive for THC. The hair was added to the library for further use if THC was found to be present from previous analyses and if enough hair was still available. If the corresponding documents in the database mentioned any cosmetic treatment of hair, samples were excluded. This is necessary to avoid misinterpretations on results of our own cosmetic hair treatments.
The resulting library of THC-positive, non-treated hair contained 36 subjects. Some hair samples were large enough to divide them further into separate segments. The final amount of hair segments that were fit to be analyzed resulted in 75 samples. Three cosmetic hair treatments were tested, so each hair treatment was assigned approximately 15 samples (a reserve was kept in case retesting or additional testing was required). Each hair sample was subdivided into 2 different locks: the first was subjected to the assigned hair treatment (as explained in sections 5.1 - 5.3 in more detail) while the latter served as its own control. Each of those samples was studied for their THC/CBN/CBD and THC-COOH content at least once, but preferably twice or even triple-fold to confirm results. Treated hair as well as control hair samples were decontaminated by washing in deionized water during approximately 1 minute to prevent any salts to interfere with the

measurements. Afterwards the hair was washed in acetone to remove any other type of contamination and was dried with paper.
Next, the hair had to be pulverized into much smaller pieces which was achieved by transferring it to a metal cage and cutting the hair with scissors (for about 2 minutes). In between cutting different samples, the scissor was cleaned with methanol to avoid transfer of components to other samples and confound results. The hair was placed in tubes with assigned serial number for later use and the bowls were cleaned afterwards with water, methanol and acetone sequentially.

5.1. Bleaching

Bleaching was performed as the first cosmetic hair treatment. Bleaching is an oxidative-alkaline treatment and is carried out by combining ammonium/potassium persulfate and 6 – 15 % hydrogen peroxide, but concentrations up to 30 % can also be used.
For this experiment, we used a commercial available bleaching product (Schwarzkopf, L1++ Extreme Lightener Plus, lightening for up to 5 to 7 shades). All preparation and application were performed according to the attached manual. The revealing emulsion and accelerator were both added to the decoloring cream and the whole was shaken horizontally and vertically to create a homogenous mixture. The resulting mixture was applied on the hair locks spread out equally. The hair was left for 45 minutes to achieve the maximum bleaching effect. Afterwards, the hair was washed briefly with normal tap water (as would be the case when the procedure was carried out at home by any person). Finally a "care"-cream was applied for 3 minutes prior to washing the hair with water and drying. Afterwards, hair was cut and pulverized to powder.

To study the effect of hydrogen peroxide on cannabinoids, an *in-vitro* study was carried out. 100µL of H-mix was taken and put in a solution of 500 µL hydrogen peroxide (30 %) and 500 µL H_2O. The solution with cannabinoids was evaporated prior to incubation to avoid any dilution effect. The solution was left for 45 minutes at room temperature to mimic the effect of the bleaching product on hair. Finally 2 mL of 1M NaOH and 20 µL (0.1 ng/mg) D^3-mix were added and the samples were loaded on STRATA-X33W columns. The procedures on conditioning, elution, evaporation and derivatization are explained below (see 5.4).

5.2. Perming

For our perming treatment, only the influence of the chemical products (Ammonium thioglycolate, ammonium hydroxide and hydrogen peroxide) on the hair are of importance. The mechanical application of curls isn't expected to contribute to any changes in cannabis content in hair. A commercial available perming kit was obtained (Schwarzkopf Poly Lock Normal Permanent). All preparation and application steps were carried out as described in the accompanied manual. The perming solution was firstly applied on the hair and spread out so the full hair was exposed. The hair was left for 30 minutes so the product would achieve its maximal effect. Sequentially, hair was washed and a fixation lotion was added twice (the second application was performed after 10 minutes), before being washed again and dried clean before cutting and pulverizing.

Additional to perming on hair, an *in-vitro* study with thioglycolate was also performed to determine its chemical effect on cannabinoids. 50 mg thioglycolate was incubated in 1 ml of water with 100 µL H-mix during 30 minutes at room temperature. As described for the bleaching study,

the cannabinoid solution was evaporated to avoid any effect of dilution. After, 2 mL of 1M NaOH and 20 µL D^3-mix were added to the solution and samples were loaded on STRATA-X33W columns. As already mentioned for the *in-vitro* study for bleaching, the following procedure are explained below (see 5.4).

5.3. Coloring (temporary and permanent)

Coloring products usually contain small traces of hydrogen peroxide to enhance the coloring effect and maintain it for a longer period of time. However, to solely study the effect of coloring on cannabinoids found in hair, it is important to apply a dye product that does not contain hydrogen peroxide. According to these criteria, a commercial available hair coloring product was bought (Schwarzkopf Brillance T862 color shine gel natural brown which holds up to 15 hairwashes). In contrast to bleaching and perming, coloring without hydrogen peroxide does only require a single application step. Application was carried out according to the provided manual which stated that the gel should be applied and left to rest for 20 minutes before washing and drying.

An *in-vitro* study was also carried out to determine possible effects of the product on cannabinoids. A pipet tip was dipped in the product and diluted in methanol. H- and D^3-mixtures of CBD, THC and CBN were added to 0.5 mL of the diluted product and left for 20 minutes at room temperature. A blank consisting of pure methanol was added to compare results. Two mL of 1M NaOH was added to all samples before loading them on the STRATA-X33W columns. The procedure on purification and derivatization is explained below (see 5.4).

In addition to the coloring product without hydrogen peroxide, we also included a product with hydrogen peroxide to compare both the permanent and the temporary dyeing. For this study, a commercial available coloring kit (Schwarzkopf Country Colors 40 Nevada Dark Blond lasting between 6 to 8 weeks) was bought. All preparation and application steps were carried out according to the accompanied manual. The coloring cream was added to the emulsion tube, the tube was closed off and shaken vigorously until a homogenous mixture was generated. The mixture was applied to each hair sample and massaged shortly for a uniform application. After, the hair was left for 30 minutes to achieve the maximal coloring effect. Finally, the hair was washed in warm tap water, the conditioner was applied, massaged and left for 2 minutes before washing it in water. The Hair was dried before being cut and pulverized.

Also for the permanent coloring, an *in-vitro* test was adopted to estimate the effects of the coloring product itself on cannabinoids. However, the conditioner was used for the *in-vitro* experiment itself. One drop of the coloring mixture was added to 1 mL MeOH with 20 µL H-mix and incubated for 30 minutes at room temperature. A blank (1 mL MeOH without coloring product) was added to compare results afterwards. After incubation, 20 µL D^3-mix was added and the samples were loaded on the STRATA-X33W columns. The procedures for further analysis are explained below (see 5.4)

5.4. Sample preparation and extraction for detection of CBD, THC and CBN

Approximately 5 mg of pulverized hair was taken and placed in a 5 mL Eppendorf tube. Because limited hair was provided, resources had to be used optimally. A weight of 5 mg proved to be sufficient to detect various cannabinoids. An analytical balance was used to achieve exact measurements. Two mL of 1M NaOH was added to each Eppendorf tube to allow hydrolysis and extraction of the capillary matrix. The samples were incubated at 60 °C for a period of 90 - 120 minutes.

After incubation, the tubes were taken out and spiked with internal deuterated standard (IS). Internal standards are needed to correct for lost analytes during sample preparation and will allow the calculation of the final concentrations of cannabinoids. Positive controls with each series of samples were spiked with a different volume of H-mixture (0.1 ng/µL of THC, CBN and CBD) and a deuterated mixture D^3 (containing 0.1 ng/µL of THC, CBN and CBD) was later added to all samples. Next, samples were centrifuged at 5000 rpm during 10 minutes. During centrifugation, STRATA-X33W reverse phase columns (nonpolar stationary phase) were conditioned with twice 3 ml MeOH which was pressed through the column under vacuum conditions. Next 3 ml de-ionized water was added and again a vacuum was applied to force the liquid through the stationary phase. When the columns were conditioned and the centrifuge was ready, samples could be loaded on the columns. The sample was passed through the column under normal atmospheric conditions to allow the analytes to bind with the stationary phase. When samples reached the filter, the column was closed off by turning it clock-wise to prevent complete drying of the column. When all samples were run, a rinsing procedure was applied with 3 mL 0.1M NaOH and 100 µL acetone sequentially. A vacuum was used to force both solutions through the column and the vacuum was maintained for 8 minutes so no watery substance remained in the column, which could complicate further analysis. All compounds of interest bound to the column wouldn't be washed out with this procedure.
After, the column was placed on a 21G needle which was positioned on top of a vial. Two mL dichloromethane (CH_2Cl_2) was added to elute all bound substances from the column. Next, the vials were placed under a nitrogen flow to make sure the organic phase (resulting from the elution with CH_2Cl_2) was evaporated. Lastly, the dried samples were incubated with 50 µL N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) for 30 minutes at 60 °C to allow derivatization before transferring the liquids to GC-MS-vials. This derivatization step is needed to increase sensitivity and allows detection of the molecules of interest in the GC-MS^2. The whole procedure, as described above, is visualized in figure 4.

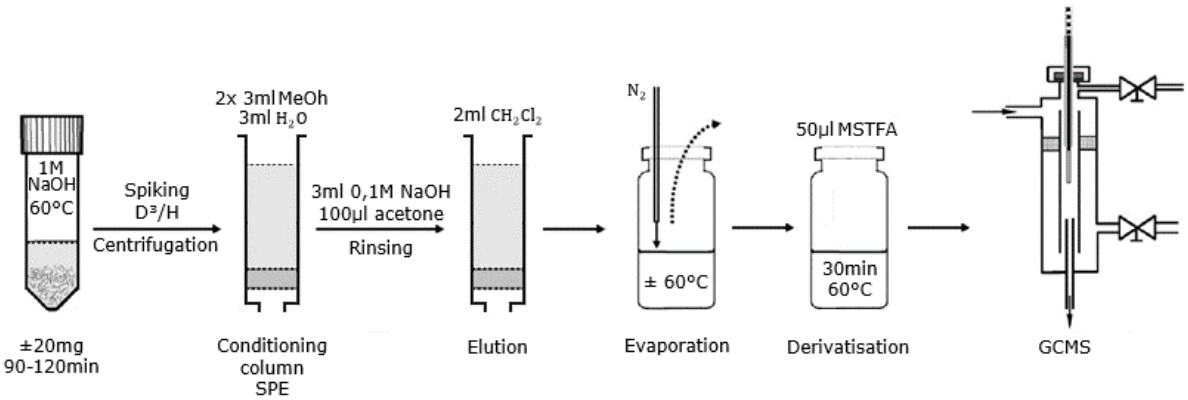


Figure 4. Workflow overview for extraction and measurement of THC, CBN and CBD in hair.

All results were obtained using a gas chromatograph 7890A with a 7693 autosampler coupled to a 7000C GC/MS triple quad (Agilent, USA). 3 µL was taken up, injected in the capillary column and heated to form a gas. The starting temperature was set at 100 °C (1 minute hold) which was further increased with a first ramp to 260 °C at 40 °C/min (no hold) and lastly increased to 300 °C at 15 °C/min (1.5 minute hold). The total run time was 9.17 min. An Agilent J&W GC DB-5MS UI column ((5 % -Phenyl)-methylpolysiloxane, 30 m × 0.250 mm × 0.25 µm) was used, linked to the ion source. An EI-source (70 eV) was used to ionize the cannabinoids so they could enter the mass analyzer where they were detected and selected for their specific mass/charge-values (m/z). EI is the most widely used technique due to its high sensitivity, specificity and separation power [42]. The injection mode was set to "Pulsed Splitless" with an Injection Pulse Pressure at 70 psi (valve off time of 45 seconds) and a Purge Flow to Split Vent at 50 mL/min as of 1 minute. The temperature of the source was kept at 230 °C.
The precursor or parent ions were selected by the first quadrupole and underwent division into smaller ions due to collision with Argon gas in the collision chamber before entering the second quadrupole. Only specific parent/precursor and product ions were chosen as represented in table 1. This application of selection specific ions is also called multiple reaction monitoring (MRM). The results were analyzed after the run was completed.

