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^{1}H NMR spectroscopy combined with chemometrics for detection of turmeric adulteration in Italian saffron (*Crocus sativus* L.)

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ABSTRACT

Saffron, derived from the stigmas of *Crocus sativus* L., is a high-valued spice often adulterated due to its high price and economic profit. Among adulterants, one of the most common is turmeric (*Curcuma longa* L.). Detecting saffron adulteration with untargeted and non-destructive analytical approaches is important for food authenticity. In our study, 400 MHz proton NMR spectroscopy was used to analyse 138 extracts of authentic saffron and saffron adulterated with turmeric at three levels (2.5, 5 and 10 % w/w). Chemometric classification using Partial Least Squares Discriminant Analysis enabled discrimination between pure and adulterated saffron samples, with classification sensitivity of 98 % for pure saffron and 95 % for adulterated samples. This approach successfully detected adulteration at levels as low as 2.5 %, demonstrating the potential of ^{1}H NMR combined with multivariate analysis for saffron authenticity assessment, even at low concentrations of adulterants.

1. Introduction

Saffron is obtained from the dried red stigmas of *Crocus sativus* L. and it is the most expensive spice in the world, also referred to as the "red gold" (Varliklioz Er et al., 2017). It is widely used in the food industry as a colouring and flavouring agent and is also employed as a remedy in traditional medicine (Hosseinzadeh & Nassiri-Asl, 2013). Saffron is mainly cultivated and produced in countries with specific climates, including Afghanistan, Azerbaijan, China, Iran, India, Greece, Morocco, and Spain. Among all these countries, Iran is the world's largest producer with a market share of 90 % of the global production of saffron (Hegazi et al., 2022). Recently, there has been an increasing interest in saffron grown at different latitudes, including Italy (Cagliani et al., 2015). In Italy, saffron is mainly cultivated near L'Aquila (Piana di Navelli), in the regions of Sardinia (Province of Medio Campidano), Tuscany (San Gimignano, Florence Hills, and Maremma), and Umbria (Cascia and Città della Pieve) (Armellini et al., 2018). However, due to climate change, saffron is nowadays also grown at higher altitudes, such as in the regions of Piemonte, Lombardy, and Trentino-Alto Adige (Angeli et al., 2024).

The key quality markers of *Crocus sativus* L. are crocins, which impart color; picrocrocin, which contributes to the bitter taste; and safranal, which is responsible for the distinctive odor and aroma of saffron. (Kanakis et al., 2004). The quality and commercial value of saffron are defined by the specifications outlined in the ISO/TS-3632 standard (ISO 3632–1, 2011; ISO 3632–2, 2010). This regulation establishes the spectrophotometric quantification of crocins in aqueous saffron extracts, along with picrocrocin and safranal, thanks to their typical absorbance at 440, 257 and 330 nm. However, this method presents some limitations due to the low water-solubility of safranal and its absorption in the 320–340 nm range, that is the same as for cis-crocin isomers (Avula et al., 2022; Si et al., 2022).

Among other spices, saffron is often subject to adulteration to increase economic profit, mainly due to its high price in the market and limited production. Main adulterants include turmeric, paprika, saffron stamens, safflower, and marigold (Sabatino et al., 2011). Thus, there is considerable interest in developing methods to verify the authenticity of saffron (Kumari et al., 2021; Sobolev et al. 2014, 2022). Specifically, the focus is on untargeted approaches capable of detecting a broad spectrum of unknown adulterants, particularly those that require minimal sample preparation and are easy to implement. Infrared spectroscopy has been explored for assessing saffron adulterated with various plant-based

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adulterants (Amirvaresi et al., 2021; Biancolillo et al., 2020; Petrakis & Polissiou, 2017) and for determining the authenticity of traded saffron (Ordoudi et al., 2014). Untargeted metabolite fingerprinting methods based on UPLC-MS coupled with multivariate data analysis were used to distinguish saffron samples according to their geographic origin or adulteration level (Angeli et al., 2024; Rubert et al., 2016). In addition, thin-layer chromatography coupled to chemometrics has been used to detect saffron adulteration (Sereshti et al., 2018).

Nuclear magnetic resonance (NMR) spectroscopy is widely used as an advanced analytical technique due to its velocity and reproducibility, offering the potential for high-throughput analysis with minimal sample preparation (Samaha et al., 2021; Sobolev et al., 2022). Various studies have involved low-field and high-field (400 MHz and above) ^{1}H NMR for investigating authenticity and quality of saffron, often in combination with chemometrics. Studies reported in the literature include the detection of adulteration using plant-based materials. For instance, saffron adulterations with *Arnica montana*, *Calendula officinalis*, and *Carthamus tinctoris* were detected comparing results obtained with a 60 MHz benchtop NMR and a high field NMR (Gunning et al., 2023). In another study, ^{1}H NMR was used to successfully detect adulteration of turmeric and safflower at 5, 10, and 20 % of adulterant (Musio et al., 2022). Dowlatabadi et al. (2017) used ^{1}H NMR to detect up to 10 % adulteration with tartrazine and safflower. High-field NMR spectroscopy is well-suited for this purpose, as it provides comprehensive information on the soluble constituents of a sample without prior separation. This approach preserves the original ratios of compounds present in the sample, ensuring an accurate analysis of the chemical composition (Cagliani et al., 2015; Petrakis et al., 2015). However, the NMR is limited by its low sensitivity, making it less suitable to detect adulteration levels at very low concentrations (below 5 % w/w) (Dowlatabadi et al., 2017). For instance, mass spectrometry offers significantly higher sensitivity, enabling the detection of adulterants at levels below 0.2 % (Aiello et al., 2018). Moreover, the complexity of NMR data in food metabolomics needs its integration with multivariate statistical analysis, which facilitates data interpretation and improves the detection of small compositional differences.

Detection of low levels of adulteration in saffron remains a significant challenge, prompting the food industry to look for fast, robust and reliable analytical techniques. Given the known limitations in NMR sensitivity, this study aims to leverage a chemometric-enhanced approach to detect turmeric adulteration at levels as low as 2.5 %. The hypothesis is that ^{1}H NMR technique combined with multivariate analysis is effective in distinguishing pure saffron from samples containing 2.5 % of turmeric powder with a suitable level of accuracy.

