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Prediction of species and freshness of caspian caviar during storage by front-face fluorescence spectroscopy

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The present study is conducted to characterized three types of Iranian Caviar (Beluga, Aserta and Sevruga) over a storage period up to 45 days. Twenty two samples of caviar from three species of sturgeon fishes were monitored by Front face fluorescence Spectroscopy to distinguish different types of caviar and also to identify oxidation in each. Oxidation was followed during storage of caviars. Emission fluorescence spectra were recorded at 360-700nm and 420-720nm region for excitation wavelength 332nm and 382 nm respectively. The PLS discriminant analysis (PLS-DA), linear discriminant analysis (LDA) and principal component analysis (PCA) were applied to the spectral data set. Correlation coefficients of 0.88% to 0.93% and 83% to 97% were obtained in classification of freshness and distinguishing these three species respectively. Results indicated that front face fluorescence is a rapid and accurate method to discriminate between fresh and oxidized caviar.

Key words: Front-Face Fluorescence; Freshness Classification; Caspian Caviar Species

Introduction

Sturgeons are subjected to extreme over-exploitation for the reason of caviar production throughout their range of distribution (DeMeulenaer and Raymaker, 1996). Caviars are the salt-cured and preserved eggs of sturgeon species that have been separated from the supporting connective tissue. The most famous and valuable caviar derivate from wild harvested sturgeons in the area of Caspian Sea are; namely Beluga (huso huso), Asetra (Acipenser persicus) and Sevruga (Acipenser stellatus) caviars (Caprino *et al*., 2008). Caviar contain 4-8 % salt which is added after sieving the egg from the ovaries,

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with the better varieties generally containing less salt (Wang *et al*., 2008). Fatty acids and amino acids are important structural component in the sea foods.

Various classes of these components fulfil different biological functions in the organism. However during the fatty caviar storage, quality may decline as a result of several reactions affecting lipid fractions and decreasing the nutritional properties of product. Due to their high level of unsaturated long chain polyunsaturated fatty acids (PUFA), caviar products are very sustainable to oxidation.

The recently reported studies about caviar are concentrated on metal contaminated (Wang *et al*., 2008), microbiological analysis (Altug and Bayrak, 2003) and fatty acid composition (Gessner *et al*., 2002; Wirth *et al*., 2002; Caprino *et al*., 2008). They usually used chemical methods on Caviar of farmed and wild sturgeons (Brunner *et al*., 1995; Chen *et al*., 1995; Himelbloom and Crapo, 1998; Garcia-Gallego *et al*., 1999; Wirth *et al*., 2000; Sengor *et al*., 2002) and nothing has been reported about non-invasive spectroscopic methods in wild source caviar. More advanced methodologies such as scanning electron microscopy (SEM), magnetic resonance imaging (MRI) and spectroscopy T1 and T2 relaxation techniques have been used to study structural properties and conservation states of salted caviar during storage (Gussoni *et al*., 2006). Lastly, quality of cod caviar paste was assessed in terms of light exposure and concentration of oxygen in the headspace by fluorescence spectroscopy and sensorial attributes (Airado-Rodriguez *et al*., 2010).

Fluorescence is a spectro-chemical method of analysis where the molecules of the analytic are excited by irradiation at a certain wavelength and emit radiation of a different wavelength. The emission spectrum provides information for both qualitative and quantitative analysis. It is reported to be 100-1000 times more sensitive than other spectro-photometric techniques (Strasburg and Ludescher, 1995). Front face fluorescence consist of illuminating the surface of the sample with the excitation light and measuring the emitted fluorescence from the same surface. This method is useful for concentrated samples such as meat, fish and diary products. Front face fluorescence has earlier demonstrated to be a suitable technique to determine the extent and distribution of lipid oxidation in different food matrices (Wold and Kvaal, 2000; Veberg *et al*., 2006; Wold *et al*., 2006; Veberg *et al*., 2007) and also in cod caviar paste (Airado-Rodriguez *et al*., 2010).