Table 1. Overview of all precursor and product ions of CBD, THC and CBN that were scanned in the MRM application run for the MS^2. (IS = internal standard)

Compound Name	IS?	Precursor Ion	Product Ion	Dwell	Collision Energy
CBD TMS	No	458	351	10	10
CBD TMS	No	458	268	20	25
CBD TMS d3	Yes	392	229	20	35
THC TMS	No	386	371	30	15
THC TMS	No	386	305	30	15
THC TMS	No	371	305,1	30	5
THC TMS d3	Yes	389	374	30	15
CBN TMS	No	382	367	10	15
CBN TMS	No	382	310	10	40
CBN TMS	No	366,6	310	10	40
CBN TMS d3	Yes	385	370	10	30

5.5. Sample preparation and extraction for detection of THC-COOH

To detect THC-COOH a very similar protocol was used, but with some additional steps necessary to do a optimize extraction from the hair matrix and allow more sensible detection. Approximately 10 mg of hair was weighed in an Eppendorf tube and was incubated with 500 µL MeOH and 400 µL 10M NaOH at 60°C for 30 minutes to allow a complete hydrolysis. Next, 500 µL acetic acid (100 %) and 600 µL acetate buffer (100mM) were added. The acetate buffer was made by adding 1.4 g sodium acetate trihydrate ($C_2H_3NaO_2$) to 100 ml H_2O and 5.3 ml MeOH. Spiking was performed with H and D^3 mixtures (0.1 pg THC-COOH/µL). Attention was payed to keep the sample at a pH between 4.5 and 6.5 before centrifugation was performed (5000 rpm during 5 - 10 minutes).

To extract THC-COOH, a different column (Strata Screen A, reverse phase) was needed. Conditioning of the column was required prior to elution. First, 2 mL MeOH was forced twice through the column with a vacuum and secondly 2 mL 100mM acetate buffer was forced twice through the column. The samples were loaded after centrifugation was complete. Because the acetate buffer had a high viscosity, a small vacuum was applied so samples could be run faster through the column (the vacuum was applied so a single drop was eluted from the column every 3 seconds). Extra assistance could be provided if necessary by applying pressure with a "pear". After, the column was cleaned with 2 mL de-ionized water and 2 mL MeOH/H_2O (50:50) under vacuum conditions. Columns were left 5 minutes under the vacuum to allow the column to dry and any unwanted substance or liquid was removed. Elution was performed with 2 mL freshly prepared n-hexane/ethyl acetate/acetic acid (75:25:1) and was transferred from the column to a vial with a 21G needle.
When all samples were collected, the remaining solvent was heated at 60 °C and dried under nitrogen flow for complete evaporation. Next a double derivatization step was needed with 70 µL Pentafluoropropanol (PFPOH) and 100 µL Pentafluoropropionic anhydride (PFP). To increase sensitivity, samples were incubated for 30 minutes at 60 °C. An additional evaporation step with nitrogen was included and the remaining content was transferred in a GC-vial with 50 µL of ethyl acetate so the sample could be injected and evaporated in the gas chromatography. An overview of all the preparation steps is given in figure 5.

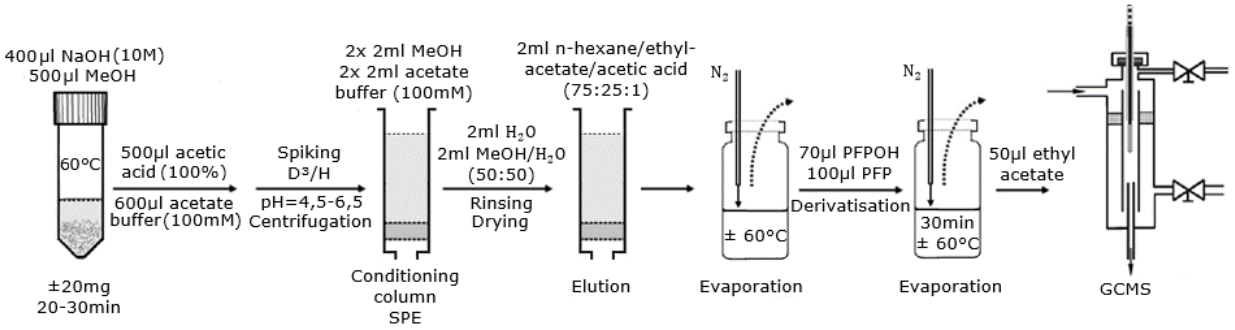


Figure 5. Workflow overview to extract THC-COOH for GC-MS² application.

All results were obtained with the same gas chromatograph used for detection of CBD, THC and CBN (gas chromatograph 7890A with a 7693 autosampler coupled to a 7000C GC/MS triple quad (Agilent, USA) and a capillary column (Agilent J&W GC DB-5MS UI column ((5 % -Phenyl)-methylpolysiloxane, 30 m × 0.250 mm × 0.25 µm)). 2 µL was taken up and injected. The starting temperature was set at 80 °C (1 minute hold) which was increased to 275 °C at 40 °C/min (2 minutes hold) and lastly increased to 300 °C at 20 °C/min (2 minutes hold). The total run time was 11.125 minutes. In contrast to the detection of the other cannabinoids, another type of ionization source is used: a NCI-source (Negative Ion Chemical Ionization, 240 eV). Negative ions are formed to greatly increase the sensitivity, even up to a thousand fold compared to other ionization methods like EI [42]. This is essential as THC-COOH is only present in very low concentrations. However, the source has to be changed manually.
In addition to the NCI-source, a special injection method called "Programmed Temperature Vaporizer" (PTV) has to be used. Larger volumes of samples are injected as a liquid at cold temperature which are then rapidly heated to 300 °C to vaporize the samples. The introduction of large volumes of samples for analysis greatly increases sensitivity (so an improvement of the

detection limits is achieved). PTV-injection is advised when larger molecules have to be studied [43]. The injection mode is set to "Pulsed Splitless" with an Injection Pulse Pressure at 55 psi (valve off time of 1 minute) and a Purge Flow to Split Vent at 50 mL/min as of 1.8 minute. The source temperature is kept at 150 °C.
As for the detection of the other cannabinoids, only specific mass over charge (m/z) values were of interest. The precursor/parent ions and product ions with all details are listed in table 2.

Table 2. Overview of all precursor and product ions of THC-COOH that were scanned in the MRM application run for the MS^2. (IS = internal standard)

Compound Name	IS?	Precursor Ion	Product Ion	Dwell	Collision Energy
D3-THC-COOH-2hfb	Yes	605	516	20	15
D3-THC-COOH-2hfb	Yes	605	477	20	15
D0-THC-COOH-2hfb	No	602	513	20	15
D0-THC-COOH-2hfb	No	602	474	20	15

5.6. Method Validation CBD, THC and CBN

To obtain a calibration curve, 8 samples were prepared with approximately 5 mg hair per tube. Two mL of 1M NaOH was added to each tube and samples were incubated at 60 °C for 90 minutes. After incubation, two samples were used as a blank while the other samples were spiked with 0.1 ng (0.01 ng/µL), 0.2 ng (0.01 ng/µL), 0.4 ng (0.01 ng/µL), 1 ng (0.1 ng/µL), 2 ng (0.1 ng/µL) and 4 ng (0.1 ng/µL) of the H-mixture containing CBD, THC and CBN. Additionally, all samples were also spiked with 20 µL (0.1 ng/µL) of the D^3-mixture (with CBD, THC and CBN). Also, intra-day precision was carried out (n = 10) using approximately 5 mg hair, spiked with 5 ng (0.1 ng/µL) H-mixture and 2 ng (0.1 ng/µL) D^3-mixture. Additionally, relative extraction recovery was determined (n = 6) by comparing corresponding peaks of both CBD, THC and CBN for a concentration of 5 ng (0.1 ng/µL). The limit of detection (LOD) and the lowest limit of quantification (LLOQ) were evaluated with 0.1 ng (0.01 ng/µL), 0.2 ng (0.01 ng/µL) and 0.4 ng (0.01 ng/µL) of the H-mixture. The LOD was calculated being 3 times greater than the noise signal. The LLOQ was deducted from the same values, but this value was 10 times greater than the noise.

5.7. Method Validation THC-COOH

To obtain a calibration curve for THC-COOH, 5 samples were prepared with approximately 10 mg. Each sample was enriched with 0.5 mL MeOH and 0.4 mL 10M NaOH before being incubated at 60 °C for 30 minutes. After the incubation step, all samples received 0.5 mL acetic acid and 0.6 mL of the before mentioned 100mM acetate buffer. Additionally, samples were spiked with 20 µL (0.1 pg/µL) H-mixture, 4 samples were spiked with 4 pg, 8 pg, 16 pg and 32 pg (0.1 pg/µL) D^3-mixture respectively and one last sample served as a blank.
Intra-day precision was determined (n = 10) using approximately 10 mg hair which was spiked with 5 pg (0.1 pg/µL) H-mix and 2 pg (0.1 pg/µL) D^3-mix. Also, relative extraction recovery was determined (n=3) by comparing corresponding peaks of THC-COOH for a concentration of 5 pg (0.1 pg/µL). The limit of detection (LOD) and the lowest limit of quantification (LLOQ) were evaluated with 4 pg, 8 pg and 16 pg (0.1 pg/µL) of the H-mixture. The LOD was calculated as being 3-fold higher compared to the noise and the LLOQ was 10-fold higher than the noise.

6. Results

6.1. Method Validation CBD, THC and CBN

Results obtained from the different concentrations (0.01-4 ng/µL) were used to form calibration curves for all three cannabinoids (CBD, THC and CBN) and all were subjected to linear regression. The resulting graphs are represented as figure 6, 7 and 8. For the calibration curve of CBD, there was no signal for the sample spiked with 1 ng of the H-mixture. Additionally, the correlation coefficients derived from the calibration curves are 0.9946, 0.999 and 0.9978 for CBD, THC and CBN respectively. Intra-day precision and accuracy were also determined for all samples. Precision is 91 % for CBD, 97 % for THC and 98 % for CBN while accuracy is determined to be 85 % for CBD, 97 % for THC and 94 % for CBN. The relative extraction recovery resulted in various percentages. A mean of 6 samples was chosen and resulted in 75.1 % for CBD, 77.5 % for THC and 81.1 % for CBN. Despite being lower than the requested 85 %, this method is very sensitive so the lower recovery is accepted. The LOD values are also calculated for the 3 lowest concentrations ranging from 0.1 to 0.4 ng/µL for all three cannabinoids. An average value is taken to compromise between the different values. The LOD values are; 4.5 pg/mg hair for CBD, 0.6 pg/mg hair for THC and 0.3 pg/mg hair for CBN. Corresponding to these values, the LLOQ can be calculated: 14.9 pg/mg hair for CBD, 1.9 pg/mg hair for THC and 1.2 pg/mg hair for CBN.

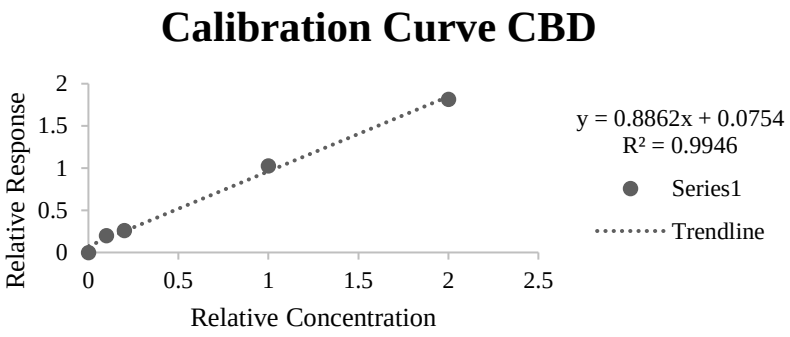


Figure 6. Calibration curve for CBD made with 4 different concentrations and a blank.

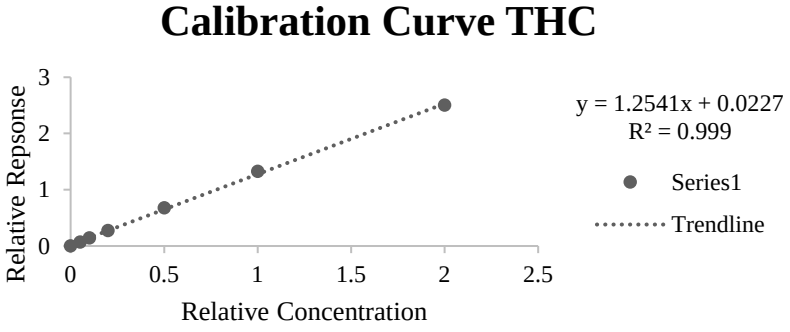


Figure 7. Calibration curve for THC made with 6 different concentrations and a blank.

