



Classification of Sidr honey and detection of sugar adulteration using right angle fluorescence spectroscopy and chemometrics

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Abstract

Sidr honey is vulnerable to adulteration with low-grade honey and sugar syrups, which compromises its nutritional and medicinal value, demanding fast and reliable analytical tools for quality assessment. In this study, fluorescence spectroscopy was employed to assess the quality of a honey samples, specifically, Sidr, unifloral (Acacia) and multifloral (Acacia, Carisa and Justicia) honey. Fluorescence spectroscopy revealed characteristic spectral signatures of Sidr honey, compared to Acacia and multifloral honey. In addition, cane sugar syrup was artificially added to Sidr honey at different concentrations. These spectral signatures were exploited for the machine-assisted classification of Sidr, sugar syrup and different concentrations of Sidr–sugar mixture. The bagging classification algorithm generated values of sensitivity and specificity close to unity, indicating its ability for efficient discrimination of the samples. Fluorescence spectroscopy in tandem with chemometrics could potentially be used as a rapid analytical tool to identify Sidr honey and its sugar adulteration.

Keywords Unifloral honey · Multifloral honey · Fluorescence spectroscopy · Ensemble learning · PCA

Introduction

Honey has been a part of human food since centuries as a nutrient as well as therapeutic agent. Nowadays, in the period of COVID-19, many people are consuming it in combination with lime or green tea due to its known anti-inflammatory and antimicrobial effects. The honey consists of carbohydrates, mainly monosaccharides (fructose and glucose) and minute amount of disaccharide (sucrose) and other types of sugars (oligo and tetrasaccharides). Besides, it contains a small amount of protein, phenolic substances, HMF (hydroxyl methyl furfural), vitamins, minerals and water. The composition of honey varies depending upon floral origin (plant type), climatic variation, geographical

region and contribution by beekeepers (Ankalam 1998). Honey from a defined botanical origin is unifloral honey and is distinctive from multifloral honey due to specific organoleptic properties [26]. The anti-inflammatory and antioxidant properties of honey are due to its phenolic compounds, flavonoids, carotenoid derivatives and organic acids [1].

Among all honey, Sidr honey is expensive and Yamen is famous for its Sidr honey since centuries due to its nutritional and therapeutic qualities. Sidr is also known as Jujuba, these trees grow in the desert areas of Yamen, belonging to the genus *Ziziphus*, are the major source of collecting nectar for pollinating bees. In Pakistan, seven species of *Ziziphus* have been reported and jujuba trees are grown mainly in Karak region. Sidr honey is collected from this region, from where it is exported to Arab countries. Pakistani flora is represented by *Ziziphus* species from Sindh, Pujab, Khyber Pakhtunkhwa (KPK) and Balochistan provinces [9].

According to the European Council directive (Codex 2001; Council Directive 2001/110/EC), honey is a natural sweet substance produced by honeybees from the nectar of plants and transformed by combining with specific substances of their own. Honey is adulterated directly or indirectly with inexpensive sugar syrups like high fructose corn syrup, corn syrup, inverted sugar syrup, sugarcane syrup and jaggery syrup. Direct adulteration is done by

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adding sugar syrup to honey after production and indirect adulteration is done by feeding sugar syrup to honeybees during nectar flow period to get more honey [11]. Commercial sugar is extracted mainly from sugar cane or sugar beet and is mainly composed of sucrose (99.8%) and 0.2% of polysaccharides, organic and inorganic non-sugar compounds and colorant polymers [5].

Different methods have been reported to detect sugar adulteration in honey [25, 28] like chromatography (HPAEC–PAD) [8], thin-layer gas chromatography (GC–MS) [20], carbon isotope ratio ($^{13}\text{C}/^{12}\text{C}$) technique [11] and nuclear magnetic resonance (NMR) [17]. These classical methods are tedious, costly and destructive with several limitations [4]. Fluorescence spectroscopy being non-destructive and rapid food analytical technique is known for its wide use in food characterization and determining molecular components, additives and adulterants [21]. Intrinsic fluorophores in food absorb ultraviolet or visible light at a specific wavelength and emit fluorescence at higher wavelength [14].

