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The feasibility of applying NIR and FT-IR fingerprinting to detect adulteration in black pepper

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Highlights

- Non-targeted fingerprinting of ground black pepper by NIR and FT-IR
- Detection of adulterants from black pepper plant itself (husk, pinheads and spent)
- Detection of foreign plant material (papaya, chili)

Abstract

Black pepper is the most widely used spice in the world. Spices are highly vulnerable to economically motivated adulteration as they are high value products and traded along complex supply chains. The main fraud opportunity is to add cheaper bulking materials. Near and Fourier-Transform Infrared Spectroscopy has been combined with chemometrics to screen for the substitution of black pepper with papaya seeds, chili and with non-functional black pepper material such as black pepper husk, pinheads and defatted spent materials. A good separation performance between black pepper and adulterated samples could be shown. After running a binary classification model with an external test set an area under the receiver operator characteristic curve of 0.98 for both, the NIR and FT-IR model was obtained. This study shows the huge potential for a fast and rapid screening method that can be used to prove the authenticity of black pepper and detect adulterants.

Key words

Black Pepper

Authenticity

Adulteration

Fourier transform infrared

Near infrared

Screening method

1 Introduction

Known as the "King" of spices, black pepper (*Piper nigrum*) is economically the most important and the most widely used spice crop globally. It is cultivated in a total of about twenty-six tropical countries, among which Vietnam, India and Indonesia are the main producers. Black pepper has extensive culinary uses for flavouring and preserving processed food. Moreover, due to its antioxidant effects it is important in the Indian traditional medicine manufacturing sector (Ravindran & Kallupurackal, 2012). The worldwide consumption and also the price of black pepper are increasing constantly. In 2016 about 430.000 tons were consumed worldwide, from which 63.000 tons were exported to Europe for a total business volume of 514 million Euro (CBI, 2016).

The globalisation of our food makes it more vulnerable to food fraud with both unintentional and intentional fraud being perpetrated. The latter is very often used for economic gain, also called "economically motivated adulteration" (EMA) (Everstine, 2017). The risk for food fraud increases proportionately with the complexity of the supply chain. This in turn leads to opportunities to make huge financial gains with a very low risk of detection. Spices are expensive commodities that meet the criteria for a high risk of being affected by fraud. Especially the addition of cheaper bulk materials provides a route to commit fraud (Silvis, van Ruth, van der Fels-Klerx, & Luning, 2017). The prediction of possible adulterants is not always an easy or sometimes even a possible task. However, the cost of the adulterant, ease of obtaining sufficient quantities of the adulterant, compatibility with the matrix and having a viable method to adulterate the matrix are key aspects to consider in the assessment of possible adulterants (US Pharmacopoeia, 2017). Papaya seeds (*Carica papaya L.)* are assumed to be one of the most common adulterants used for black pepper, due to structural resemblance, low price, and easy availability. (Dhanya, Syamkumar, & Sasikumar, 2009), (Curl & Fenwick, 1983) (Bhattacharjee, Singhal, & Gholap, 2003). Chili has been found in two of nine tested black pepper market samples (Parvathy et al., 2014). Moreover has the adulteration with buckwheat (*Fagopyrum esculentum*) and millet [finger millet (*Eleusine coracana*); pearl millet (*Pennisetum glaucum*)] been investigated. (McGoverin, September, Geladia, & Manley, 2012). Finally, suspicious pepper samples have been inspected by microscopic analysis (Tremlovà, 2001). The Food and Drink Federation recommends in their guidance on authenticity of herbs and spices to consider the addition of extraneous matter from the same plant as well as exhausted also referred as spent, defatted and depleted material (Food and Drink Federation, 2016). To the best of the authors' knowledge methods to detect a broad range of potential adulterants in black pepper are not available. Ground black pepper is the most common form of black pepper available to the consumer in the western countries (Ravindran & Kallupurackal, 2012). It is particularly vulnerable to fraud, as an

additional processing step is included and adulteration cannot easily be detected by visual examination alone.

There are several methods available to characterize ground black pepper samples, but these methods are expensive and time consuming (Aurich, 2009). In relation to the globally traded amount of pepper an adequate number of controls by several traditional analytical methods is not realistic. In contrast, spectroscopic fingerprinting techniques have been shown to be low cost, rapid and comprehensive characterisation tools (Casale, Bagnasco, Casolino, Lanteri, & Leardi, 2016). Along with chemometrics a resolution of unique chemical information is provided, which allows rapid monitoring of subtle compositional changes (Rodriguez-Saona, Giusti, & Shotts, 2016). Therefore the comparison of the fingerprints obtained from authentic samples to adulterated samples can reveal mis-description or adulterations (Riedl, Esslinger, & Fauhl-Hassek, 2015).