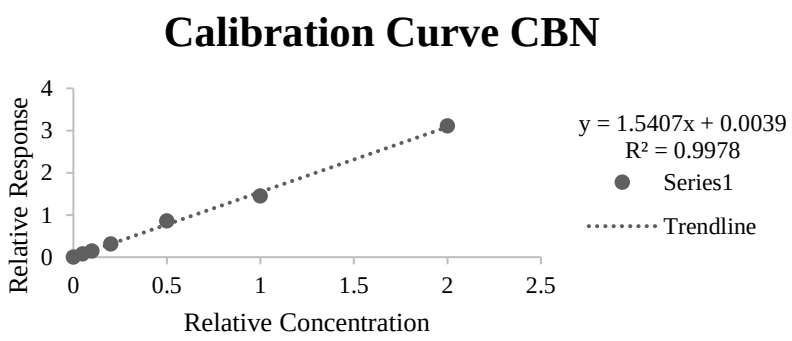


Figure 8. Calibration curve for CBN made with 6 different concentrations and a blank.

6.2. Method Validation THC-COOH

All results obtained from different concentrations (0 - 32 pg) are used to form a calibration curve for THC-COOH which is subjected to linear regression (figure 9). The correlation coefficient derived from the calibration curve is 0.9721. Intra-day precision is 91 % and accuracy is 93 %. The relative extraction recovery is estimated to be 86 % on average. The LOD values are also calculated for the 3 lowest concentrations (4 pg, 8 pg and 16 pg). The mean LOD value is chosen as a compromise and is 0.034 pg/mg hair. Additionally, the LLOQ is 0.113 pg/mg hair on average.

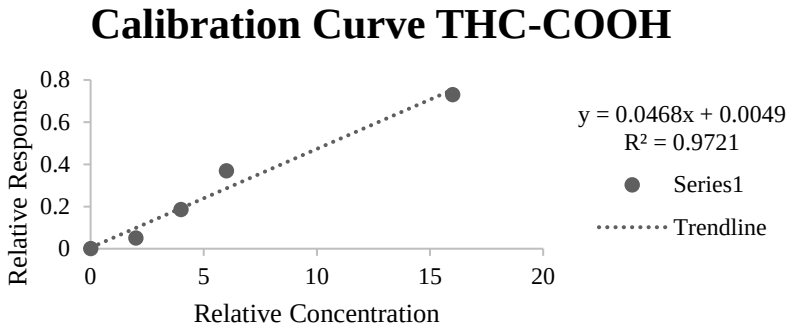
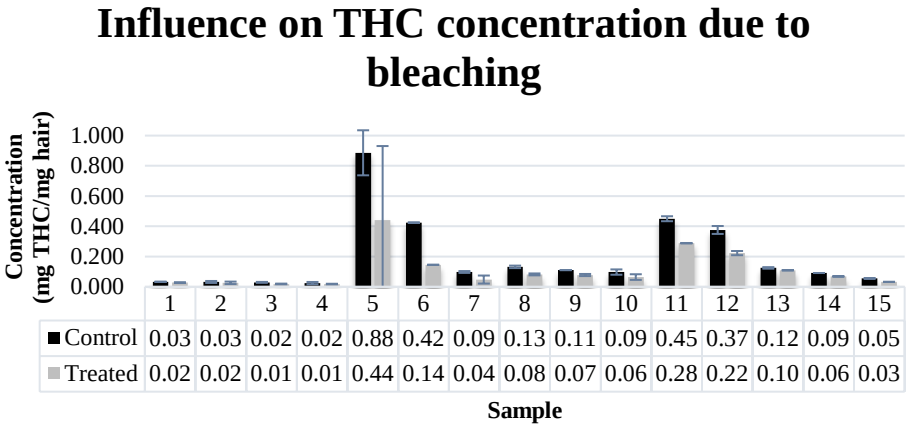


Figure 9. Calibration curve for THC-COOH with 4 different concentrations and a blank.

6.3. Bleaching

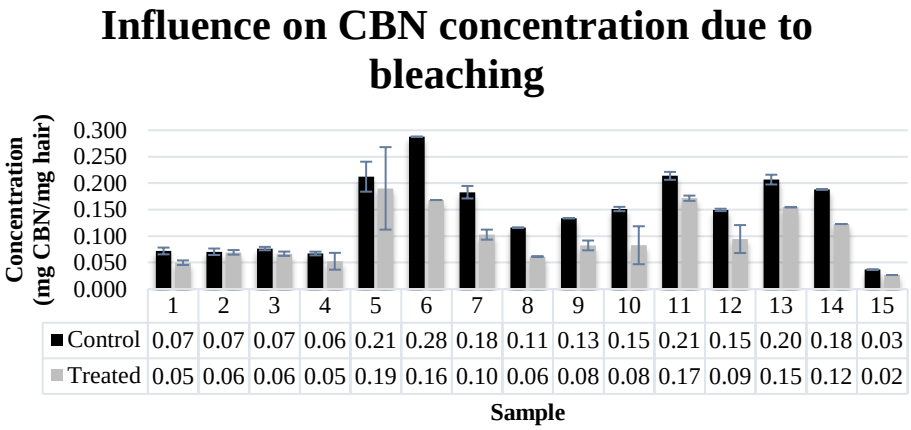
Fifteen hair samples were used from 8 different persons. The influence of bleaching on THC is represented in figure 10. The control samples that didn't undergo any treatment are represented in black while the corresponding treated (bleached) samples are shown in grey. All hair samples show a decrease in THC content.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Control	0.03	0.03	0.02	0.02	0.88	0.42	0.09	0.13	0.11	0.09	0.45	0.37	0.12	0.09	0.05
Treated	0.02	0.02	0.01	0.01	0.44	0.14	0.04	0.08	0.07	0.06	0.28	0.22	0.10	0.06	0.03

Figure 10. The influence on THC concentration after bleaching (black = control samples, grey = bleached samples)

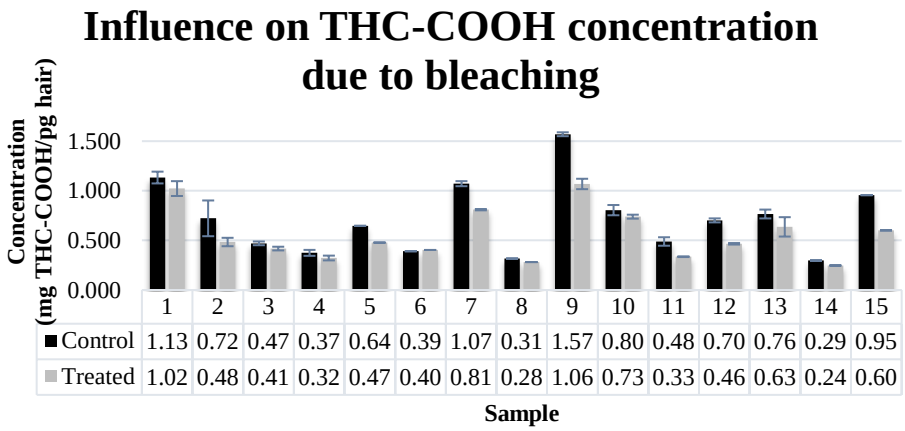
The bleaching effect on CBN content in hair is represented in figure 11. Compared to THC, CBN concentrations are relatively higher in all samples. The decrease in CBN content is more even amongst the hair samples, but less in comparison to THC. Again, control samples are represented in black while the bleached hair samples are shown in grey.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Control	0.07	0.07	0.07	0.06	0.21	0.28	0.18	0.11	0.13	0.15	0.21	0.15	0.20	0.18	0.03
Treated	0.05	0.06	0.06	0.05	0.19	0.16	0.10	0.06	0.08	0.08	0.17	0.09	0.15	0.12	0.02

Figure 11. The influence of bleaching on CBN content in hair (black = control samples, grey = bleached samples)

Results on the influence of bleaching on THC-COOH are represented in figure 12. As for THC and CBN, also for THC-COOH a decrease can be observed for all samples, despite being less prominent compared to THC and CBN.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Control	1.13	0.72	0.47	0.37	0.64	0.39	1.07	0.31	1.57	0.80	0.48	0.70	0.76	0.29	0.95
Treated	1.02	0.48	0.41	0.32	0.47	0.40	0.81	0.28	1.06	0.73	0.33	0.46	0.63	0.24	0.60

Figure 12. The influence of bleaching on THC-COOH in hair (black: control samples, grey: bleached samples)

The results of figure 10, 11 and 12 are combined to calculate the percentages of decrease. The resulted graph is shown as figure 13. Additionally, extra data is shown on the changes in CBD concentrations due to bleaching.

Overall bleaching causes a decrease for all cannabinoids (with exception of CBD in sample 2 were an increase is observed). In general, a mean decrease of 34.8 % in THC concentration is observed amongst all samples. CBN (shown in red) decreases more equal amongst samples (except sample 2). An average decrease of 29.2 % of CBN is estimated. In samples where sufficient CBD was measured (i.e. the concentration was greater than the LLOQ), the results (green) shows a decrease in CBD concentration of 32.9 %. Finally, also THC-COOH concentrations (purple) decrease in all samples, except in sample 6. The mean decrease is calculated to be 20.2 % (21.8 % if sample 2 is excluded).

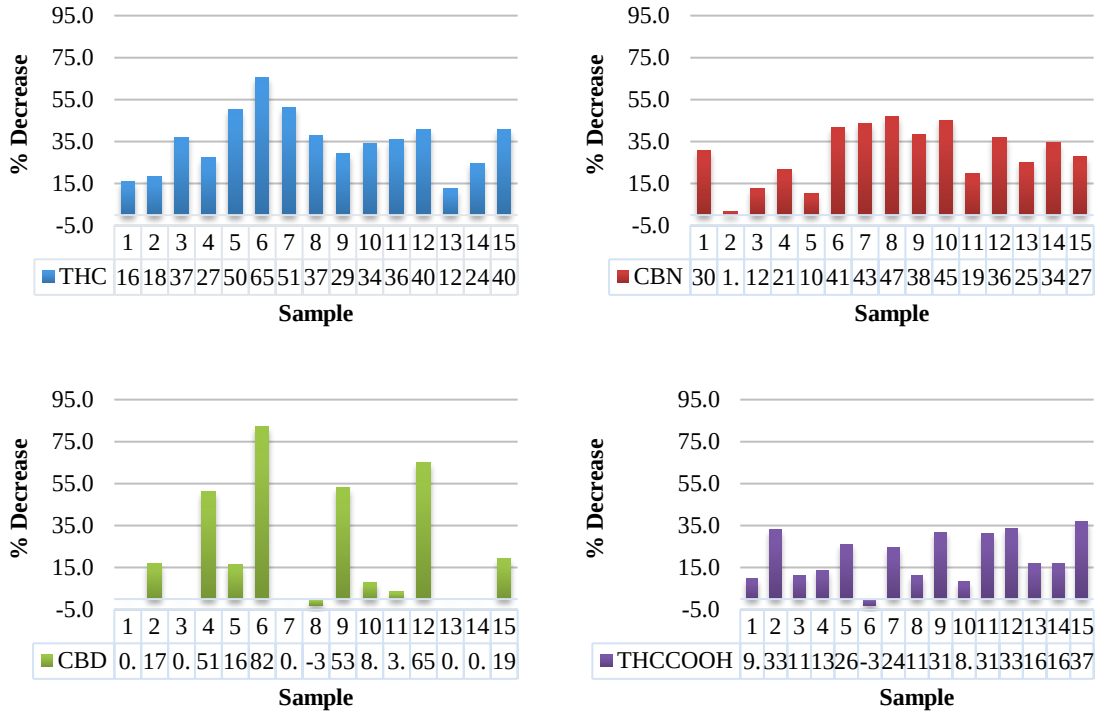


Figure 13. Influence of bleaching on THC (blue), CBN (red), CBD (green) and THC-COOH (purple) concentrations.

Additionally, THC/CBN and THC/THC-COOH ratio can be calculated for each sample. The ratios of control (non-treated) hair and bleached hair can be compared to look for trends.

In general, a decrease in THC/CBN ratio is found from 1 to 0.8 when samples are bleached (calculated from 1.2 (control) to 1.0 (bleached)). However, this general decrease does not occur for every sample. Six samples had an increase of their THC/CBN ratio after bleaching. This increase occurs to samples with low THC and slightly higher CBN content. After bleaching, CBN loss is greater than THC loss, creating a shift in the calculated ratio.

A THC/THC-COOH ratio decrease is also found from 1 to 0.7 after bleaching (calculated from 0.36 in control samples to 0.25 in bleached samples). Only 3 samples show an actual shift in their ratio (samples 5, 6 and 8), due to a greater loss of THC compared to THC-COOH. In all other samples there is no change in the THC/THC-COOH ratio.

The *in-vitro* study of hydrogen peroxide with cannabinoids showed a strong decrease for all compounds. H and D^3-mixtures were added separately to enable usage of the calibration curves as shown in 6.1. If H and D^3-mixtures are added simultaneously, only the area can be used to compare cannabinoids before and after their in-vitro treatment. CBD concentrations decreased by 89 %, THC by 93 % and CBN by 78 %. Variation between samples is represented with the error bars. All results are shown in figure 14.

