



A simple strategy for the detection of an undeclared hydrothermal treatment of poppy seeds using FT-IR spectroscopy

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Abstract

The cultivation of low-opium varieties of poppy (*Papaver somniferum* L.) in Central Europe has a very long tradition. The blue seeds, thanks to their delicious taste, have several uses, both in the food industry and culinary practice. However, when seeds are mechanically damaged and/or ground, lipase is released, and a bitter taste develops due to the accumulation of free fatty acids. To prevent hydrolytic rancidity, seeds can be treated with hot steam before grinding. In this way, inactivation of native lipase is achieved. Unfortunately, in some cases, fraudsters misuse the thermostabilization process to remove opium alkaloids from seeds of pharmaceutical poppy varieties, which are then marketed as a food poppy variety. Distinguishing these mislabeled seeds from authentic ones is a rather difficult task. In this study, we have developed a simple Fourier-transform infrared spectroscopy (FT-IR) based strategy for identifying heat-treated poppy seeds. Instead of poppy oil isolation for monitoring the acid value increase, which would occur in case of native seeds, the assessment of spectral changes in the 1730–1690 cm⁻¹ region after poppy seed grinding was employed to recognize thermostabilized seeds. The validation of this novel approach was performed on several poppy seed varieties from various harvest years and also on the set of 108 samples provided by the industrial partner, resulting in 2% of false positives and 7% of false negatives when using the decision criterion of lipase activity factor [LAF] >10%. The working hypothesis was supported by U-HPLC-HRMS/MS analysis of the oil component of the tested seeds.

Keywords Poppy seeds · Adulteration · Authenticity · Lipase activity · FT-IR · Thermal processing

Introduction

Papaver somniferum L. is a flowering plant grown, both legally and illegally, in many regions around the world for various purposes. Number of its forms, differing in various parameters including opium alkaloids content, the colour of seeds, etc., are known [1, 2]. Breadseed poppy is a traditional agricultural crop widely cultivated in Central European countries as a source of seeds, which are used either for oil production or as a decoration and/or filling of bakery products [3–5]. In contrast to high opium poppy varieties (e.g. Buddha, Lazur, Fortemo etc.) [2, 6] used by the

pharmaceutical industry for analgesics production [7], those grown for food uses are specially bred to contain low amounts of opium alkaloids in the poppy straw. Although these bioactive secondary metabolites are not primarily present in seeds, their contamination may be caused by latex. This milky sap containing opium alkaloids is released when an unripe poppy capsule is mechanically damaged, e.g., by insects [8]. Alternatively, when not removed during the cleaning process, pieces of poppy straw stuck to the surface of seeds during the harvest can cause contamination. Importantly, blue poppy seeds obtained from certified varieties grown in the Czech Republic are considered of premium quality due to their unique flavor and low contamination of seeds by opium alkaloids [9, 10]. In 2021, the Czech blue poppy was registered as a Protected Geographical Indication.

Like other high-value commodities, the Czech blue poppy can be a target for fraud. Dilution, or even replacement of premium seeds with cheap pharmaceutical seeds waste, represents an apparently high profit for a deceiver

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and seemingly low risk for a consumer. However, in addition to its unpleasant taste, pharmaceutical poppy is not suitable for human consumption because of its typically high content of opium alkaloids (achieving up to hundreds of mg/kg) [11]. More than 80 different alkaloids have been isolated from the poppy plant to date; nevertheless, the most abundant are typically morphine, codeine, and thebaine [12]. It should be noted that consuming even a small amount of food containing contaminated seeds may pose a health risk for consumers. The European Food Safety Authority (EFSA) panel on Contaminants in the Food Chain (CONTAM) established the Acute Reference Dose (ARfD) for morphine equal to 10 µg/kg body weight (bw). For codeine, the 'morphine equivalent' is calculated using the conversion factor of 0.2 on its concentration [8]. A similar approach is employed in the current EU legislation; the Commission Regulation (EU) No 2023/915 has set maximum limits for morphine equivalents 20 mg/kg in poppy seeds and 1.5 mg/kg in bakery products [13].

Given that the presence of opium alkaloids in food generally poses a safety risk, recommended pre-treatment and processing methods that reduce the alkaloid content in poppy and poppy products have been summarized in the European Commission (EC) Recommendation 2014/662/EU [14]. Besides applying good agricultural practices, one of the recommended mitigation measures is the treatment of seeds with high temperatures and/or hot steam. In addition to decontamination from opium alkaloids, such procedures result in the inactivation of lipase, an enzyme that may cause hydrolytic rancidity of the oil component associated with the development of a bitter taste of ground/mechanically damaged poppy seeds during storage. At the same time, such a thermal treatment may, presumably, induce oxidation processes of polyunsaturated fatty acids, and, consequently, a loss of typical poppy seed flavor.

Recently, the Czech Agriculture and Food Inspection Authority raised the issue of possible misuse of the thermostabilization process for decontamination of pharmaceutical seeds, which are further marketed as Czech poppy seeds. On this account, the need for a relevant laboratory method enabling the detection of this type of economic fraud has become urgent. As the concentrations of opium alkaloids in thermostabilized pharmaceutical poppy seeds are, thanks to their washing out, presumably low, not significantly differing from those in authentic seeds intended for food use [11, 15], therefore, the analysis of these secondary metabolites is not applicable for fraud detection. Under such conditions, another marker/typical parameter must be used for distinguishing untreated and heat-treated poppy seeds. Needed to mention that, despite a thorough literature search, no suitable solution for the given purpose was found. Therefore, it was concluded that the characterization of lipase activity

is the only conceivable parameter that would allow distinguishing between native and heat-treated seeds.

In this study, we demonstrate the development of a new, simple, and cost-efficient Fourier transformation mid-infrared (FT-IR) spectroscopy method for distinguishing thermostabilized and untreated (native) poppy seeds based on the monitoring of spectral changes induced by grinding. The working plan was based on the following assumption: while in untreated seeds the increase of free carboxylic groups commonly occurs, this should not be the case in thermostabilized poppy. To verify the occurrence of those processes, an ultra-high performance liquid chromatography coupled to high-resolution tandem mass spectrometry U-HPLC-HRMS/MS method was employed to analyze and compare the profile of lipids isolated from ground treated and untreated seeds after storage.

Materials and methods

Chemicals and reagents

Dichloromethane, isopropyl alcohol, and methanol for U-HPLC-HRMS/MS were of HPLC grade and obtained from Merck (Darmstadt, Germany). Petroleum ether, hexane, methanol, and KOH were of analytical grade and obtained from Penta (Prague, Czech Republic). Phenolphthalein was obtained from Fluka (Buchs, Switzerland).

Poppy seed samples

Authentic blue breadpoppy seeds were provided by the Czech Central Institute for Supervising and Testing in the Agriculture, which is responsible for their registration. Variety Major (2000 g) was used for experiments aimed at the construction of a model for thermal treatment detection. For the evaluation of factors potentially influencing lipase activity, 29 untreated poppy seed samples and 13 thermostabilized seeds were used. The samples included 14 different poppy varieties (Aplaus, Bergam, Gerlach, Major, Maratón, MS 1–15, MS 4–15, MS 521, Onyx, Opal, Opex, Orbis, Racek), including one sample of a high-thebaine (pharmaceutical) variety with thebaine content 390 mg/kg, and one high-morphine variety with morphine content 202 mg/kg. Pharmaceutical seeds were provided by the Czech University of Life Sciences in Prague. Samples originated from 10 locations in the Czech Republic (localities Bratkovice, Domanínec, Horažďovice, Klecany, Lednice, Libochovice, Milčevce, Praha, Pusté Jakartice, Trutnov), and 6 harvest years (2014–2019). The complete list of samples is available in the Supplementary material S1. Similarly to conditions employed in industry, thermostabilization of seeds was

performed by steaming for one hour in a steam pot or by quick (30 s) washing with boiling water.

To validate the method, a set of 108 anonymized (coded) test samples was provided by the Czech Agricultural and Food Inspection Authority (CAFIA). The only available information about the delivered poppy seeds was that half of them were thermostabilized. The seeds were examined in the same way as described above. The assessment of the data (false positive/false negative classification of respective sample category) obtained by analysis was performed in cooperation with CAFIA based on the report.