The differential diagnostic potential of spectroscopic methods to determine the deliberate addition was shown for illegal dyes like Sudan dyes to paprika (Horn, Esslinger, Pfister, Fauhl-Hassek, & Riedl, 2018) and chili (Haughey, Galvin-King, Ho, Bell, & Elliott, 2015) and also plant-derived adulterants to saffron (Petrakis & Polissiou, 2017) and oregano (Black, Haughey, Chevallier, Galvin-King, & Elliott, 2016), (Wielogorska et al., 2018). A big advantage of spectrometric methods combined with chemometrics lies in the fact that once a database is established and a suitable data analysis protocol is determined, a new sample can be screened within a few minutes. With a suitable user interface, even non-specialist personnel can undertake sample analysis in QC laboratories and factories (Wilson, Colquhoun, & Kemsley, 2001).

The present research investigated the feasibility of using NIR and FT-IR fingerprinting to detect economically motivated adulteration in black pepper. Based on a detailed literature review about fraud incidents in black pepper and advice from the industry, a supervised classification model with papaya seeds, chili, black pepper husk and black pepper spent material has been developed. The potential of a non-targeted spectroscopic screening method for the detection of economically motivated adulteration in black pepper is presented.

2 Material and Methods

2.1 Sample collection and preparation

A total of 115 samples of black pepper were investigated in this study. Samples from India (n=3), Vietnam (n=33), Indonesia (n=6), Sri Lanka (n=3), Brazil (n=19) and Malaysia (n=2) were provided by five different organisations. For nine samples the country of origin is unknown, 40 samples are a mix from Vietnam, Indonesia and Sri Lanka. In addition, black pepper husk (two from India, one from Vietnam), spent material

(two from India), pinheads (two from India), papaya seeds (two), and chili powder (three) were sourced from the internet and local retailers. All samples were stored at room temperature in the dark under clean and dry conditions. Prior to spectroscopic analysis the samples and adulterants were ground to a homogeneous powder using a ball mill (Planetary Ball Mill: PM-100 (Retsch, Haan, Germany). Approximately 5 g solid material were added into grinding jars and milled at 600 rpm for 5 min. For the adulterated sample three pepper pools (obtained by the combination of different black pepper samples) have been spiked at levels of 10, 20, 30 and 40 g/100g with husk, spent, chili and papaya powder, respectively. In total 48 spiked samples were prepared.

2.2 FT-IR and NIR data acquisition

FT-IR and NIR spectra were collected on a Thermo *iS50* spectrometer (Thermofisher, Dublin, Ireland) at ambient temperature. Prior to sample acquisition an internal background has been collected. The FT-IR samples were analysed on the benchtop FTIR with ATR accessory, diamond crystal and DLaTGS detector. The samples were placed on the diamond crystal sample area, while equal pressure was applied by the slip clutch pressure tower. Spectra were acquired in the 4000-400 cm^{-1} range with 32 scans and a resolution of 4 cm^{-1}. The NIR samples (5g) were poured onto the sample cup (diameter: 3.2 cm, height: 1.5 cm) spinner on the Integrating Sphere module of the instrument. Spectra were acquired in the 12000-4000 cm^{-1} range with 32 scans with a resolution of 8 cm^{-1}. Three replicates of each sample were measured and averaged prior to data pre-processing.

2.3 Chemometrics

Chemometric analysis was carried out using the software SIMCA 15 (Umetrics, Sweden). Prior to model development pre-processing was conducted. The spectral regions between 2800-1800 cm^{-1} in the FT-IR spectra and the region between 12000-9000 cm^{-1} in the NIR spectra contain no relevant sample information and were removed. There upon common scaling and pre-processing methods and combination thereof were applied to the reduced data sets. The pre-processing methods were derivatives, Savitzky-Golay (SG) and Standard normal variate (SNV) (Table 1).

Table 1: Pre-processing methods and different combinations applied to the NIR and FT-IR data

1st step	2nd step	scale
none	none	none
none	none	pareto
SNV	none	pareto
1st derivative, SG	SNV	pareto
SNV	1st derivative, SG	pareto

