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Research article

Rapid discrimination of *Nigella Sativa* oil from different geographical origins using ATR-FTIR-unsupervised and supervised pattern recognition techniques

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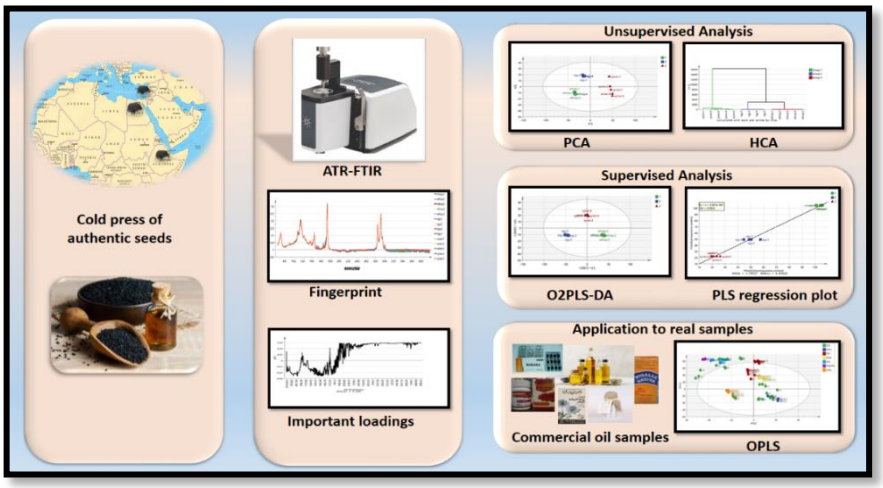
Abstract:



Previous metabolomics studies have proven the efficiency of infrared (IR) spectroscopy as a tool for authenticity assessment. Attenuated total reflectance – Fourier transform infrared spectroscopy (ATR-FTIR), with principal component analysis (PCA) and bidirectional orthogonal projection to latent structures-discriminant analysis (O2PLS-DA), was used to create a model for the discrimination of *N. sativa* oil samples gathered from three geographical areas; Egypt, Ethiopia and Syria. It is reported that, for herbal drug treatments even from the equal species, the quality and efficacy are somewhat different according to their growing conditions. The unsupervised pattern recognition of variable authentic samples utilizing PCA and HCA (Hierarchical Cluster Analysis) revealed that each oil type was separately clustered from the others.

The data matrix of ATR-FTIR was handled by the supervised pattern recognition technique O2PLS-DA to give a better clustering and a better separation between sample types than given by PCA. For the first time, this study built a partial least square (PLS) model used to predict thymoquinone concentration in any new *N. sativa* seed oil only by its ATR FT-IR spectrum. Furthermore, OPLS and its HCA model of authentic samples have been used to predict types of commercial oil samples obtained from the Egyptian market and compare them with sellers' claims.

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As demonstrated in this study, the recommended method might be used to regularly evaluate the phytochemical variability
in *N. sativa* seed oil varieties sourced from various regions, which could aid in meeting the demand for their quality and safety.

Keywords: ***Nigella sativa seed oil, ATR-FTIR, Geographical origin, Multivariate analysis.***

1. Introduction

Nigella sativa L. seed (commonly named black cumin or black seed) oil was utilized traditionally, particularly in the Middle East and India, for the cure of cough, bronchitis, asthma, headache, rheumatism, influenza, fever, and eczema [(1)]. Recently, these seeds have been used commercially for a variety of goods, including soaps, oils, and shampoos [(2)]. The oil and seeds are both used as food supplements to provide nutrients [(3)]. One of the more recent sources of edible oils that is crucial to human nutrition and health is *N. sativa* seed oil [(4)]. According to certain theories, seed oil possesses immune-stimulating properties [(5)], anticancer activity [(6)], antioxidant [(1)], anti-inflammatory [(7)], and antibacterial efficacies [(8)]. Additionally, it supports the health of the kidneys, liver, stomach and intestines [(9)]. *N. sativa* is said to have antihistamine-like properties that help relieve congestion [(10)]. Many of these activities have been referred to quinone components in the seed[(11-13)].