2. Materials and methods

2.1. Samples

Saffron samples (0.5 g x 2) harvested in 2022 were purchased directly from eleven Italian local producers and two local supermarkets, according to Table 1. Two samples were purchased for each producer and were treated as biological replicates. Turmeric powder for adulteration was purchased from a local supermarket.

2.2. Sample preparation

Saffron samples were initially ground with a Precellys Evolution (VWR, Darmstadt, Germany) at a speed of 8000 rpm, with 3 cycles of 25 s and intervals of 20 s. The obtained powders were freeze-dried. For each biological replicate, at least two aliquots of 20 mg were weighed and used as independent samples for analysis, thereby enhancing the robustness of the data. To prepare the adulterated samples, specific amounts of turmeric were added to each aliquot as follows: 19.5 mg of saffron with 0.5 mg of turmeric for 2.5 % adulteration; 19 mg of saffron with 1 mg of turmeric for 5 % adulteration; and 18 mg of saffron with 2 mg of turmeric for 10 % adulteration (Table 1). In total, 61 pure saffron samples and 77 adulterated samples were prepared by combining different proportions by weight of ground saffron with turmeric (nominally 2.5, 5, 10 % w/w of adulterant). Then, 20 mg of powder (pure saffron and mixtures) were extracted in 1 mL deuterated dimethylsulfoxyde containing tetramethylsilane as internal standard (DMSO-*d6*, 99.9 % D, 0.03 % (v/v) tetramethylsilane (TMS), Merck Life Sciences, Milano, Italy). The samples were vortexed for 30 min, centrifuged (22 °C, 10000 rpm, 10 min) and the supernatant was filtered and transferred in tubes for the ^{1}H NMR analysis (Wilmad-LabGlass High-Throughput NMR Tube, diameter 5 mm, ATS Life Sciences Wilmad, Cambridge, Ontario, Canada). Therefore, 138 samples were considered: 61 samples were labelled as pure, while 77 samples as adulterated, of which 25 adulterated with 2.5 % of turmeric, 27 with 5 % and 25 with 10 % of turmeric.

2.3. ^{1}H NMR analysis

The one-dimensional ^{1}H NMR spectra acquisition were recorded on a Jeol ECZ-400 R MHz spectrometer (Jeol, Basiglio, MI, Italy), equipped with a high resolution 5 mm wideband Z gradient probe. All spectra were collected at 25 °C, with 16384 complex points, using a pulse length of 90°. A total of 256 scans per sample were acquired in 2.732 s with a recycle delay of 20 s. The acquisition parameters were the following: spectrum width (SW) = 12 ppm, frequency offset = 5 ppm, 90° ^{1}H pulse = 6 μs, receiver gain = 56, relaxation delay = 20 μs. The ^{1}H NMR spectra were processed by Delta software and MestReC 6.0.2 software (Santiago de Compostela, Spain, EU): all spectra were Fourier transformed, zero filled to 64,000 data points and 0.2 Hz line broadening function was applied. Phase and baseline corrections were automatically performed. Alignment was relative to the TMS standard (0 ppm). The signal of water in the region at 3.4 ppm and of DMSO at 2.5 ppm were suppressed using the dante presaturation algorithm of the Delta software. ^{1}H NMR spectra were standardised with respect to the TMS reference peak at 0 ppm. Area of the standardised NMR peaks was calculated with MestReC 6.0.2 software (Santiago de Compostela, Spain, EU). Both standardised ^{1}H NMR spectra and areas of the standardised ^{1}H NMR peaks were used as input variables for the subsequent chemometric modelling.

2.4. Multivariate data analysis

Initially, data structure was analysed by means of Principal Component Analysis (PCA), which is a well-known multivariate technique for exploratory data analysis (Bro & Smilde, 2014). PCA projects the data in a reduced hyperspace, defined by orthogonal principal components, which are linear combinations of the original variables, with the first principal component having the largest variance, the second principal component having the second-largest variance, and so on. Loadings are the variable coefficients in the linear combinations which define the components, while scores represent the coordinates of samples in the principal component space. Subsequently, Partial Least Square Discriminant Analysis (PLSDA) was used as multivariate classification method to discriminate between pure and adulterated saffron samples (Barker & Rayens, 2003). PLSDA is one of the most used linear classification approaches among machine learning classifiers and it is essentially based on the PLS2 algorithm that searches for latent variables with a maximum covariance with the Y-variables. In PLSDA, the Y-block encodes class labels of samples with a dummy binary matrix. PLSDA predictions are basically quantitative, with values in-between 0 and 1, from which class probabilities can be estimated (Pérez et al., 2009; Pérez et al., 2009). Samples can thus be assigned to the class associated with the maximum probability. The optimal number of latent variables (LVs) was selected by means of internal cross validation procedures, by minimising the cross-validation error in classification. When modelling standardised ^{1}H NMR spectra, data were mean-centred, while areas of peaks were autoscaled.

Table 1
Sample name, company location, type of material, year of harvesting and adulteration levels of saffron samples.