Poppy seed oil extraction

Oil used for the determination of acid value was extracted from poppy samples as follows: 25 g of ground seeds were placed into a 250 ml Erlenmeyer flask, then 75 ml of dichloromethane were immediately added, and the suspension was shaken for 15 min at 240 rpm using a laboratory shaker (IKA Labortechnik, Staufen im Breisgau, Germany). After that, the supernatant was filtered by a glass frit (pore size S1), and the remaining solid portion was re-extracted with 75 ml of dichloromethane. The solvent from combined extracts was removed using a rotary vacuum evaporator (Rotavapor R-100, BÜCHI, Flawil, Switzerland) (250 mbar, 10 min; then 150 mbar, 5 min). The oil obtained was placed into 15 ml cuvettes and centrifuged (Rotina 35 R, Hettich Zentrifugen, Mülheim an der Ruhr, Germany) for 5 min (10 000 rpm) to separate any residual seed particles from the extracted oil.

Oil preparation for lipidomic screening

Extracted poppy seed oil was further used for lipidomic screening. 10 mg of oil was placed into a 1.5 ml Eppendorf tube and diluted using 1 ml of isopropanol: methanol (65:35, v/v). The sample was then placed into the vial and analyzed using U-HPLC-HRMS/MS.

Determination of acid value of poppy oil based on the titrimetric method

A titrimetric method based on the ISO 660:2019 standard was used for the acid value determination. Briefly, 1 g of the extracted oil was weighed into a titration flask. After that, 50 ml of isopropanol: petroleum ether (1:1, v/v) and 2 drops of phenolphthalein solution (1%, w/v) were added as a pH visual indicator. The sample was then titrated with 0.1 M KOH in methanol. The acid value was expressed as the number of milligrams of KOH needed for the neutralization of free fatty acids in 1 g of the sample.

Fourier-transform infrared spectroscopy-based analysis of ground poppy seeds

For the monitoring of the changes accompanying free fatty acid release in ground poppy seeds, FT-IR spectroscopy was used. The Nicolet iS50 FT-IR (Thermo Fisher Scientific, USA) instrument with deuterated triglycine sulphate (DTGS) thermal detector and KBr as a beam splitter used for this purpose was operated in attenuated total reflectance (ATR) mode. The spectrometer was run under the Omnic v 9 software (Thermo Scientific, USA). Ground samples of poppy seeds were applied to the diamond ATR crystal. All spectra were acquired in the range of 4000–400 cm^{-1} in triplicates, each time with a freshly applied sample. The number of scans for background and sample collection was set to 32, the resolution to 4 cm^{-1} , the digital data spacing to 1.928 cm^{-1} , and absorbance was selected as the output. The background spectra were collected before every new sample measurement.

Creation of the partial least squares (PLS) regression model

To create a model for detecting the free fatty acid release, samples of seeds differing in acid value (AV) of extracted oil were used. A 40-day storage experiment was carried out to obtain material for the construction of the model. In detail, the storage and sampling were performed as follows: a 1000 g aliquot was taken from a 2000 g poppy seed (Major variety) sample and was thermostabilized using water steam for 60 min. After that, thermally treated seeds were dried in a laboratory drier at 30 °C for 8 h. Subsequently, 500 g of the thermostabilized seeds and 500 g of untreated seeds were ground using a laboratory knife mill. After this step, four different types of the original sample were obtained: (i) untreated whole, (ii) untreated ground, (iii) treated whole, and (iv) treated ground seeds were placed into a metalized polyethylene terephthalate/biaxially oriented polypropylene (PET/BOPP) film package with a thickness of 45 μm . During storage, seeds were sampled for spectroscopic analysis by FT-IR and acid value determination. The storage experiment design is shown in Fig. 1. The sampling process is shown in Table 1. Using TQ Analyst v 9 software (Thermo Scientific, USA), the acid values of the extracted oils were assigned to the respective IR spectra. A total of 200 spectra were then used to build the PLS regression model, with 171 spectra used for calibration and 29 for model validation. The acid values (AVs) of oils, used to assign to the FTIR spectra, used for calibration and validation, determined by the titrimetric method described in Sect. [Determination of acid value of poppy oil based on the titrimetric method](#), ranged from 2.36 mg KOH g^{-1} fat (AV of freshly ground seeds) to

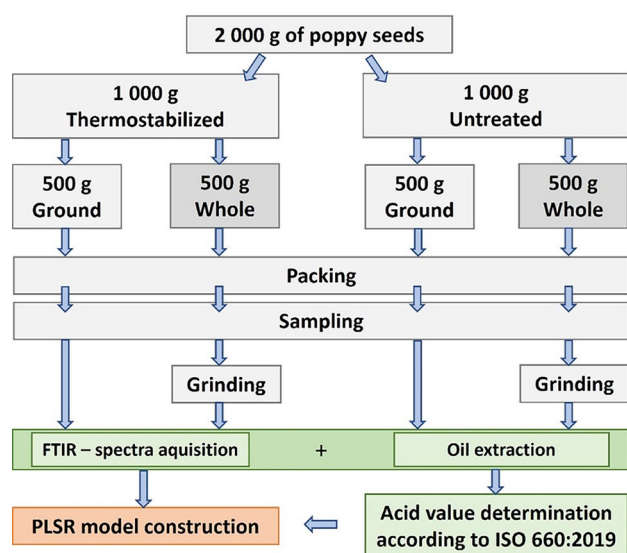


Fig. 1 Set-up of storage experiment and data collection for the PLS regression model construction

Table 1 Sampling process during the storage experiment

Sampling No.	Day of storage
1	0
2	1
3	2
4	4
5	7
6	10
7	14
8	18
9	28
10	40

69.57 mg KOH g⁻¹ fat (AV of untreated ground seeds after 40 days). The pathlength type was set to constant. The spectrum region was chosen based on the correlation spectrum function of the TQ Analyst software.

Data were normalized by the mean centering technique. The spectrum outlier function was used to determine the outliers. The deviations were expressed as a root mean square error (RMSE). RMSE represents the absolute fit of the created model to the data [16]. The number of factors was determined by finding minimum values for the predicted residual error sum of squares (PRESS) and the root mean square error of cross-validation (RMSECV). The calculations of PRESS and RMSECV are given in Eqs. 1 and 2.

$$PRESS = \sum_i^n (\hat{y}_i - y_i)^2 \quad (1)$$

$$RMSECV = \sqrt{\frac{\sum_i^n (\widehat{y_{i,CV}} - y_{i,CV})^2}{n}} \quad (2)$$

Where $\hat{y}_i$ represents the predicted values, y_i represents the actual values of the acid value and n represents the number of observations.

Detection of active lipase using Fourier-transform infrared spectroscopy

A representative amount of a poppy seed sample was ground, and its infrared spectrum was then measured immediately. To obtain the average spectrum, the measurement of spectra was performed in triplicate, each time with a newly applied sample on the ATR crystal. The ground sample was then left in a laboratory thermostat at 25 °C (± 3 °C). After 24 h, the infrared spectra were measured again. Using the created PLS regression model, the estimated acid value was calculated from the average spectrum obtained using Omnic v 9 software. The estimated acid values after grinding (AV_0) and after the 24-hour incubation (AV_{24}) were then compared and, based on the difference, the lipase activity was evaluated. For the expression of the difference, the lipase activity factor was introduced. This factor represents a percentage change in the estimated acid value after 24 h. The calculation of the factor is given in Eq. 3.

$$\text{lipase activity factor (LAF \%)} = \frac{AV_{24} - AV_0}{AV_0} \cdot 100 \quad (3)$$

The AV_0 represents the calculated ‘acid value’ immediately after grinding the seeds, and the AV_{24} represents the ‘acid value’ after 24 h.

Validation of acid value estimation

The method for estimating acid value using the PLS regression model was validated by evaluation of the repeatability and day-to-day reproducibility. The repeatability was calculated as the relative standard deviation (RSD) of 6 estimated acid value determinations using FT-IR. The day-to-day reproducibility was performed as repeated estimated acid value determination of one sample for 14 days, and was calculated as RSD as well.

Characterization of extracted oil using U-HPLC-HRMS/MS

U-HPLC separation was performed using the Dionex UltiMate® 3000 RSLC U-HP (Thermo Scientific, USA), equipped with a binary solvent delivery system and an autosampler with Acquity UPLC BEH C18 column, 100 Å, 100 mm×2.1 mm; 1.7 µm particles (Waters, USA). The mobile phase consisted of solvent (A) 0.1% formic acid with 5mM ammonium formate in water: methanol (95:5,

v/v) and (B) 0.1% formic acid with 5mM ammonium formate in water: methanol: isopropanol (5:30:65, v/v). The following elution gradient was used in both positive and negative ionization modes: 0–1 min (10–50% B; 0.35 mL min⁻¹), 1–5 min, (50–80% B; 0.35 mL min⁻¹), 5–11 min (80–100% B; 0.35 mL min⁻¹), 11–17 min (100% B; 0.35 mL min⁻¹), 17.1–20 min (10% B; 0.35 mL min⁻¹). The sample injection volume was set at 1 µL, the column temperature was maintained at 60 °C, and the autosampler temperature was permanently set at 10 °C.