South Europe, India, Pakistan, Syria, Turkey, and Saudi Arabia are just a few of the countries that grow *N. sativa*, a plant native to Southern Europe, North Africa, and Southwest Asia. [(14)]. Reports state that the quality and efficacy of herbal medicinal treatments, even those derived from the same species, differ slightly depending on the climate and soil conditions in which they are grown, primarily due to their geographic origins [(15-17)]. Our previous study has proved that the studied oils' geographical origin in the Egyptian market significantly impacted their biological efficacies; Ethiopian oil showed the most impressive biological activity, followed by Egyptian and Syrian oil [(18)]. Thus, the development of analytical procedures for identifying the geographical origin of herbal medications is an important area of study, and the results of this endeavour can assist in meeting the demand for their quality and safety[(15-17)].

ATR mode-FTIR imaging demonstrates a supplementary approach to transmission or reflection mode FTIR imaging. In essence, ATR-FTIR spectroscopy relies on the curvature of light beams traveling through various media as well as molecular vibration. Transmitting radiations such as UV, IR, and visible are used to obtain the ATIR spectrum. To ascertain the incident radiation attenuated by the sample, these radiations are transmitted through the sample, which is placed in an optical crystal [(19)]. The fact that ATR-FTIR imaging requires little to no sample handling prior to spectral measurements is one of its primary advantages. This is because, for ATR measurements, the depth of infrared light penetration in the sample is independent of sample thickness. As a result, this method works particularly well for measuring materials like water that absorb a lot of infrared light.

Multivariate analysis is a powerful statistical technique that plays a crucial role in discriminating the geographical origin of various products depending on their chromatographic and spectroscopic metabolic data [(20)]. Several studies applied different chemometric models that help in pattern recognition, biomarker discovery, quantitation, authentication and allocation of marketed products to their destination [(20-23)].

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This study intends to expand the use of ATR-FTIR transmission spectroscopy in conjunction with the relevant chemometric techniques (PCA, HCA, PLS, OPLS, and O2-PLS) in order to categorize *N. sativa* seed oil according to its place of origin using the distinctive infrared spectrum that is acquired. The study's conclusions can assist in meeting the demand for their quality and safety.

2. Materials and methods

2.1. Materials and reagents

Cold pressing of *N. sativa* seeds has produced nine genuine samples: three grown in Egypt, three imported from Ethiopia, and three imported from Syria **Table S1**. The Department of Medicinal and Aromatic Plants at the National Research Institute in Cairo, Egypt, identified, authenticated, and processed all of the seed samples using a cold press method.

Twenty seven different black seed oil commercial samples were collected from several local suppliers and manufacturers (exportation companies). Samples from the same manufacturer with different batch numbers were also considered. Pharmaceutical products containing black seed oil as capsules and their raw oils were also collected. Black seed oil in the Egyptian market was of Egyptian, Ethiopian or Syrian origin. All black seed oil commercial samples used in the study were letter-coded appropriately as shown in **Table 1**. Adequate concentrations of black seed oil authentic and commercial samples were made by dissolving 0.5 ml of the oil in 10 ml of ethyl acetate in a 10 ml volumetric flask.

2.2. Samples preparation

Oil from *N. sativa* seeds has been extracted using the cold press extraction method [24]. To preserve the physicochemical properties of the oil, the temperature was maintained below 40 ºC and no heating or chemical processes were used. The process speed was initially fixed at 17 rpm, and the cold press oil machine head diameter was selected at 8 mm. To remove sediments, oil was kept in stainless steel containers for a day. Filtration paper with 1µ pores was used to purify the oil the following day. The final stage involved filling 200 ml amber glass bottles with *N. sativa* oil.

2.2.1. ATR- FTIR measurement

FTIR–ATR experiments were performed using Agilent cary 630 Geranium attenuated total reflectance (Ge ATR) FT-IR spectrometer accommodated with a Smart iTR accessory for ATR sampling and a deuterated triglycine sulfate (DTGS) detector (Agilent Technologies, USA). A tiny drop of *N. sativa* oil was pipetted onto the ATR baseplate. Before measuring the subsequent sample, the ATR base was meticulously cleaned in situ using methanol scrubbers and soft tissue drying. By gathering a background spectrum and comparing it to the prior one, the cleaning technique was confirmed. At each data point, these spectra were captured as absorbance values. For genuine samples, every measurement was made three times, and the spectra were averaged [25]. Each spectrum had sixteen co-added images and was obtained at a spectral resolution of 4 cm^{-1}. Spectrum recording took place between 4000 and 650 cm^{-1}, with a data collection time of 23 s for each spectrum. With the aid of FT-IR spectrometer software, the FT-IR spectrum data of the various *N. sativa* oil samples were subsequently transformed into American Standard Code for Information Interchange (ASCII) files, which were subsequently exported to an Excel file.