In the present study, distinguishing between species of caviar and lipid oxidation of each one were investigated on three types of Iranian sturgeon caviars; Beluga (huso huso), Asetra (Acipenser persicus) and Sevruga (Acipenser stellatus) – over storage period of 45 days. The aims were focused on: Establishing front face fluorescence in the emission spectral region of 370

to 720 nm (at two different excitation wavelengths) as a tool to discriminate different species of caviars. This rapid, reliable and accurate technique can be useful for the caviar exports.

Establishing how storage time after opening the container of caviar samples can affect lipid properties which mainly influenced the caviar quality and authenticity. This study would provide a useful method for these three types of caviar freshness evaluation based on different lipid oxidation during the storage time.

Materials and methods

Caviar Samples

Twenty two caviar samples from three types of wild Caspian caviar (Beluga, Aserta and Sevruga) were provided from Iranian agri-service Company. This was withdrawn from mature female sturgeon fishes that were caught in the spring of 2011 from the south coast of Caspian Sea (Iran). The samples were transported to the Institute of food, fisheries and aquaculture of research (Nofima Mat), Ås, Norway in their original export packages with in the glass of 16g, 20g and 30g. Commercial samples were supplied in glass vials and stored around 0°C to 4°C after arrival to Nofima Mat. Front face fluorescence measurements were done every fifteen days and it was continued during the 45 days. The first measurement which we called it fresh here was done after 8 days and it repeated every 15 days.

Front Face Fluorescence Measurements

Fluorescence spectroscopy is a sensitive, rapid and non-invasive analytical method which provides information on the presence of fluorescent molecules and on their environment in biological and food samples (Marangoni, 1992; Dufour *et al*., 2000).

Caviar samples were monitored by fluorescence emission spectra. The samples of 3mm thickness were placed into cuvettes, which exposed a flat dark circular surface with a diameter of 5cm for the measurements. Emission fluorescence spectra were recorded at 4°C. The spectra of each sample were recorded twice, and the mean of them was used for further analysis. The illumination was not perfectly homogeneous; therefore, samples were rotated 90 degree between the two exposures. An optical setup system optimized for measuring large sample surfaces was used. The samples were illuminated with 332 and 382 nm excitation lights, and fluorescence emission spectra were measured in the ranges of 370-700 nm and 420-720 nm, respectively.

Excitation wavelengths of 332nm (λ_{ex}=332) and 382nm (λ_{ex}=382) were justified by the fact that they covered information related to the level of oxidation of products in different food matrices (Wold and Kvaal, 2000; Veberg *et al*., 2006; Veberg *et al*., 2007; Wold *et al*., 2006; Airado-Rodriguez *et al*., 2010;). The excitation light was generated by a 300 W xenon light source (Oriel 6258; Oriel Corporation, Stratford, CT) and passed through a 10nm bandwidth interference filter (Oriel 59920). The incidence angle of excitation radiation was set at approximately 45 degree to ensure that reflected light scatter radiation and depolarisation phenomena were minimised. The spectra were collected by a sensitive charge-coupled device (CCD) camera (Roper Scientific NTE/CCD-1340/400-EMB; Roper Scientific, Trenton, NJ). Cut-off filters at 360nm and 400 nm (Melles Griot 03FCG049; Melles Griot, Rochester, NY) were positioned in front of the spectrograph slit to suppress the excitation light reflected from the sample with 332 nm and 382 nm respectively. The exposure time was 10 s for all spectroscopic measurements. The spectrograph and detector were controlled by the software WinSpec 1.4.3.4 (Roper Scientific).