TripleTOF® 6600 mass spectrometer (SCIEX, Concord, ON, Canada) equipped with the ion source Duo Spray™ with separated electrospray ion (ESI) source and atmospheric-pressure chemical ionization (APCI) was used. For the measurements of poppy seeds metabolomic fingerprints ESI probe was used, while the APCI probe worked as the second gas heater and at the same time, it was used to calibrate mass accuracy of the TripleTOF® 6600 system. The mass spectrometer was operated in positive and negative mode and the interface parameters were as follows: nebulizing gas pressure of 55 psi, drying gas pressure of 55 psi, curtain gas of 35 psi, capillary voltage of +5000 V (ESI+) and -4500 V (ESI-), temperature of 480 °C and declustering potential of 80 V. At the same time, a TOF MS method and information-dependent acquisition (IDA) method were used to collect MS and MS/MS spectra. The method consisted of a survey TOF MS experiment ranged from *m/z* 100 to 1200, and in parallel, Product Ion (PI) spectra from *m/z* 50 to 1200. PI spectra were collected for the ten most intense ions of the survey spectra throughout the chromatographic run. The collision energy was set to 35 V with collision energy spread of ±15 V. In this way, both low and high-energy fragment ions were present in a single spectrum.

An automatic *m/z* calibration was performed by the calibration delivery system (CDS) for every ten samples using a positive or negative APCI calibration solution (SCIEX, Canada) according to the batch polarity. The achieved resolving power was greater than 40,000 full width at half maximum (FWHM), *m/z* 829.5393 for positive ionization mode and *m/z* 933.637 for negative ionization mode. The PI spectra were measured in high-sensitivity mode, which provides half the resolving power.

The qualitative analysis was performed using PeakView 2.2 (SCIEX, Canada) equipped with MasterView and Formula Finder functions for molecular formula estimation and structural elucidation. The identification of detected compounds was carried out based on the exact *m/z* value (tolerance 5 ppm), isotopic profile, and fragmentation spectra.

Results and discussion

As mentioned in the Introduction, the native lipase in poppy seeds is released by any mechanical damage, including grinding. To avoid worsening of sensory properties, mainly bitterness, originating due to free fatty acids accumulation, so-called thermostabilization is employed by industry for the treatment of poppy seeds before storage. In any case, the heat treatment has to be declared on the packaging label. However, as thermostabilization removes opium alkaloids from seeds surface, it can be misused for decontamination of low-quality seeds, a side product of pharmaceutical poppy plant processing.

One of the ways to identify that fraudulent practice is to evaluate the presence of active lipase in seeds after grinding. While in native (untreated) seeds successive increase of free fatty acids normally occurs; this enzyme-catalyzed reaction is inhibited in thermostabilized seeds. The routine way to assess the extent of hydrolytic rancidity would be the monitoring of the acid value of poppy oil. However, when performed according to the ISO 660:2019 standard, isolation of a relatively high amount of oil from seeds is required, making the analysis rather time-consuming and low-throughput. To eliminate the need for oil extraction, we developed a simple FT-IR spectroscopy-based method that requires minimal sample preparation (only seed grinding), does not require organic solvents, which makes it green, and, at the same time, targets the same group of analytes. In the next paragraphs, the method development and its validation are comprehensively described.

Chemometric model optimization for acid value estimation

In the first phase, a PLS regression model was built employing the values obtained by ‘classic’ determination of AV and FT-IR measurements of signal relevant to changes in carboxylic acids content associated with a release of free fatty acids. Based on FT-IR spectra interpretation, the region 1730–1690 cm⁻¹ was identified as suitable for AV estimation (Fig. 2). The stretches of the free fatty acid carbonyl group (C=O) can be observed in this spectral region [17, 18]. The changes at 1710 cm⁻¹ corresponded to the increasing amount of free fatty acids. The spectral changes in this region also showed the best correlation (higher than 0.9) [19] with the acid value, as shown in the correlation spectrum (Fig. 3).

Minimal values of the PRESS and RMSECV were used to determine the optimum number of factors (principal components) for the model construction. These factors describe the variation in spectra used for the model calibration. The optimization is necessary because using too many factors can lead to model over-fitting. In such cases, the constructed

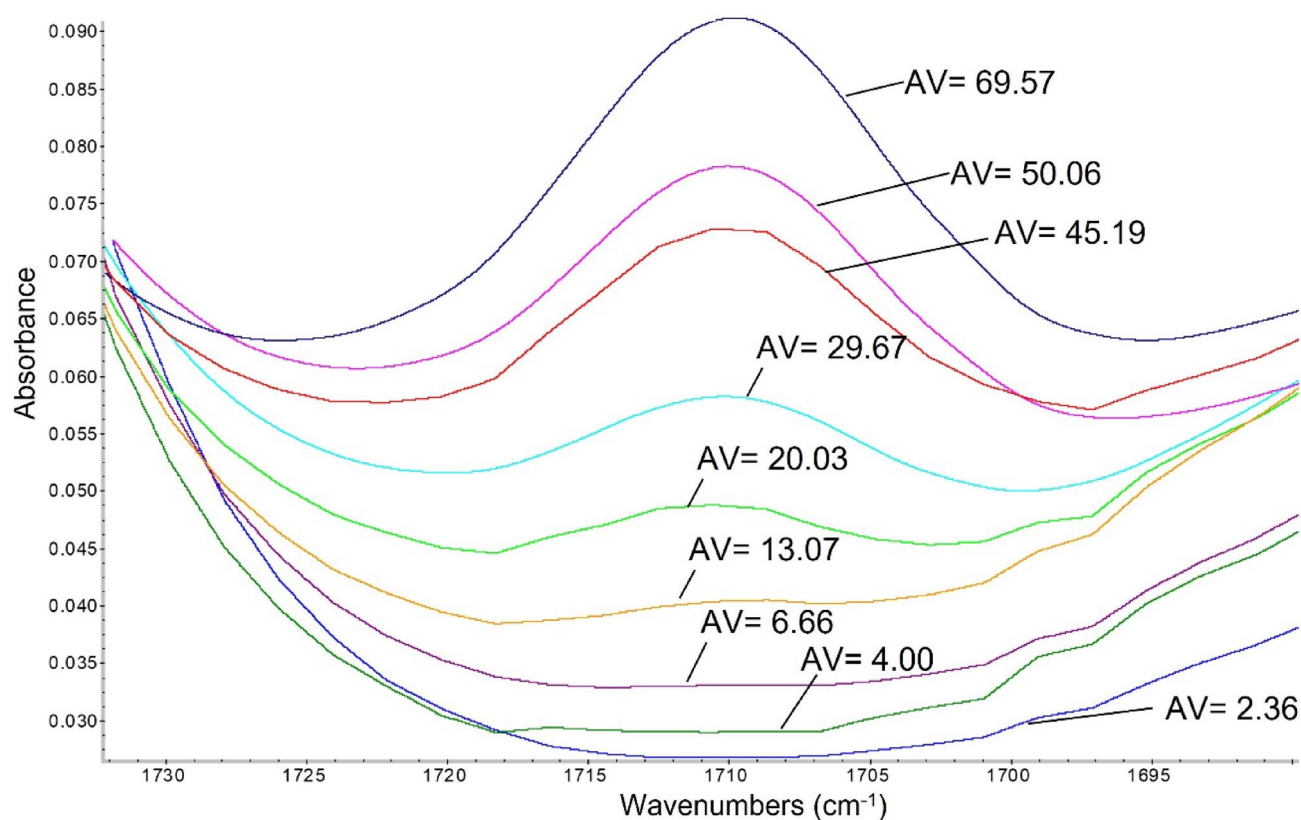


Fig. 2 Difference in the spectral response of the ground poppy seeds with acid value (AV) of oil ranging from 2.36 to 69.57 mg KOH g⁻¹ fat. The 1730–1690 cm⁻¹ spectral region is displayed