2.2.2. Data preprocessing

The developed IR data matrix was thereafter preprocessed and the following transformations were applied: Baseline correction which is mostly used for spectroscopic purposes to adjust the spectral offset to the minimum point in the data, subtracting the value of the spectrum's lowest

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point from each variable, leaving the minimum value at zero and the remaining

Table 1: list of commercial samples used in the study letter coded together with the supplier's claimed geographical source.

Supplier Name	Sample Coding	Formula, some data	Claimed Geographical origin
Oils for Exportation			
Supplier (A)	A	Oil	Egyptian
Supplier (B)	B	Oil	Egyptian
	B- aged	Oil	
Supplier (C)	C	Oil	Egyptian
Supplier (D)	D	Oil	Egyptian
Supplier (E)	E	Oil	Egyptian
Supplier (F)	F	Oil	Egyptian
Raw oils in local markets			
Supplier (G)	G	Oil	Egyptian
Supplier (H)	H	Oil	Egyptian
Supplier (I)	I	Oil	Syrian/ Ethiopian mix.
Supplier (J)	J	Oil	Egyptian
Supplier (K)	K	Oil	Egyptian
Supplier (L)	L	Oil	Egyptian
Supplier (M)	M	Oil	Syrian
Supplier (N)	N-1	Oil	Syrian
	N-2	Oil	Syrian/ Ethiopian mix.
Supplier (O)	O-1	Oil	Syrian
	O-2	Oil	Ethiopian
	O-3	Oil	Syrian/ Ethiopian mix.
Finished Pharmaceutical products and/or their raw oils			
Supplier (P)	1	Oil, (body care product)	Egyptian
Supplier (Q)	2	Capsules, (B No. 10799)	Unknown
	3(aged)	Capsules, (B No. 7211)	
	4	Capsules, (B No. 10897)	
	5(aged)	Capsules, (B No. 5820)	
	6	Raw oil of capsules	
Supplier (R)	7	Capsules, (B No. 10439)	Ethiopian
	7-aged	Raw oil of capsules	

variables at positive values[26]. Multiplicative Scatter Correction (MSC) and Standard Normal Variate (SNV) spectral filters, and average spectra were computed for every genuine sample [27]. Unit vector normalization has been utilized to normalize sample-wise data to unit vectors which is required for pattern normalization to delete the differences between the spectra because of the different amounts of analyzed samples [28]. Triplicate measurements of each individual *N. sativa* oil sample were averaged to increase signal-to-noise and also to limit the number of the samples to the original size. The FT-IR spectra of all *N. sativa* oil samples were then subjected to the un-supervised pattern recognition technique principal component analysis (PCA). Supervised O2PLS-DA modelling was performed later using the ATR-FTIR data. Chemometric analyses was performed using SIMCA® version 14.1 software by MKS UMETRICS.

3. Results and Discussion

3.1. *Nigella sativa* oil FTIR spectrum characterization

Previous metabolomics studies have proven the efficiency of IR as a tool to identify natural markers in authenticity assessment [29]. Accordingly, the IR spectral profiling of the *N. sativa* oil was experimented using the neat oil without any prior sample preparation steps and thus the generated IR spectra, shown in **Fig. 1A**, will be expressing and profiling only the exact *N. sativa* oil features without any background interferences. Spectrum had significant vibrational bands at 3008, 2922, 2853, 1742, 1656, 1458, 1377, 1161- 1097 and 760 - 900 cm^{-1} assigned for C-H stretching (aromatic) or C=C-H (cis) stretching, C-H stretching (alkyl), O-H stretching (carboxylic acids), C=O stretch (mainly from quinones), C=C stretch (aromatic) or (cis), C-C stretching (aromatic), C-H bending and C-O stretching and C-H

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bending out of plane (aromatic) **Fig. 1B**. These bands were associated with the chemical groups of constituents in the chemical structure of *N. sativa* oil components as shown in **Fig. 1B** [30]. The most important one is lying at 1742 which is attributed to the carbonyl group that can mainly be credited to thymoquinone.

