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Abstract: Saffron, the red dried stigmas of Crocus sativus L., is considered as one of the most expensive spices worldwide, and as such, it is prone to adulteration. This study introduces an NMR-based approach to identify and determine the adulteration of saffron with Sudan I-IV dyes. A complete 1H and 13C resonance assignment for Sudan I-IV, achieved by two-dimensional homonuclear and heteronuclear NMR experiments, is reported for the first time. Specific different proton signals for the identification of each Sudan dye in adulterated saffron can be utilised for quantitative 1H NMR (qHNMR), a well-established method for quantitative analysis. The quantification of Sudan III, as a paradigm, was performed in varying levels (0.14-7.1 g/kg) by considering the NMR signal occurring at 8.064 ppm. The high linearity, accuracy and rapidity of investigation enable high resolution 1H NMR spectroscopy to be used for evaluation of saffron adulteration with Sudan dyes.

COVER LETTER

Title: "Sudan dyes in adulterated saffron (*Crocus sativus* L.): identification and quantification by ^{1}H NMR "

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4. Explanation of the manuscript significance:

The present study shows the application of ^{1}H NMR to detect the presence of artificial colourants such as Sudan I-IV dyes, being a potential adulterants affecting saffron, the most expensive spice on the global market. These colourants, that may be used for improving appearance of old and inferior quality saffron, should not be present in saffron according to the ISO 3632 standards; however, ISO 3632-2 provides chromatographic (TLC, HPLC) test methods for artificial water-soluble acid colourants but not for fat-soluble compounds, such as the Sudan dyes. The $CDCl_3$ extract of saffron allowed to identify in the aromatic region of ^{1}H NMR spectrum, specific resonances due to the adulteration with each Sudan dye. As the matter of fact that region resulted depleted of any significant signal attributable to saffron. Specifically, the typical signals of Sudan dyes, could be safely quantified by qHNMR. As a paradigm, in this study, the quantification of Sudan III by an internal and an external reference have been performed in the range of 0.14-7.1 g/kg. In both cases a high linearity was achieved enabling this approach useful for evaluating saffron adulteration with Sudan dyes. Moreover for the first time, a complete ^{1}H and ^{13}C NMR assignment for all Sudan (I-IV) are reported.

Highlights

- Complete ^{1}H and ^{13}C resonance assignment for Sudan I-IV dyes by NMR
- ^{1}H NMR spectroscopy for the direct identification of Sudan I-IV dyes in saffron
- Determination of Sudan III in saffron at a low of 0.14 g/kg by qHNMR

Sudan dyes in adulterated saffron (*Crocus sativus* L.): identification and quantification by ^{1}H NMR

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Abstract

Saffron, the red dried stigmas of *Crocus sativus* L., is considered as one of the most expensive spices worldwide, and as such, it is prone to adulteration. This study introduces an NMR-based approach to identify and determine the adulteration of saffron with Sudan I-IV dyes. A complete ^{1}H and ^{13}C resonance assignment for Sudan I-IV, achieved by two-dimensional homonuclear and heteronuclear NMR experiments, is reported for the first time. Specific different proton signals for the identification of each Sudan dye in adulterated saffron can be utilised for quantitative ^{1}H NMR (qHNMR), a well-established method for quantitative analysis. The quantification of Sudan III, as a paradigm, was performed in varying levels (0.14-7.1 g/kg) by considering the NMR signal occurring at 8.064 ppm. The high linearity, accuracy and rapidity of investigation enable high resolution ^{1}H NMR spectroscopy to be used for evaluation of saffron adulteration with Sudan dyes.

Keywords: Saffron; Quality control; Sudan dyes; ^{1}H and ^{13}C NMR; qHNMR; Adulteration

Chemical compounds studied in this article

Sudan I (PubChem CID: 5858445); Sudan II (PubChem CID: 5354447); Sudan III (PubChem CID: 5379348); Sudan IV (PubChem CID: 5876571); Chloroform-d (PubChem CID: 71583)

1. Introduction

Sudan dyes constitute a family of lipophilic azo-compounds, widely used in industries as colouring agents in products such as textiles, plastics, waxes, and polishes. The International Agency for Research on Cancer categorizes the dyes Sudan I to IV as Group 3 carcinogens (IARC, 1975) and their use in food is not approved in most countries, including the European Union (Commission decision, 2005). However, numerous notifications reported to the EU rapid alert system (RASFF) have demonstrated the illegal presence of these dyes in a range of foodstuffs, including saffron, since 2003.