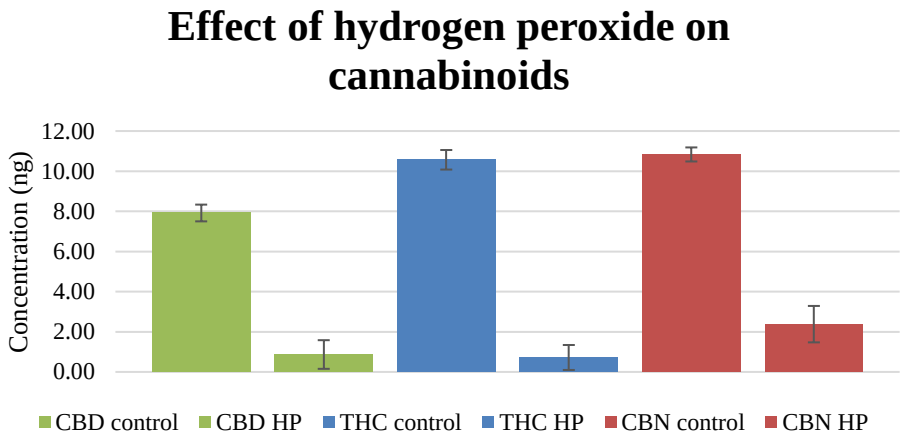
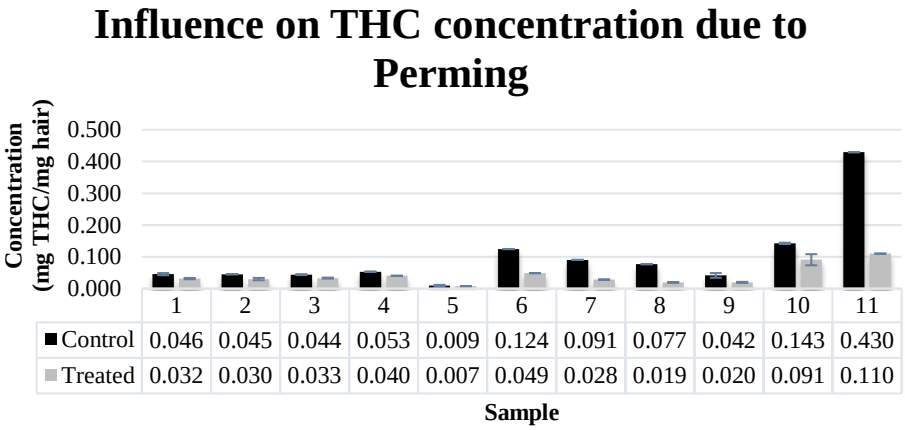


Figure 14. Influence of hydrogen peroxide on CBD (green), THC (blue) and CBN (red).

6.4. Perming

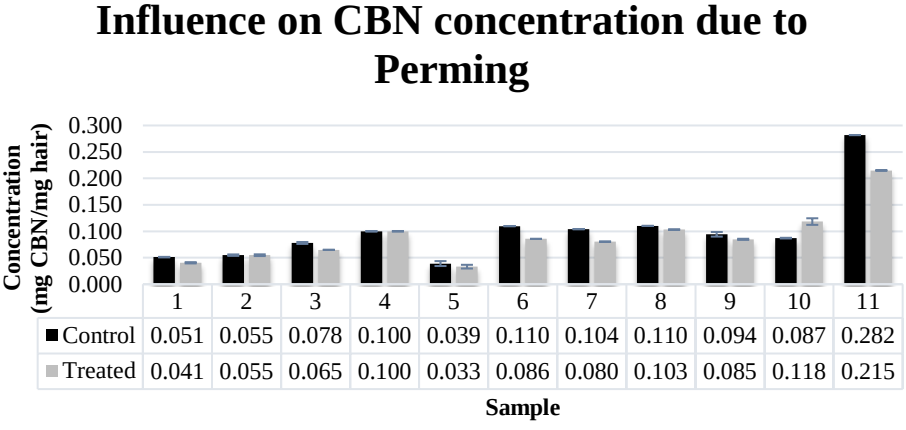
Thirteen hair samples were used from 4 different individuals, compared to the samples in the bleaching study. Figure 15 represents the influence of Ammonium thioglycolate on THC concentration in hair. Control samples are shown in black and the treated samples are shown in grey. 2 of the 13 samples are not shown due to interferences to the CBN signals. All samples tested showed a decrease in THC concentration (even the 2 samples that were excluded).



	1	2	3	4	5	6	7	8	9	10	11
Control	0.046	0.045	0.044	0.053	0.009	0.124	0.091	0.077	0.042	0.143	0.430
Treated	0.032	0.030	0.033	0.040	0.007	0.049	0.028	0.019	0.020	0.091	0.110

Figure 15. The influence of perming on THC concentration in hair
(black: control samples, grey: permed samples)

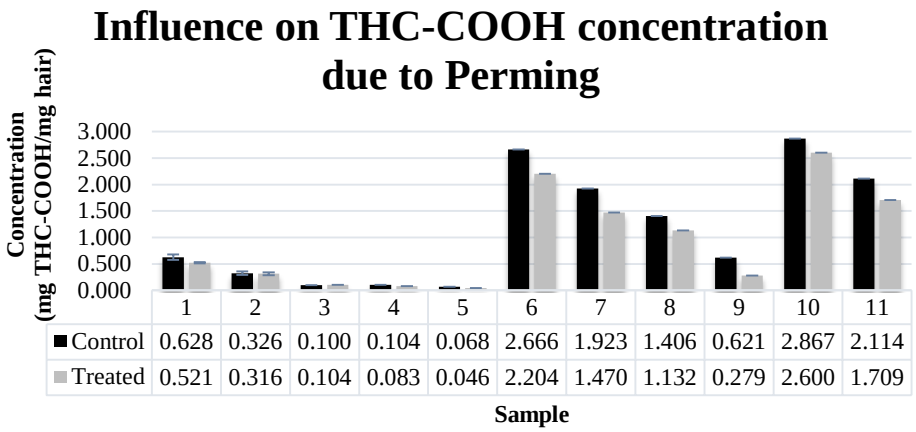
The effect of perming on CBN content in hair is shown in figure 16. Non-treated hair samples are represented in black, whilst permed samples are shown in grey. As mentioned before, samples 12 and 13 were excluded. CBN is relatively less affected by perming. Two samples (3 and 10) give a small increase in CBN concentration. Sample 3 was retested and results show that the increase detected for CBN was false (the newly measured concentration was used for further analysis). Sample 10 had an increase due to an outlier value. Retesting this sample resulted in a decrease in CBN concentration. However, in figure 16, the original values are kept.



	1	2	3	4	5	6	7	8	9	10	11
Control	0.051	0.055	0.078	0.100	0.039	0.110	0.104	0.110	0.094	0.087	0.282
Treated	0.041	0.055	0.065	0.100	0.033	0.086	0.080	0.103	0.085	0.118	0.215

Figure 16. The influence of perming on CBN in hair
(black: control samples, grey: permed samples)

THC-COOH concentrations in control (black) and permed (grey) samples are represented in figure 17. All samples show a similar decrease for THC-COOH with exception of sample 2 - 5.



	1	2	3	4	5	6	7	8	9	10	11
Control	0.628	0.326	0.100	0.104	0.068	2.666	1.923	1.406	0.621	2.867	2.114
Treated	0.521	0.316	0.104	0.083	0.046	2.204	1.470	1.132	0.279	2.600	1.709

Figure 17. Influence of perming on THC-COOH in hair
(black: control samples, grey: permed samples)

Figures 15, 16 and 17 are combined to form a new graph (figure 18) to calculate the percentages of decrease. As for the bleached hair samples (figure 13), the changes in CBD concentrations (green) are also shown.
All cannabinoids concentrations (THC, CBN and CBD) show a reduction after perming treatment. THC, represented in blue, has a greater decrease in concentration compared to CBN (black). The average decrease in THC concentration is 46.5 % (44 % when the 2 rejected samples are included to calculate the average reduction). For CBN concentrations, a mean decrease of 13.4 % is noted. Both average reduction percentages don't change when rejected samples were in- or excluded.
CBD (green) was not measured in sample 4 and only once for sample 8. Additionally, we failed to detect CBD in samples 6 and 7. The overall decrease in CBD concentration was 36.2 %.
THC-COOH also decreases in every sample except in sample 3, whereas in sample 2 the decrease is low. The mean decrease was calculated to be 18.9 % (a decrease of 21.9 % is found if samples 2 and 3 are not included).

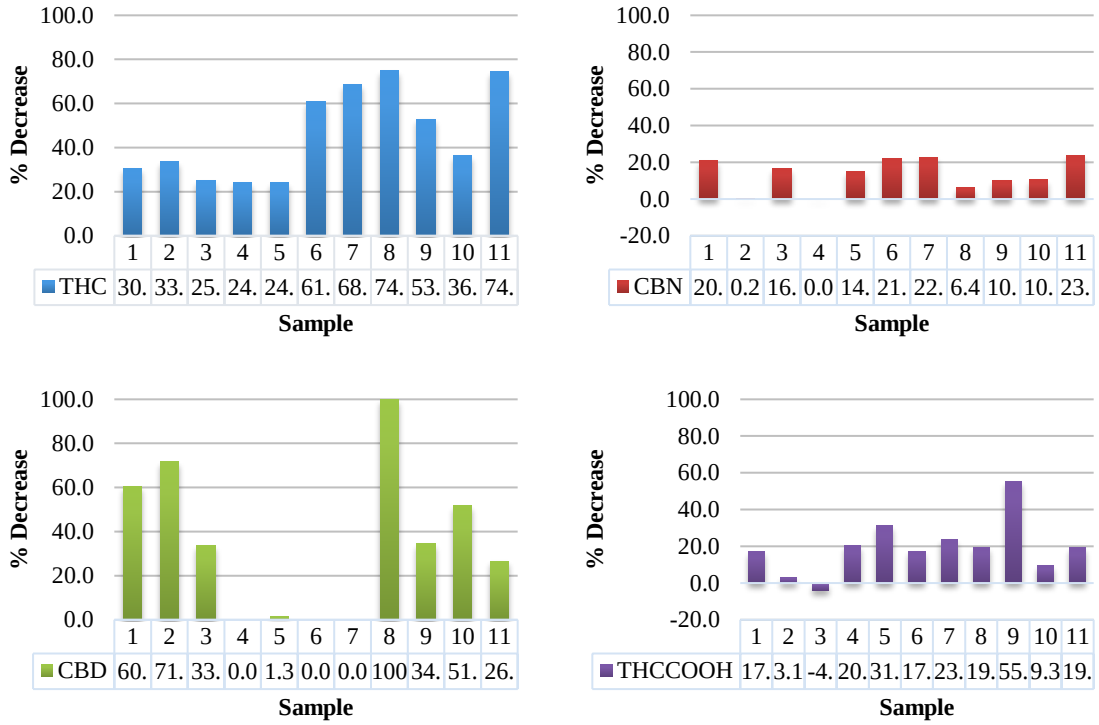


Figure 18. Influence of perming treatment on THC (blue), CBN (red), CBD (green) and THC-COOH (purple) concentrations.

THC/CBN and THC/THCCOOH ratios can be deducted from the results shown in figures 15 - 17. Overall, a decrease in THC/CBN ratio was found for all samples. CBN is far less affected by perming in comparison to THC. This causes the THC/CBN ratio decrease from 1 to 0.53 (calculated from 0.9 (control) to 0.5 (permed)). The ratio didn't change when the 2 rejected samples (12 and 13) were added.
There is also a decrease in THC/THC-COOH ratio, mostly contributed by high decreases in THC/THC_COOH ratios in 2 samples (3 and 11). The calculated ratio decreased from 1 to 0.75 (calculated from 0.16 to 0.12 after perming).

The *in-vitro* study with thioglycolate showed no effect on neither CBD, THC nor CBN. Error bars are also included to show inter-sample variations. H- and D^3-mixtures were added separately, so the generated calibration curves were used to evaluate the results. All results are shown in figure 19.