Data Analysis

In this study an original spectra processing system based on Unscrambler X (v.10, Camo As, Oslo, Norway) was used to analysis the spectral data. The principal component analysis (PCA) and linear discriminant analysis (LDA) were the two main functions used for classification and by using PLS-DA the classification models extracted and validated. As mentioned above, measurements were done twice for each sample then by using average method, if there is an equal number of replicates for each sample in the data table, the replicates can be averaged to get one row for each sample. Another pre-treatment method that was performed was standard normal variate (SNV). The practical result of SNV is that it removes multiplicative interferences of scatter effects from spectral data. PCA is well suited to optimize evaluation of large data set with a minimum loss of information. The PCA transforms the original wavelengths into new axes, or principal components (PCs), which are orthogonal, so that the area set presented on these axes are uncorrelated with each others. Therefore PCA expresses as much as possible the total variation in data set. PCA method was used to identify the species and date of measurement for each type of caviar. The objective of LDA is to determine the best fit parameters for classification of samples by a developed model. The model can then be used to classify unknown samples. It is based on the normal distribution assumption and the assumption that the covariance matrices of the two (or more) groups are identical. LDA was performed on the first 4 PCs (which

contains the whole information observed in the 370-700 and 420-720 emission spectra data set in 332 and 382 excitation filters, respectively), resulting from PCA applied to the emission fluorescence spectral data.

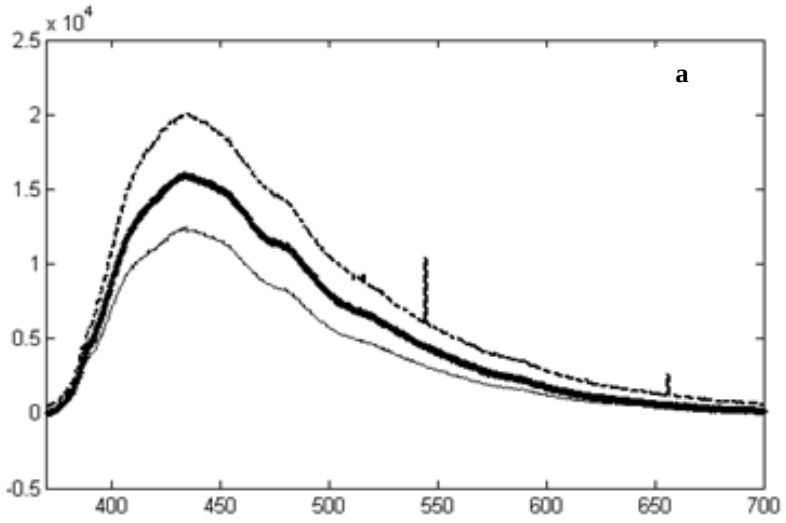
When samples have to be assigned to qualitative groups, prediction can be handled by discrimination techniques which involved building a model from a calibration set and choosing a rule to assign the calibration samples to the qualitative groups. Two groups were created for each species (Beluga, Asetra and Sevruga) and different storing time of caviar, i.e., fresh (8 days after arriving to Laboratory when the tests started), 15 (days after first measurement), 30 and 45 days old. These were done by PLS-DA method. PLS-DA is the use of PLS regression for discrimination purposes. Once the PLS model has been checked and validated, one can run a Prediction in order to classify new samples.

Results and discussions

Fluorescence Spectra

It has been reported that fluorescence spectroscopy is a very accurate technique able to measure trace substances in cod caviar paste samples (Airado-Rodriguez *et al*., 2010). As most of spectra had very similar shapes and can therefore be very difficult to make visual discrimination, some spectra for samples recorded at fresh, 15, 30 and 45 days of storage. The fluorescence emission spectra of caviar samples (i.e. Asetra and Sevruga) stored at 4°C for the excitation wavelength 332 and 382 nm are shown in Fig. 1a and Fig. 1b. Oxidation products are represented by spectra with maxima centred at 430 and 475 nm and shoulder in 472 and 516nm respectively. The region from 410 to 500 nm arises from different stable fluorescence oxidation products; among them, products formed by reaction of unsaturated aldehydes with proteins as has been reported by Veberg *et al*. (2006). The peak at 475 nm (λ_{ex}=382 nm) has been associated with amino acid oxidation products, that maybe related to glycine here. Yamaki *et al*. (1992) also investigated the fluorescence of glycine with 2, 4 heptadienal at 37°C. Glycine is one of the most important amino acid compositions of the protein in the caviar that is responsible for flavour and taste. Having peak at 475 nm (λ_{ex}=382) is common to different food matrices of oxidation, such as minced turkey, pork and cod meat (Veberg *et al*., 2006), chicken meat (Wold and Kvval, 2000), salmon pate (Olsen *et al*., 2006) and dairy products (Wold *et al*., 2006; Veberg *et al*., 2007) among others. The peak around 430nm (λ_{ex}=332) in all samples spectra has been seen. The peak at 425 to 433 nm have been reported in oxidized soybean flour (Liang and Lin, 2000) and also reported on lysine with 2 heptadienal at 37°C by Yamaki *et al*. (1992).