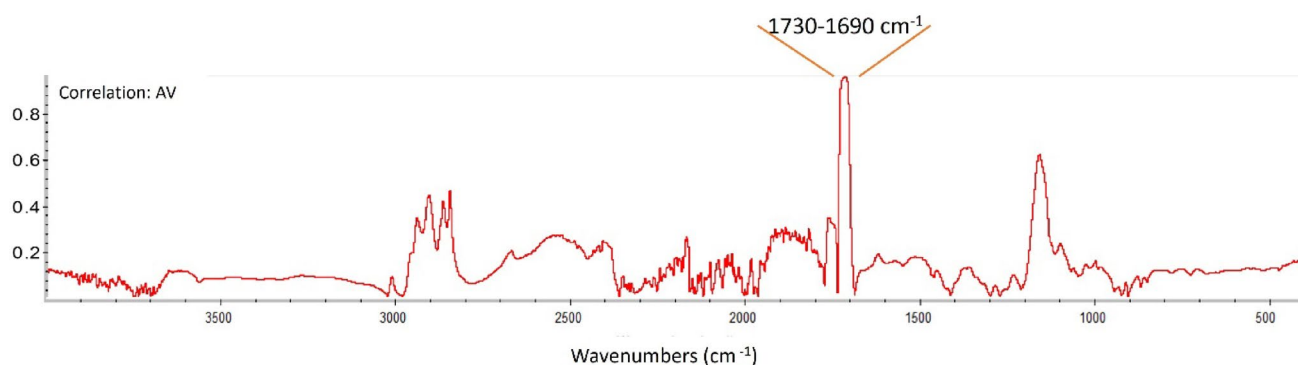


Fig. 3 The correlation spectrum documenting the correlation of spectral variation to the change of oil AV. The region 1730–1690 cm⁻¹ showed a correlation higher than 0.9

model describes mainly too much specific information about the calibration samples, which then causes good accuracy of the model when quantifying samples from the training set, but cannot reach the same level of accuracy when quantifying new samples [20]. Based on the PRESS and RMSECV values given in Tables 2, 6 factors were chosen for the model. The factor loadings are available in Supplementary material S2. One factor used 89.48% of the spectral and 87.54% of the concentration information. All six factors used 99.99% of the spectral and 96.84% of the concentration information.

The correlation coefficients, which express the accuracy of the obtained model, were as follows: $R_{\text{calibration}} = 0.9848$, $R_{\text{prediction}} = 0.9892$, $R_{\text{cross-validation}} = 0.9779$. All coefficients were higher than 0.95, which indicates a strong correlation [19, 21]. The root mean square errors were as follows: $\text{RMSE}_{\text{calibration}} = 2.97$, $\text{RMSE}_{\text{prediction}} = 1.64$, $\text{RMSE}_{\text{cross-validation}} = 3.57$. Based on these data, the model can be considered satisfactory for screening ongoing hydrolytic rancidity as an indicator of lipase activity. The calibration curve of the model is shown in Fig. 4A. Further improvement can be

Table 2 Values of PRESS and RMSECV depending on the number of factors

Factor	PRESS	RMSECV
0	26,752.03711	12.47137
1	3,363.87061	4.42237
2	1,554.59595	3.00639
3	1,170.48987	2.60867
4	1,143.16296	2.57804
5	1,121.87000	2.55392
6	1,117.55273	2.54900
7	1,139.16528	2.57353
8	1,169.89795	2.60801
9	1,211.37524	2.65384
10	1,218.42908	2.66156

achieved by adding more standards into the model, especially with higher acid values. Fig. 4B shows the residual plot, which shows the relative differences between the calculated (estimated) acid value and the correct acid value of the samples used for the model construction. The relative differences were less than 20% in all cases.

Validation of acid value estimation

The repeatability, expressed as a relative standard deviation (RSD), of the FT-IR method was calculated from 6 replicates of the estimated acid value determination. The resulting RSD value was 11%. Sample homogeneity, its placement on the ATR crystal, or slight changes in ambient conditions during measurement (mainly temperature) are

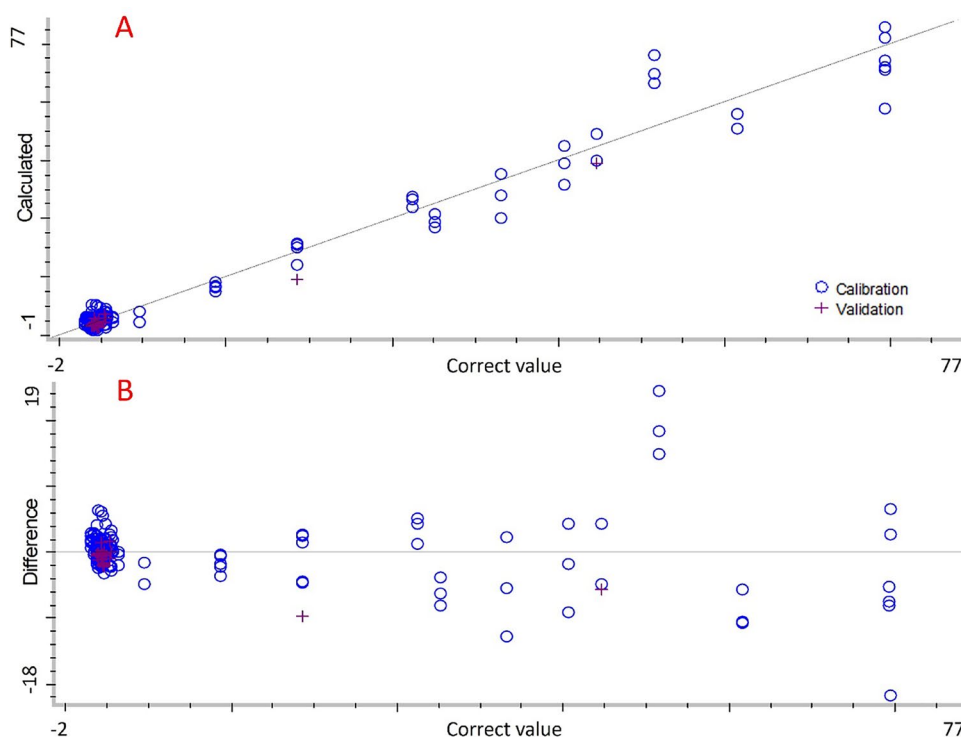
examples of variation sources. The day-to-day reproducibility expressed as RSD was 13% and was calculated by measuring the estimated acid value of a sample ten times during a 14-day period.

Investigation of important factors that may affect lipase activity

Effect of the incubation temperature and time

Two incubation temperatures were tested to determine suitable conditions for detecting hydrothermal treatment based on the change in the FT-IR estimated acid value. Each sample of ground seeds was incubated at 25 °C and 35 °C (± 2 °C), and seven measurements were performed in one-hour intervals and one after 24 h since the experiment beginning (Supplementary material S3). The last measurement time (24 h) was selected as suitable for the incubation time due to the pronounced change out of the 11% interval representing the repeatability expressed as RSD. In line with a study [22] concerned with the impact of various factors on lipase activity in poppy seeds, the most suitable incubation temperature under which the most rapid changes occurred was 25 °C (Fig. 5). The introduced lipase activity factor (LAF) was 82% (Bergam variety) and 66% (Maratón variety) when using 25 °C temperature, while it was only 41% (Bergam) and 47% (Maratón) when using 35 °C. The 25 °C temperature was used in all subsequent experiments.