Principal Component Analysis (PCA) an unsupervised technique was used to explore differences between samples and adulterants based purely on spectral differences. Moreover, the variations between black pepper samples from different geographical origins have been investigated. Afterwards a binary model was built with the supervised technique orthogonal partial least square discriminant analysis (OPLS-DA). The classification model was optimised by single cross validation. 6/7th of the dataset were used to create a model, while 1/7th was removed as validation set. For each validation set the prediction error is stored. This procedure was repeated for all subsets. The total prediction error for all the models is calculated and the model with the lowest total prediction error selected. R2 is a measure that describes how well the data are explained by the model. Q2 indicates for the prediction ability of the model and is here used as a measure to describe the model predictability after internal validation (Sartorius-Stedim, 2017). The model predictability has further been evaluated by running a test-set with samples that has not been involved in the model development (external validation). Thirty pure black pepper samples (typical samples) and 48 spiked samples (atypical) were included in the test set. The sensitivity is the percentage of the correctly detected pure black pepper in relation to all pure black pepper samples in the test set. It can be expressed as TPR = TP/(TP+FN), where TRP describes the true positive rate, TP the true positive (correctly identified samples), and FN the false negative (incorrectly rejected samples). The specificity is the percentage of detected adulterated samples in relation to the adulterated samples in the test set. It can be expressed as TNR= TN/(TN+FP), where TNR describes the true negative rate, TN the true negative (correctly rejected) and FP the false positive samples. The ROC (receiver operator characteristic) curve has been used to assess the prediction ability of the model after external validation. The ROC combines the true positive rate (TPR, sensitivity) with the False positive rate (FPR, 1-specificity). A ROC curve plots TRP of a test set versus FPR. The work flow for the exploratory data analysis, model development, internal and external validation is summarised in Fig.1.

Fig. 1. Workflow for the binary o-PLS-DA classification model, including PCA reliability check, pre-processing, model development, internal and external validation

3 Results and discussion

3.1 NIR and FT-IR spectra of black pepper and adulterants

The visual inspection of the FT-IR and NIR spectra of black pepper and the adulterants reveals that both techniques enable the observation of spectral differences. Spectra A1 (Fig. 2) shows the FT-IR spectra of black pepper, husk, pinheads and spent material. Relevant sample information about functional groups are in the region between 3800-2800 cm^{-1}, whereas the fingerprinting region is found

between 1800-400 cm^{-1}. Black pepper husk is the skin of black pepper, pinheads are the under-developed and broken berries of black pepper and the so-called spent material is obtained after the oleoresin is extracted from the black pepper berries. Oleoresin contains piperine, the most pungent main compound in black pepper. From FT-IR spectral information, pepper oleoresin has shown to have mainly bands due to =C-O-C stretching vibrations at 1194 cm^{-1} and 1252 cm^{-1} as well as CH_2 wagging vibrations at 996 cm^{-1}, which is in correspondence with the specific signals at 1633 cm^{-1}, 1580 cm^{-1}, 1252 cm^{-1} and 996 cm^{-1} for piperine (Schulz, Baranska, Quilitzsch, Schütze, & Lösing, 2005). The respective bands are regarded as diagnostic signals, which distinguish the black pepper fruits from husk, pinheads and spent material. The latter are expected to contain no or very little rests of oleoresin (Fig. 2, Spectra A1). The differences between black pepper fruits and foreign plant material like papaya and chili are more obvious, the most significant variations are in the fingerprinting region (Fig. 2, Spectra A2). In NIR spectra absorptions of overtones or combinations of fundamental stretching bands occur. The bands, usually caused by C, H, N or O stretching are weak in intensity and very often overlapping. Therefore the NIR is often not very useful for identification purposes. However, important differences between the samples can be seen in the raw spectra (Fig. 2, Spectra B1-2) and in combination with chemometrics these differences have the potential to be exploited for classification models (Stuart, 2000).

Fig. 2. FT-IR (A1-A2) and NIR (B1-B2) averaged raw spectra of black pepper, husk from Vietnam, husk from India, pinheads, spent, chili, papaya. Spectral regions without relevant sample information are grey shaded

3.2 Pre-processing

Pre-processing aims to enhance the feature sought in the spectra by reducing the variability that is not related to the sample information of interest (Rinnan, Berg, & Engelsen, 2009). First, the raw spectra were inspected and regions without sample information have been removed. Mathematical pre-processing methods like derivatives, Savitzky-Golay (SG) and Standard normal variate (SNV) are used for scatter corrections, resolution of peak overlap and elimination of linear baseline drifts. There is no straight-forward strategy to apply mathematical pre-processing techniques that is suitable for all data and purposes. It is rather difficult to assess a proper choice of pre-processing prior to model validation. Therefore, several pre-processing methods have been tested. The prediction measure error Q2 value, which is a measure for the prediction ability of the model obtained by internal cross-validation of the multi-class training set has been used as a parameter to assess the suitability of pre-processing method. Derivatives, SG and SNV are very commonly used techniques and have been tested individually as well as the combinations thereof (data not shown). Finally, SNV, 1st Derivative and SG were found as a suitable choice for the model generated by NIR and FT-IR data.