Saffron, the spice obtained from the red dried stigmas of *Crocus sativus* L., is particularly liable to deliberate adulteration because it is considered as one of the most expensive spices worldwide (Moore, Spink, & Lipp, 2012). This spice is traditionally used for colouring and flavoring food and is endowed with a range of health promoting benefits (Winterhalter & Straubinger, 2000). Because of its high price and the production constraints, saffron has experienced various types of adulteration throughout the years (Hagh-Nazari & Keifi, 2007). Nowadays, the addition of artificial colourants is a common way of fraud. The quality control of saffron typically involves the determination and quantification of colourants suspected as potential adulterants. As a general rule, artificial colourants should be absent from saffron according to the ISO 3632 standards (ISO, 2010; ISO, 2011). However, ISO 3632-2 (ISO, 2010) provides chromatographic (i.e. TLC, HPLC) test methods for artificial water-soluble acid colourants but not for fat-soluble compounds, such as the banned Sudan dyes, that may be used for improving appearance of old and inferior quality saffron. Adulteration of saffron with synthetic dyes has been the subject of rather limited studies, with keen emphasis on water-soluble colourants such as erythrosine, ponceau 4R, and tartrazine (Hajimahmoodi et al., 2013; Lozano, Castellar, Simancas, & Iborra, 1999; Ordoudi & Tsimidou, 2011; Zalacain et al., 2005; Zougagh, Ríos, & Valcárcel, 2005). The only method reported so far for the detection of Sudan dyes in saffron has been

developed by Ates, Mittendorf and Senyuva (2011) using liquid chromatography/mass spectrometry (LC/MS) and cloud point extraction.

Today, there is a steady growth in reports concerning Sudan dyes detection mainly in chilli or curry powder and related food products. The majority of the analytical methods developed are based on LC coupled with UV-Visible (UV-Vis), photodiode array (PDA), or MS detection (Rebane, Leito, Yurchenko, & Herodes, 2010; Zhu et al., 2014). The main drawbacks of such chromatographic techniques are that they may be time-consuming and usually require sample manipulation. To overcome these handicaps, spectroscopic techniques that provide rapidity, reliability and ease of use could be applied.

Recent studies have successfully examined the potential of spectroscopic techniques such as Fourier transform mid-infrared (Ordoudi, De Los Mozos Pascual, & Tsimidou, 2014), near-infrared (Zalacain et al., 2005), Raman (Anastasaki et al., 2010), UV-Vis (Maggi et al., 2011) and nuclear magnetic resonance (NMR) spectroscopy (Cagliani, Culeddu, Chessa, & Consonni, 2015; Ordoudi, Cagliani, Lalou, Naziri, Tsimidou, & Consonni, 2015; Petrakis, Cagliani, Polissiou, & Consonni, 2015; Sobolev et al., 2014; Yilmaz, Nyberg, Mølgaard, Asili, & Jaroszewski, 2010; Yilmaz, Nyberg, & Jaroszewski, 2011) to assess saffron quality and authenticity parameters. UV-Vis spectroscopy, which is routinely used in saffron quality control, can be used for detecting Sudan dyes in saffron although it provides rather low sensitivity, as the detectable adulteration is 5 g/kg for Sudan I and II and 2 g/kg for Sudan III and IV, respectively (Sánchez, Maggi, Carmona, & Alonso, 2011). The ability of high resolution ^{1}H NMR combined with chemometrics to detect possible contamination of culinary spices including turmeric, curry, and mild or hot paprika with Sudan I-IV dyes has been recently demonstrated (Di Anibal, Ruisánchez, & Callao, 2011a, 2011b).

Over the past years, ^{1}H NMR spectroscopy has also attracted considerable attention as an efficient quantitative tool (Pauli, Gödecke, Jaki, & Lankin, 2012). The recent development of NMR spectrometers with high-field magnets as well as the advances in the probe technology have enabled the analysis of numerous compounds in low concentrations, with high precision and accuracy

(Bharti & Roy, 2012; Ohtsuki, Sato, Furusho, Kubota, Sugimoto, & Akiyama, 2013). As the signal integral is directly proportional to the associated number of protons, the quantification of the molecule under investigation can be easily performed by evaluating the integral ratio with a reference molecule. The lack of necessity for isolation and purification of the target component and the quantification capability using solely a well-resolved signal are also among the key advantages that quantitative ^{1}H NMR (qHNMR) offers. To our knowledge, the feasibility of qHNMR for the determination of Sudan dyes in spices and other foodstuffs has not yet been explored.