3.2. Un-supervised pattern recognition of variable authentic samples utilizing PCA and HCA

Nine genuine samples—three Egyptian, three Ethiopian and three Syrian—had their ATR-FTIR spectra obtained, and each sample was measured five times. ATR-FTIR spectra obtained between 4000 and 650 cm^{-1} were subjected to MSC and SNV pretreatment, and average spectra were computed for every genuine sample. The spectra were then mean centered and vector normalised. MKS UMETRICS then used SIMCA® version 14.1 software to apply a PCA to this spectral matrix.

When ellipse hotelling is adjusted to 95%, the scores of the several genuine *N. sativa* oil samples in the space defined by the first and second principal components, which explained 75.1% (PC1) and 11.1% (PC2) of the variability in the data, are displayed in **Fig. 1C**. The overall variance is 86.2%. The samples in the scatter plot are denoted by various shapes and color codes that correspond to the various classes of our oil. Syrian samples were grouped in the positive side of PC1 and the negative side of PC2, Ethiopian samples were grouped in the negative side of both PC1 and PC2, and Egyptian samples were grouped in the positive side of PC2 and the negative side of PC1.

The weight of each original variable is displayed in the loading plot of PC1 in **Fig. 1D**, indicating that variables with greater loadings contribute more to the creation of a particular principle component. The spectral range of 1300 to 2000 cm^{-1} exhibits significant changes in loadings, which may have an impact on the grouping of genuine samples. Real samples were divided into two groups based on HCA, the first group clustered the Ethiopian and the Egyptian *N. sativa* oil samples together while the second group contained only the Syrian *N. sativa* oil samples **Fig. 1E**. The Egyptian and the Ethiopian oil samples clustered together due to close chemical composition of both types, while the Syrian oil samples clustered away from them due to the difference of its chemical composition.

3.3 Supervised pattern recognition of different authentic samples using O2PLS-DA

ATR FT-IR data matrix of authentic oil samples has been utilized to build up O2PLS-DA model by introducing class ID of the three oil types. The O2PLS-DA was adopted to give a robust classification models, with a clear explanation of the systematic difference beneficial to characterize each class. Unlike PCA which depends on similarities between samples for clustering, O2PLS-DA depends on dissimilarities [31]. O2PLS-DA as a highly sophisticated technique, gives much better clustering of oil types as shown in **Fig. 2A** in which Ethiopian samples were closely clustered in the positive side of factor 1 and negative side of factor 2, Syrian samples in the positive side of both factors while Egyptian samples were clustered in the negative side of both factors. Compared to PCA model, O2PLS-DA model enables samples of the same type to be clustered much closer to each other causing higher discrimination between different types of samples. **Fig. 2B** shows the variable influence on the projection approach for O2PLS-DA models, also known as VIPO2PLS. Using VIPO2PLS profiles for the data set, the variables were arranged in order of significance for the model interpretation. The variables are displayed along the horizontal axis of the VIP

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plots, while the VIP values (in arbitrary units) are localized along the vertical axis. The key variables can be identified with the naked eye at VIP value equal to 1. Variables with VIP values higher than 1 are related to the model interpretation, whereas variables with VIP values less than 0.5 can be considered for possible model removal [32].