3.3 Exploratory data analysis

When using multiple linear regression techniques with a dataset where the number of variables exceeds the number of samples, there is a risk of building a too optimistic model (Brereton & Lloyd, 2014). Spectral datasets usually contain much more variables than samples and it must be ensured that correlation with the classifier is not just by chance, but due to reliable differences (Westerhuis et al., 2008). Principle component analysis (PCA) as an unsupervised method can serve as an indicator for model reliability. The principle components in a PCA model point in the direction of maximum variance (Bro & Smilde, 2014). Therefore differences between the groups are exclusively caused by the major contributors to the total variability, which are considered as more reliable. In contrast to that, OPLS-DA can find smaller sources of variation in order to maintain group separation. These variations might be less reliable and therefore less viable for a generalised model (Worley & Powers, 2016). However, the score plot of the first two components of the PCA models generated by the FT-IR and NIR data show separation between the groups of black pepper and the other adulterants (Fig. 3, score plot A1 and B1). Thus, it is indicated that the differences between the groups shown by the OPLS-DA model (Fig. 3, score plot A2 and B2) are based on reliable variations in the dataset.

Fig. 3. Multiclass unsupervised PCA (A1 and B1) and supervised OPLS-DA (A2 and B2) score plots of the FT-IR and NIR data (pure black pepper and pure adulterants)

The 115 black pepper samples originate from six different countries and were provided by five different organisations. The pepper samples from a quite heterogeneous origin are tightly clustered together and show a clear distance to the adulterants (Fig. 3). The OPLS-DA models generated by NIR and FTIR respectively, reveal that adulterants from the pepper plant (spent, husk, pinheads) are related to positive scores, while adulterants from foreign material (chili, papaya seeds) are related to negative score values on the second factor.

Considering the information about the geographical origin the PCA model illustrates that the different black pepper samples are similar and can therefore not be clearly seperated by the origin (Fig. 4). Thus, these methods are not indicative to describe the geographical origin of black pepper. However, given that spectral characteristics of the ground black pepper are not mainly determined by the geographical origin, it indicates that the available samples set is useful even though it reprents only six of the twenty-six countries that cultivate black pepper.

Fig 4. PCA score plots of FT-IR (A) and NIR (B) with 66 black pepper samples from different geographical origins

Non-targeted binary classification model

Due to the nature of food fraud it is not known which adulterants may be added to black pepper. Thus, a rapid quality check will determine, whether a sample meets the specifications of black pepper or not. A non-targeted binary model with typical samples (black pepper) and atypical samples (adulterants and spiked black pepper) has been developed and validated. The model has been assessed by internal cross-validation followed by an external validation. A harmonised validation procedure for non-targeted fingerprinting approaches is still missing (Esslinger, Riedl, & Fauhl-Hassek, 2014), (McGrath et al., 2018). However, in order to test whether the model is suitable for general applicability it is required to run a test-set that has not been involved in the model development (US Pharmacopoeia, 2017).

Fig. 5. Binary supervised OPLS-DA score plots of the FT-IR (A) and NIR (B) data (pure black pepper and pure adulterants)

The training set has been used to generate the binary OPLS-DA model (Fig. 5). The optimal number of components has been determined by a 7-fold internal cross validation. High values for measure of fit R2 and the prediction ability Q2 indicate that the model describes and predicts the data very well (Table 2).

Table 2: Measure of fit R2 and prediction ability Q2 of the FT-IR and NIR binary model

	R2	Q2
NIR	0.93	0.98
FT-IR	0.83	0.97

The binary classification model was generated with pure black pepper samples and pure adulterants. The test-set, consisting of 48 spiked black pepper samples and 30 pure black pepper samples, was run against this model. For generating atypical samples spiking level of 10-40% (w/w) has been chosen. Based on the authors' extensive knowledge of food fraud levels of bulking from 10% upwards makes adulteration economically viable, whereas a bulking level above 40% can become very suspicious.

The area under the ROC curve (AUROC) serves as a quality measure that enables the comparison between different models. A random decision method is represented by an AUROC-value of 0.5, whereas a good non-targeted method should have an AUROC-value close to 1 (US Pharmacopoeia, 2017). The areas under the ROC curve for the model generated by the NIR and the FT-IR dataset are 0.98, respectively (Fig.6). The high values indicate, that both binary classification models are equally suitable for the detection of adulterants in black pepper samples.

Fig. 6. ROC curve for the prediction of the test sets run against the binary classification models based on FT-IR (A) and NIR (B) data. The vertical line (J) shows the maximum value of Youden's-index for the ROC-curve.