Fig. 4 **A** Correlation between the correct and calculated acid value of calibration and validation standards. **B** Percent difference between the correct and calculated acid value



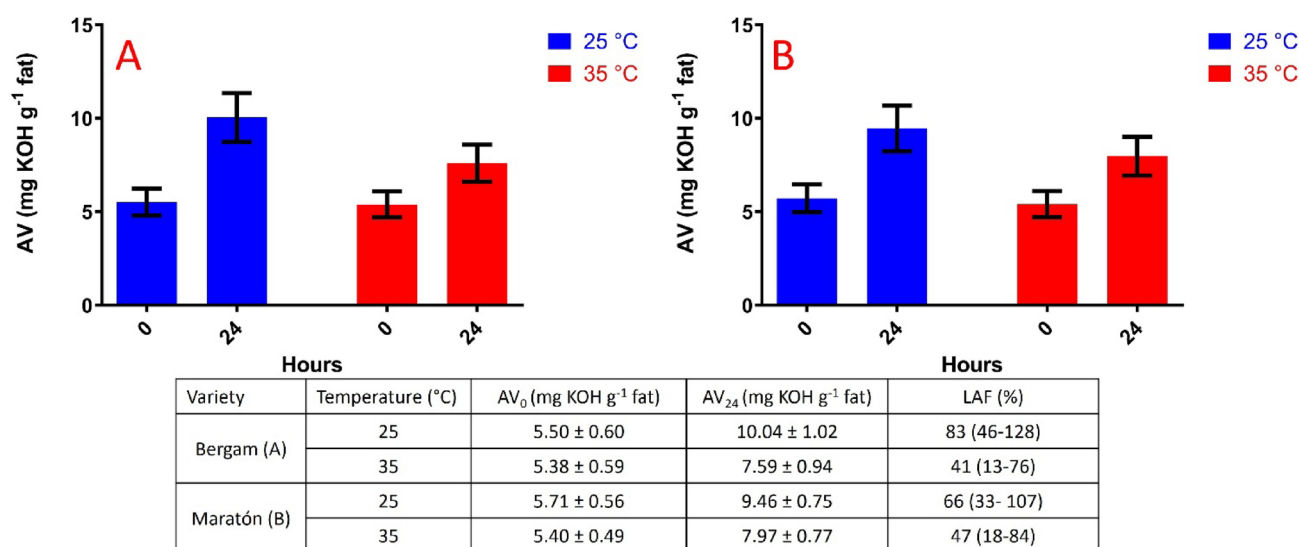


Fig. 5 Increase in estimated acid value when using different incubation temperatures for ground poppy seeds **A** Bergam variety, locality Pusté Jakartice, 2018 harvest, and **B** Maratón variety, locality Pusté Jakartice, 2018 harvest. Error bars represent repeatability expressed as RSD = 11%

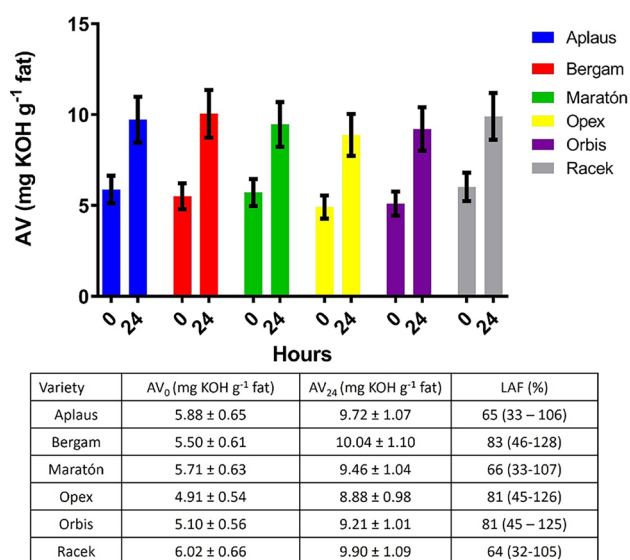


Fig. 6 Increase in estimated acid value in six different poppy seed varieties harvested in 2018 in locality Pusté Jakartice

Effect of poppy seeds variety, harvest year, and growing locality

The activity of lipolytic enzymes was evaluated based on FT-IR spectra using untreated poppy seed samples of different varieties, origins, and years of harvest. As shown in Fig. 6, no pronounced differences in the estimated acid value change (LAFs) were observed among the examined varieties harvested in the same locality in the same year. The LAFs were in the range of 64–82%.

As it comes to different harvest years, the main concern was the possible decrease of the lipase activity with the

age of the sample. The results of measurement performed on two different varieties (Opal from Trutnov locality and Gerlach from Trutnov and Pusté Jakartice) and two harvest years (2015 and 2017), displayed in Fig. 7, did not indicate any pronounced difference between these samples. The LAFs were in the range of 34–71%. However, when comparing the lipase activity in seeds of the same variety, but grown in different localities, significant differences were observed (Fig. 8). In the case of both tested varieties, Opal and Gerlach, higher LAFs were found for seeds from the Lednice locality (145 and 163%, respectively) compared to the other two localities (Trutnov, Pusté Jakartice), the LAFs were in the range 34–89% only. Besides of soil and other agrotechnical factors might play a role, it should be noted that the Lednice locality has a higher average temperature during the growing season than the other two. The average temperatures and precipitation for individual locations were compared based on the database of the Czech Hydrometeorological Institute and are given in Supplementary material S4 [23].

Assessment of FT-IR method application on thermostabilized and non-treated poppy seed samples

The validated FT-IR method was used to analyze two sets of poppy seed samples, both thermostabilized and non-treated (native). As expected, regardless of the variety, harvest year, and growing locality, in all native poppy seed samples, a distinct increase in the estimated acid value occurred (the data are summarized in Supplementary material S5). The LAFs ranged from 19% to 485%. The standard deviation

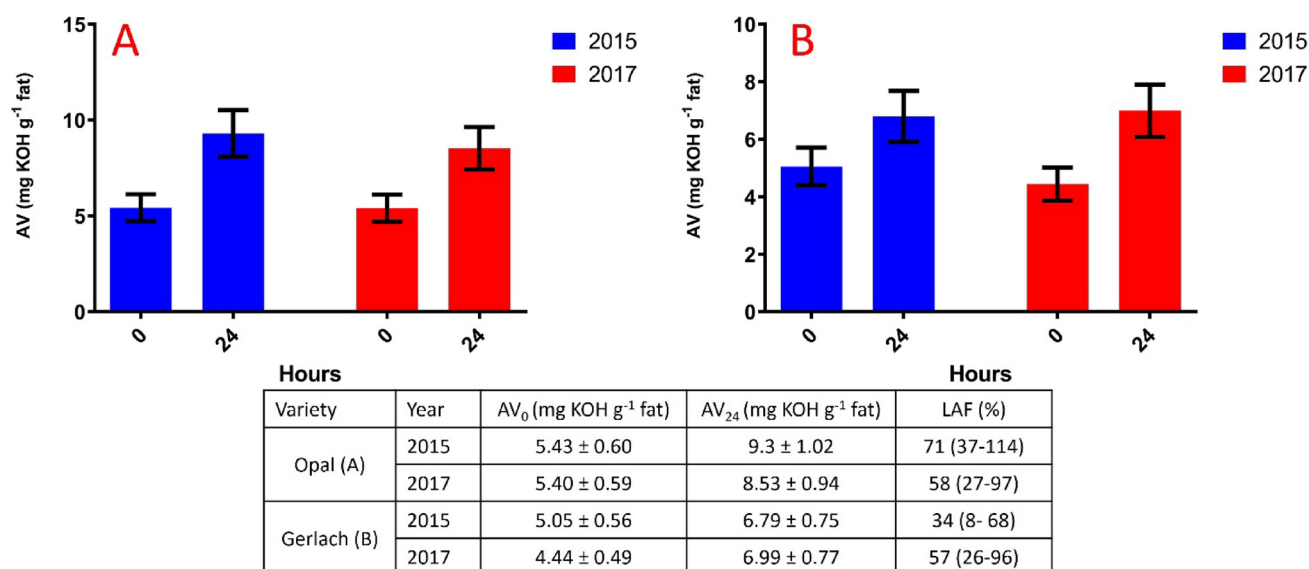


Fig. 7 Lipase activity of poppy seeds harvested in the years 2015 and 2017, **A** Opal variety, **B** Gerlach variety