Sample	Saffron (mg)	Turmeric (mg)	Total weight (mg)	Saffron Origin	Type of material	Year
A1_1	20.12		20.12	South Tyrol (BZ)	dried stigmas	2022
A1_2	20.12		20.12			
A1_2.5 %	19.55	0.5	20.05			
A1_5 %	19	0.99	19.99			
A1_10 %	18	1.99	19.99			
A2_1	20.04		20.04			
A2_2	20.15		20.15			
A2_2.5 %	19.5	0.5	20			
A2_5 %	18.99	1.06	20.05			
A2_10 %	17.98	2.02	20			
B1_1	20.08		20.08	Trentino (TN)	dried stigmas	2022
B1_2	20		20			
B1_2.5 %	19.5	0.5	20			
B1_5 %	19.05	0.97	20.02			
B1_10 %	18	2.02	20.02			
B2_1	20.2		20.2			
B2_2	20.1		20.1			
B2_2.5 %	19.49	0.51	20			
B2_5 %	18.98	1.05	20.03			
C1_1	20		20	Lombardia (CO)	dried stigmas	2022
C1_2	20.05		20.05			
C1_2.5 %	19.53	0.62	20.15			
C1_5 %	19	1.36	20.36			
C1_10 %	17.99	2.02	20.01			
C2_1	20.08		20.08			
C2_2	20.05		20.05			
C2_2.5 %	19.51	0.48	19.99			
C2_5 %	19.01	0.98	19.99			
C2_10 %	18.1	1.92	20.02			
D1_1	20		20	Calabria (RC)	dried stigmas	2022
D1_2	19.99		19.99			
D1_2.5 %	19.53	0.46	19.99			
D1_5 %	19.04	0.96	20			
D1_10 %	18.06	2	20.06			
D2_1	20.1		20.1			
D2_2	20.05		20.05			
D2_2.5 %	19.5	0.49	19.99			
D2_5 %	19.07	0.93	20			
D2_10 %	18	2.02	20.02			
E1_1	20.05		20.05	Umbria (PG)	dried stigmas	2022
E1_2	20.02		20.02			
E1_2.5 %	19.49	0.51	20			
E1_5 %	19.01	1.19	20.2			
E1_10 %	18.04	1.97	20.01			
E2_1	20.06		20.06			
E2_2	20		20			
E2_2.5 %	19.5	0.89	20.39			
E2_5 %	19.01	1	20.01			
E2_10 %	18	2.06	20.06			
F1_1	20.06		20.02	Abruzzo (CH)	dried stigmas	2022
F1_2	20.02		20.02			
F1_3	20.16		20.16			
F1_2.5 %	19.51	0.49	20			
F1_5 %	18.99	1.03	20.02			
F1_10 %	18.04	1.95	19.99			
F2_1	20		20			
F2_2	20.03		20.03			
F2_3	20.11		20.11			
F2_2.5 %	19.5	0.5	20			
F2_5 %	19	1	20			
F2_10 %	18.04	1.97	20.01			
G1_1	20.02		20.02	Piemonte (AT)	dried stigmas	2022
G1_2	20		20			
G1_3	20.11		20.11			
G1_2.5 %	19.5	0.5	20			
G1_5 %	18.98	1.04	20.02			
G1_10 %	18.03	1.99	20.02			
G2_1	20.06		20.06			
G2_2	20.05		20.05			
G2_2.5 %	19.51	0.48	19.99			
G2_5 %	19.03	0.96	19.99			
G2_10 %	18.03	1.97	20			
H1_1	20		20	Lazio (RO)	dried stigmas	2022
H1_2	19.99		19.99			

(*continued on next page*)

Table 1 (*continued*)

Sample	Saffron (mg)	Turmeric (mg)	Total weight (mg)	Saffron Origin	Type of material	Year
H1_2.5 %	19.51	0.51	20.02			
H1_5 %	19.03	0.97	20			
H1_10 %	18.02	2.04	20.06			
H2_1	20.06		20.06			
H2_2	19.98		19.98			
H2_3	20.11		20.11			
H2_2.5 %	19.49	0.5	19.99			
H2_5 %	19	1.02	20.02			
H2_10 %	18.03	2.26	20.29			
I1_1	19.99		19.99	"Primia" (supermarket)	powder	na
I1_2	20		20			
I1_2.5 %	19.52	0.48	20			
I1_5 %	19.02	0.97	19.99			
I1_10 %	18.05	1.96	20.01			
I2_1	20.01		20.01			
I2_2	20.04		20.04			
I2_2.5 %	19.52	0.49	20.01			
I2_5 %	18.99	1	19.99			
I2_10 %	18	2	20			
J1_1	20		20	"Tre cuochi" (supermarket)	powder	na
J1_2	20.04		20.04			
J1_2.5 %	19.49	0.49	19.98			
J1_5 %	19	1.05	20.05			
J1_10 %	18.04	1.97	20.01			
J2_1	20.02		20.02			
J2_2	20.04		20.04			
J2_2.5 %	19.5	0.49	19.99			
J2_5 %	19.05	0.97	20.02			
J2_10 %	18.05	1.94	19.99			
L1_1	20.08		20.08	Marche (AN)	dried stigmas	2022
L1_2	20.08		20.08			
L1_3	20.06		20.06			
L1_2.5 %	19.51	0.5	20.01			
L1_5 %	19.03	0.97	20			
L1_10 %	18.03	1.98	20.01			
L2_1	20		20			
L2_2	20.04		20.04			
L2_3	19.99		19.99			
L2_2.5 %	19.5	0.5	20			
L2_5 %	19.04	0.96	20			
L2_10 %	18.02	1.98	20			
M1_1	20.03		20.03	Sicilia (CT)	dried stigmas	2022
M1_2	20.06		20.06			
M1_3	20		20			
M1_2.5 %	19.52	0.48	20			
M1_5 %	19.02	0.98	20			
M1_10 %	18.08	1.93	20.01			
M2_1	20		20			
M2_2	20.07		20.07			
M2_2.5 %	19.54	0.47	20.01			
M2_5 %	19.02	1.11	20.13			
M2_10 %	18.05	1.99	20.04			
N1_1	20		20	Abruzzo (AQ)	dried stigmas	2022
N1_2	20.08		20.08			
N1_3	20.05		20.05			
N1_2.5 %	19.51	0.49	20			
N1_5 %	19	1.01	20.01			
N1_10 %	18.02	1.98	20			
N2_1	20.04		20.04			
N2_2	20.03		20.03			
N2_3	20.08		20.08			
N2_2.5 %	19.48	0.5	19.98			
N2_5 %	19.02	0.98	20			
N2_10 %	18.06	1.94	20			

However, similarities between ^{1}H NMR spectra of pure and adulterated samples were expected, especially when dealing with low levels of turmeric as adulterant and therefore overlaps between classes were expected too. When using classification models on complex data with overlapping classes, the traditional approaches can fail. One way to better handle such discrimination tasks is to build hierarchical models, where subclasses are gradually separated, one or a few at a time (Marchi et al., 2022), thereby facilitating the task of the models in separating the most overlapping classes. The following PLSDA-based hierarchical-approach was therefore used to better deal with the discrimination of samples with adulteration at 2.5 % level of turmeric; in particular, we trained two independent models: a) model A was trained with PLSDA to discriminate samples of pure saffron vs samples with adulteration equal or higher than 5 %; b) model B was instead trained with PLSDA to separate samples of pure saffron from samples with adulteration equal to 2.5 %. Unknown or test samples can be predicted as follows: first, model A is applied and if a target sample is classified by model A as "adulterated", then it is labelled with that class; on the

opposite, if model A predicts the target sample as “pure”, then it is predicted by model B: if model B predicts the sample as “pure” too, this label is confirmed, otherwise the target sample is labelled as adulterated.