We also found emission maximum in the same region which are due to the reaction between amino group and aldehydes formed during the oxidation mechanism. Lysine is an amino acid of caviar that has important role during spoilage. This amino acid can produce biogenic amines by decarboxylation which is very powerful from toxicity point of view and as quality control index for seafood spoilage (Ozden, 2005). The emission maximum was observed at 430 nm and 475 nm and shifted slightly as a function of the storage time. It appeared that fresh caviars presented less intense fluorescence at about 430nm (λ_{ex}=332) and 475 nm (λ_{ex}=382), while old caviars had the highest intensity in that region. Even without any chemical reference analysis it is possible to suggest probable reactions leading to changes in the fluorescence of spectra throughout storage. The spectra recorded on caviar samples at difference storage times showed differences suggesting that a fluorescence spectrum may be considered as a fingerprint.



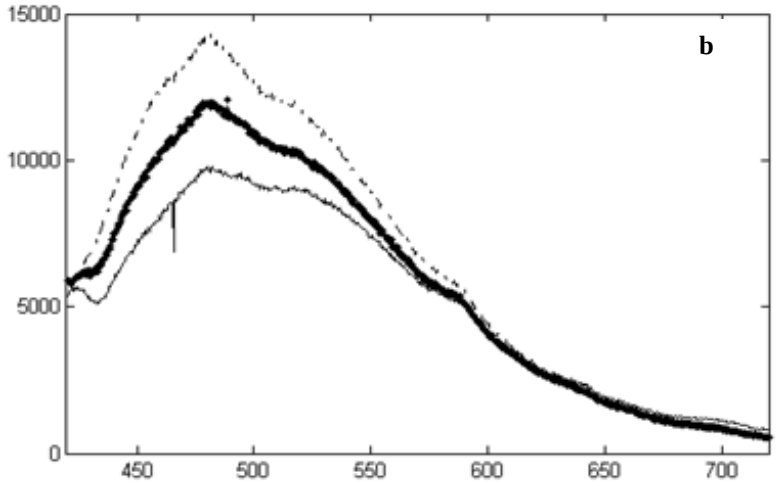


Fig. 1. Fluorescence spectra of caviar during 45 days of storage shows increased intensity, a. Asetra (excitation wavelength 332nm), b. Sevruga (excitation wavelength 382nm).

Multivariate analysis of caviar spectra

Multidimensional statistical analysis such as PCA, LDA and PLS-DA make it possible to extract information from spectral data bases. Principal component analysis (PCA) was applied to the each spectral data set in order to investigate its correlation with the storage time course of the caviar. Using this statistical technique, the emission spectra can easily be compared with each other in the way that two similar spectra are represented by two neighbouring points on the plot. PCA was performed on the Caspian caviar (Beluga, Asetra and Sevruga) spectra during the storage time. The score scatter plot of PC1 versus PC2 of emission fluorescence spectra of Asetra species represented 99.63% of the total variance with predominance of PC1 accounting for 90% of the total variance (Fig. 2). Examining the two dimensional scores plot in the space defined by PC1 and the PC2, a good discrimination of Asetra samples was observed throughout the storage periods. Indeed considering the PC2 accounting for 9% of the total variance, Asetra samples of fresh had negative value (indicated by circle in Fig. 2) and 15 days old had positive score value, while those of 30 days old separated by PC1 and PC2 from 15 days old and 45 days old samples separated by PC2 from fresh one. The score plots of the first two PCs show a slight discrimination between Asetra samples of fresh and higher than 30 days old.