Moreover, the ROC curve is used to assist with setting the model boundaries, which is a trade-off between the sensitivity and specificity rate of the method. The risk of not-detecting adulterated samples must be

balanced against possible incurred cost by discarding or double checking unadulterated samples. This method is intended to detect adulterants that are added as cheaper bulk material, which causes a decrease of the quality of the product, but are not expected to be a food safety risk. Therefore a very high specificity rate, which ensures the detection of all even low adulteration level at all costs is not reasonable for this specific case. The Youden-Index *J* describes the threshold where the separation between the groups is most efficient, it can be calculated by $J = \max_c \{\text{Sensitivity}(c) + \text{Specificity}(c) - 1\}$, where c describes all possible threshold values. The Youden index ranges from 0 to 1, while a value of 0 indicates no separation between the pure and the adulterated samples and a value of 1 perfect separation. A Youden-Index of 0.95 and 0.96 have been determined for the FT-IR model and NIR model, respectively. The Youden-index has been chosen as exemplary threshold for the FT-IR and NIR model, the obtained specifity and sensitivity rates are presented in Table 3. The specificity rates for respective subgroups of spent/husk and the foreign plant material papaya/husk are the percentages of samples that have been correctly classified within this subgroup. The specificity rates for respective subgroups of spent/husk and the foreign plant material papaya/husk are the percentages of samples that have been correctly classified within this subgroup.

Table 3: Sensitivity and specificity rates of exemplary thresholds obtained by the Youden-Index

	Adulteration level	Percentage of correct classification (NIR model)	Percentage of correct classification (FT-IR model)
Black Pepper (Sensitivity)	0%	90%	93%
Adulterants (Specificity)	10-40%	100%	98%
Spent/Husk (Specificity)	10-20%	100%	100%
	20-30%	100%	100%
Papaya/Chili (Specificity)	10-20%	100%	92%
	20-30%	100%	100%

The model performance allows to choose thresholds that leads to high sensitivity and specificity rates. As the differences in the spectrum between adulterant and black pepper are more pronounced at a higher adulteration level the prediction reliability increases with a higher spiking level. The method has shown to be capable of detecting adulterants in ground black pepper and provides an impetus to working towards the introduction of this non-targeted fingerprinting approach to the routine testing environment. The model can be further extended by the addition of more adulterants and black pepper samples from different regions. Moreover, the system should be challenged by long term stability tests and robustness checks like e.g. analysis by different operators and different instruments (Riedl et al., 2015)(Riedl et al., 2015).

4 Conclusion

In this feasibility study, FT-IR and NIR spectroscopy were combined with chemometrics to test the potential of revealing economically motivated adulteration in ground black pepper. A non-targeted fingerprinting approach was applied to detect adulterants such as papaya seeds and chili, but also adulterants from the black pepper plant itself like husk, pinheads and spent materials. The latter of these are highly likely to be added to ground black pepper by unscrupulous companies and are impossible to detect by methods that are based on DNA analysis. Internal and external validation indicate that classification models built on FT-IR as well as NIR spectroscopic data are capable of detecting these forms of adulteration. This study has shown the potential for a fast and rapid screening of ground black pepper and gives a strong impetus to expand the approach by further validation and system challenges. Considering the huge volume of worldwide traded black pepper, a fast, cheap and easy to use surveillance method is needed to counterfeit fraud in an efficient way.

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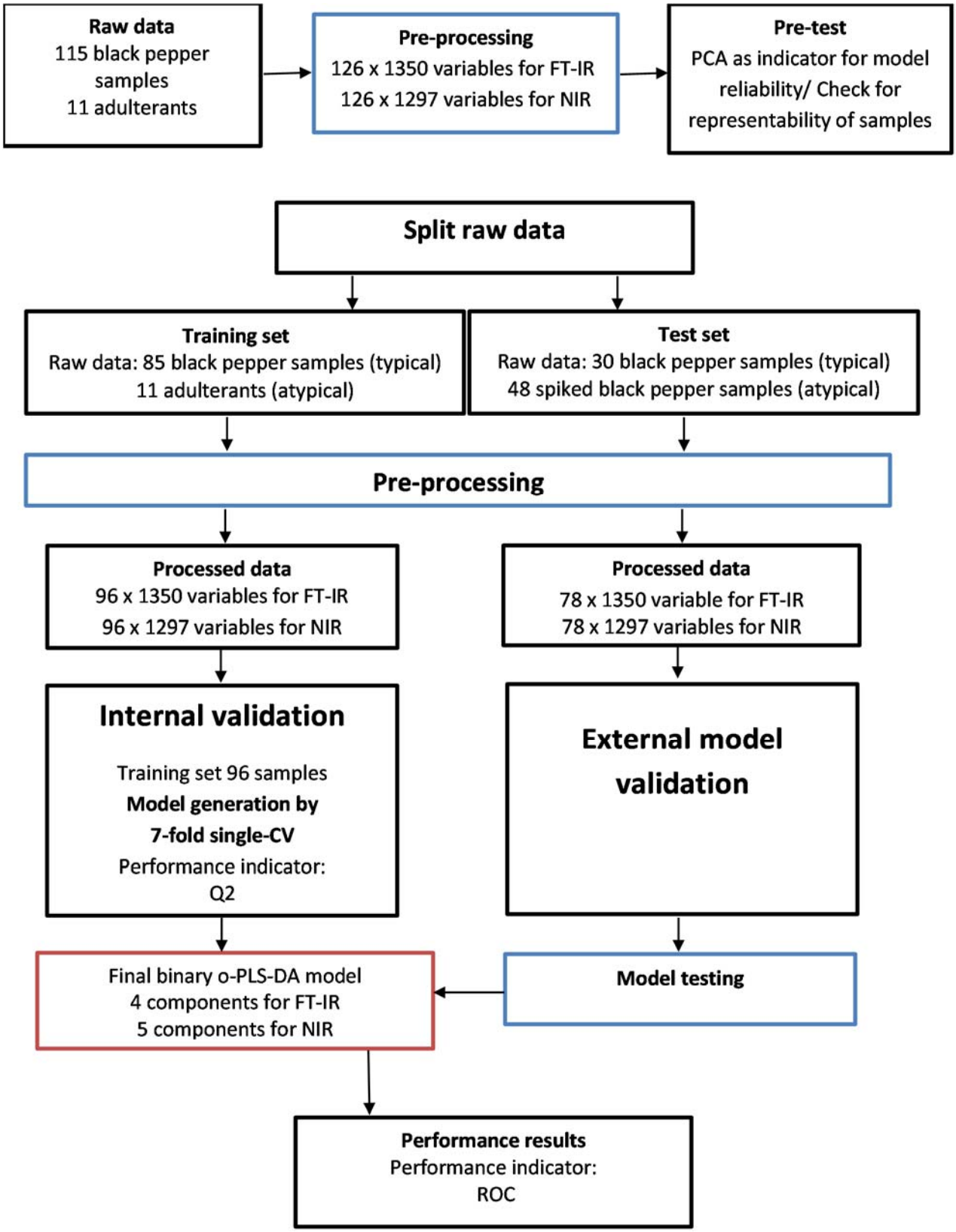