Hierarchical models were calculated with ad-hoc MATLAB functions; PLSDA models were calculated by means of the classification toolbox for MATLAB (D. Ballabio and Consonni, 2013).

2.5. Validation of classification models

The performance of the overall hierarchical classification approach was tested with a double validation procedure (Ballabio, Robotti, et al., 2018; Varmuza & Filzmoser, 2009), which was iteratively repeated for 100 times. At each iteration, samples were randomly divided into temporarily test (20 %) and training (80 %) sets, maintaining the proportion between classes. For any given pure sample randomly assigned to either the test or training set, all of its replicates and corresponding spiked sub-samples were constrained to be assigned exclusively to the same set. This ensures that the assumption of independence is respected and avoids over-optimistic assessment of the predictive capability of the models. Training samples were used to calibrate PLSDA models A (pure vs 5 and 10 % adulterants) and B (pure vs 2.5 % adulterants). At each iteration, the selection of the number of LVs for each model was carried out by internal cross validation of training samples (thus independently from the test samples). Test samples were then predicted with models A and B and labelled as “pure” or “adulterated” by means of the hierarchical procedure previously described. Sensitivity for pure and adulterated classes were used as figures of merit to evaluate the model discrimination capability: sensitivity refers to the percentage of samples belonging to a class and correctly classified in the class. In addition, non-error rate (NER) was calculated as the average of the sensitivity values (Ballabio, Grisoni, & Todeschini, 2018).

3. Results and discussion

3.1. NMR spectral windows for saffron authentication

The typical ^{1}H NMR spectrum of an authentic saffron extract (A1) is shown in Fig. 1. To minimize the variability, the spectral regions corresponding to the DMSO-d6 peak at 2.5 ppm and the water peak at 3.4 ppm were suppressed, as they are influenced by water absorption in DMSO-d6 and the dehydration state of the samples. Given the need for a detailed fingerprint despite the presence of minute adulterant amounts, it is advisable to exclude regions containing confounding information. This approach aligns with the previously published study on seized controlled substances, where unknown or poorly controlled storage conditions caused similar challenges (Antonides et al., 2019).

According to Fig. 1, the peaks appear well resolved. The resonance corresponding to the peaks of most important hydrogens are marked according to the assignments by Cagliani et al. (2015). This includes several resonances originating from key metabolites in saffron. Picrocrocin produces prominent peaks at 1.16, 1.18, 2.10, and 10.05 ppm, with the last being a singlet from the aldehydic proton of the 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde group (Gunning et al., 2023). This signal appears in an unusual region of the ^{1}H NMR spectrum, making it a potential helpful visual indicator of saffron. The group of peaks between 6.50 and 7.40 ppm represent the resonances of the conjugated double bonds of crocins. Kaempferol signals were recognized by resonances at 8.05, 6.91, 6.43 and 6.19 ppm, even though this compound is present in very low concentration, as previously reported. At high field intense singlets from the methyls of picrocrocin at 1.16, 1.18 and 2.10 ppm and of crocins at 1.97 and 2.00 ppm are the most characteristic in the ^{1}H NMR spectrum (Cagliani et al., 2015). Although crocins are typical compounds in saffron, they cannot be used alone as authenticity markers due to the low sensitivity of the NMR technique. Indeed, turmeric also contains crocins.

A further selection of peaks at specific regions of the ^{1}H NMR spectra was carried out as they showed increasing relationships to the addition of turmeric. The selected peaks are listed in Table 2. The selection was

Table 2
Selected NMR peaks for multivariate analysis.

Spectral window ID	Spectral window (ppm)	Peak
1	7.55–7.57	7.56
2	7.32–7.35	7.33
3	7.13–7.15	7.14
4	6.03–6.07	6.06
5	6.03–6.07	6.04
6	3.83–3.84	3.84
7	1.81–1.83	1.825

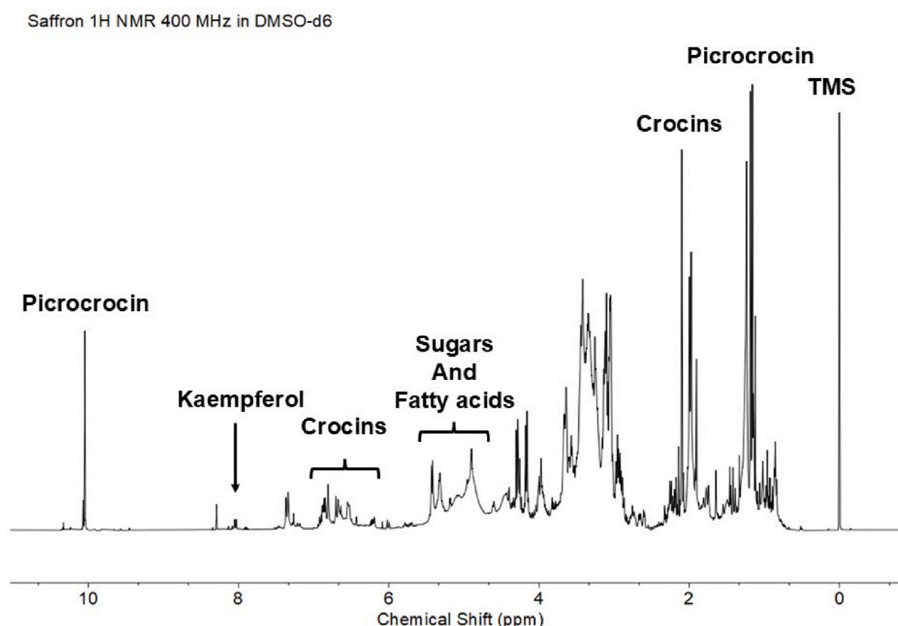

Fig. 1. ^{1}H NMR spectrum of an authentic saffron sample.

based on the information reported in previous studies related to the vinyl area of curcuminoids (6.0–7.6 ppm) in turmeric extracted with DMSO-*d6* (Praveen et al., 2021). In particular, curcumin has a signal at 6.04, 6.73, 6.77 ppm, 7.56 and 7.61, demethoxycurcumin has signals at 6.67, 6.71, 6.74, 6.78 ppm, 7.56 and 7.61, while bisdemethoxycurcumin has signals at 6.67, 6.71, 7.56 and 7.61 ppm, as reported in Fig. 2. (Gören et al., 2009; Praveen et al., 2021). The chemical shift at 7.14 ppm was attributed to tyrosine (Nurani et al., 2021), while the shift at 7.33 ppm belongs to the resonance peaks of crocins (Musio et al., 2022). Moreover, a difference in signal at 3.84 ppm was attributed to the sucrose multiplet resonance (Nurani et al., 2021) while the chemical shift at 1.82 ppm is attributed to picrocrocin (Cagliani et al., 2015).