Chemical profiling, quality assessment and botanical origin of honey have been reported using fluorescence spectroscopy in combination with multivariate analysis. Front face or reflectance fluorescence spectroscopy (FFFS) has been used by many researchers due to the viscous and opaque nature of honey [13, 18, 19]. Classical right angle fluorescence methodology was also evaluated to discriminate honey samples solubilized in buffer and methanolic solution from the region of Croatia [24]. In these studies, fluorescence spectroscopy coupled with PCA and different regression models were applied. Owing to the complex nature of honey, different statistical tools for the characterization of honey and adulterant in it are required. Normally, unsupervised multivariate data analysis tools such as PCA or hierarchical cluster analysis (HCA) are used. In this study, in addition to PCA, we performed ensemble learning to achieve high accuracy for classification of dataset. An ensemble method combines the predictions from multiple machine learning models for more accurate predictions as compared to any individual model. Bootstrap aggregation (or Bagging for short) is a very powerful ensemble method that independently trains several machine learning algorithms using randomly chosen subsets of data. It reduces the variance for algorithms with high variance [16].

The research in our group was started with the aim to establish quick and non-destructive analytical test for characterization of Sidr honey and detection of adulteration in it. Sidr honey collected from various beekeepers and villagers was fingerprinted and distinctive fluorescence spectral signatures were identified in comparison with other monofloral honey samples [2] using synchronous fluorescence spectroscopy (SFS) and PCA. SFS is the multicomponent

fluorophore analysis as compared to right angle fluorescence spectroscopy [10].

In the present work, application of right angle fluorescence spectroscopy along with machine learning method has been applied to identify a single wavelength that can be used quickly to fingerprint Sidr honey and sugar adulteration in it. Development of quick, eco-friendly and non-destructive analytical tool may also be useful for the development of handheld device for honey researchers, producers, exporters, consumers and retailers.

Materials and methods

Sample preparation

The study was conducted on 25 samples of Sidr honey (S1–S25) along with 25 samples of Acacia (A1–A25) and 25 samples of multifloral (Acacia, Carisa and Justicia) honey (MF1–MF25), provided by Honey Bee Research Institute, Pakistan Agricultural Research Council, (PARC), Islamabad, Pakistan. Physicochemical, microscopic and sensory analysis of these honey samples was performed at PARC and listed in Supplementary Tables 1, 2, 3. The adulterated set included ten solution mixtures. Sugar syrup was prepared by adding 50 g of cane sugar in 50 mL of distilled water and heated in a water bath at 50 °C to dissolve sugar crystals. Different concentrations (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50%) in weight were prepared by mixing sugar syrup in pure Sidr honey. The Sidr-sugar mixtures were then heated in a water bath and vortexed for homogenous mixing.

Fluorescence spectroscopy

Spectra of all different types of honey including Sidr, Acacia, multifloral and Sidr-sugar mixtures were analyzed by fluorescence spectroscopy using FluoroMax®-4 (HORIBA Scientific, Jobin Yvon, USA) spectrophotometer fitted with a xenon lamp and a Fluorescence TM software. Right angle geometry was used for samples in a 1 cm quartz cuvette for recording emission spectra ranging from 365 to 690 nm. Two excitation wavelengths, i.e., 350 and 405 nm, were used with excitation and emission slits set at 2 nm.

Data processing and chemometrics

The spectra were processed and vector normalized using Matlab software (2014a). The fluorescence data of honey samples was subjected to principal component analysis (PCA) to differentiate groups using Origin software (OriginPro 2017, OriginLab.Corp., Northampton, MA).

In this study, the bootstrap aggregating—bagging for short—algorithm, was used for machine-assisted

classification of Sidr, sugar and mixed sidr–sugar samples. A total of seven different classes were considered—one each for Sidr and sugar and five mixtures of Sidr–sugar. The complete cohort of data comprised 140 samples (i.e., 20 for each of the seven classes). The predictive capabilities of the bagging technique are leveraged on the famous classification and regression tree (CART) algorithm, where the input data space is transformed into a classic binary decision tree, as detailed below:

- The position of maximum intensity signal in the fluorescence spectra (i.e., λ_{max}) collected for each sample was used as input data for the automated multiclass classification.
- The data elements from the original input data space D were transferred to a new, equal dimensional data space D_n ; this transformation was a random process so that some data points were not transformed at all while others were transformed repeatedly.
- Based on the characteristics of the dataset D_n , the algorithm generated multiple classifiers H_n , where $n = 1, 2, \dots, N$ (= number of characteristics of D_n).
- Training followed by testing of each H_n was executed on the dataset D_n so that $H_n : D_n \rightarrow R$.
- The training and testing phase of the classifier was executed with tenfold cross-validation.
- The weighted aggregate of each classifier H_n contributed toward the final compound classifier H as

$$H(d_i, c_i) = \sum_{n=1}^N \mu_n H_n(d_i, c_i),$$

- H where the weighting factor μ_n quantified the role of individual base classifier H_n for the final prediction H , such that it takes higher value for more precise classifiers as compared to less precise classifiers, while c_i and d_i indicate the given segment of data and class, respectively. The need of apprehending the efficiency of a classifier as a reliable and repeatable measure has been emphasized as fundamental for automated classifications. That said, there is a general agreement that sensitivity and specificity are the two most common, informative and convenient metrics for assessing and monitoring the quality of any classification algorithm. A variety of other quantitative metrics have been introduced for expressing the reliability of any classifier and ensuring objective comparison among contemporary classification algorithms; such commonly used evaluation metrics include positive and negative predictive values (PPV and NPV), F measure and Matthews correlation coefficient (MMC) [7]. These metrics altogether were computed for quantitative appraisal of the bag-

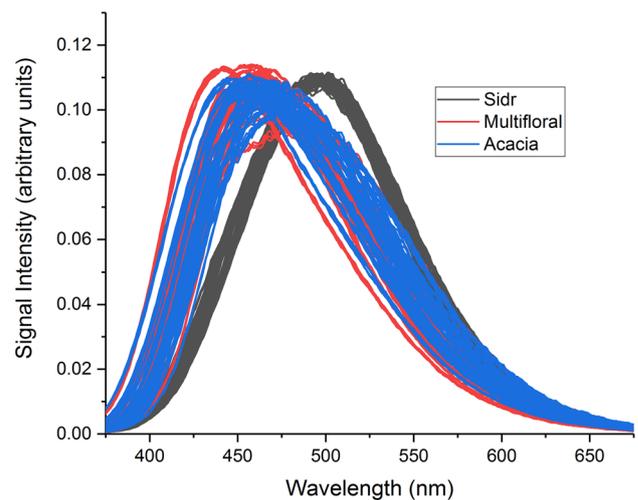


Fig. 1 Normalized fluorescence emission spectra of different honey samples—Sidr, Acacia and multifloral (Acacia, Carisa and Justicia) with excitation wavelength of 350 nm

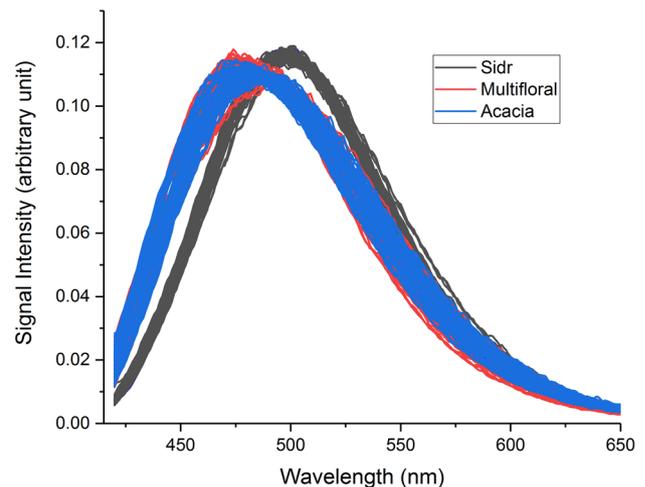


Fig. 2 Normalized fluorescence emission spectra of different honey samples—Sidr, Acacia and multifloral (Acacia, Carisa and Justicia) with excitation wavelength of 405 nm

ging classification model for the fluorescent spectra of the seven-class problem presented herein.

Results and discussion

The normalized fluorescence emission spectra of all investigated honey samples (Sidr, Acacia and multifloral) with both excitation wavelengths (350 and 405 nm) are presented in Figs. 1, 2. The emission from pure Sidr honey can be seen in the range of 494–508 nm with λ_{exc} 350 nm, whereas similar spectra was observed for Sidr with λ_{exc} 405 nm (Fig. 2). On the other hand, MF and Acacia honey exhibited overlapping

spectra from 440–484 nm to 464–488 nm, respectively. Emission maxima shifted to longer wavelengths from 474 to 490 nm in both Acacia and MF honey when excitation wavelength was changed from 350 to 405 nm. Such shifts to longer wavelengths point to emission from increased molecular size of fluorophore [12]. However the fluorescent cluster in pure Sidr honey remained unchanged at both excitation wavelengths.