Fig. 1. Workflow for the binary o-PLS-DA classification model, including PCA reliability check, pre-processing, model development, internal and external validation

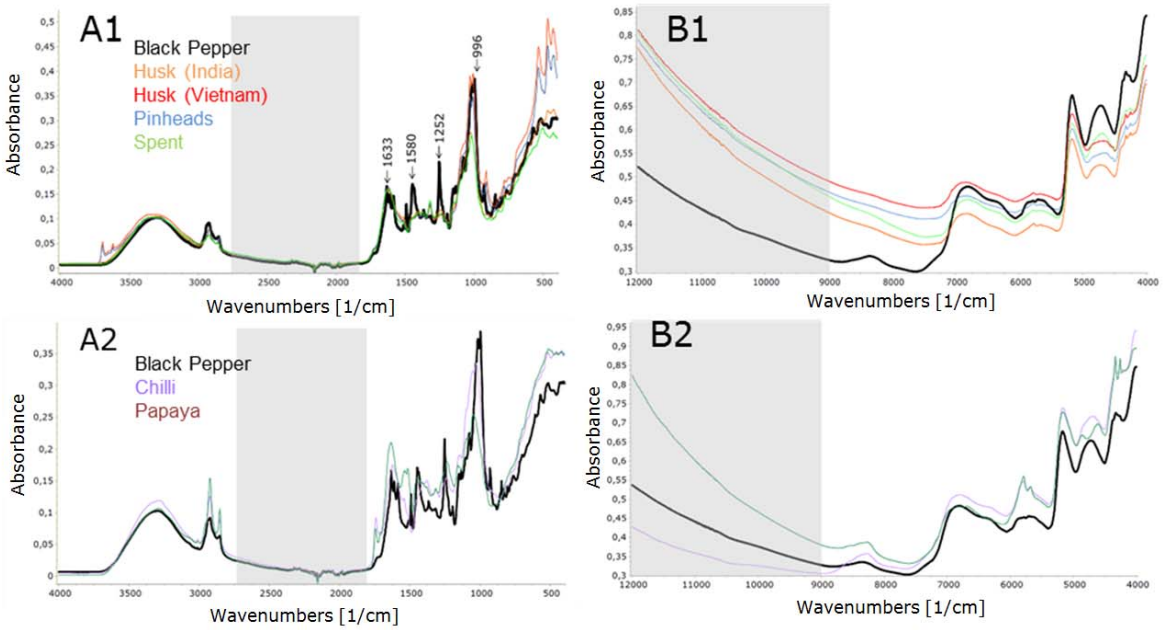

Fig. 2. FT-IR (A1-A2) and NIR (B1-B2) averaged raw spectra of black pepper, husk from Vietnam, husk from India, pinheads, spent, chilli, papaya. Spectral regions without relevant sample information are grey shaded