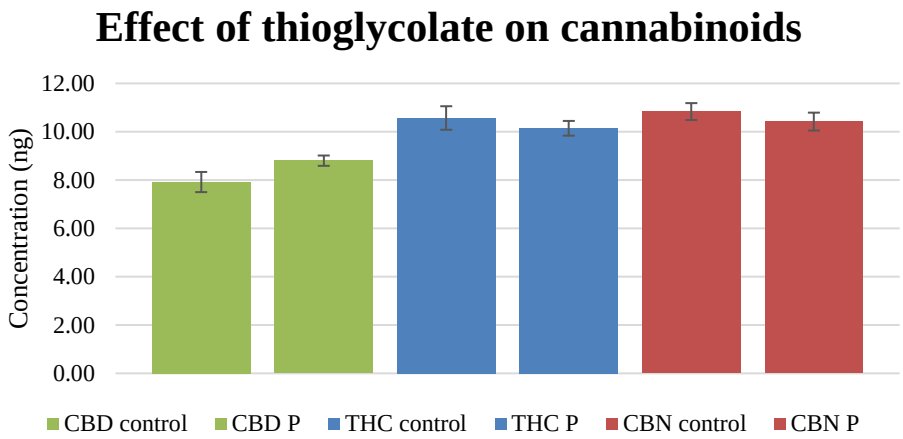


Figure 19. Influence of thioglycolate on CBD (green), THC (blue) and CBN (red).

6.5. Temporary coloring (1)

Fifteen hair samples were used from 11 different test subjects. Temporary coloring is achieved by applying the product on the hair and does not require use of chemicals. As mentioned in the introduction, we specifically chose a product without hydrogen peroxide to solely study the dyeing effect. As no damaged is caused to the hair by chemicals, limited to no loss in cannabinoids concentration is expected. A first analysis of the colored samples showed an increase in cannabinoid concentration, except for 3 samples where CBD concentrations had decreased.

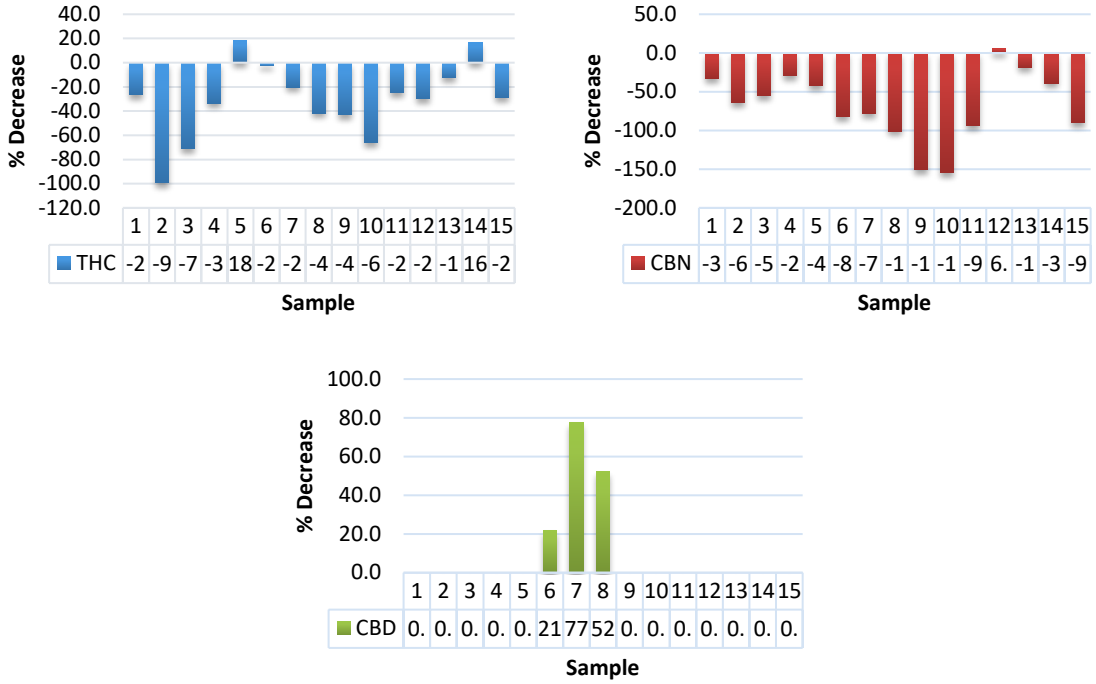


Figure 20. Influence of coloring on THC (blue), CBN (red) and CBD (green) content in hair samples.

Cannabinoid concentrations should not (cannot) increase. We hypothesized that interferences could occur on different critical steps in the extraction method as well as in the chromatograph itself. The coloring product could augment detection in the GC-MS², the extraction could be enhanced in presence of the coloring product or interferences with the IS could impact our findings.

The *in-vitro* study as mentioned in the introduction with the temporary coloring product (see 5.3) showed no effect of the product on cannabis content. H- and D³-mixtures were added simultaneously, so instead of the area has to be used instead of the calculated concentration. Results are shown in figure 21. Additionally, error bars are also present to indicate inter-sample variances.

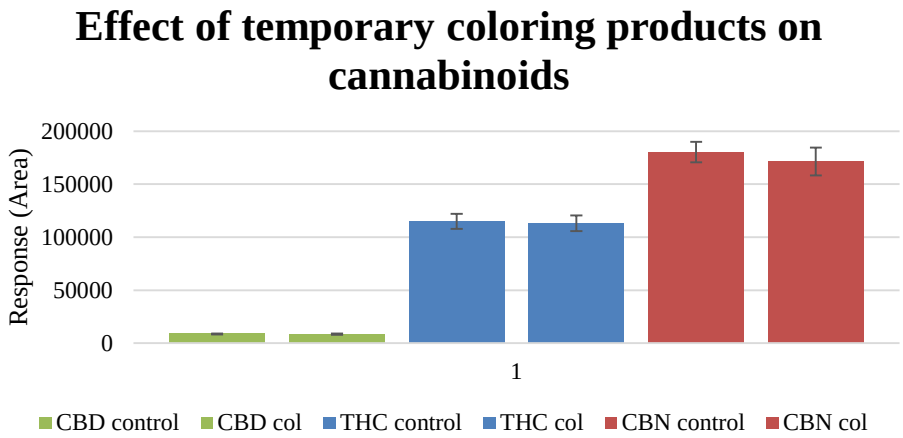


Figure 21. Influence of a temporary hair coloring product on CBD (green), THC (blue) and CBN (red).

Another coloration product (L'Oréal – Dédicace No. 54 Light Chestnut Brown) was used on 5 new prepared hair samples (from 2 different subjects) in order to define if our observations were product-related.
The new dyeing product was applied for 30 minutes. Hair was firstly washed in lukewarm water and afterwards in water and acetone before being cut and pulverized. The 5 samples were all tested once to compare the results with the first coloration product. The results were not uniform, indicating both increases and decreases for all tested cannabinoids. No conclusion could be drawn after testing both products.
We tested if the first coloring product enhanced the extraction of the hair matrix. 2 mL of different NaOH concentrations (1M, 5M and 10M) were tested according the same procedure as applied for the hair samples. A concentration of 1M NaOH dissolved the hair matrix almost completely, so we could eliminate this hypothesis. One of the dyed hair samples (sample 7) and its control were also subjected to 3 different extraction methods in addition to the standard operation method. An overview is given in table 3.

Table 3. Different methods on extraction of the hair matrix, with the main focus on differences in pH.

Product	Molar concentration	pH	Duration (h)	Temperature (°C)
NaOH	1M	14	1.5	60
NaOH	1M	14	> 16	20
Ammoniac buffer	3.5M	9.5	1.5	60
Ammoniac buffer	3.5M	9.5	> 16	20

Results showed that a higher pH is more suited for extraction of the hair matrix. In addition, the use of heat allows to achieve the most optimal extraction. The order of best incubation to worst was the same order (top to bottom) of the methods summarized in table 3. The same increases in CBD, THC and CBN persisted, indicating that none of the extraction methods is enhanced.
Lastly, we investigated a possible interference on the IS during extraction. Samples 13 and 15 were re-used for this experiment and the IS was added before incubation. All cannabinoid concentrations had slightly increased compared to when the IS was added after extraction, but the overall increase in THC and CBN still persisted.
Finally, we decided to investigate if there was an interference within the GC-MS^2. The results generated from our first analysis were loaded in the MassHunter Analyzer Software and special attention was given to the generated areas. All the areas of the IS were lower for colored hair samples compared to the non-treated hair samples. The original samples were run on 2 separate days. To eliminate inter-day variance we split the data according to the day of analysis. The area for CBD decreased by 56 % for samples 1 - 9 and 78 % for the other samples. The area for THC decreased by 46 % in samples 1 - 9 and 58 % in the other samples. Finally also the area for CBN decreased for sample 1 - 9 by 42 % and for the other samples by 59 %. These findings were compared to results from the perming and bleaching studies. In these series no decrease in the area of the IS is observed. The decrease in the area of the IS is thus only related to dyed hair samples. Due to the “loss” of IS, the resulting peaks in the software are much lower. When the IS peak decreases, the measured concentration increases, giving thus a false impression that the cannabinoid concentrations have increased after temporary coloring. The percentage of decrease found for the samples were used to correct the according samples. The findings resulting from this correction were used instead to explain the effect of coloring on cannabinoids in hair.

6.6. Temporary coloring (2)

The result on the influence of coloring on the THC concentration is shown in figure 22. The THC concentration in these subjects is low compared to results of other hair treatments. THC concentrations show a slight decrease in most samples (except in sample 2). Samples 5 and 6 show the highest reduction.

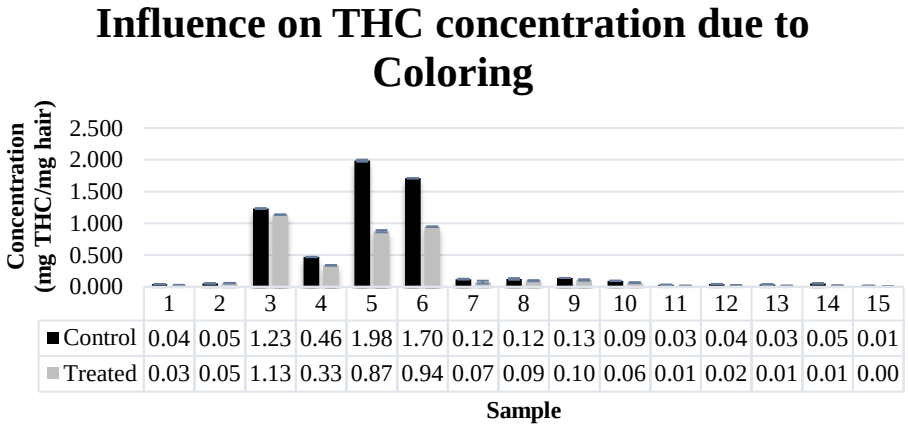


Figure 22. The influence of temporary coloring on THC in hair (black: control samples, grey: colored samples).

Regarding CBN content after coloring, results show that concentrations are increased, decreased or not affected at all. A decrease in CBN concentration is found in samples 1 - 5 and 11 - 14. Increases are found in samples 6 and 7, no CBN was measured in sample 8 - 10 and no alteration was detected in sample 15 due to the low and almost unmeasurable concentration. All results are shown in figure 23.