Summarising, the subsequent multivarate models based on the PLSDA classification approach were calculated considering as input variables: a) the windows of the standardised ^{1}H NMR spectra associated to the seven selected peaks or b) the areas of these peaks. In the former case, a total number of 187 variables were used, while only 7 variables were used when considering the peak areas.

3.2. Exploratory analysis based on windows of the standardised NMR spectra and normalized NMR peak areas

A preliminary exploratory analysis of the two was carried out by means of Principal Component Analysis (PCA). In particular PCA scores are shown in Fig. 3. Samples are coloured in a grey-scale according to the % of adulteration, from white (pure samples) to black (10 % adulteration). When calculating PCA considering ^{1}H NMR windows, data were mean-centred; when looking at PC1 and PC3 (Fig. 3a), which explains 91.5 and 1.3 % of variability, a pattern of distribution of samples with respect to the level of adulteration is evident, even if some overlaps exist between groups with different levels of adulterations. When considering the PCA results achieved when analysing the area of NMR peaks (in this case data were auto-scaled), the same pattern is evident, as shown in the score plot of PC1 (63.5 % explained variance) and PC2 (19.9 % explained variance) (Fig. 3b).

3.3. Detection of saffron adulteration based on windows of the standardised NMR spectra

The dataset consisted of 138 samples, labelled as pure (61 samples) or adulterated (77 samples). Initially, classification models based on PLSDA were calculated considering as input variables the standardised ^{1}H NMR spectra associated to the selected peaks. The hierarchical PLSDA modelling was validated through 100 iterations of the double validation procedure previously described. Predictions of test samples were used to estimate the predictive capability of such an approach to detect saffron adulteration and distinguish pure from adulterated saffron samples.

The hierarchical modelling resulted in a sensitivity of 90 % and 97 % for pure and adulterated test samples, respectively, meaning that the majority of test samples were correctly classified (Non-Error Rate equal to 94 %). These results indicate an excellent capability of the model in discriminating authentic saffron samples from samples adulterated with turmeric with percentages ranging from 2.5 to 10 %. A detailed analysis of the predictions achieved with the double validation procedure over the test samples is summarized in Table 3, where the percentage of correct classifications of test samples is shown as a function of the adulteration level. As expected, the classification of samples adulterated with 2.5 % turmeric was more challenging. However, 91.7 % of samples were correctly predicted (misclassification rate of 8.3 %). On the contrary, adulterations associated with the addition of higher turmeric levels (5 % and 10 %) were always correctly predicted. This result is better compared to the previously reported for other spectroscopic techniques. A study reported a good linearity in the range between 10 and 50 % of saffron adulteration with attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy, Raman spectroscopy, and laser-induced breakdown spectroscopy (LIBS), with a LOQ of 9.32 % (Varliklioz et al., 2017). In another study performed with thin-layer chromatography coupled with Raman spectroscopy, the lowest level of adulteration with turmeric that could be detected, was 41 % (Dai et al., 2020). Vis-NIR hyperspectral imaging coupled with independent component analysis showed the sensitivity of 95 % for adulteration levels between 5 and 35 % (Hashemi-Nasab & Parastar, 2022).

To better inspect the classification models constituting the

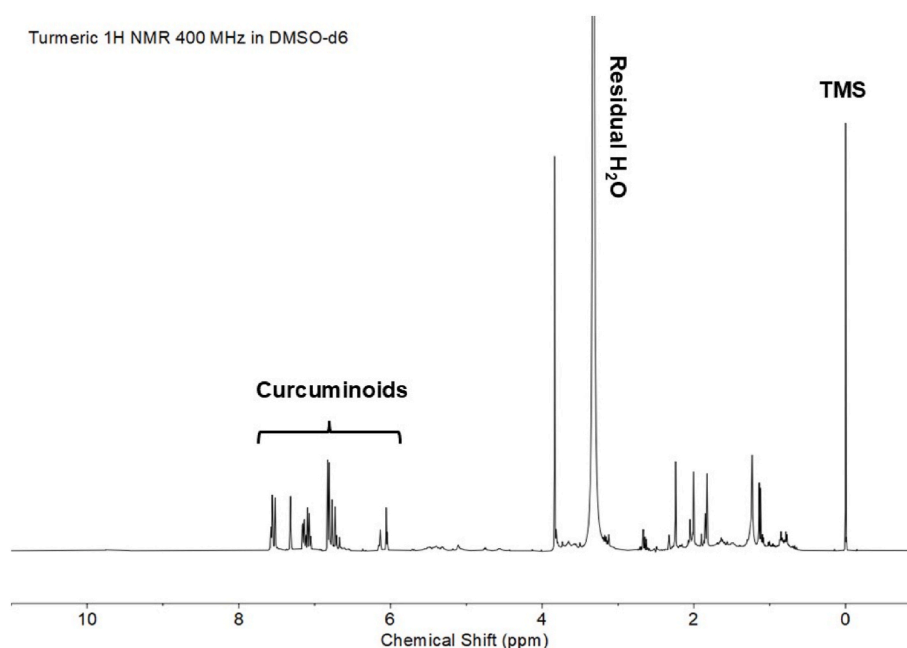

Fig. 2. ^{1}H NMR spectrum of turmeric sample.