This paper introduces a simple, rapid and reliable method for the identification of the banned Sudan dyes I-IV in adulterated saffron by employing high resolution ^{1}H NMR spectroscopy. A complete ^{1}H and ^{13}C NMR assignment for all Sudan dyes was achieved by extensive use of NMR experiments, like two-dimensional total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), and two-dimensional heteronuclear single quantum coherence - total correlation spectroscopy (HSQC-TOCSY). The application of qHNMR is proposed herein for the quantification of Sudan III in adulterated saffron in levels that may practically impact the visual colour, without requiring elaborate sample preparation.

2. Materials and Methods

2.1. Chemicals

Sudan I standard (dye content: ≥95%) was acquired by Sigma-Aldrich (St. Louis, MO, USA) while Sudan II (90%), and Sudan IV (≥80%) were obtained from Aldrich (Steinheim, Germany). Sudan III (analytical standard purity: ≥96% HPLC) was acquired from Fluka (Steinheim, Germany). Chloroform (99.96 atom % D) was purchased from Euriso-Top (Saclay, France).

2.2. Standard solutions

The Sudan III standard was accurately weighed in the amount of 4 mg and dissolved in 1 mL of $CDCl_3$ as a stock solution, which was stored at 4 °C in the dark. Intermediate working solution at 0.36 mg/mL was prepared by serially diluting the stock solution with $CDCl_3$.

2.3. Sample preparation

An authentic Greek saffron sample (harvest 2012) of commercial category I, according to the ISO 3632 specifications, was provided by Kozani Saffron Producers Cooperative (Cooperative De Safran). Spiked saffron samples were prepared by adding the Sudan III working solution to the selected sample (10 mg), firstly at 1.4, 3.6, and 7.1 g/kg concentration levels (Di Anibal et al., 2011a,b) and then at 0.14, 0.36, and 0.71 g/kg. The latter three concentration levels were used to check the sensitivity of the ^{1}H NMR method. All concentrations are within the range of adulterated spices, as suggested by the American Spice Trade Association (ASTA, 2005). Accurately weighed quantities (10 mg) of pure Sudan I-IV dyes and the spiked saffron samples with Sudan III were dissolved in 600 μL of $CDCl_3$ and stirred for 3 min at room temperature. After 10 min, they were submitted to centrifugation at 12100 *g* for 10 min and then 500 μL aliquots of the supernatant were transferred into 5 mm NMR tubes for analysis.

2.4. NMR analysis

All spectra were recorded on a Bruker AV600 spectrometer (Bruker Biospin GmbH, Rheinstetten, Karlsruhe, Germany), operating at 14.09 T and equipped with a 5-mm inverse probe with a z-gradient at 300 K.

2.4.1. Quantitative ^{1}H NMR (qHNMR)

^{1}H NMR spectra were recorded under quantitative conditions using a 90° pulse experiment, collecting 128 scans over 32K data points and a relaxation delay of 40 s. The quantification of Sudan III dye in the $CDCl_3$ extracts of spiked saffron samples, at various concentration levels, could be carried out with a molarity-based quantification approach by using a calibration curve with a standard solution of whichever Sudan dye acquired under identical conditions used for all spiked

saffron samples; in the present case Sudan II has been considered. Moreover, the residual solvent signal (0.04% of $CHCl_3$ in $CDCl_3$) in spiked saffron samples was adopted as internal reference and the Sudan III (purity grade ≥96%) content was calculated by using the following equation (Bharti & Roy, 2012):

$$W_{SIII} = \frac{I_{SIII}}{I_{std}} \times \frac{N_{std}}{N_{SIII}} \times \frac{M_{SIII}}{M_{std}} \times \frac{P_{std}}{P_{SIII}} \times W_{std} \quad (1)$$

where *I*, *N*, *M*, *P* and *W* are signal integral value, number of protons generating the selected signal for integration, molar mass, purity and weight of Sudan III (*SIII*) and the reference standard (*std*) used, respectively.

The Sudan III content (g/kg) of spiked saffron samples was calculated using the following formula:

$$\text{Content (g/kg)} = \frac{W_{SIII} \times 1000}{W_{sample}} \quad (2)$$

with W_{sample} being the weight of the spiked saffron sample (0.010 g).

2.4.2. ^{13}C NMR

^{13}C NMR spectra of the Sudan I-IV samples were acquired with 1024 transients and 64K points covering 34000 Hz and a pulse width of 11 μs (90° pulse) with the inverse-gated decoupling pulse sequence from a Bruker library, to avoid the hetero-nuclear Overhauser effect derived from proton decoupling.

2.4.3. Two-dimensional NMR

Two-dimensional homo- and hetero-nuclear correlation NMR spectra (DQF-COSY, TOCSY, HSQC, HMBC, and HSQC-TOCSY) were acquired for Sudan I-IV spectra assignment. Spectra were typically acquired with 11 and 231 ppm over 2048 and 256 data points in proton and carbon

dimensions, respectively. TOCSY spin lock was set at 80 ms with the direct heteronuclear coupling constant at 145 Hz.