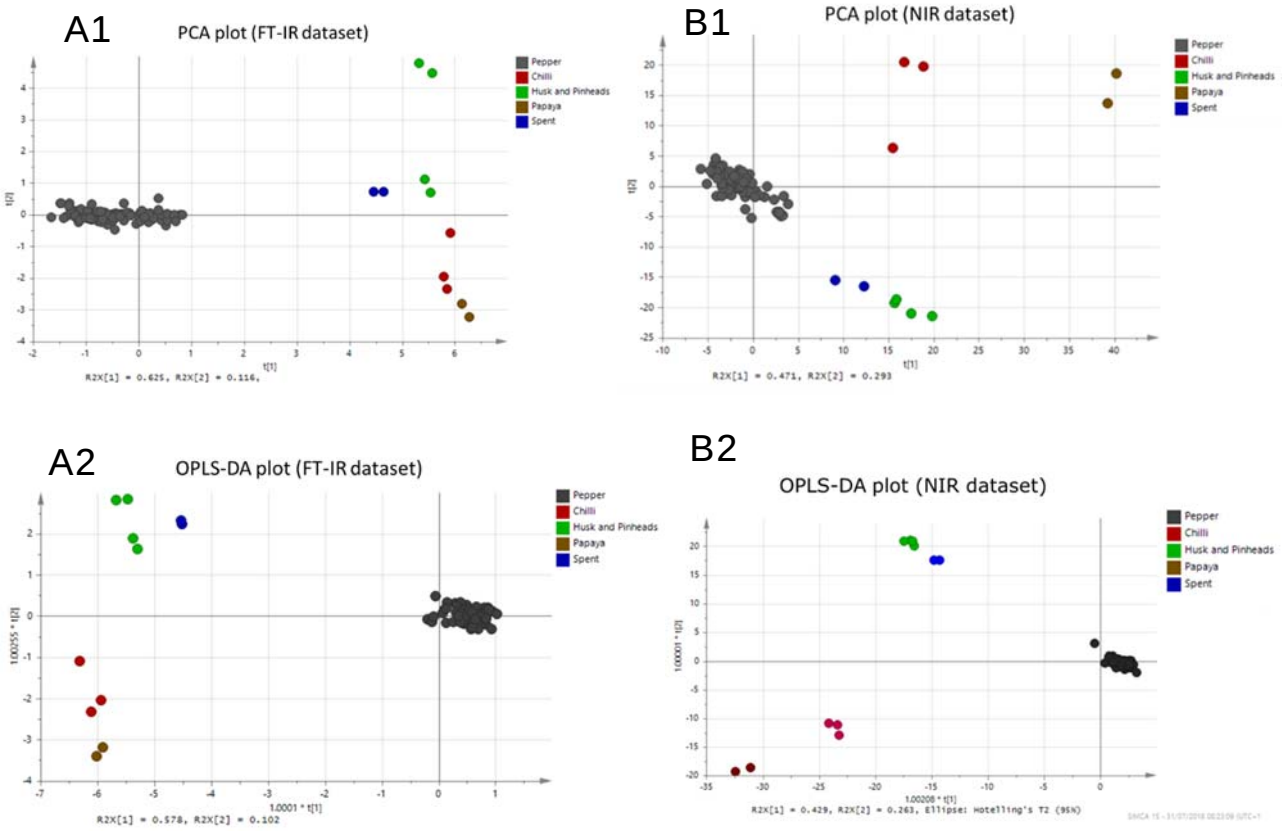

Fig. 3. Multiclass unsupervised PCA (A1 and B1) and supervised OPLS-DA (A2 and B2) score plots of the FT-IR and NIR data (pure black pepper and pure adulterants)

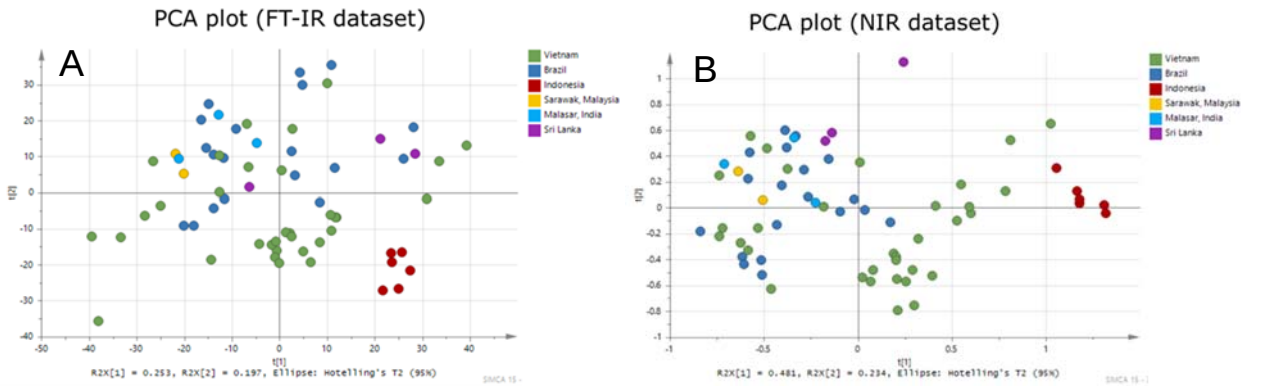

Fig 4. PCA score plots of FT-IR (A) and NIR (B) with 66 black pepper samples from different geographical origins