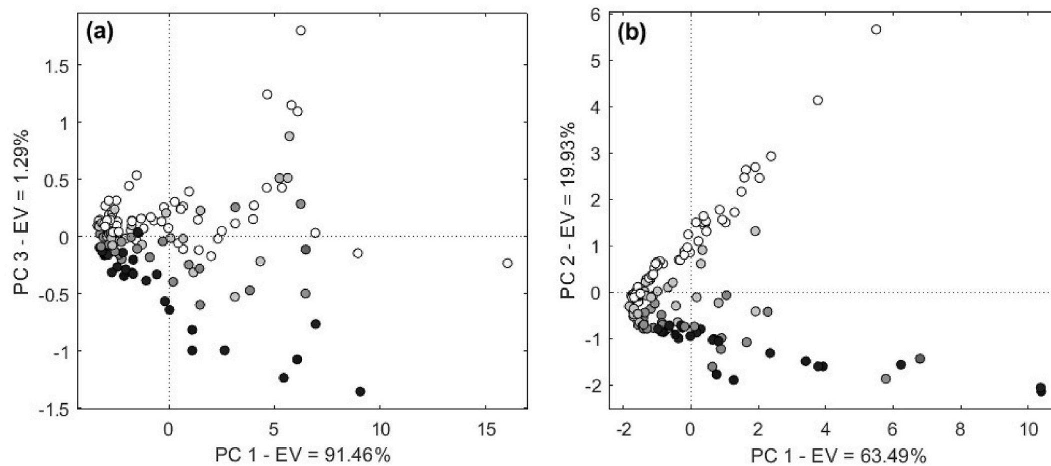

Fig. 3. Scores of the PCA carried out on a) selected ^{1}H NMR windows and b) area of the selected peaks. Samples are coloured according to the % of adulteration (white: pure samples; light grey: 2.5 % adulteration; grey: 5 % adulteration; black: 10 % adulteration).

Table 3
Percentage of correct predictions in validation as a function of the adulteration level for the PLSDA model based on windows of the standardised ^{1}H NMR spectra.

Adulteration level	Predicted as pure saffron	Predicted as adulterated
pure saffron	90.3 %	9.7 %
adulteration 2.5 %	8.3 %	91.7 %
adulteration 5 %	0 %	100 %
adulteration 10 %	0 %	100 %

hierarchical approach, the performance of the individual PLSDA models A and B were analysed. As previously described, to better address the discrimination of samples with adulteration at 2.5 % level of turmeric, we trained model A to discriminate pure saffron and adulteration equal or higher than 5 %, while model B was calibrated to separate pure saffron from adulteration with the lowest level of turmeric (2.5 %). To this end, the full set of samples was used as training data, while validation in this case was carried out by means of a Monte Carlo procedure (100 iterations with random selection of 20 % of samples as test samples in each iteration) (Ballabio, Robotti, et al., 2018; Varmuza & Filzmoser, 2009). Sensitivity for the pure and adulterated classes, as well as their average non-error rates (NER) are listed in Table 4. When looking at both individual models, classification results are very satisfactory both in fitting and validation, with NER values higher than 94 %. In particular, the pure class is always associated with better sensitivity, meaning that individual models can better classify pure samples of saffron.

Table 4
Classification performances of PLSDA model A (pure saffron vs. 5–10 % turmeric) and PLSDA model B (pure saffron vs. 2.5 %) on the training (fitting) and Monte Carlo validation for the PLSDA model based on windows of the standardised ^{1}H NMR spectra. Sensitivities (Sn) refer to the pure and adulterated (Adu) class.

Model		Fitting			Validation		
	LV[a]	NER[b] (%)	Sn Pure (%)	Sn Adu (%)	NER (%)	Sn Pure (%)	Sn Adu (%)
A (pure vs adulteration 5 %–10 %)	3	94	98	90	95	100	90
B (pure vs adulteration 2.5 %)	5	96	100	92	95	99	91

[a] Latent variable.
[b] Non-error rate.

The PLSDA score plots of both model A and B (Fig. 4) once again confirm the good degree of separation between classes. However, when looking at the scores of the PLSDA model for the discrimination between samples adulterated with 2.5 % of turmeric and pure samples (Fig. 4b), the first and third latent variables (LVs) are more effective for the visual class separation. On the opposite, a good discrimination of pure and adulterated samples (5–10 % of turmeric) is already visible in the space defined by the first two LVs for model A.

Finally, the average standardised spectrum of pure saffron samples (thicker blue line, ±1 standard deviation) and adulterated samples (thicker red line) are shown in Fig. 5, to better understand importance of the selected ^{1}H NMR peaks for discrimination of pure and adulterated samples. For most of the considered ^{1}H NMR peaks (spectral windows 1, 3, 4, 5, 7), adulterated samples have on average higher intensities than pure samples, which can be associated to the increasing amount of curcuminoids present in turmeric powder.

3.4. *Classification based on normalized NMR peak areas*

In addition to models carried out with the selected windows of the standardised ^{1}H NMR spectra, the proposed hierarchical classification approach was calculated also considering the normalized areas of the seven considered peaks (Table 2). Even in this case, the overall performance of the hierarchical model was evaluated by double validation procedure, resulting in an increase in sensitivity values of 94 % and 98 % for pure and adulterated classes, respectively (Non-Error Rate equal to 96 %). As before, both individual models constituting the hierarchical approach (model A and model B) were analysed too. Their classification performances are listed in Table 5. Model A demonstrated to be able to perfectly discriminate pure samples (sensitivity equal to 100 % and 98 % for training and validation), whereas in model B the discrimination is very good (NER equal to 97 % and 95 % for training and validation) considering the low level of adulteration (2.5 % turmeric).

This classification performance based on normalized areas was better than those based on the spectral ^{1}H NMR windows (sensitivity increases from 90 % to 100 % for 5–10 % adulterated samples), suggesting that modelling based on peak areas may facilitate class separation. For this reason, instead of using the hierarchical PLSDA approach, direct discrimination between pure and adulterated saffron (2.5 %, 5 % and 10 % of turmeric) was carried out by means of a single PLSDA model. This classification model achieved sensitivity for pure saffron samples equal to 98 % for both training and Monte Carlo validation (100 iterations, random selection of 20 % of samples as test samples in each iteration), while sensitivity for the adulterated samples was equal to 95 % (Non-Error Rate equal to 96 %). The small error in classification was due to the