2.4.4. ^{1}H-NMR data processing

Exponential line broadening of 0.3 and 1.2 Hz were applied as resolution enhancement function for ^{1}H and ^{13}C spectra, respectively; zero-filling to 32K (^{1}H) and 64K (^{13}C) prior to Fourier transformation (TOPSPIN v. 3.0 software; Bruker Biospin GmbH Rheinstetten, Karlsruhe, Germany) was used. Manual phase adjustment and baseline correction were carefully performed. The spectra were referenced to the residual solvent signal at 7.26 ppm for ^{1}H and 77.16 ppm for ^{13}C spectra, respectively.

3. Results and discussion

The analysis of the ^{1}H NMR spectra of $CDCl_3$ extracts for Sudan I-IV dyes showed the majority of signals in the aromatic region with the only exception for methyl signals of Sudan II and Sudan IV dyes located in the aliphatic region. Notwithstanding the crowded aromatic region resulted increased according to the molecular complexity, the full resonance assignment has been achieved for both proton and carbon atoms, for the first time, by the analysis of homo- and hetero- nuclear bi-dimensional spectra of all Sudan dyes dissolved in $CDCl_3$, as reported in Table 1 (labelling according to Fig. 1). The combined use of HSQC, HMBC and HSQC-TOCSY allowed us to resolve assignment ambiguities due to spectral overlap in ^{1}H NMR spectra. All the aromatic signals of Sudan I-IV dyes occur in the spectral region between 6.750 and 8.650 ppm where no significant signals in the pure saffron spectrum are present (Fig. 2), thus enabling to monitor a selected spectral region for the detection of aromatic dyes. Additionally, Fig. 2 reports a red grid highlighting specific proton resonances that allowed univocal identification of each Sudan dye in adulterated saffron: proton 3 at 6.870 ppm for Sudan I, proton 8 at 8.635 ppm for Sudan II, protons 2' and 6' overlapped at 8.064 ppm for Sudan III, and proton 6' at 8.181 ppm for Sudan IV.

The quantification of the dye was evaluated by adopting the qHNMR requirements, concerning the NMR acquisition and processing procedures (Bharti &Roy, 2012). The primary requirement, the presence of one non-overlapping signal of the molecule to be quantified by integration (Beyer, Schollmayer, & Holzgrabe, 2010) was met in our case for at least one signal within all Sudan dyes, revealing high specificity and selectivity.

To check the linearity of the quantification within the adopted range for the presence of colourant addition, solutions consisting of Greek saffron sample of certain origin were spiked with Sudan III working solution, at six different concentrations: 0.14, 0.36, 0.71, 1.4, 3.6, and 7.1 g/kg. The quantification of Sudan III was carried out by considering the resonance at 8.064 ppm accounting for two diphenylazo protons (De Nino, Di Donna, Maiuolo, Mazzotti, & Sindona, 2008), namely 2' and 6'. This signal was integrated for all saffron samples spiked with Sudan III, and referred to the residual solvent signal. The calibration curve shown in Fig. 3A was generated based on the integral values and the corresponding amount of dye, obtaining a correlation coefficient of 0.999, thus confirming the high linearity of the measurements. These integral values were used to calculate, according to the equations (1) and (2) previously reported, the concentration of Sudan III with respect to the internal reference represented by the residual solvent signal of $CHCl_3$ (0.04%) in $CDCl_3$, as shown in Fig. 3B, and similarly resulted in a correlation coefficient of 0.999.

Alternatively, the quantification of Sudan III could be achieved by considering an external quantified reference standard. The results obtained considering a Sudan II standard solution (purity grade 90%) were here reported. In particular, the well isolated resonance of the naphthol proton 8, occurring at 8.635 ppm, was chosen for the molarity-based quantification. Also in this case, the results obtained in the concentration range of 0.14-7.1 g/kg indicated high linearity. Fig. 4 reports the calibration curve resulting in a correlation coefficient of 0.998. In both procedures the quantification of Sudan III in saffron was calculated evaluating a single isolated resonance of the aromatic moiety for this dye and this principle will be achievable for the other members of Sudan family, easily identifiable in saffron by monitoring specific isolated proton signals, as previously

discussed and highlighted in Fig. 2. The further quantification could be feasible by taking into proper account the number of corresponding protons, according to the full resonance assignment reported here for the first time. As a matter of fact, the use of an organic solvent like chloroform did not allow detection of any possible interfering signals for components of saffron containing aromatic rings. The proposed procedure presents some advantages in comparison with data fusion approaches (Di Anibal et al., 2011b) that requires meta-spectrum and multivariate data analysis; in the present work a single spectrum is required, obtained with high reproducibility and linearity of the response, allowing a single evaluation, without the use of a set of representative samples necessary for statistical treatment. Moreover, the use of high resolution ^{1}H NMR measurements allowed the detection of a lower concentration of Sudan III (0.14 g/kg) in saffron, when compared to other spectroscopic techniques like UV-Vis where the dye was detected at levels as low as 2 g/kg (Sánchez et al., 2011). Thus, the outcome of this procedure can be complementary to previous achievements (Ates et al., 2011).