The fluorescence spectral profile in honey is due to the contributions of main fluorophores, such as phenolic acids, flavonoids, amino acids, and vitamins. Thus, the presence of slightly broader hump in MF and Acacia as compared to Sidr honey represents overlapping fluorescent compounds owing to multifloral nature of sample predominantly from Acacia. Acacia plant was one of the honey sources in MF samples and seems like all the three sources (Acacia, Carisa and Justicia) shared the same fluorophores and thus showed overlapping emission spectra. Research studies have confirmed the presence of polyphenols in honey that are responsible for sensory properties (color, taste and odor) as well as anti-oxidative, antimicrobial, antitumoral and anti-inflammatory properties [15] depending upon the floral origin from which they are extracted.

Previously, we found a blue green fluorescence emission represented by broad hump in the range of 395–459 nm in commercial multifloral samples and 420–445 nm from samples provided by National Agriculture Research Council farms linked with HBRI using synchronous fluorescence spectroscopy (λ_{exc} range 250–450 nm, $\Delta\lambda = 60$ nm). In Sidr honey, the main difference from the multifloral honey can be seen in the spectral region at 397 and 451 nm, represented by phenolic acids [2]. Synchronous fluorescence spectra reduce spectral overlaps and thus display broad fluorescence bands that contain chemical, physical and structural information of components. Alzubier & Okechukwu [3] reported that Sidr honey from Yemen has different phytochemicals such as flavonoids, steroid, alkaloids, saponins and tannins and possess strong anti-inflammatory, analgesic and antipyretic effects.

Though it was not difficult to compare the fluorescence emission spectra by visual inspection, still data were subjected to chemometrics for authentication. Principal component analysis is applied to reduce the dimensionality of data and analyze statistical differences between spectral data of different samples [23]. The resulting score plot for PCA applied on the normalized data of honey samples is presented in Fig. 3. The total variance for the two components, i.e., PC1 and PC2, corresponds to 93.4% and 5%, respectively. Sidr honey formed a cluster that is distant from Acacia and MF samples, suggested due to the presence of different phenolic compounds represented by longer emission wavelength. Most of the samples from Acacia and MF showed overlapping that can be explained by the presence of similar fluorophores in these honey types.

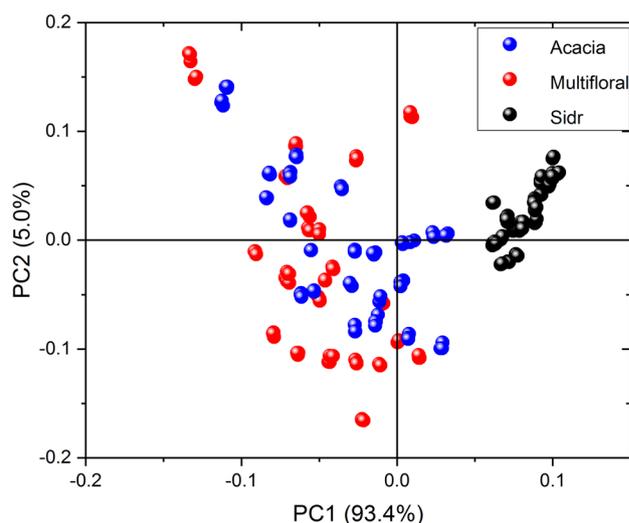


Fig. 3 PCA score plot of the first principal component (PC1) versus the second principal component (PC2) of the fluorescence data for Sidr, Acacia and multifloral honey samples excited at 350 nm

Sidr honey mixed with different concentrations (5, 10, 15, 20, 25, 30, 35, 40, 45 & 50%) of sugar syrup was analyzed using excitation wavelength of 350 nm. We used 350 nm instead of 405 nm, as more distinct emission spectra were observed relating to Sidr and non-Sidr (MF and Acacia) honey types. Figure 4 shows the specific qualitative display of Sidr honey fluorescence emission maxima at 507 nm with gradual shift toward shorter wavelength subsequent to the addition of sugar syrup in honey. In this study, sugar syrup was expressed with a fluorescence emission band with maxima at 435 nm, also reported by Ghosh et al. [10] with excitation at 340 nm and emission at 430 nm that was suggested to be generated from nicotinamide adenine dinucleotide (NADH). Baunsgaard et al. (2006) analyzed beet sugar and cane syrup just before sugar crystallization and found emission from amino acid and colored precursors with higher wavelength in cane sugar as compared to beet sugar. The fluorescence in sugar syrup in visible wavelength might be generated from colored compounds (Maillard reaction products) during thermal processing of cane sugar [6] (Fig. 4).