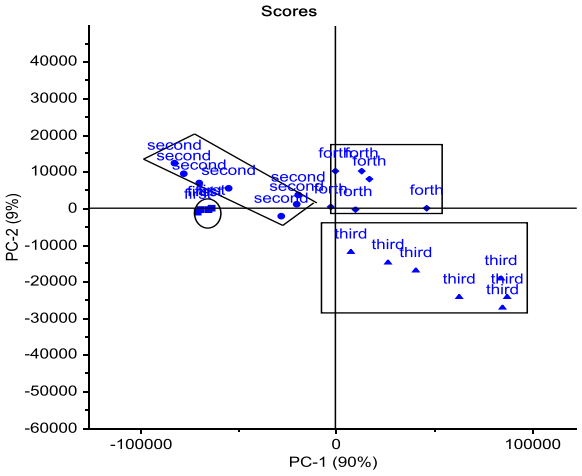


Fig. 2. PCA analysis scores for Asetra samples in four different dates of measurement by 382nm excitation filter

PCA was applied on all spectral data extracted from three different spices of Caviars to reduce the high dimensionality and to check qualitative discrimination in the spectra among the caviar's types. The interpretation of the result of PCA is usually carried out by visualization of its PC scores. Figures 3 show the score plot of PC1 against PC2 of three different types of caviars in excitation wavelength 382 nm, which reveals the feasibility of discrimination between caviars. Three different clusters are obviously observed without overlap among them.

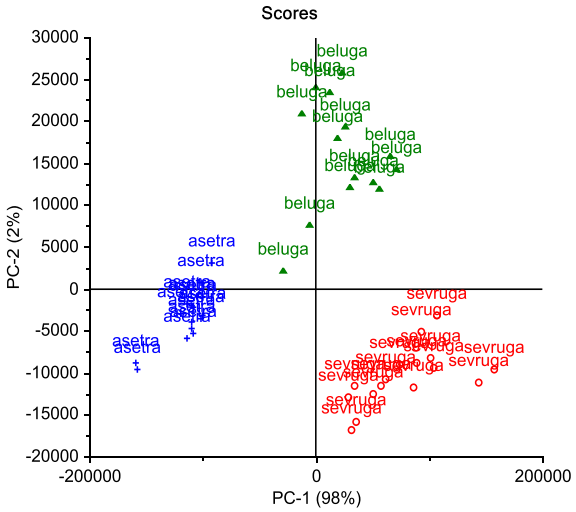


Fig. 3. PCA score plots show distinguished three species of caviar in excitation wavelength of 382 nm

For the next step, the ability of the investigated emission fluorescence spectra to differentiate caviar throughout storage was investigated by applying LDA on the 4 PCs of the PCA performed on the both regions of emission fluorescence spectra recorded at two different excitation filters. Four groups (fresh, 15, 30 and 45 days old) were created for the distinguished samples before applying the LDA. Correct classification rate was observed for 100%, 92.86% and 96.43% of the cross validation spectra of three types of caviar at λ_{ex}=332, respectively (Table 1). According to cross validation data set 100% correct classification rate was observed for spectra recorded on Asetra caviar in every date. Considering the spectra scanned on Beluga one of 15 days old spectrum misclassified as 30 days old and three other spectra failed to discriminate as 15 days old. For Sevruga, one spectrum of 30 days old samples was considered as a spectrum of 15 days old, and then 92.85% of cross validation spectra were correctly classified. Regarding to these results, emission fluorescence spectra of 370-700 nm recorded at 332 nm excitation wavelength can be considered as a promising method for monitoring the oxidation of wild caviar throughout storage and consumption.