Finally, the quantification of Sudan dyes could be performed by considering either an internal ($CHCl_3$) or an external reference, most likely a standard solution of any Sudan dye. In our opinion, the direct use of solvent as a reference for quantification is preferable to preparing a standard solution of dye, as experimental time is being reduced and both calibrant (solvent) and analyte (Sudan dye) are subjected to the identical experimental conditions, minimizing possible deviations. The lowest concentration quantified by qHNMR was as low as 0.14 g/kg; considering that the levels of adulteration that make practical economic sense exceed 0.12 g/kg and may reach 1 g/kg (ASTA, 2005), the method here proposed seems to be a valid tool for the detection of Sudan (I-IV) adulteration in saffron. The high linearity, accuracy and rapidity of investigation, enable this approach to be used for evaluation of saffron adulteration with Sudan dyes. The main advantage of using NMR is the minimal sample preparation and null chemical treatment. In this respect, water-soluble artificial colourants, usually analysed by other spectroscopic techniques, such as UV-vis, and liquid chromatography, could be investigated as well, avoiding the drawbacks of sample pre-

treatment and additional problems derived from the matrix effect that might influence the chromatographic resolution.

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Figure captions

Figure 1. Structure and labelling scheme for Sudan I-IV dyes analysed.

Figure 2. Aromatic region of ^{1}H NMR spectra of Sudan I-IV dyes and the pure Greek saffron analysed. From the bottom to the top pure saffron, Sudan I, Sudan II, Sudan III, and Sudan IV are represented. Specific signals for the identification of each Sudan dye in adulterated saffron are highlighted with the red grid.

Figure 3. Concentration by weight of Sudan III (g/kg) in adulterated saffron (in the range of 0.14-7.1 g/kg) *vs* (A) integral values and (B) concentration calculated (g/kg) for Sudan III considering the signal at 8.064 ppm scaled to the residual $CHCl_3$ signal at 7.260 ppm.

Figure 4. Concentration by weight of Sudan III (g/kg) in adulterated saffron (in the range of 0.14-7.1 g/kg) *vs* concentration calculated (g/kg) for Sudan III considering the signal at 8.064 ppm scaled to the standard solution of Sudan II considering proton signal at 8.635 ppm.

Table

Table 1. ^{1}H and ^{13}C NMR assignments for Sudan I-IV dyes dissolved in $CDCl_3$.

Atom	SUDAN I		SUDAN II		SUDAN III		SUDAN IV	
	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm	^{1}H ppm	^{13}C ppm
1	-	129,94	-	130,54	-	130,89	-	131,77
2	-	171,67	-	170,39	-	175,35	-	176,47
3	6,870	124,67	6,936	124,30	6,818	125,59	6,810	125,93
4	7,713	140,06	7,738	139,18	7,719	141,43	7,690	141,64
5	7,598	128,30	7,633	128,35	7,584	128,75	7,554	126,54
6	7,393	125,58	7,399	125,18	7,421	126,46	7,401	129,36
7	7,552	128,75	7,564	128,77	7,556	129,21	7,555	128,97
8	8,564	121,52	8,635	121,54	8,547	121,96	8,537	122,15
9	-	133,50	-	133,39	-	133,33	-	133,56
10	-	127,82	-	127,84	-	128,22	-	128,41
1'	-	144,54	-	140,99	-	150,72	-	143,74
2'	7,738	118,34	-	137,85	8,064	124,69	-	151,01
3'	7,481	129,66	7,113	131,56	7,828	118,31	7,825	125,73
4'	7,304	127,41	-	129,60	-	145,89	-	128,39
5'	7,481	129,66	7,170	127,89	7,828	118,31	7,931	123,01
6'	7,738	118,34	7,980	115,69	8,064	124,69	8,181	116,13
1''	-	-	-	-	-	152,78	-	138,36
2''	-	-	-	-	7,946	122,86	-	150,94
3''	-	-	-	-	7,531	129,07	7,351	131,43
4''	-	-	-	-	7,486	131,01	7,275	126,58
5''	-	-	-	-	7,531	129,07	7,351	131,43
6''	-	-	-	-	7,946	122,86	7,648	115,53
Me'	-	-	2,386	21,06	-	-	2,602	17,72
Me''	-	-	2,539	17,46	-	-	2,748	17,76