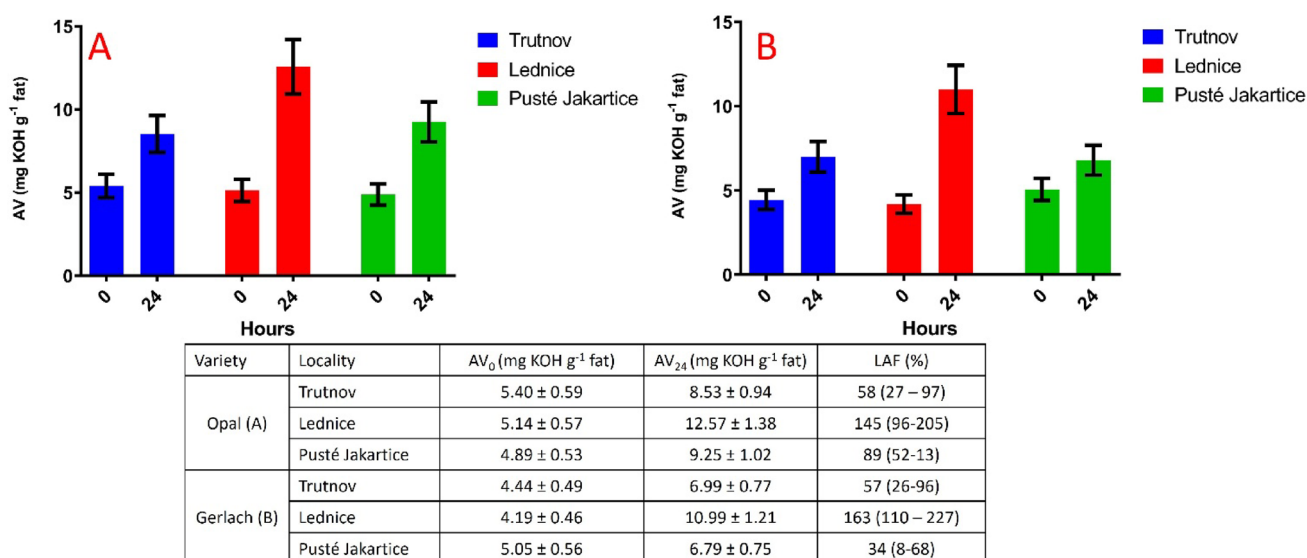


Fig. 8 The impact of locality on lipase activity for **A** Opal and **B** Gerlach varieties

of LAFs of the dataset, including pharmaceutical varieties (29 samples in total), was 90%. Excluding the two samples of pharmaceutical seeds, which showed far higher LAFs than food varieties, the standard deviation was 35%. The highest LAF was documented for the sample of high-morphine pharmaceutical poppy seeds. The lowest LAF was documented for the sample of Major variety from the 2015 harvest originating from Bratkovice. The mean LAF of the dataset, including pharmaceutical varieties, was 88%. Without pharmaceutical seeds, the mean LAF of the dataset was 68%. The results obtained for thermostabilized poppy seeds are presented in Table 3. The validity of our concept is clearly documented here. Due to inhibited lipase, the FT-IR

measurement showed either a negligible increase of the estimated acid value, or, in most cases, even a decrease, including the pharmaceutical varieties, which showed far higher LAF in their untreated states in comparison with food varieties. The LAFs ranged from -46% to 1%. The lowest LAF was documented for the sample of Major variety from the 2014 harvest originating from the locality Klecany. The highest LAF was documented for two samples, one sample of Major variety (harvest 2015, Libochovice) and one sample of MS 1-15 variety (harvest 2017, Trutnov). When including pharmaceutical varieties, the standard deviation was 15%. The mean LAF of the dataset was -14%. Without

Table 3 Changes in the estimated acid value and LAF of thermostabilized samples

Sample	AV ₀	AV ₂₄	LAF (%)
Sample 1	8.40 ± 0.92	4.55 ± 0.50	-46%
Sample 6	9.03 ± 0.99	6.73 ± 0.74	-25%
Sample 7	7.21 ± 0.79	7.27 ± 0.80	1%
Sample 17	4.45 ± 0.49	4.21 ± 0.46	-5%
Sample 18	4.65 ± 0.51	4.68 ± 0.51	1%
Sample 19	4.90 ± 0.54	4.28 ± 0.47	-13%
Sample 20	4.69 ± 0.52	3.95 ± 0.43	-16%
Sample 22	5.64 ± 0.62	3.30 ± 0.36	-41%
Sample 23	5.46 ± 0.60	5.32 ± 0.59	-3%
Sample 25	6.27 ± 0.69	5.28 ± 0.58	-16%
Sample 27	3.12 ± 0.34	2.97 ± 0.33	-5%
Sample 28 ^a	2.94 ± 0.32	2.95 ± 0.32	0%
Sample 29 ^a	1.86 ± 0.20	1.61 ± 0.18	-13%

AV₀ acid value after grinding, AV₂₄ acid value after 24 h

^aPharmaceutical varieties

pharmaceutical varieties, the standard deviation was 16% and the mean of the dataset was -15%.

Rather limited information is available on the parameters set under industrial conditions to achieve poppy seed thermostabilization. Therefore, an experiment was conducted to compare the effects of the approach used within this study (one hour steaming) and a 30-second treatment of seeds by a stream of boiling water (washing) on the extent of lipase inactivation. Orbis (Pusté Jakartice, 2018) and Major (Klecany, 2014) varieties were used. Fig. 9 shows that the effect was comparable; the enzyme was inactivated, so the latter

approach may represent a faster option for thermostabilization. The decrease of the estimated acid value of seeds was in the range of 16–46%.

Validation of the classification strategy on the set of industrial samples

The results of the validation study, within which a set of samples obtained from an industrial processor was analyzed, are shown in the Supplementary material S6. A total of 54 samples of untreated poppy seeds and 54 samples of seeds subjected to the industrial heat stabilization process were evaluated. According to common rules set by official laboratories, the false positive rate should not exceed 5%. To meet this threshold, a decision criterion of $|LAF| > 10\%$ was chosen. Under such conditions, a total of 2% of samples were determined to be false positives, and 7% of samples were determined to be false negatives. It should be noted that after the harvest, a multi-step procedure is employed for seeds handling during which they are exposed to various impacts during transport, drying, storage, processing itself (cleaning), and subsequent distribution. Specifically, unexpectedly elevated temperatures (apart of thermostabilization), insufficient drying, or mechanical damage, can change the nature of native seeds, which may result in their inconclusive or even biased classification results. Poppy seeds for which $|LAF| \leq 10\%$ were recognized as inconclusive and accounted for 17% of the total number of samples. To avoid any classification bias, a more sophisticated,

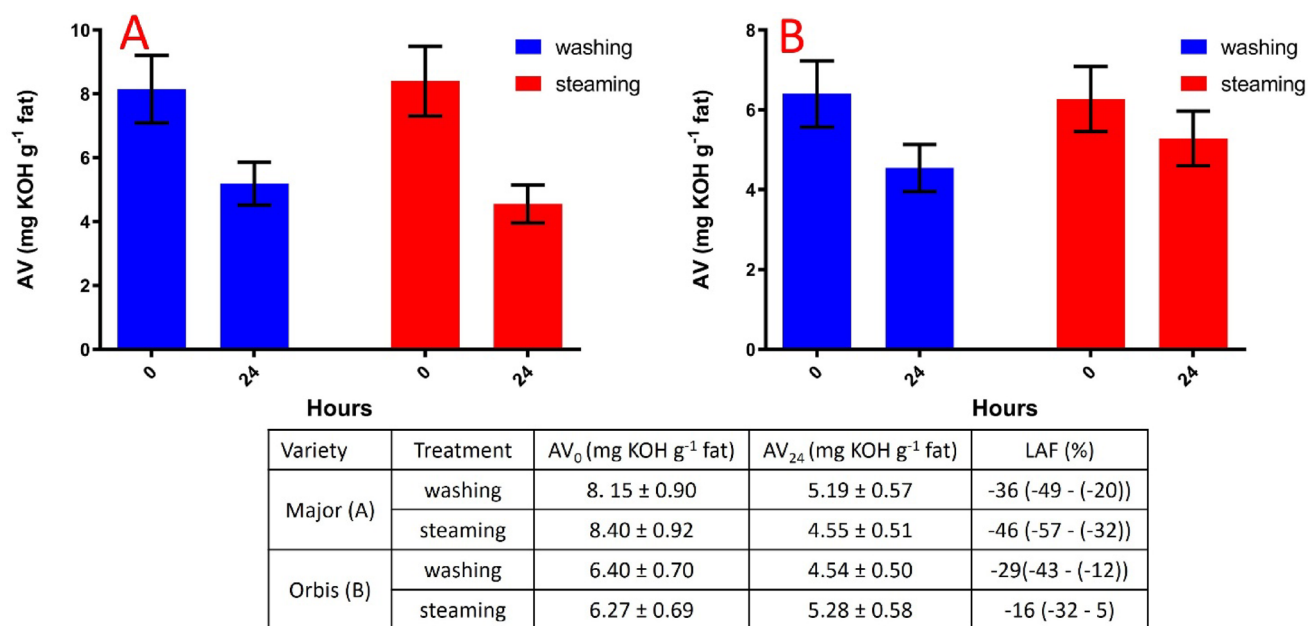


Fig. 9 Comparison of two poppy seeds thermal treatment methods: **A** for variety Major (Klecany, 2014) **B** variety Orbis (Pusté Jakartice, 2018)