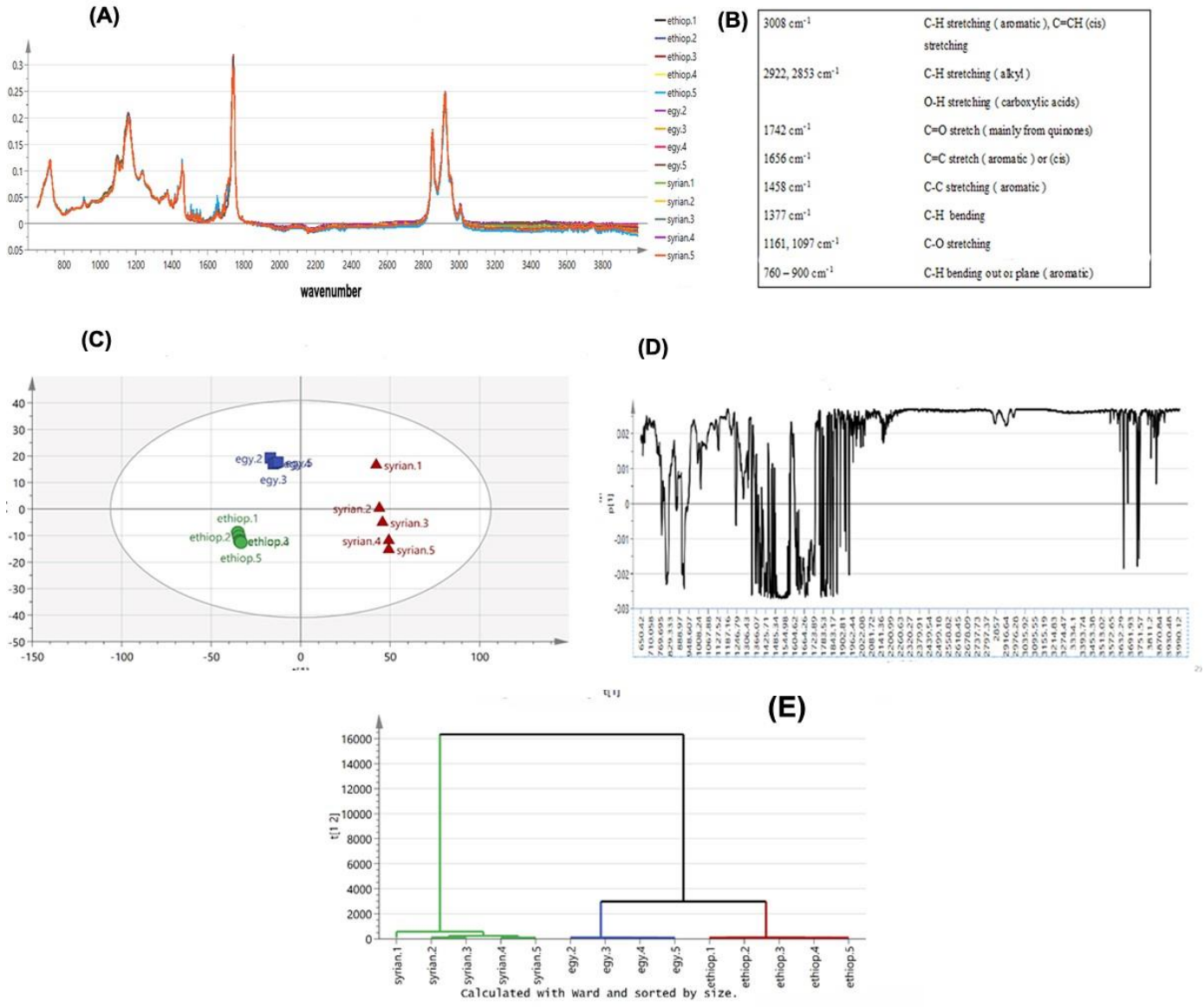

Fig. 1: ATR-FTIR spectra of the different oil samples (A) along with the significant bands detected (B), PCA score scatter plot based on the ATR-FTIR data of the samples (C) and the corresponding line loadings plot (D). Dendrogram of HCA of ATR FT-IR data matrix of authentic samples (E).

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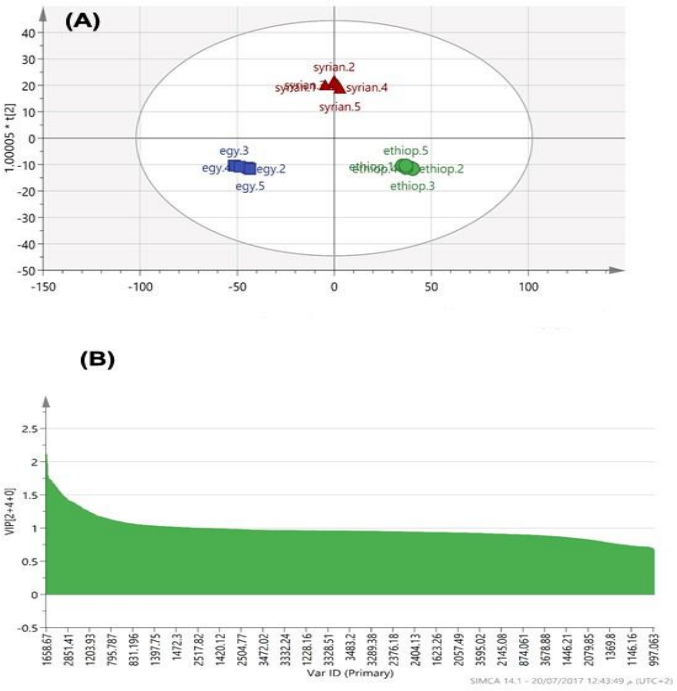

Fig. 2. Score plot of O2PLS-DA performed on ATR-FTIR spectra recorded from black seed oil authentic samples (A). The VIP-O2PLS plot (B). The VIP threshold is represented at VIP=1.