Figures

Figure 1

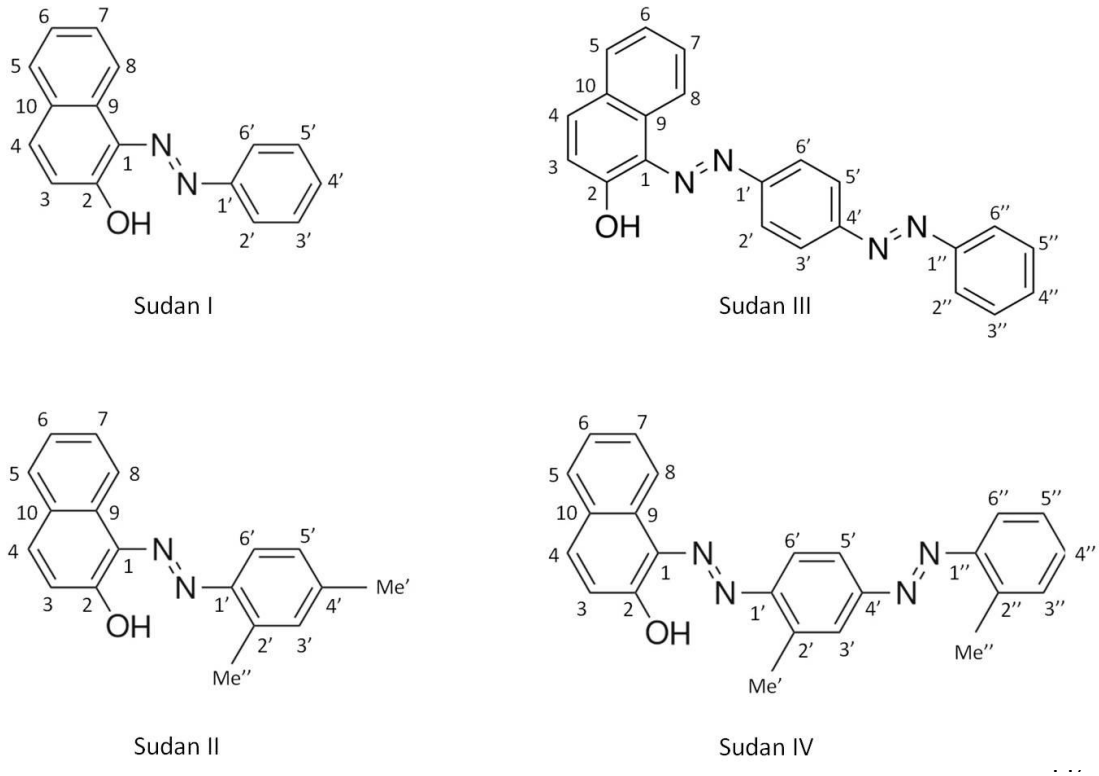


Figure 2

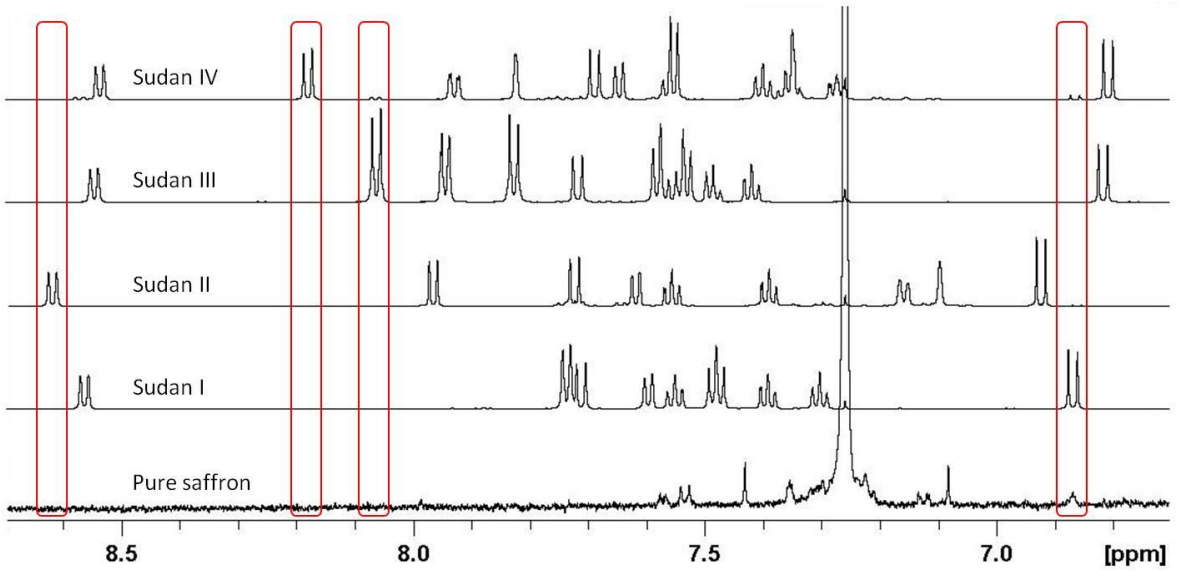


Figure 3A