Table 1. Freshness classification of emission spectra of caviar in excitation wavelength of 332 nm

Caviar Types	**Storage time (days)**	**Storage time (days)**				**Correct classification (%)**
		fresh	15	30	45	
Asetra	Fresh	14				100
	15		14			100
	30			14		100
	45				14	100
	Total				-	100
Beluga	Fresh	14	3			100
	15		10			71,43
	30		1	14		100
	45				14	100
	Total				-	92,86
Sevruga	Fresh	14				100
	15		15			92,85
	30			13		92,85
	45				14	100
	Total				-	96,43

LDA was then applied to the 4 PCs of PCA performed on the 420-720 nm emission fluorescence spectra (Table 2). Correct classification rate was observed for 100%, 96.86% and 97.50% for Asetra, Beluga and sevruga respectively. Considering Beluga samples, spectra recorded on 30 days old

were not classified quite satisfactory, since only 87.50% of them were correctly classified. Considering Sevruga samples of 15 days old, one of 10 spectra were classified as belonging to fresh group. For spectra recorded on the fresh samples on each type, all spectra were correctly classified. From the results obtained, it was shown that misclassification occurred between samples of 15 and 30 days old. No misclassification was observed between fresh and old (45days) caviars. Throughout storage, it seemed that amino acid components such as lysine and glycine, and aldehydes were affected by oxygen as reported by Wold *et al*. (2002). Good results obtained from spectra scanned at these three types of caviar showed that the 420-720 nm emission spectra could be used as a powerful tool for monitoring the oxidation of wild Caspian caviars throughout storage.

Table 2. Classification of emission spectra of caviar in excitation wavelength of 382nm according to date of storage

Caviar Types	**Storage time (days)**	**Storage time (days)**				**Correct classification (%)**
		fresh	15	30	45	
Asetra	Fresh	8				100
	15		8			100
	30			8		100
	45				8	100
	Total				-	100
Beluga	Fresh	8				100
	15		8	1		100
	30			7		87,5
	45				8	100
	Total				-	96,86
Sevruga	Fresh	10	1			100
	15		9			90
	30			10		100
	45				10	100
	Total				-	97,50

Front face fluorescence spectral data were also used by LDA to classify caviar types. It performs classification based on finding optimum boundaries among classes by maximizing the ratio of between class variance and minimizing the ratio of within class variance. Table 3 shows classification results in the form of a confusion matrix in excitation wavelength of 332 nm and 382 nm. The number of correctly classified caviar is shown on fresh and aged Sevruga in both excitation wavelengths. Some misclassification occurred for Beluga and Asetra in excitation wavelength of 332 and 382 nm. The results

suggest that front face fluorescence has the potential to discriminate different types of caviars without any chemical information combined with spectral data.

Table 3. Classification of different types of caviar by using front face fluorescence

Wavelength	Date	Caviar types	Predicted Class			Correct classification (%)
			Asetra	Beluga	Sevruga	
332nm	fresh	Asetra	10			100
		Beluga		8		80
		Sevruga		2	10	100
		Total				93,34
382nm		Asetra	10			90
		Beluga		10		100
		Sevruga			10	100
		Total				96,67
322nm	After 45 days of storage	Asetra	10			100
		Beluga		10		100
		Sevruga			10	100
		Total				100
382nm		Asetra	8			80
		Beluga		10		100
		Sevruga	2		10	100
		Total				93,34

For the last step, PLS-DA was applied to the all spectra recorded on three types of caviar samples and at forth times of measurements. The spectra of three wild Caspian caviar spices and the forth times of storage were pooled in two different matrices. PLS discriminant analysis was applied on the spectral collection divided on two data sets for calibration and validation. The two sets were obtained by splitting the spectral collection, i.e. for each type of samples and for each storage time, 14 spectra were put in calibration group and the other spectrum was used to create the validation group. Then PLS-DA used to generate calibration models in order to determine unknown samples and date of storage in two different models by front face fluorescence. To provide a reliable measure of the calibration model to predict species and age of unknown caviars, a careful validation procedure was required. Full cross validation and quadratic discriminant analysis could give optimistic results. The score map of Asetra samples defined by discriminant factors in Fig. 4a considering discriminant factor1 accounting for 97% of the total variance, fresh caviar were observed on the far right and also negative, whereas those of old (30 and 45 days old) were located on the left. Also samples of 15 days old exhibited on the positive side of right which are completely separated from fresh samples.