3.4. Partial least square regression (PLSR) model for prediction of thymoquinone content

ATR FT-IR data matrix of authentic samples was used to build up a PLS regression model after setting the thymoquinone concentration as a class and Y variables. Thymoquinone was revealed to be the efficacy-related marker according to our previous study predetermined by HPTLC [18]. This model can be used to predict thymoquinone concentration in any new *N. sativa* seed oil only by its ATR FT-IR spectrum. Model validation is done by leave-one-out cross-validation. In leave-one-out cross-validation, each sample is excluded one at a time, and the response attribute of the excluded sample is predicted using the remaining samples. The model with the lowest root mean square error of cross-validation (RMSECV), root mean square error of estimation (RMSEE) and the highest coefficient of determination (R2) served as the basis for the optimal model selection criteria. A very good PLS regression model with a strong correlation between the measured and projected thymoquinone concentration values are displayed in **Fig. 3** (R^2 = 0.9922, RMSEE = 3.70225 and RMSECV = 6.83918). **Table 2** shows observed versus predicted values of thymoquinone content. PLS components and their eigenvalues are shown in **Table S2**.

Table 1. ATR-FTIR PLS observed versus predicted thymoquinone concentration.

Sample	Observed thymoquinone content	Predicted thymoquinone content
ethiop.1	104.59	98.7086
ethiop.2	104.59	102.573
ethiop.3	104.59	105.437
ethiop.4	104.59	106.635
ethiop.5	104.59	106.427
egy.2	49.4714	50.6436
egy.3	49.4714	52.6028
egy.4	49.4714	49.1985
egy.5	49.4714	48.4837
syrian.1	22.0137	13.6632
syrian.2	22.0137	19.2356
syrian.3	22.0137	22.8064
syrian.4	22.0137	25.9958
syrian.5	22.0137	28.9663

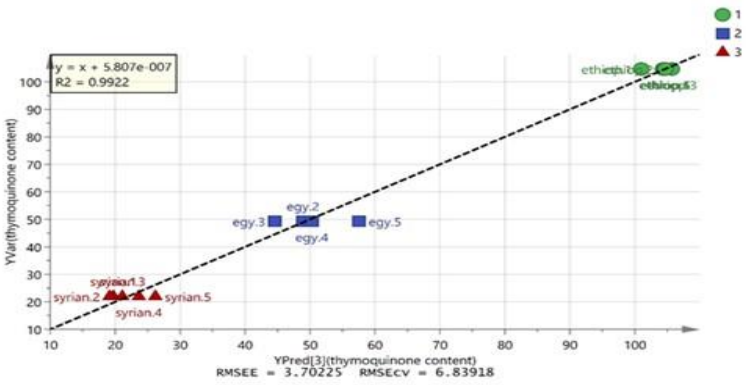

Fig. 3. PLS regression model between observed and predicted thymoquinone content.

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3.5. Application to real samples

OPLS and HCA model of authentic samples have been utilized to predict types of commercial oil samples gathered from the Egyptian market and compare them with sellers' claims. ATR FT-IR data matrix of commercial oil samples pretreated and derivatized by MSC and SNV spectral filters and predicted by OPLS model of authentic samples. **Fig. 4A** shows OPLS clustering of authentic and commercial samples. Hierarchical clustering analysis (HCA) was also used to study our data as shown in **Fig. 4B**. PCA plot and HCA dendogram reveal that samples H, J and F clustered with authentic Egyptian oil sample and they were claimed as Egyptian *N. sativa* oils. While samples 7, 7-aged, 4 and 5-aged clustered with authentic Ethiopian oil sample and they were claimed to be Ethiopian *N. sativa* oils. Samples G and M clustered with authentic Syrian oil sample, sample M was claimed to be Syrian *N. sativa* oil, but sample G was claimed as Egyptian *N. sativa* oil. It means that we have 8 samples of 27 collected from the Egyptian market (about 29.6%) that are considered well-claimed by sellers. Only sample G is considered a false authentication, it was claimed as Egyptian, but it is a Syrian *N. sativa* oil. The rest of the samples are considered outliers, they may be adulterated by cheaper oils, or they may be mixtures of different types of *N. sativa* oils.