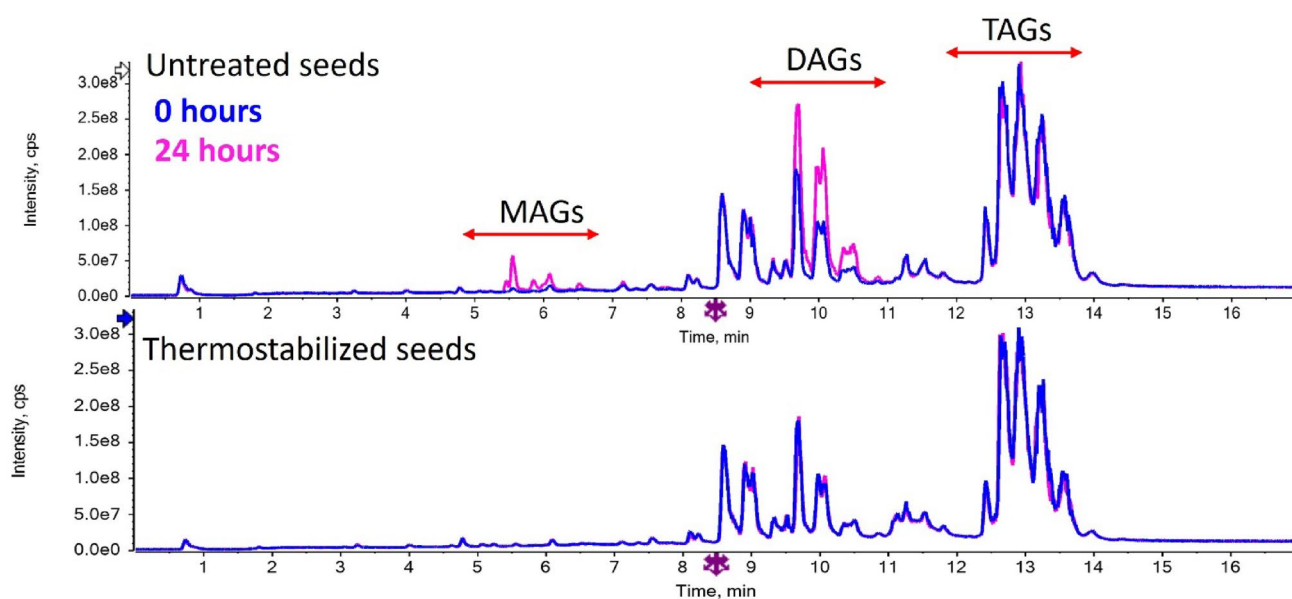


Fig. 10 Total ion HRMS chromatogram of the lipid component of freshly ground seeds (blue) and ground seeds after 24 h (purple) in ESI +

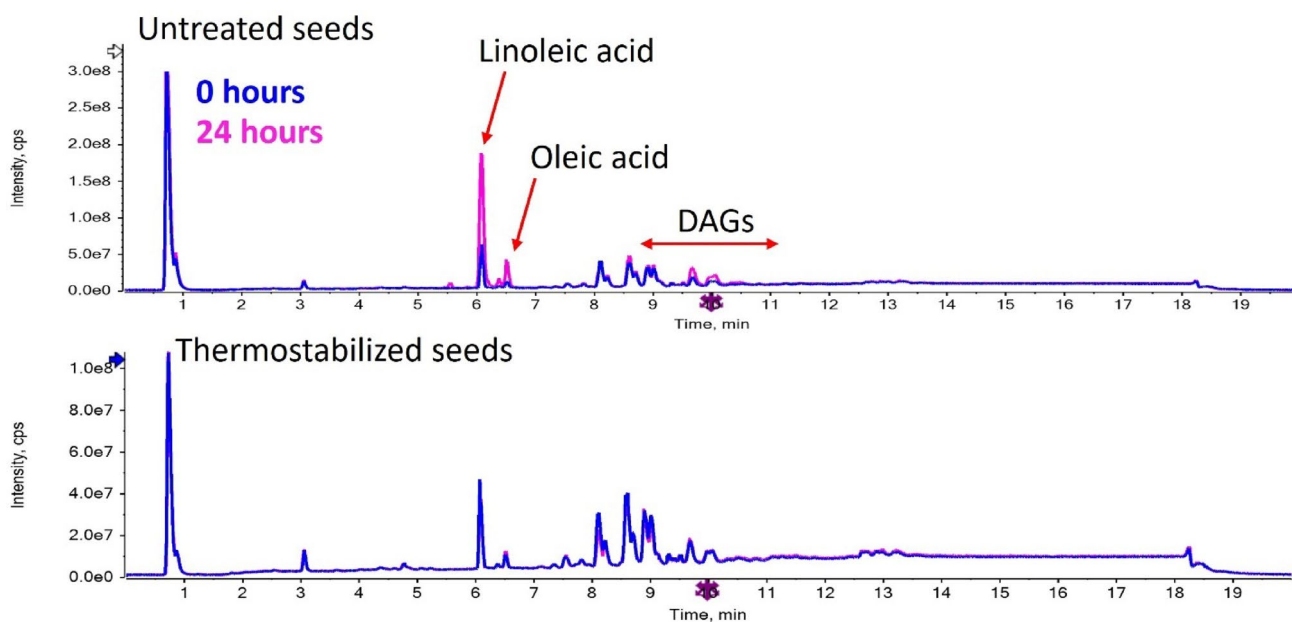


Fig. 11 Total ion HRMS chromatogram of lipid component of freshly ground seeds (blue) and ground seeds after 24 h (purple) in ESI-

complementary method, such as characterization of the lipid fraction based on LC-MS/MS analysis, should be used for those samples. From these inconclusive samples, 10 belonged to the untreated group of samples, and 8 to the treated group. The LAFs of inconclusive untreated samples ranged from -6% to $+9\%$, and the LAFs for the inconclusive treated samples were in the range of -10% – 10% .

Characterization of the lipid component changes by U-HPLC-HRMS/MS

To get a more detailed insight into changes occurring in ground poppy seeds during 24 h of storage, the isolated lipid component was analyzed using U-HPLC-HRMS/MS technique. Lipids were extracted from seeds by dichloromethane,