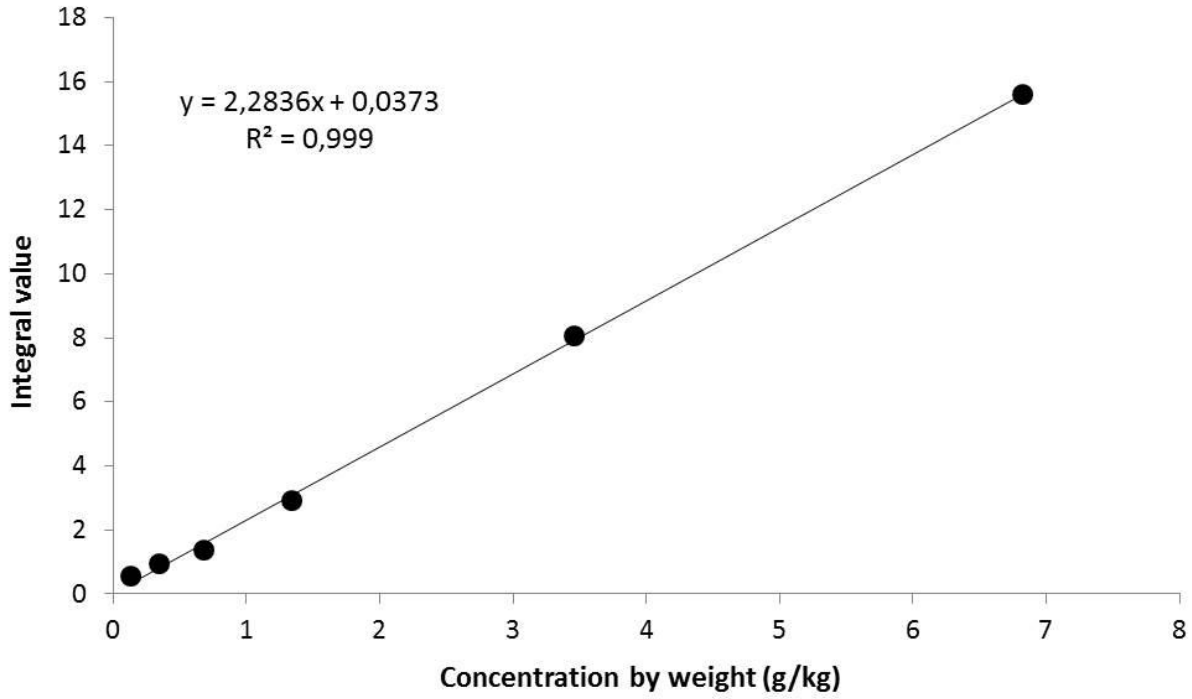


Figure 3B

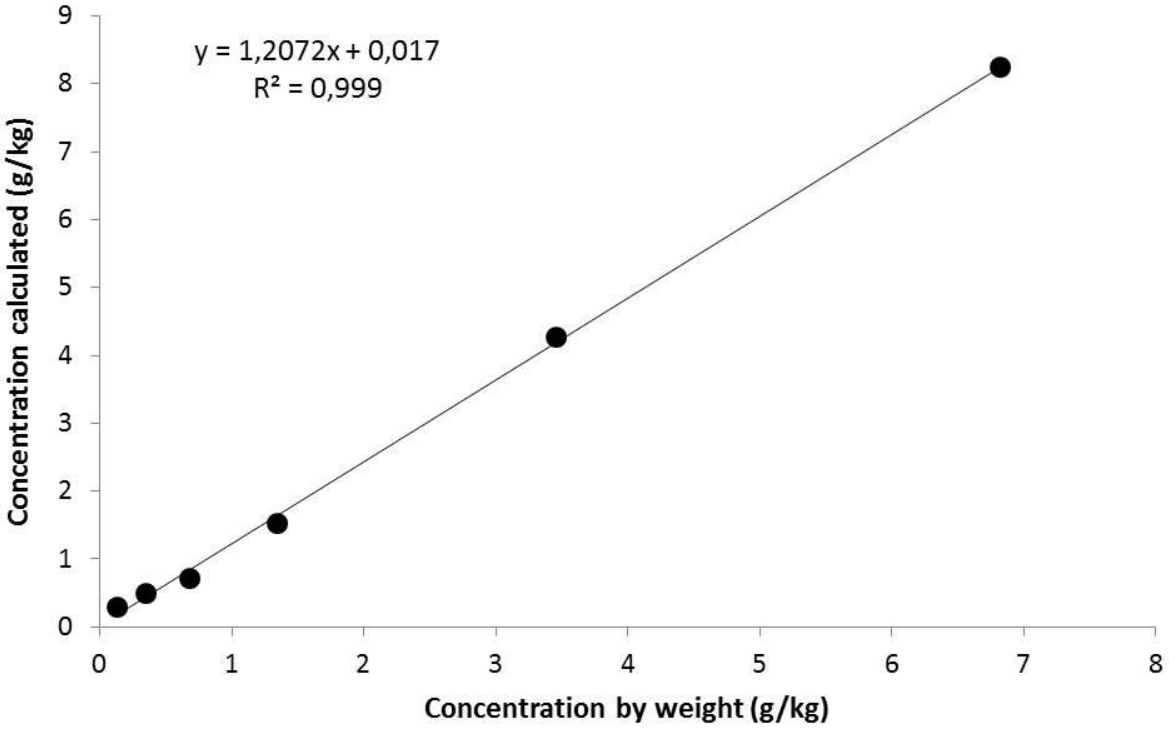
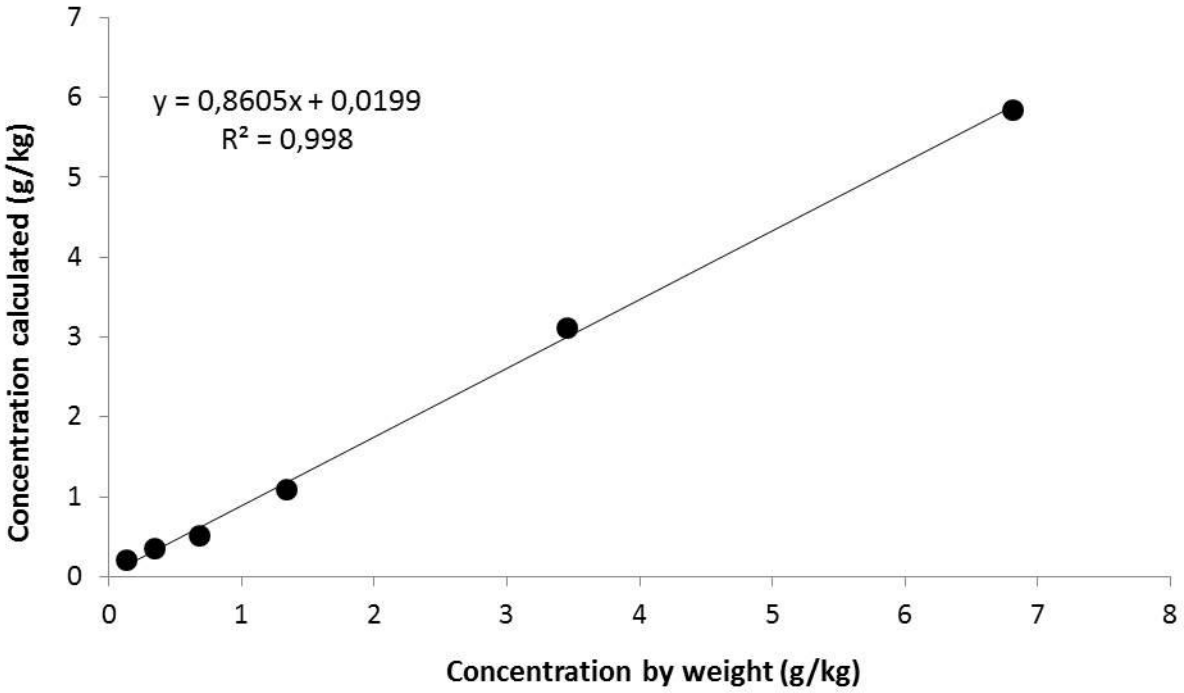


Figure 4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R² = 0,998
Concentration calculated (g/kg)
Concentration by weight (g/kg)
0
1
2
3
4
5
6
7
8