PCA was applied to the five selected sugar adulterated concentrations of Sidr honey that showed discrimination of pure honey subjected to adulteration with cane sugar syrup. The score plot (Fig. 5) defined by two components is represented by a separate cluster consisting of Sidr and adulterated samples (10–30% sugar syrup adulteration) with dominance of PC1 (89.7%). The pure honey samples were well separated from adulterated samples (40% and 50% sugar syrup adulteration) and pure sugar syrup at PC1, but there was small variation for separation of adulterated samples up to 30%. The remaining component of the two-dimensional score plot, i.e., PC2, showed small contribution

Fig. 4 Normalized fluorescence emission spectra of Sidr honey, sugar and adulterated honey samples prepared by mixing different concentrations of sugar syrup with Sidr honey. *SS* sugar solution, *SH* Sidr honey

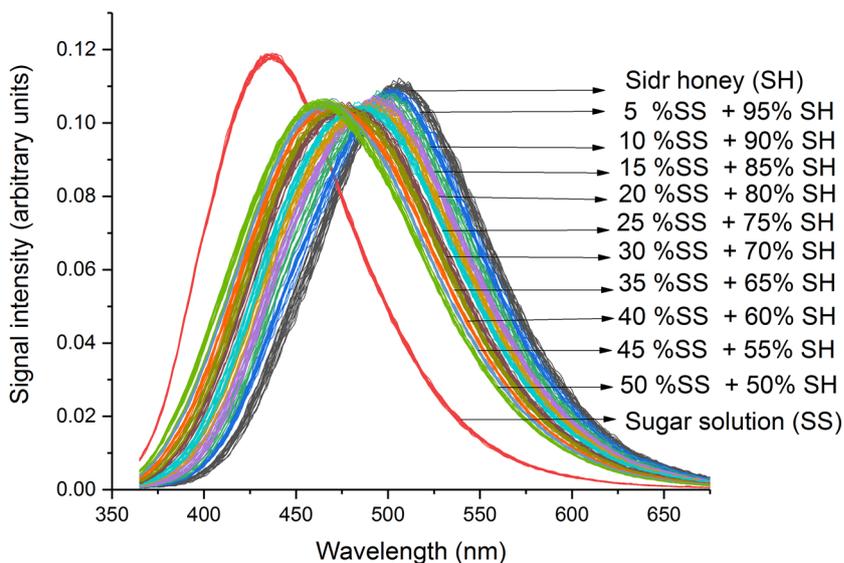
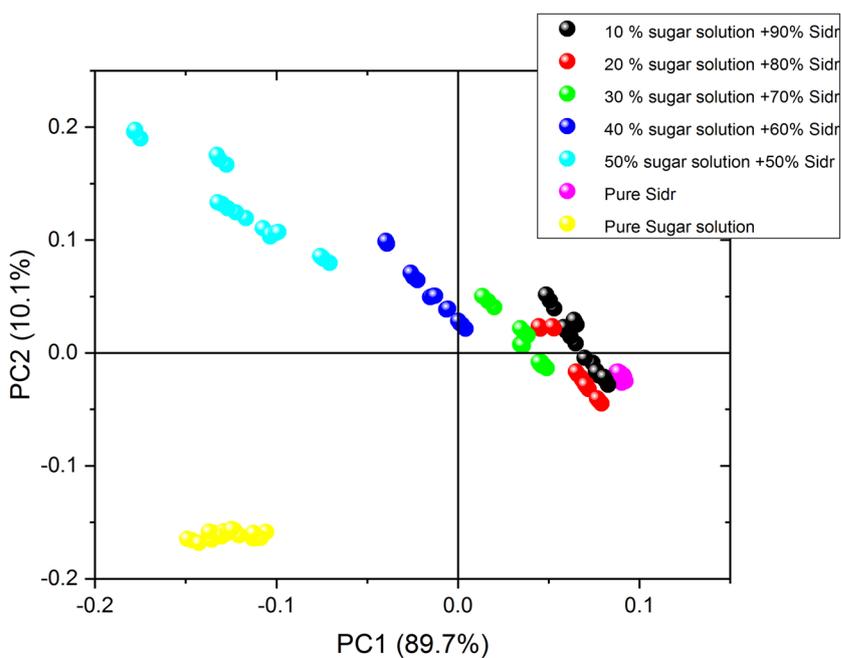