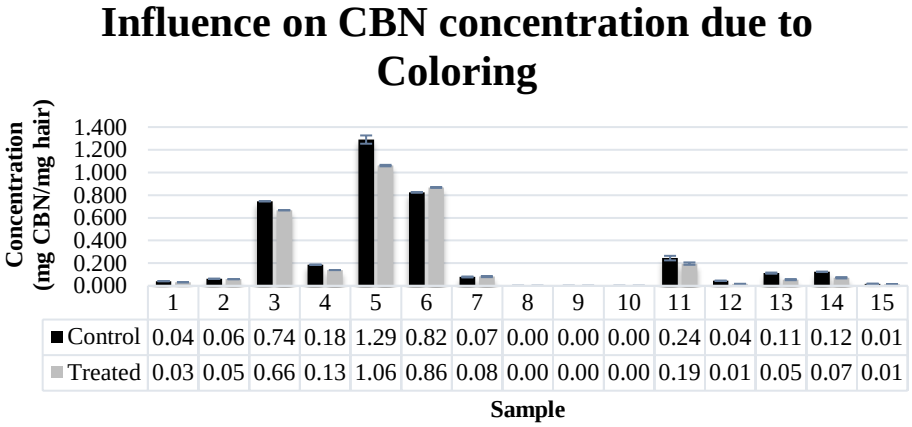
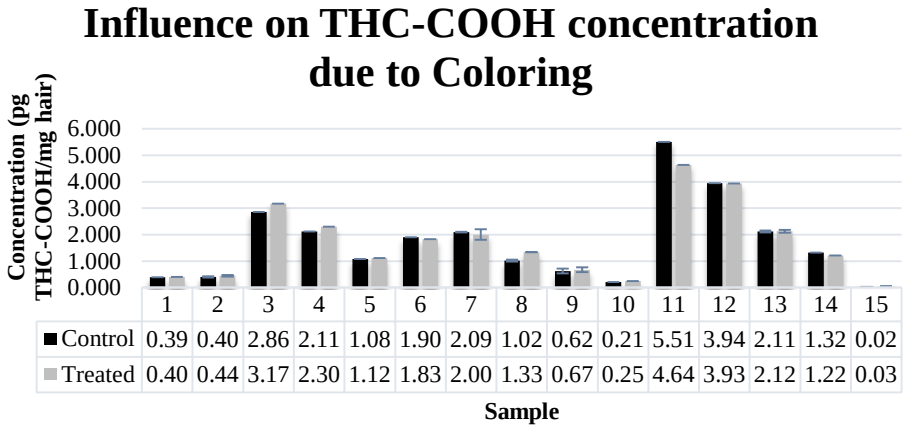


Figure 23. Influence of temporary coloring on CBN (black: control samples, grey: colored samples)

Also THC-COOH concentrations were analyzed and show no changes after coloring. Sample 11 has the most noticeable decrease whilst an increase in THC-COOH concentration is observed in sample 3 and 4.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Control	0.39	0.40	2.86	2.11	1.08	1.90	2.09	1.02	0.62	0.21	5.51	3.94	2.11	1.32	0.02
Treated	0.40	0.44	3.17	2.30	1.12	1.83	2.00	1.33	0.67	0.25	4.64	3.93	2.12	1.22	0.03

Figure 24. Influence of temporary coloring on THC-COOH
(black: control samples, grey: colored samples)

Results of figure 22, 23 and 24 are further combined into a new graph (figure 25) to calculate the percentages of decrease. CBD results (green) are also included and concentrations showed a decrease of 8.0 %. THC is decreased in all samples except sample 2, where a small increase is noted. The average decrease for THC is 35.5 %. CBN concentrations are found to be increased as well as decreased. A mean decrease of 10.4 % is calculated when using all values (19.1 % is calculated when sample 9 and 10 are left out, which have the greatest measured increase in CBN). In general, an average increase for THC-COOH of 6.2 % is calculated (2.2 % is noted when the increases for samples 10 and 15 are excluded).

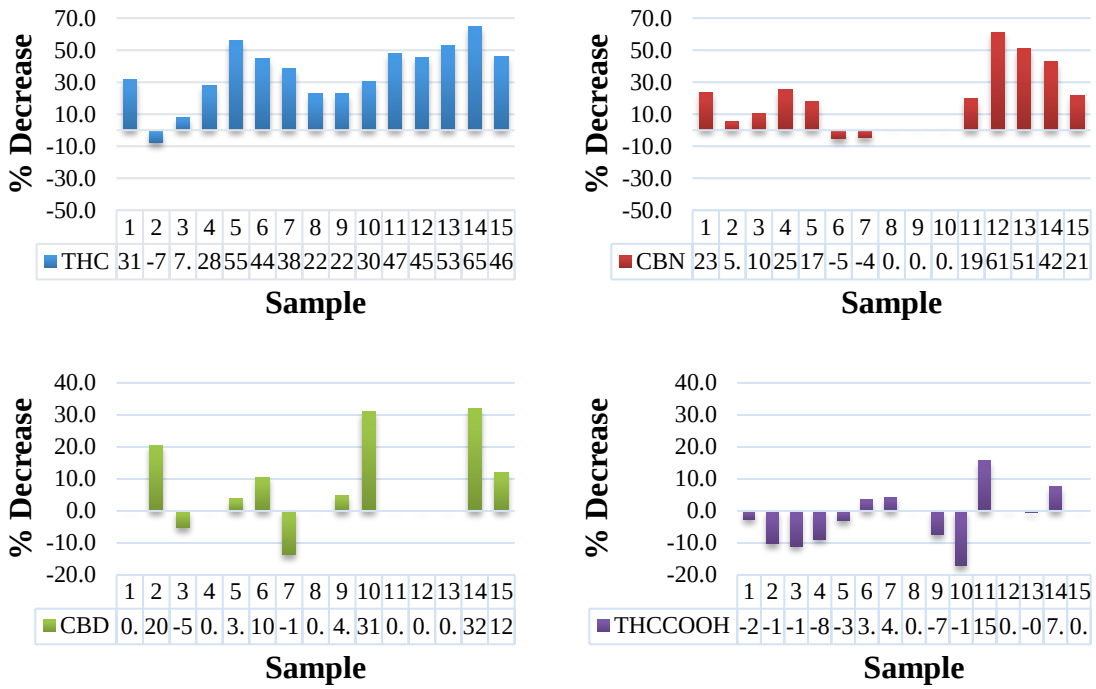


Figure 24. The influence of temporary coloring on THC (blue), CBN (red), CBD (green) and THC-COOH (purple) in hair samples.

However, when the THC/CBN ratio is investigated, all samples show a small decrease in this ratio (except sample 2 where a small increase of CBN after coloring results in an increase in THC/CBN ratio). The THC/CBN ratio decreases from 1 in control samples to 0.8 for dyed samples (calculated from 0.9 to 0.8 when samples 5, 6 and 7 are excluded).
Also the THC/THC-COOH ratio was studied. Samples 5 and 6 had larger decreases in THC, reducing the average calculated ratio. The ratio decreased from 0.12 to 0.09 after coloring. Samples 10 and 15 were not taken into account when this mean ratio was calculated.

As mentioned in 6.5, the *in-vitro* study showed no great effect on cannabinoids: CBD (96 %), THC (98 %) and CBN (95 %). Small changes observed in cannabinoid concentrations (10 ng for CBD, 10 ng for THC and 30 ng for CBN) are due to variances between measurements.

6.7. Permanent coloring

Results on the influence of permanent coloring on THC concentration is shown in figure 26. THC concentrations showed a slight decrease amongst all samples, but the effect on THC is rather low.

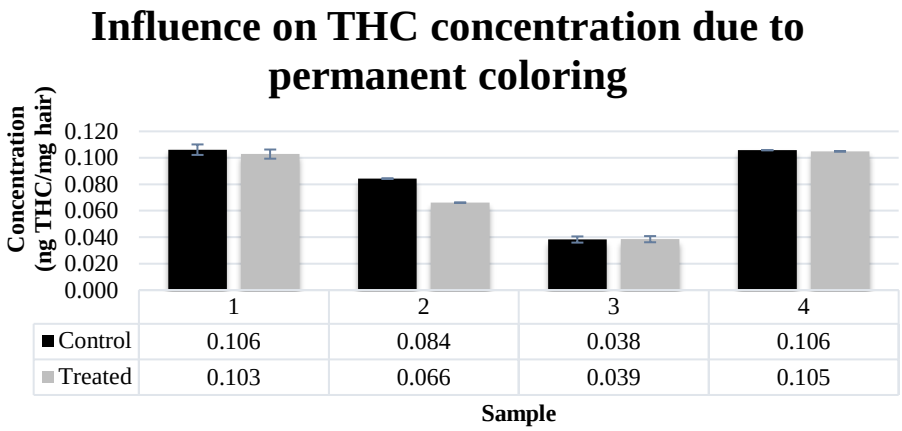


Figure 26. The influence of permanent coloring on hair THC (black: control samples, grey: colored samples).

CBN concentrations after permanent coloring have slightly increased compared to control samples. Results are show in figure 27 below.

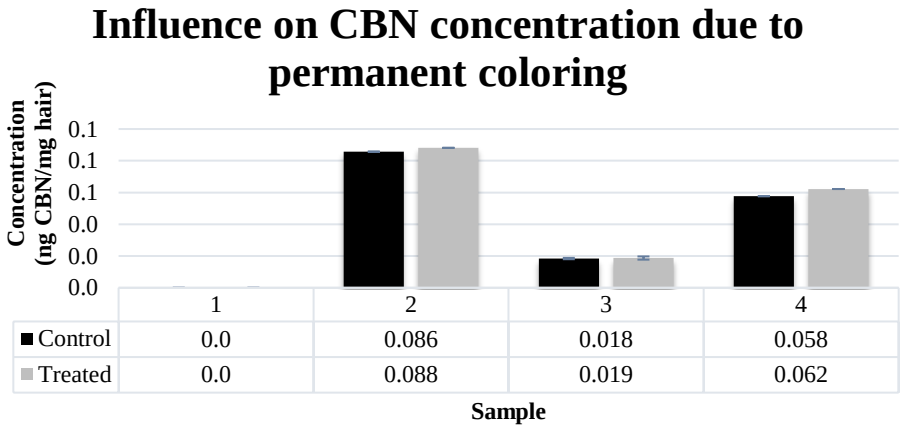


Figure 27. Influence of permanent coloring on CBN (black: control samples, grey: colored samples)

Results on the effect of permanent coloring on THC-COOH are represented in figure 28 and show a small decrease. The smallest decrease was observed for sample 2.

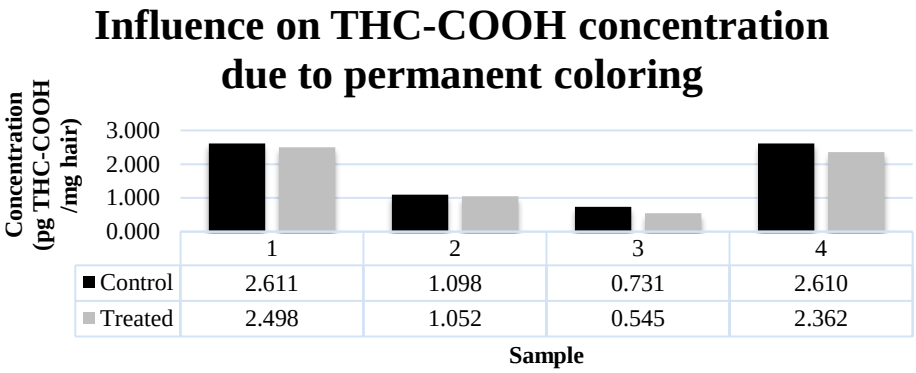


Figure 28. Influence of permanent coloring on THC-COOH (black: control samples, grey: colored samples)

Figure 29 summarizes the results of figures 26-28, and results are shown as a percentage of decrease. The effect on CBD concentrations (green) is also shown and has an average decrease of 13.1 %.

THC was less affected: only sample 2 showed a higher decrease, whereas other samples were almostl unaffected. An average decrease of 6.2 % was noted (1.1 % without sample 2). CBN concentrations had slightly increased, which was more noticeably in sample 1. A mean increase of 16.0 % (4.1 % without sample 1) was calculated. Finally, THC-COOH concentrations had decreased amongst all samples: a mean decrease of 10.9 % was noted.

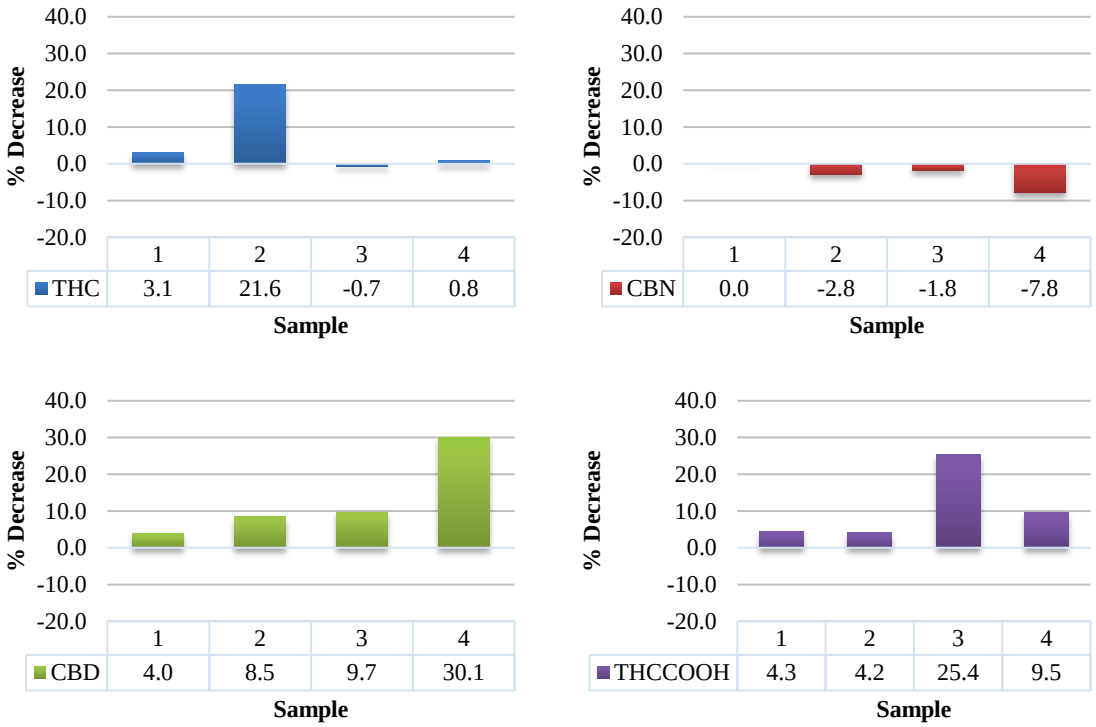


Figure 29. The influence of temporary coloring on THC (blue), CBN (red), CBD (green) and THC-COOH (purple) in hair samples.