Figure 4b shows the score plot of factors which explained 80% of total variance. It appears that the intrinsic fluorescence spectra could be considered as sensitive method that may allow discriminating between fresh and aged caviar (Fig 4. a) and between different types of samples (Fig 4. b). The results of all species reported in Table 4 and Table 5.

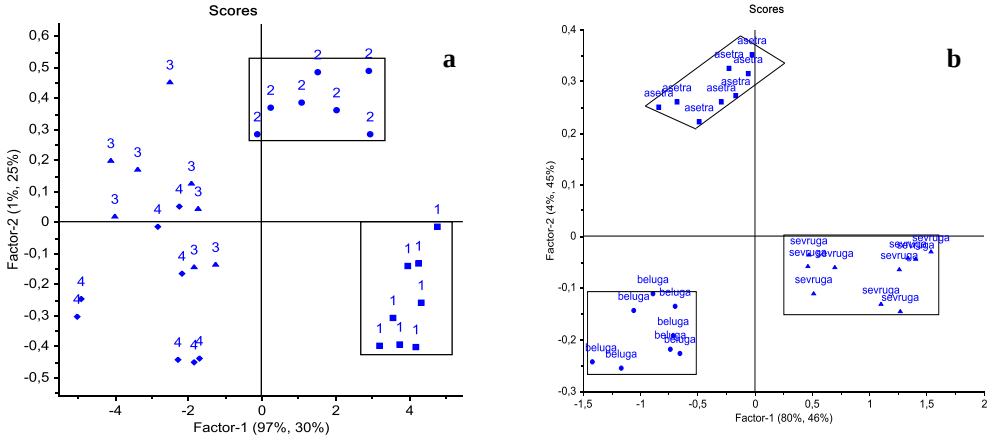


Fig. 4. PLS discriminant analysis similarity map of cross validation data set performed on emission fluorescence of spectra recorded on a. Asetra samples at excitation wavelength 382nm, b. second date of measurement of different types of Caviar at excitation wavelength 332nm

Table 4. R-square classification of PLS-DA calibration and validation models to validate fresh and aged of Caspian caviars

Samples	Excitation wavelength (nm)	Calibration (%)	Validation (%)
Asetra	332	0,99	0,93
	382	0,99	0,92
Beluga	332	0,98	0,88
	382	0,97	0,89
Sevruga	332	0,98	0,93
	382	0,97	0,90

Table 5. R-square classification of PLS-DA calibration and validation models to validate different types of Caspian caviars

Excitation wavelength (nm)	Date of measurment	Calibration (%)	Validation (%)
332nm	Fresh	99	94
	15days	96	93
	30days	98	89
	45days	92	83
382nm	Fresh	98	92
	15days	96	93
	30days	98	87
	45days	99	97

This study demonstrated that fluorescence properties of fluorophores are very sensitive to change in the age and type of samples. To the authors' knowledge, it is the first time that wild Caspian caviars are classified by non-destructive front face fluorescence spectra. It is now well documented that these three different caviars (Beluga, Asetra and Sevruga) can modify difference in market price especially for export. Lipid and protein oxidation developed in different caviar during the storage. There two reactions developed independently but they can also interact with each other. Jarenback and Liljemark (1975) demonstrated that linoleic acid hydroperoxides can interact with cod proteins inducing denaturation. Aldehydes groups formed during lipid oxidation can interact with amino groups of proteins (Chio and Tappel, 1996). In contrast the effect of protein oxidation products on lipid is not documented. Østdal *et al*. (2002) showed in model system that protein radical could attack free fatty acids, which resulted in an increased level of primary oxidation products. Oxidation in Caspian caviars could be monitored by front face fluorescence method during storage period. According to results presented in Table 4 and Table 5 correct classifications of calibration and cross validation data sets for three different species of caviar of wild sturgeons at different age was observed. Both region of 370-700nm (λ_{ex}=332nm) and 420-720nm (λ_{ex}=382nm) emission spectra success for monitoring different species of Caspian caviars and oxidation process in fresh and old samples. Front face fluorescence spectroscopy has the potential to dramatically reduce analytical time and cost of traditional measurement used for evaluation of oxidation in foods.

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