Fig. 5 2D score plot of PCI and PC2 generated for Sidr, sugar and adulterated honey samples



with variance of 10.1%. The main variable that contributed for grouping of Sidr and sugar syrup is the fluorescence emission from phenolic compounds from Sidr and colored products from sugar syrup.

A discriminant analysis based on bagging algorithm was also developed to differentiate pure and adulterated Sidr honey. The ability of the bagging classifier to handle multifaceted data, while concurrently diminishing the noise, variance and bias in the classification, has motivated its widespread use in applications pertaining to biomedical optics [22, 27]. Moreover, the implementation of the bagging algorithm for data containing gaps, i.e., missing data points, is

equally efficient in terms of accuracy. In perspective of these strengths, a custom version of the bagging algorithm was utilized in this study.

Table 1 presents a summary of the classification of the samples and the quantitative metrics used for performance evaluation of the bagging classifier. Specifically, the algorithm was modeled and executed for classification of seven-class problem—one class each for Sidr and sugar and five classes for different Sidr–sugar mixtures. Table 1, columns 2–5, depicts the confusion matrices for all seven classes as computed with the bagging classifier; the confusion matrix contains the most primitive information of classification,

Table 1 Summary of quantitative metrics used for the performance evaluation of Bagging Classifier

	TP	FP	FN	TN	Sn	Sp	PPV	NPV	F-measure	MCC
Sidr	18	4	2	116	0.900	0.967	0.818	0.983	0.857	0.833
Sugar solution	20	0	0	120	1.000	1.000	1.000	1.000	1.000	1.000
SS + SH (10% + 90%)	16	3	4	117	0.800	0.975	0.842	0.967	0.821	0.792
SS + SH (20% + 80%)	18	3	2	117	0.900	0.975	0.857	0.983	0.878	0.857
SS + SH (30% + 70%)	14	7	6	113	0.700	0.942	0.667	0.950	0.683	0.629
SS + SH (40% + 60%)	11	6	9	114	0.550	0.950	0.647	0.927	0.595	0.536
SS + SH (50% + 50%)	17	3	3	117	0.850	0.975	0.850	0.975	0.850	0.825

TP true positive, FP false positive, FN false negative, TN true negative, Sn sensitivity, Sp specificity, PPV positive predictive value, NPV negative predictive value, MCC mathew correlation coefficient, SS sugar solution, SH sidr honey

which is subsequently exploited to compute different metrics that identify and quantify the classifier's efficiency. In particular, the range of sensitivity and specificity values of the bagging classifier for the seven classes was 0.55–1.0 and 0.94–1.0, respectively. For pure Sidr and pure sugar, the bagging classifier generated the highest values for these two metrics (i.e., sensitivity and specificity), indicating that the machine-based differentiation of samples in these two classes is substantially accurate. A relatively low values of sensitivity (=0.55) and specificity (=0.95) was observed for sugar 40. Other metrics computed for the appraisal of the classifier included PPV, NPV, F-measure and MCC; the values of these metrics for all seven classes have been given in Table 1. Albeit all metrics demonstrated efficient performance of the bagging classifier (as the values are close to unity) for the seven-class problem, the details of specific trends related to each individual metric can be analyzed from Table 1. In future, such model will also be developed for other adulterants in Sidr honey such as fructose corn syrup, beet sugar syrup and glucose corn syrup.

Conclusion

The study revealed that right angle fluorescence spectroscopy, being a non-destructive and rapid technique, is a promising tool to fingerprint Sidr honey with no sample processing. This fast and simple technique along with chemometrics can be practically applied for the identification of Sidr honey at massive scale and detection of direct sugar adulteration in it. Moreover, this technique can be utilized in the development of handheld device that will be very beneficial not only for honey researchers, but also for producers, exporters, consumers and retailers for the authentication of Sidr honey.

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Declarations

Conflict of interest There is no conflict of interest among authors.

Compliance with ethics requirements This research involves no Human Participants and/ or Animals.

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