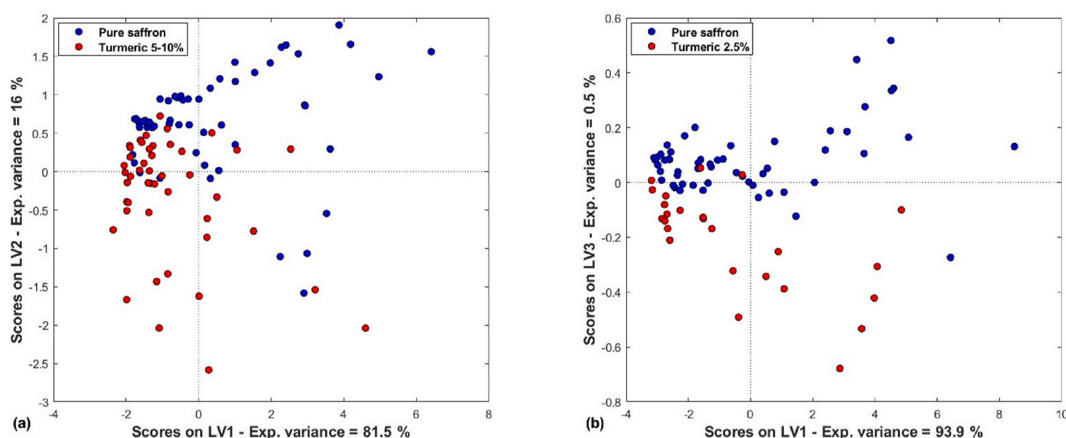

Fig. 4. PLSDA scores plots for a) model A (pure vs 5–10 % turmeric adulteration) and b) model B (pure vs 2.5 % turmeric adulteration). Pure samples are coloured in blue, adulterated samples in red.

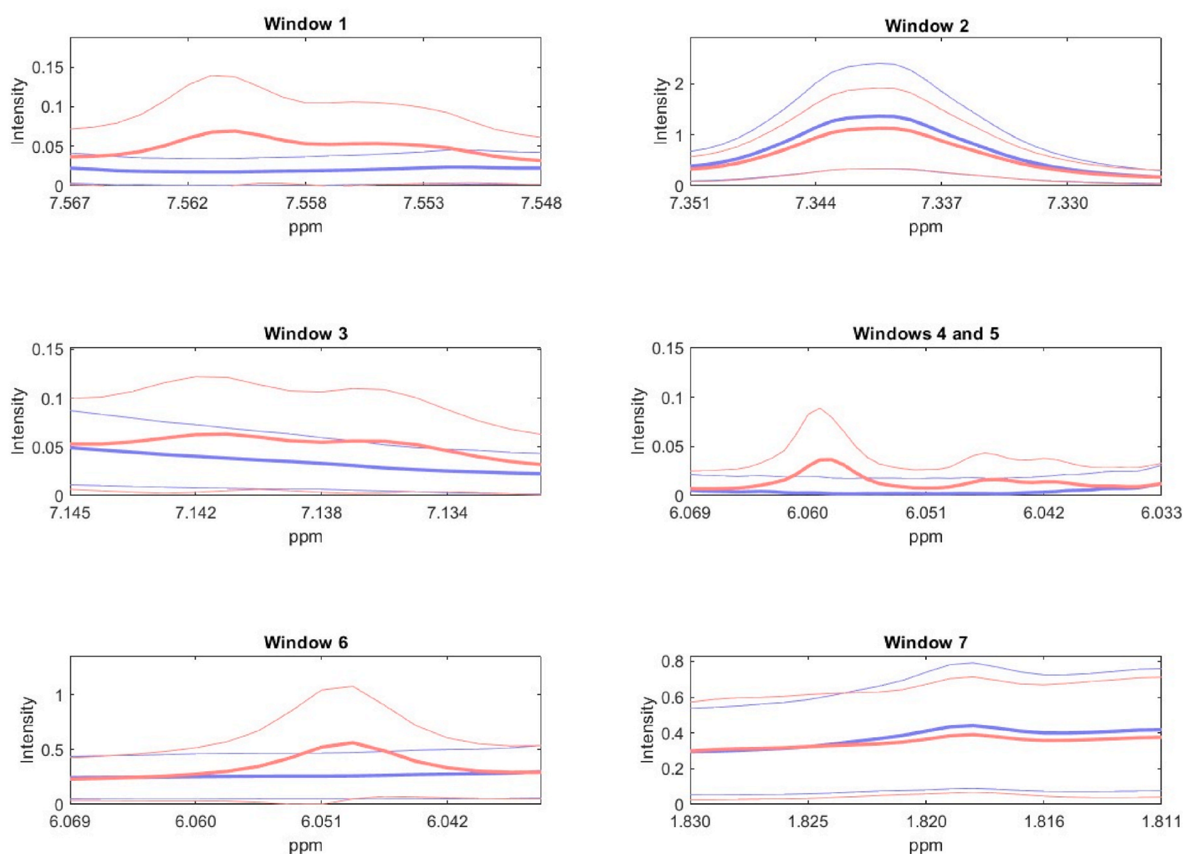

Fig. 5. Average standardised ^{1}H NMR spectra of pure saffron (thicker blue line) and adulterated samples (thicker red line) for each ^{1}H NMR window; thinnest lines denote ± 1 standard deviations.

minority samples adulterated with 2.5 % turmeric, which were classified as pure saffron. Therefore, results demonstrated the capability of the information encoded in NMR data to discriminate between pure and adulterated saffron samples.

PLSDA coefficients were inspected to estimate the contribution of each ^{1}H NMR peak to class discrimination. Higher absolute values indicate greater importance of the corresponding peak area for the discrimination of the pure saffron samples. Moreover, positive coefficients are associated with variables that are expected to be high in the pure samples, while the opposite for negative ones. In Fig. 6, the coefficients of the pure class are shown (those of the adulterated class are just specular and therefore not shown): the discrimination between pure and adulterated samples is mainly due to the area of peaks at 7.56, 7.14, 6.06 and 3.84 ppm. As an example, the plot of areas of peaks at 7.56 and 7.14 ppm is shown in Fig. 7, with adulterated samples coloured on the basis of the level of adulteration. These two features appear to be very effective for the identification of pure saffron samples, being classes well discriminated in the plot. The area at 7.56 is increasing proportionally to the level of adulteration. This result is justified by the increasing levels of curcuminoids proportional to the level of adulteration. Indeed, the signal of the spectrum detected at 7.56 ppm, is related to the presence of curcumin, demethoxycurcumin and

Table 5
Classification performances based on normalized ^{1}H NMR peak areas: PLSDA model A (pure saffron vs. 5–10 % turmeric) and PLSDA model B (pure saffron vs. 2.5 %) on the training (fitting) and Monte Carlo validation. Sensitivities (Sn) refer to the pure and adulterated (Adu) class.