All samples show a decrease in THC/CBN ratio from 1 in control samples to 0.83 after permanent coloring is applied. The THC/THC-COOH ratio remains unaffected as it is only slightly increased from 1 to 1.04 after coloring, mainly due to sample 2.

The *in-vitro* study shows a 14 % decrease for CBD, a slight decrease of 2 % for THC and a small increase of 4% for CBN after incubation with the permanent coloring product. H- and D^3-mixtures were added separately, so calibration curves to calculate the concentrations could be used. As mentioned in the *in-vitro* study of the temporary coloring product, a small change in concentration can be related to variances in measurements and has no real impact. All results on the *in-vitro* study for the permanent hair coloring product are shown in figure 30. No error bars are included for the blank samples as they were only tested once.

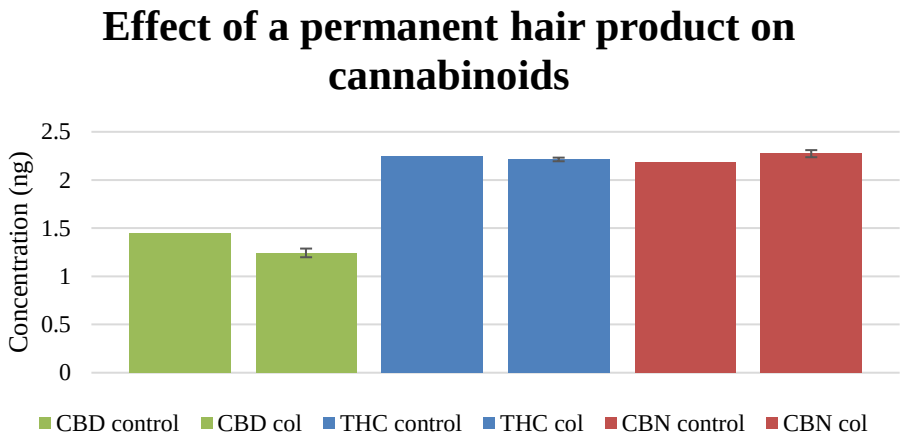


Figure 30. Influence of a permanent hair coloring product on CBD (green), THC (blue) and CBN (red).

7. Discussion

Depending on the chosen hair treatment, there can be made a clear distinction between their effects on the different cannabinoids and on the ratios calculated from their concentrations.

7.1. Bleaching

A high concentration of hydrogen peroxide found in bleaching mainly exhibits its effect on the pigments and granules in hair. Lipophilic drugs in hair like cannabinoids, adhere mostly to these pigments and granules. Since hydrogen peroxide aggressively affects both compounds, it is expected that bleaching removes cannabinoids from hair. Indeed, after tests were run it was concluded that bleached samples show a decrease for all cannabinoids content. THC decreases were observed in all samples. It can even be observed that THC concentrations are higher in more proximal segments in comparison to the more distal hair segments derived from the same subject. However, the degree of decrease depends mostly on how much THC is initially present in the sample. The more THC is initially present (i.e. in the control samples), the larger the effect of hydrogen peroxide on cannabinoids is. Changes in the lower concentration range are less drastic. Variation in the application of the bleaching product can influence the effect of hydrogen peroxide on cannabis content in hair. If the product is not equally applied, the effect of hydrogen peroxide can be limited. CBN concentrations were also decreased after hair samples were bleached, but the overall observed decrease is slightly lower. In general, it can be stated that CBN concentrations are often (except in samples 5, 11 and 12) higher compared to THC. The latter can be transformed into CBN by liver enzymes such as CYP2C9 which also converts THC to THC-OH. Thus CBN concentrations found in hair could be higher compared to THC-concentrations [44]. The THC/CBN-ratio decreases due to the higher loss of THC in comparison to CBN. Samples in which the THC/CBN ratio has not decreased, are generally samples with very low THC concentration (the less THC present, the lesser it is affected by hydrogen peroxide).
THC-COOH is one of the metabolites of THC and CBN. Due to its hydrophilic nature in comparison to other cannabinoids, it is excreted more rapidly. THC-COOH is only scarcely present in the body and the incorporation in hair is even lower. The adaptation of sensitive methods is essential to be able to detect THC-COOH. A decrease of THC-COOH concentration was noted in almost all samples. When no change in THC-COOH concentration is observed, it could be hypothesized that the bleaching product was not uniformly applied. Additionally, small differences in measurements of THC-COOH due to its very low concentration range can have a big impact. For example, the concentration THC-COOH in sample 6 in the bleaching study is increased by 3.3 %. More specifically, 0.391 pg had increased to 0.404 pg after bleaching. The difference between both samples is smaller compared to the precision value (91 %). The differences in concentration of THC-COOH amongst all other bleached samples were bigger than the precision value. It can be concluded that sample 6 is influenced by instrumental error and the THC-COOH has decreased in all samples after bleaching.
The THC/THC-COOH ratio decreases in every sample. In samples were THC was more strongly affected, the THC-COOH ratio decreases more drastically.
In general it can be concluded that bleaching affected mostly THC and CBD. THC-COOH and CBN concentrations in hair are slightly less affected after bleaching.
Our findings are in contrast with the results found by Jurado et al. regarding the effect of bleaching on THC and THC-COOH in hair [29]. They found a decrease in THC concentration of 14 %, but

no observable effect of bleaching on THC-COOH. This data was derived from only 1 test subject, which is not enough to create sufficient statistical power. With more subjects, a more exact range of variation can be obtained. The study has also been performed more than 20 years ago. Conditions and product specifications were not given, so comparison of results has to be done with caution. Although GC-MS^2 is a very robust technique, small improvements over the years could have contributed to a better and more precise detection of cannabinoids in hair. The introduction of MS^2 and new chemicals surely played a role. However, more samples should be studied for their cannabinoid content in order to have a more precise view on how bleaching affects their concentrations.

The effect on CBD concentration after bleaching was also investigated, but interpretation on our initial results was difficult due to many inconsistencies. Samples used for this study were selected based on their THC-presence as determined from previous analysis, specifically to increase the likelihood to detect THC-COOH. During our initial analysis, CBD was only detected in a few samples. Additionally, CBD concentrations were always lower compared to THC and CBN. CBD was affected by the pollution of MSTFA on the EI-source. As a result, counts for CBD were highly reduced (the count range for IS of CBD was 1 - 10 instead of >100) or the IS of CBD was sometimes absent after analysis. Samples had to be retested after an autotune was performed and to limit further pollution of MSTFA on the EI-source it was decided to regularly run a sample with pure ethyl acetate (a solvent used frequently in GC-MS^2 analysis). This extra precautions (the“wash”-step and application of an autotune) made possible that the IS was detected in every sample. CBD in hair samples (both treated and control samples) was not always detected. Changes found in CBD concentrations after hair treatment could be linked to the specific treatment, but could also be related to the fact that CBD was not detected or that CBD was not present in the sample. This can lead to falsely drawn conclusions; e.g. a complete loss of CBD (only CBD in the treated sample) or an appearance of CBD (no CBD in the control sample).
The EI-source was cleaned extensively after all series (bleaching, perming and coloring of hair) were run. All samples for which enough hair was still available for analysis were retested according to the same preparation steps as applied for the previously analyzed bleached samples. Cleaning of the EI-source increased the counts for both the IS and for CBD detected in hair. The result of the 12 retested samples showed a mean decrease in CBD after bleaching of 32.9 % as already mentioned with figure 13. Samples 1 - 3 were not included as their value was below the LLOQ.

Incubation of hydrogen peroxide with non-deuterated cannabinoids (H-mix with CBD, THC and CBN) showed a decrease in all their concentrations. As mentioned before, H- and D^3 -mixtures should be added separately to avoid any effect on the deuterated drugs. If both are added simultaneously, only their area can be used to compare results. THC was the most affected as concluded from the results obtained from the test subjects. In contrast with these findings, CBD concentrations were lower in the *in-vitro* study and CBN was the least affected. Hydrogen peroxide is highly reactive on cannabinoids and chemically degrades them in its presence. Every hair product containing hydrogen peroxide can thus lower cannabinoid concentrations when applied. However, cannabinoid concentrations in hair after treatment were not affected in a similar way. The hair matrix shields cannabinoids from being fully exposed to hydrogen peroxide so they are spared.

7.2. Perming

The effect of a perming treatment on the hair is more achieved by ammonium thioglycolate (to open the cuticle and break the disulphide bonds). Hydrogen peroxide is also present, but it is solely used to reform the disulphide bonds so a curl can be obtained. The concentration of hydrogen peroxide is lower in comparison to the hydrogen peroxide found in bleaching products. Its effect on hair pigments is minimal. It is expected that the opening of the cuticle will allow cannabinoids to be released or be destroyed. This could result in a decrease of cannabinoid concentrations in hair.
THC content decreases in every treated sample. The average loss of THC is higher compared to THC decrease after bleaching is performed. It was already hypothesized in the bleaching study that if a higher concentration of THC is present in the control sample, it is expected for a higher decrease to occur. In case of low THC concentrations, the loss will be less. The same observation was made for the perming study, especially when the last sample (sample 11 in figure 15) is studied. Here, THC concentrations were highly reduced (up to 74.4 %). Additionally, samples derived from the same test subject to prepare samples 6 – 9 show a decrease in THC content from proximal to more distal hair segments. This observation cannot be made for samples 1 – 4 were THC concentrations are equal in all samples, but the percentage of decrease in samples of the same test subject are more consistent.
CBN concentrations are less affected by perming in comparison to bleaching. Decreases are less or absent: sample 10 had an outlier value which resulted in a small increase (was confirmed to be false after retesting) and no effect on CBN concentration was found in sample 4. The strong decrease of THC in combination with the weak effect of perming on CBN causes the THC/CBN ratio to highly decrease. This observation can be made for each individual sample and indicates that this ratio can be used to identify the application of perming as a hair treatment. THC-COOH concentration decreased for every sample, except in sample 3. Here the difference between the concentration before and after perming is inferior. Since the difference between the treated and the untreated samples is less than the precision value (i.e. 4.0 % compared to 9 % (calculated from 100 - 91 %)). Additionally, it can be concluded that the THC/THC-COOH ratio is affected in a similar fashion as found in the bleaching study. Only 2 samples had no observable decrease in their THC-COOH concentration. Low concentrations of THC-COOH (like THC) are less prone to decrease. The decrease found for the THC/THC-COOH ratio is highest for sample 11 due to a greater loss of THC in comparison to THC-COOH.
As explained extensively in the discussion of the results of the bleached samples, CBD results are also included. However interpretation on the firstly obtained results had to be done with caution. The excessive use of MSTFA complicated the detection and interpretation on the results concerning CBD. A complete loss (100 %) of CBD indicates that detection of CBD might have failed. After the EI-source was cleaned extensively with aluminum oxide powder, 10 samples were retested. Counts were increased after cleaning, allowing detection of CBD in every sample. A mean decrease of 36.2 % for CBD concentration was found. It can be concluded that the firstly obtained results including complete losses of CBD after perming were false. Additionally, the results of sample 9 and 10 were below the LLOQ for CBD.

The *in-vitro* study with thioglycolate in presence of non-deuterated cannabinoids showed no difference in their concentrations. D^3-mix was added after incubation of the product to avoid any effect of thioglycolate on the deuterated drugs. In contrast to hydrogen peroxide, thioglycolate

mainly exerts its effect in presence of ammonium. From our results, it can be concluded that without ammonium, no interaction takes place between thioglycolate and CBD, THC or CBN. It has to be noted that in another study, an effect between thioglycolate and EtG was observed [41]. This study used the same experimental procedure, but EtG was the investigated drug metabolite. Additionally, EtG is a much smaller molecule and could be more sensible to thioglycolate. Even though no effect was observed when thioglycolate was incubated with cannabinoids alone, the perming procedure showed to decrease all cannabinoid concentrations. We cannot conclude that there is a chemical degradation as observed for EtG, but it could be hypothesized that there is a leaching-out effect of cannabinoids due to the perming procedure. The hair could be rendered porous after the procedure, so cannabinoids are extracted out. To confirm this hypothesis, additional studies should be performed. For example, it could be interesting to investigate the waste after hair is washed for cannabinoid concentrations.
We solely investigated the effect of thioglycolate on cannabinoids. It is possible that other chemicals used in perming solutions have negative effects on cannabinoids. For future research, it would be interesting to also investigate these parameters.