4. Conclusion

Nigella sativa L. seed oil from different geographical origins could be successfully discriminated depending on ATR-FTIR transmission spectroscopy in conjunction with the relevant multivariate techniques (PCA, HCA, O2-PLS-DA, PLS and OPLS). The models constructed could successfully discriminate the different chemotypes of *N. sativa* seed oil depending on their geographical origin. OPLS and HCA models of authentic samples have been used to predict types of commercial oil samples obtained from the Egyptian market and compare them with sellers' claims. Eight samples (H, J, F, 7, 7-aged, 4, 5-aged and M) of 27 collected samples from Egyptian market (about 29.6%) that were considered well claimed by sellers. As demonstrated in this study, the proposed method might be used to regularly evaluate the phytochemical diversity of several *N. sativa* seed oil varieties.

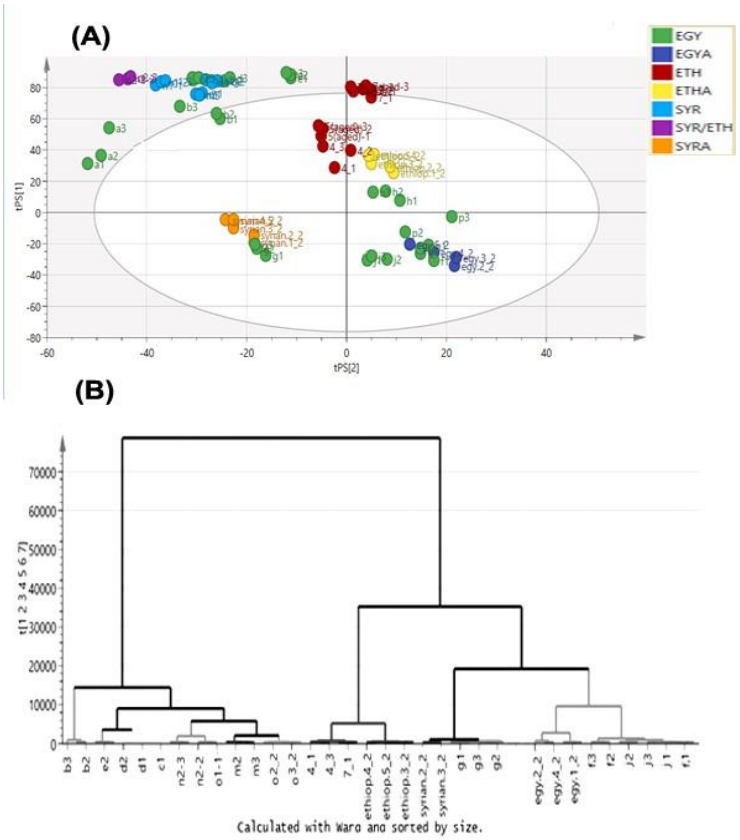

Fig. 4. OPLS Score plot of authentic and commercial samples (A). Authentic samples coded as EGYA, ETHA, SYRA and commercial samples named according to sellers` claims as EGY, ETH, SYR and SYR/ETH. Dendrogram of HCA of ATR FT-IR data matrix of authentic and commercial samples (B).

Declarations

Consent for publication

All authors have read and approved the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Data availability

All data generated or analyzed during this study are available upon contacting the authors.

Ethical approval and consent to participate

Not applicable

Authors' contributions

Nihal M. El Newehy: Conceptualization, Methodology, Data Curation, Original Draft Preparation-Writing and Editing.

Gamal A. Omran: Supervision, Original Draft Preparation and Visualization.

Fathallah M. Harraz: Supervision, Original Draft Preparation and Visualization.

Eman Shawky: Conceptualization, Methodology, Data Curation, Supervision, Validation, Writing -Reviewing and Editing.

Amira M. Beltagy: Supervision, Original Draft Preparation and Visualization.

Highlights

- ATR-FTIR has been used for the quality assessment of N.sativa oils.
- Multivariate analysis could discriminate oils from different origins.
- OPLS and HCA models were built for authentic oil samples.
- Models predict commercial oil types and compare them with sellers' claims.
- Only 29.6% of oil samples were considered well-claimed by sellers.

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