Model		Fitting			Validation		
	LV[a]	NER[b] (%)	Sn Pure (%)	Sn Adu (%)	NER (%)	Sn Pure (%)	Sn Adu (%)
A (pure vs adulteration 5 %–10 %)	3	100	100	100	99	98	100
B (pure vs adulteration 2.5 %)	2	97	98	96	95	97	92

[a] Latent variable.
[b] Non-error rate.

bisdemethoxycurcumin (Praveen et al., 2021). In addition, similar results were observed in LC-MS experiments (Angeli et al., 2024).

4. Conclusion

In this study ^{1}H NMR and multivariate classification was demonstrated as a valid approach to discriminate authentic Italian saffron from samples containing as little as 2.5 % of turmeric powder. This result is better compared to the previous studies, which reported a detection up to 5 % w/w turmeric addition (Musio et al., 2022). To the best of our knowledge, no chemometric approaches were developed to detect such a low adulteration level with ^{1}H NMR. Indeed, the proposed classification models were able to correctly classify 98 % of pure samples and 95 % of adulterated samples correctly. Considering the increasing interest in the development of untargeted and non-destructive techniques to determine saffron authentication, ^{1}H NMR was proved to be a valid and robust technique to detect adulteration providing a non-destructive and fast method that requires minimum sample preparation. As shown in this study, the application of the ^{1}H NMR spectroscopy in food

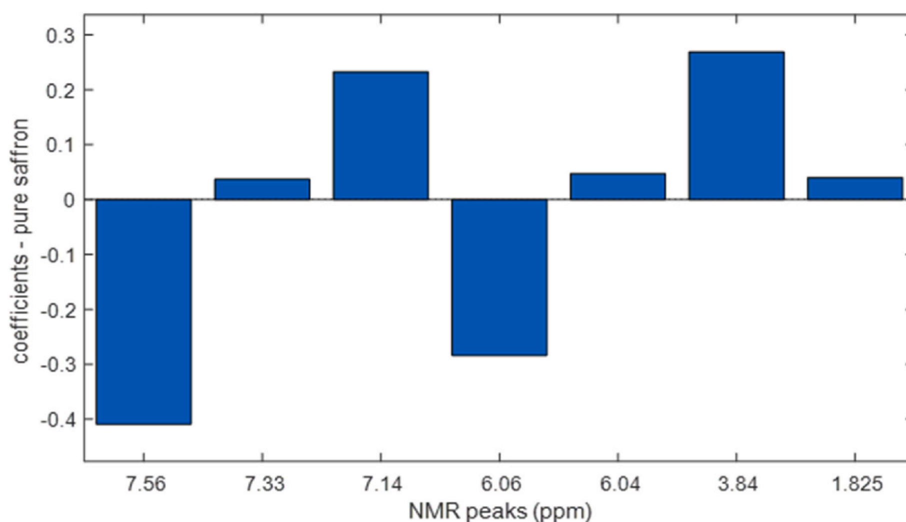

Fig. 6. Coefficients for the pure class of the PLSDA model for the discrimination between pure and adulterated saffron samples.

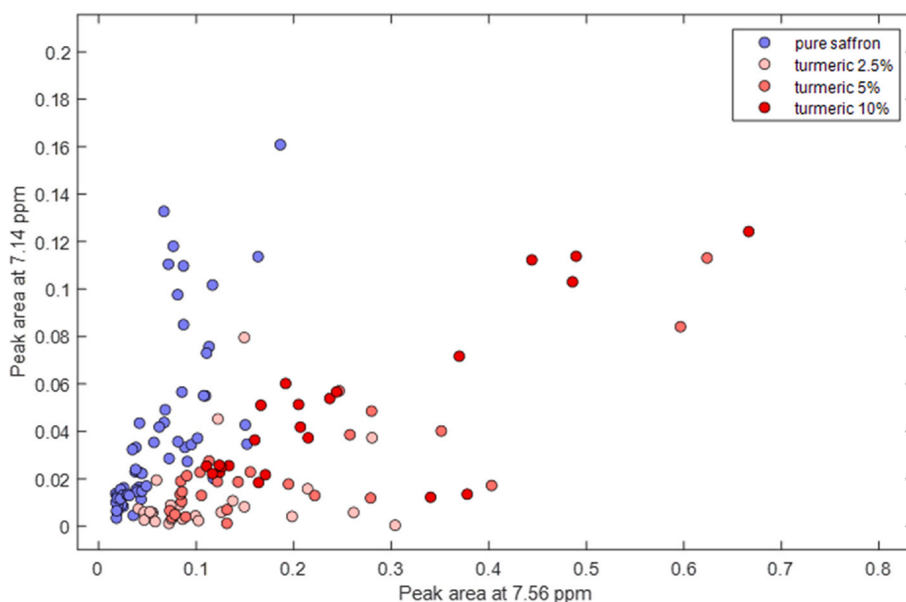

Fig. 7. Plot of areas of the peak at 7.56 ppm and 7.14 ppm. Pure saffron samples are coloured in blue; adulterated samples are coloured in red with intensity proportional to the levels of adulteration.

adulteration analysis can be considerably improved by application of the multivariate statistical analysis. Overall, ^{1}H NMR spectroscopy coupled with advanced multivariate analysis proved to be a suitable approach for detecting low levels of adulteration with turmeric. Although the available number of different saffron and turmeric samples in this study was very limited, this preliminary approach demonstrated strong potential for extension to other spices in future studies, in which the number of samples used for modelling should be increased. Nevertheless, Hotelling's T^2 and Q-residuals statistics can be used to evaluate whether predictions are reliable or not. This allows for assessing the reliability of predictions on "similar" samples. In addition, class imbalance issues need to be addressed and overcome in future sampling campaigns to develop more reliable models. Afterwards, further research should extend the range of tested adulterants to include other common saffron contaminants (e.g., safflower, marigold, paprika). Finally, given this was a preliminary feasibility study and therefore, geographical variability was not considered, future studies could include a wider variety of saffron samples from different geographical origins to develop a more robust and universally applicable model.

CRediT authorship contribution statement

Lucrezia Angeli: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Enmanuel Cruz Muñoz:** Writing – review & editing, Visualization, Software, Formal analysis, Data curation. **Davide Ballabio:** Formal analysis, Writing – review & editing, Visualization, Software, Methodology. **Ksenia Morozova:** Writing – review & editing, Visualization, Project administration, Methodology, Investigation, Formal analysis. **Matteo Scampicchio:** Writing – review & editing, Supervision, Software, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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