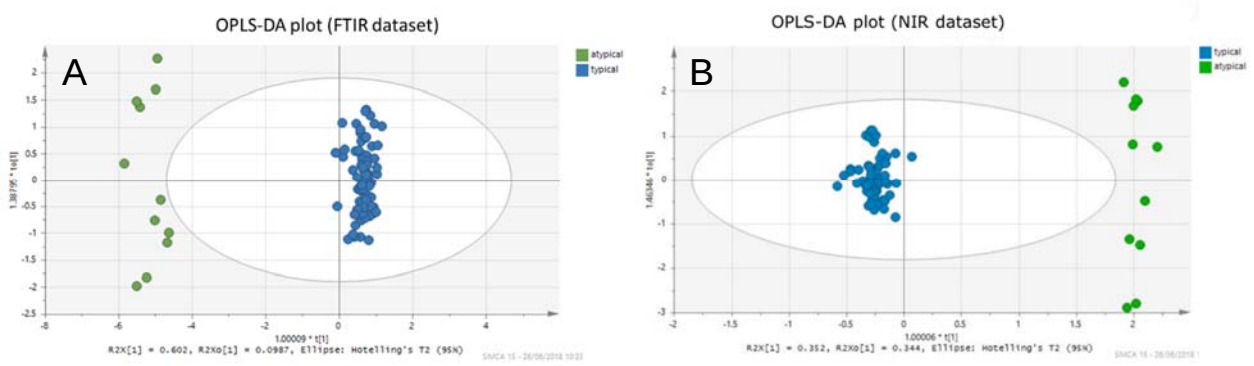

Fig. 5. Binary supervised OPLS-DA score plots of the FT-IR (A) and NIR (B) data (pure black pepper and pure adulterants)

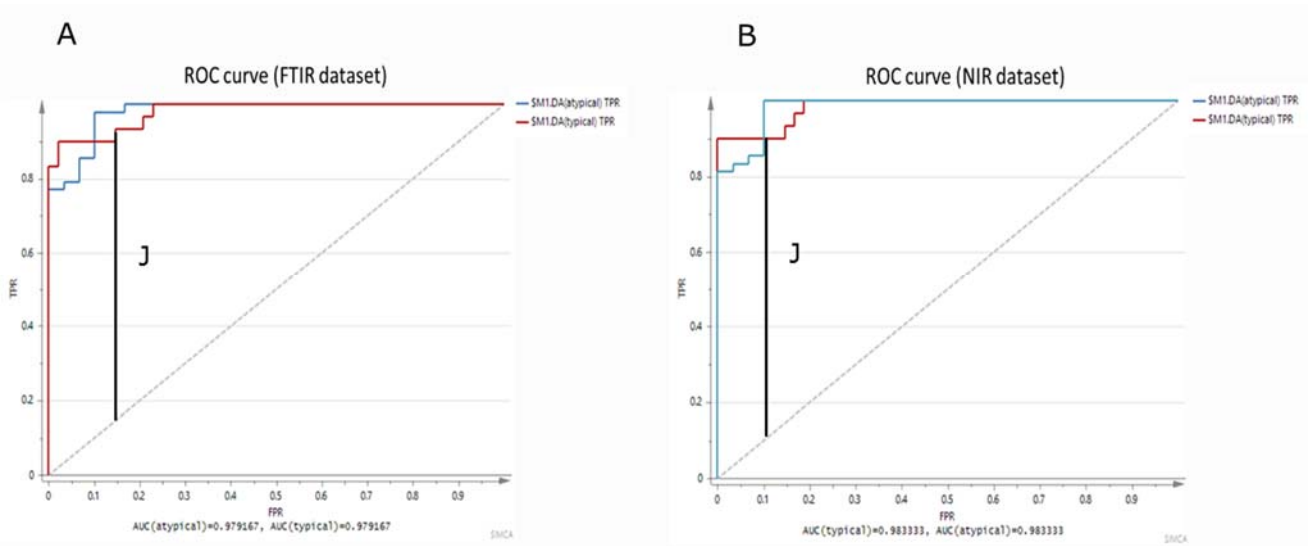

Fig. 6. ROC curve for the prediction of the test sets run against the binary classification models based on FT-IR (A) and NIR (B) data. The vertical line (J) shows the maximum value of Youdan's-index for the ROC-curve.