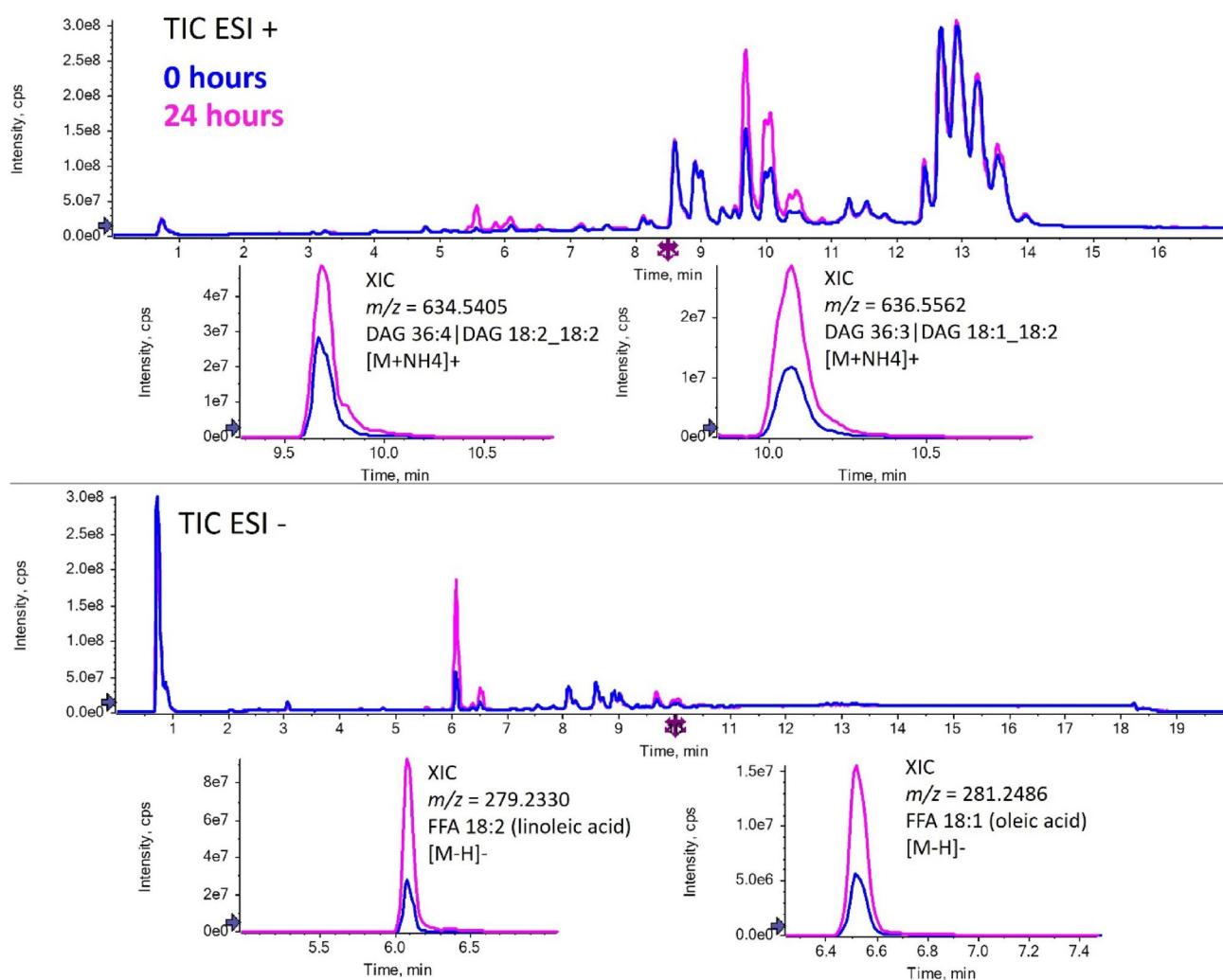


Fig. 12 Total ion chromatograms and extracted ion chromatograms of selected markers for sample 6 (batch 5, untreated), classification inconclusive using the FT-IR method

which was selected for its low boiling point (40 °C) and lower toxicity compared to chloroform. Total ion chromatograms (Figs. 10 and 11) clearly document the release of free fatty acids associated with an increase of monoacylglycerols (MAGs) and diacylglycerols (DAGs) in native poppy seeds 24 h after grinding. It should also be noted that changes in triacylglycerols (TAGs) response due to their dominating content could not be visualized this way. In line with expectation, no such changes occurred in the case of thermostabilized seeds analyzed under the same conditions.

Specific lipid changes caused by the lipase were also investigated. Identified free fatty acids and acylglycerols, which were affected by the lipase enzyme, are summarized in the Supplementary material S7. The identified lipidic markers of ongoing hydrolytic rancidity corresponded to the profile of fatty acids in triacylglycerols of poppy seed oil, in which linoleic and oleic acid are dominating [24–26]. This

list of analytes was subsequently screened in the selected inconclusive and incorrectly classified samples from the Validation of the classification strategy on the set of industrial samples. Sample 6 from batch 5 (untreated) was classified as inconclusive with LAF = −4% using the FT-IR method. Total ion chromatograms and extracted ion chromatograms of selected markers (Fig. 12) were recorded. The increase of signals in the elution region of free fatty acids and diacylglycerols was clearly documented, indicating the activity of the lipase.

In Fig. 13, the total ion chromatograms and selected extracted ion chromatograms of sample 4 from batch 4 (untreated) are shown. This sample was incorrectly classified as treated, using the FT-IR method, with the LAF = −20%. In both cases, the UHPLC-HRMS/MS method led to the correct classification of the sample. This proved the suitability of this method as a confirmatory strategy.

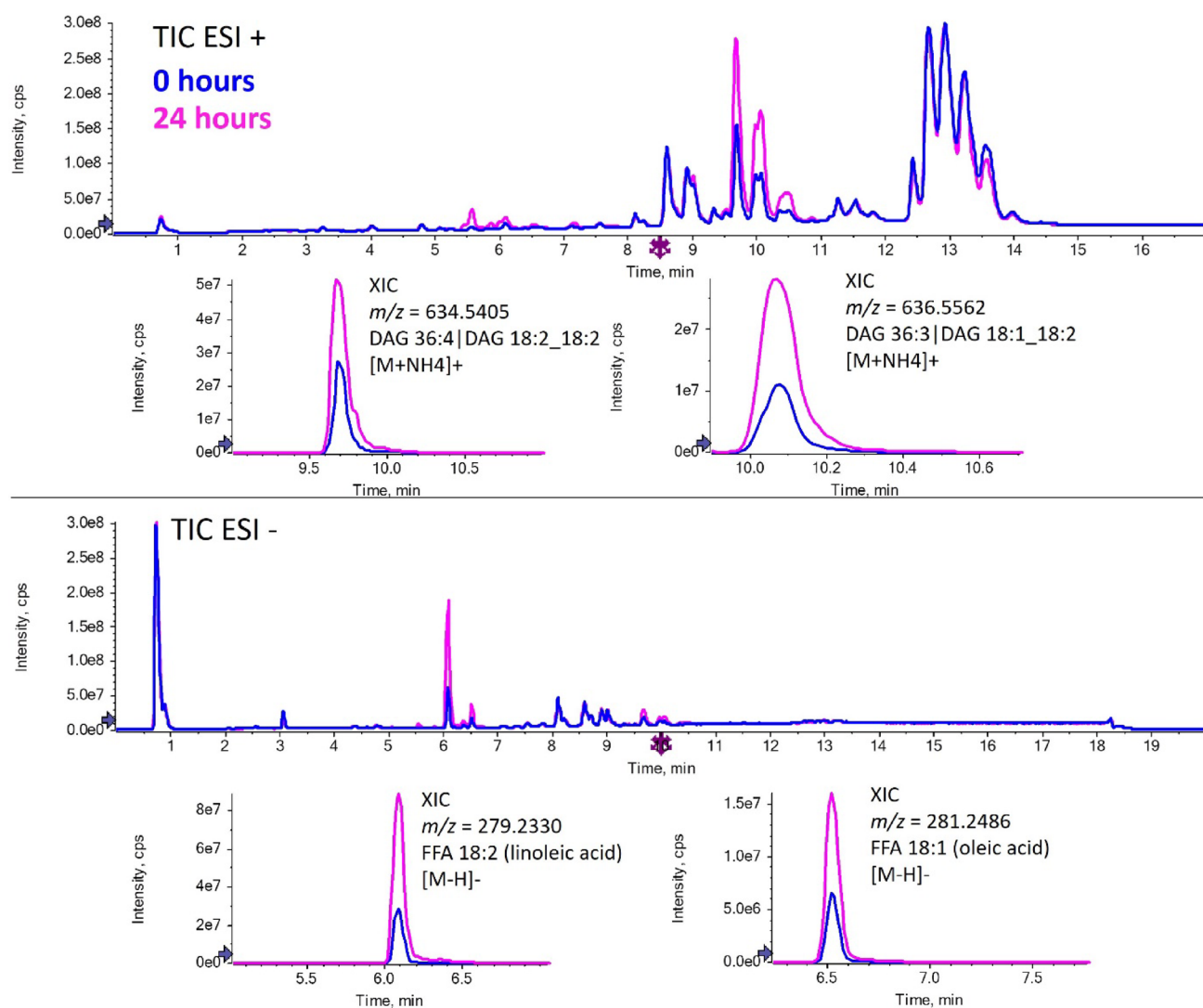


Fig. 13 Total ion chromatograms and extracted ion chromatograms of selected markers for sample 4 (batch 4, untreated), wrongly classified by the FT-IR method

Conclusion

Like other highly valued commodities, Czech poppy seeds, known for their unique flavour, are prone to fraudulent practices, one of them being undeclared hydrothermal treatment, which is, instead of declared lipase inactivation, used for removing opium alkaloids from seeds of poppy grown for the pharmaceutical industry. Although distinguishing native seeds from thermostabilized ones is not an easy task, a novel, simple, and cost-efficient FT-IR method developed in this study enables a reliable assessment of whether respective poppy seeds are native, i.e., active lipase is present. Method applicability was documented for various poppy seed varieties, obtained from different localities and harvest years. Validation of the method classification potential was performed on a set of 108 samples from industrial processing, resulting in 2% false positives and 7% false negatives. The developed method is highly effective and applicable

in routine laboratories, both those responsible for market control and units generating data for the management of industrial processes concerned with poppy seeds thermal treatment. Except for seed grinding, the new method does not require any laborious sample preparation, which is a significant advantage in comparison to the rather labor-demanding ISO titrimetric method and/or other conceivable analytical strategies such as LC-MS, employing fairly more complex instrumentation.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Human and animal rights This article does not contain any studies with human or animal subjects. Informed consent: not applicable, Clinical trial number: not applicable.

Informed consent Not applicable.

Clinical trial number Not applicable.

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