Currently, there is no research that has investigated the effect of perming on cannabinoids found in hair. Other studies on different drugs (for example EtG) do exist, but these studies are not comparable as drugs of interest are different [41].

7.3. Temporary coloring

Temporary coloring is achieved by applying the dyeing product on the hair. In general, temporary coloring products are removed from the hair after washing it 6 to 8 times. The product is administered at the outside of the hair (cuticle) and does not affect the underlying layers. The hair matrix is not exposed, which is the case when bleaching or perming is carried out. Temporary coloring is thus a weak cosmetic hair treatment and is not expected to affect drug concentrations in the hair. Drugs incorporated in the hair are mainly found in the cortex, which should not experience any effect caused by temporary coloring.

THC concentrations in hair samples were low. Only 4 samples had a high THC content (samples 3 - 6), however these samples were derived from different persons. The highest decrease for THC was found in samples 5 and 6. Since concentrations are very low in other samples, the percentages of decrease may give a false indication on how strong the decrease after coloring effectively is. The highest percentages of decrease in these low concentration range have a high impact on the average percentage of decrease.

CBN content in these hair samples was higher compared to THC, but is less affected. The average percentage of decrease corresponds to the decrease found for perming. However, small increases as well as decreases in CBN concentrations are observed. In 4 samples (1, 2, 7 and 15) no effect of coloring was observed. Besides all the inconsistencies found for THC and CBN, their ratio (THC/CBN) remains rather unaffected. Only in samples 5 and 6 a large decrease in THC was observed, the ratio decreased as well after dyeing. Compared to the experiments performed for both bleaching and perming, it can be concluded that coloring has no strong impact on the THC/CBN ratio. CBN was not detected in samples 8 - 10 as their values were below the LLOQ.

THC-COOH concentrations were slightly increased in a few samples (3 and 4). One more decrease was noted for sample 11, but all other samples showed little to no effect of coloring on THC-COOH concentration. The smallest differences are lesser than the precision value of 9 % for THC-COOH, so these samples most likely didn't experience any effect of the dyeing product on their THC-COOH content. Performing a double derivatization with PFPOH and PFP eliminated the problem with the IS and the coloring product. The results of samples that were tested multiple times for their THC-COOH concentrations were more consistent in contrast to the results obtained for the concentrations of the other cannabinoids after retesting. The derivatization method with MSTFA used for CBD, THC and CBN doesn't seem to be optimized when a coloring product is used. The presence of chromatographic interference when the product is present may complicate both the analysis and interpretation of the results. The THC/THC-COOH ratio was slightly decreased for all samples. Samples 5 and 6 however had a high decrease in THC concentration, lowering their ratios more compared to other samples. Additionally, samples 10 and 15 who had a very small increase in THC-COOH concentration (< LOD) and also had a bigger change in their THC-COOH ratio. When these values are excluded, the mean THC/THC-COOH ratio does not change in comparison to the other cosmetic treatments (bleaching and perming). The THC/THC-COOH ratio is the lowest for the coloring experiment.

We were able to detect CBD concentrations in more samples compared to the bleaching and perming study, but the average decrease of CBD was higher than expected. Since we cleaned the EI-source after using it extensively for a long period of time and CBD content was re-measured for 13 samples of the bleaching and the perming study, we retested 13 additional samples from the coloring study for their CBD concentrations. The newly calculated decrease for CBD after coloring was 8.0 %. Samples 1 and 11-13 had CBD concentrations lower than the LLOQ, meaning that no

conclusions can be drawn from these results. Consequentially, those percentages are were not included.
The results of the *in-vitro* study with the temporary coloring product were already shortly mentioned in section 6.3. Samples with and without coloring product were compared for CBD, THC and CBN after spiking them with both H- and D^3-mix. All areas had slightly decreased (4 % for CBD, 2 % for THC and 5 % for CBN). From these results, it can be concluded that there is no effect of the temporary dyeing product on cannabinoids.

The previously mentioned study of Jurado et al. also investigated the effect of dyeing on THC and THC-COOH content in hair [29]. Initially, we hypothesized that temporary coloring had no effect on cannabinoids in hair as the coloring product is applied on the hair without any type of chemical treatment. However, we did find high decreases for THC and CBN, but lesser for CBD and THC-COOH. The introduction of a correction factor due to the decreased area of the IS was a convenient tool, but not the most precise solution. This part of our study should be re-investigated and another derivatization method should be used to avoid changes in areas.
The study of Jurado et al. found also decreases in THC and THC-COOH content (respectively 30 % and 60 %). However, Jurado et al. didn't perform the coloring of hair themselves. Hair was reported to be colored for 3 test subjects, but it was not specified which type of coloring product was used. It is possible that the highest decreases found in their study for both THC and THC-COOH are caused by a more permanent coloring treatment. It should also be mentioned that there is a difference between their *in-vivo* and our *in-vitro* coloring. It is possible that the hair of the subjects in the study of Jurado et al. was colored multiple times (as coloration has to be repeated every 8 weeks to also color new-grown hair). Multiple coloring procedures could affect the hair and leave it structurally weak. Cannabinoids could then be washed out and result in a higher decrease in their concentrations compared to *in-vitro* coloring. The thickness of the hair could also play a role. Additionally, the lack of more test subjects used in their study complicate any formulation of a conclusion. It is possible that these 3 subjects are all situated in the higher ranges of variation of decrease. Therefore, more test subjects should be used and more research should be performed.

7.4. Permanent coloring

Permanent coloring is, as the name suggests, achieved by opening the hair with hydrogen peroxide and adhering dye molecules to the hair matrix for a more prolonging effect as the coloring effect can be maintained for a period of 8 weeks. A conditioner is usually applied afterwards to return the hair to its normal state. However, only a small amount of hydrogen peroxide is used. The concentration is expected to be too low to affect cannabinoids in comparison to bleaching. Repetitive coloring could have a higher impact on the detection of cannabinoids in the more proximal segments. Increased porosity of hair can cause a leakage of certain drugs, but in our study the permanent coloring was only performed once.
Concentrations of THC and CBN after the first permanent coloring had mostly increased (30.3 and 31.5 % respectively), whereas CBD had decreased by 9.8 %. This observation was against our hypothesis that permanent coloring should not affect cannabinoids so drastically. The first sample had the highest concentration of THC and CBN, suggesting that there could have been a carry-over of both components during application of the coloring product and the rinsing steps. The coloring product was applied on all samples and massaged on the hair using the same gloves.

Additionally the hair samples were also washed in the same water container. To eliminate the possibility of transfer of cannabinoids, 4 new samples were prepared. This time, gloves were changed in between every application and hair was washed separately. The newly obtained results were used for further analysis.
THC content was very slightly affected after permanent coloring. Only sample 2 had a more noticeable decrease in THC. In comparison to our previously obtained results where an increase was found for THC by 30.3 %, Mean THC content had only decreased 6.2 % for the newly prepared samples. Also, for CBN a similar observation was made. The mean CBN content increased by 4.1 % (16 % if sample 1 was also included). An increase of 4.1 % may be related to variance in measurements.

Results on the THC/CBN ratio show a mean decrease after permanent coloring was applied. THC concentration had lowered while CBN concentrations were slightly higher, which affects their ratio. CBN was not detected in sample 1 (value was below the LLOQ). The THC/CBN ratio of sample 3 was the least affected (THC and CBN concentrations were low and were barely affected). THC-COOH concentrations were decreased in all samples, most notably in sample 3 were THC-COOH was the lowest amongst all samples (< 1 pg). An effect was expected on THC-COOH concentrations, but lesser compared to bleaching as a lower hydrogen peroxide concentration is utilized. Our results confirmed our hypothesis: a 10.9 % decrease versus 21.8 % after bleaching. In addition, the THC/THC-COOH ratio was mostly unaffected after permanent coloring. Some variance can be observed amongst the samples as both THC and THC-COOH are not always affected similarly. It should be mentioned that THC and THC-COOH are not measured simultaneously as they require a different sample preparation and detection method. NCI in combination with PTV increases sensitivity for THC-COOH (to a range of 0.1 pg). Since our method for THC is not very sensitive (LOD = 0.6 ng/mg), results of the THC/THC-COOH ratio might not be as meaningful. More samples should be included in order to investigate this ratio in-depth.
Prior to the application of the permanent coloring, the EI-source was cleaned so CBD detection was possible without problems. However, like mentioned in the first attempt of the temporary coloring, counts for the IS tend to drop slightly in the colored samples (giving an increased value). Small corrections were made and a mean decrease of 13.1 % was found. All samples had concentrations above the LLOQ, so all results could be included. The decreases were low in all samples, except for sample 4. CBD concentrations were rather low, so percentage of decrease changed more drastically in this lower concentration range.
Finally, the *in-vitro* study showed a decrease of 14 % for CBD after incubation with the permanent coloring product. THC had slightly decreased (2 %), whereas CBN had slightly increased (4 %). There was only an effect of the coloring product on the H-mix as the D^3-mix was only added after incubation right before SPE. Additionally, all areas had also increased (126 % for CBD, 66 % for THC and 10 % for CBN). As mentioned for the temporary coloring product, there can be an effect of the coloring product on the area calculated by the MassHunter-software. The state of the source (e.g. how clean it is) can impact results even further. Compared to the temporary coloring, the permanent coloring product increases all areas for each of the cannabinoids. We hypothesize that a chromatographic interference is the cause of this increase. This is highly speculative and should be tested in future studies. The most important effect is the increase in CBN after incubation with the product. Small changes in concentrations can be due to measurement variances. However, here the difference between the product and blank samples is 80 ng, which is much higher in

comparison to the 10 and 30 ng mentioned in the temporary coloring *in-vitro* study (see 6.6). This coloration product enhances detection of CBN over CBD and THC. The 4 % increase in CBN concentrations was also observed amongst colored hair samples. The increase in CBN concentration is not related to the product, but to the favored detection of CBN in presence of the product. For future studies, even more hair samples should be analyzed to confirm these results.

As mentioned for temporary coloring of hair, Jurado et al. described a decrease in THC and THC-COOH of 30 % and 60 % respectively [29]. In comparison, permanent coloring has less effect THC and CBN while CBD and THC-COOH are affected more strongly. However, all results are still much lower than the decreases found by Jurado et al and also compared to our own temporary coloring results. It seems that depending on the coloring product, the effect on cannabinoids is altered. Depending on the product composition, application time and repeated use, cannabinoid concentrations are spared or decreased. Products mention their composition, but not the exact concentration of hydrogen peroxide. Thus, different products have different effects. More research is needed to confirm these findings.

8. Conclusion

Various experiments using different hair treatments such as bleaching, perming and coloring indicate that there is an effect on cannabis content in hair.
The hydrogen peroxide present in bleaching product strongly effects all cannabinoids and degrades them chemically. However, the effect of hydrogen peroxide is mitigated by the hair matrix. THC, CBN and CBD concentrations decrease strongly, while THC-COOH content is slightly less affected.
Thioglycolate in perming products reacts poorly with cannabinoids. However, the combination of various chemicals in perming solutions strongly decreases THC and CBD concentrations. On the other hand, CBN and THC-COOH concentrations are far less affected. The THC/CBN ratio seems to be of high interest to determine if a perming procedure was used. The different observations made for cannabinoid concentrations between the *in-vitro* study and the application of perming products on hair could be designated to the fact that other chemicals might influence the stability of all cannabinoids. Another possibility for decreased concentrations could be related to a leaching-out effect of cannabinoids during the treatment, but additional research is required to solidify or reject this hypothesis.
Temporary coloring products are merely applied on the exterior part of the hair (cortex) and thus should barely impact cannabinoids in hair. However, we found decreases for all cannabinoids. Additionally, one must be aware of a possible effect on the area of the deuterated drugs when a coloring product is used. This could lead to a false interpretation of results.
In case of permanent coloring, results show that only a little to no effect can be observed on cannabinoid concentrations in hair. These products contain hydrogen peroxide, but in low concentrations so that it does not strongly affect cannabinoids.
Hair treatments such as bleaching and perming do affect cannabinoid concentrations in hair. This information should always be taken into account when the hair of such subjects is analyzed to have a good interpretation of their results.

9. References

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