COMPARISON OF SPECTROPHOTOMETRIC METHODS FOR ASSESSMENT OF OXIDATION OF MILK FAT

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Abstract. Three spectrophotometric methods were compared for the assessment of milk fat oxidation: the AOCS (American Oil Chemists' Society) 2-thiobarbituric acid (TBA) value direct method, the TBA method of Aristova, and the modified method for the estimation of total carbonyl compounds in oils of Endo et al. Two cow milk fat samples – a fresh fat, obtained from fresh milk, and a rancid fat, obtained from a rancid commercial butter by means of high-speed centrifugation (15.871 × g, 30 minutes at 40 °C) – were analyzed several times in a 22-day period after sample preparation.
A positive linear correlation between absorbance and storage time was established in all used methods.
The method of Aristova is appropriate for exact determination of oxidation products. This method had rather good R^2 value (0.85) of the fresh fat sample results; however, the coefficient of variation (45.78%) showed rather large deviations between parallel analyses.
The methods of AOCS and Endo et al. can be used for the assessment of the dynamics of fat oxidation by reporting absorbance values from the assay directly. By these methods rather high R^2 values (0.86, 0.93) of the fresh fat sample results were obtained; the coefficients of variation also were satisfactory (10.13%, 11.12%). The last two methods are cheaper and require smaller sample amounts (< 0.2 g), while the method of AOCS is the most time-consuming method.
Key words: fat deterioration, malonaldehyde, TBARS, carbonyl compounds, rancidity, TBA method.

Introduction

The oxidative stability of milk and dairy products is of concern to the dairy industry. The oxidation processes can result in strong off-flavours and in deterioration of nutritional quality of dairy products, making them unacceptable to consumers (Hawemose et al., 2004). The reaction of lipids with oxygen results in flavour deterioration and creates serious problems in storage stability (Day, 1960). Inhibiting the progress of lipid oxidation is a key factor in maintaining the product quality and extending the shelf-life. Autoxidation is one of the chief processes by which lipids degrade. In addition to autoxidation, lipid oxidation can proceed along a photooxidation route or a lipoxygenase route. These differ from autoxidation at the initiation stage only. Food lipids possess an inherent stability to oxidation, which is influenced by the presence of antioxidants and pro-oxidants. After a period of relative stability (induction period), lipid oxidation becomes autocatalytic and rancidity develops. The length of the induction period is crucial for the shelf-life estimation of oils, fats and of fatty foods (O'Connor and O'Brien, 2006; Pegg, 2005).

Lipid oxidation is essentially a free-radical chain reaction involving initiation, propagation and termination stages. Unsaturated fatty acids are very susceptible to oxidation. The cause of the autoxidation is the particular reactivity with oxygen of the methylene group, situated between two double bonds. The speed of autoxidation increases with the amount of double bonds in fatty acid molecule. Unsaturated fatty acids are oxidized to form odorless, tasteless hydroperoxides. These are unstable and degrade by entering into numerous and complex breakdown and interaction mechanisms responsible for the production of myriad compounds of various molecular weights, flavour thresholds and biological significance. Since milk fat contains different minor unsaturated fatty acids, very many carbonyl products may be produced during autoxidation. Hence, the overall flavour produced during the autoxidation of milk fat is a combination of many flavours imparted by individual carbonyls present in minute concentrations (O'Connor and O'Brien, 2006; Gunstone, 1996).

One of the important polyunsaturated fatty acids in milk fat is linolenic acid. As reported by Hawemose et al. (2004), its content is depending on the type of the cow forage and in their investigation was approximately 0.2-0.7% from total fatty acids. A scheme revealing the photooxidation development of the triene group of linolenic acid is given as an example of the oxidation processes (Fig. 1).

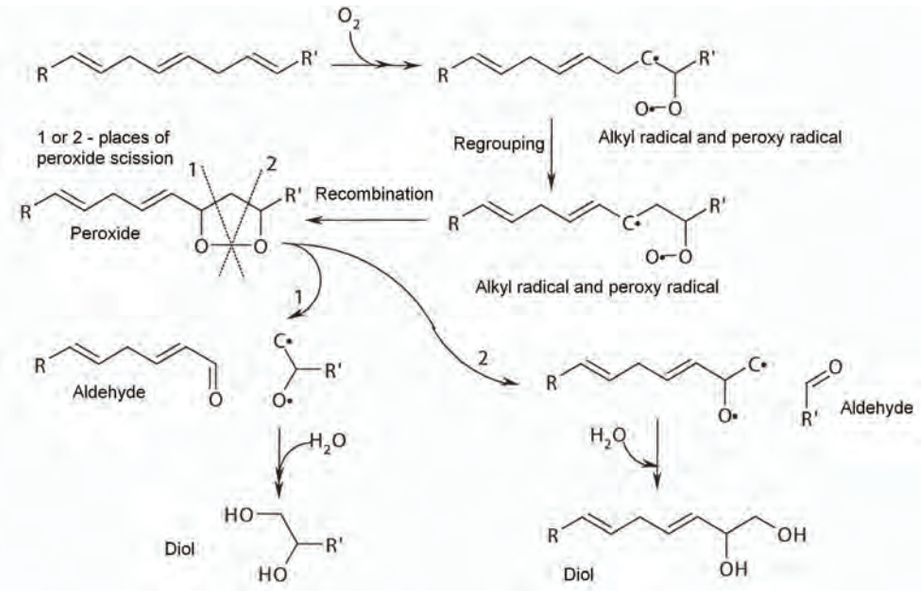


Figure 1. The scheme of the photooxidation processes of the triene group of the linolenic acid.

Among the various families of flavour compounds of dairy products, carbonyl compounds have been studied extensively. Some of them are naturally in milk fat, and some others are produced during heat treatment and autoxidation of milk fat. The flavour of dairy products depends on the nature and amounts of these compounds. Aldehydes are the main secondary oxidation products that are largely responsible for the development of undesirable taste and flavour which characterizes rancid fat. At elevated temperatures these products are further degraded to short-chain acids (tertiary oxidation products) (Bhat et al., 1981; Gunstone, 1996).

The changes in the concentration of carbonyl compounds and consequently the intensity of oxidation processes can be monitored by simple spectrophotometric techniques or by more sophisticated instrumental analysis, for example, methods using high-performance liquid chromatography (HPLC) or methods involving static and dynamic gas chromatography, so called 'electronic nose' apparatus and electron spin resonance (ESR) analysis (MacGibbon and Taylor, 2006; Pegg, 2005). The selection of methods is essential for the right monitoring of lipid oxidation processes. Advantages of spectrophotometric methods are that they are faster, cheaper and simpler regarding the necessary laboratory apparatus, personnel and reagents.

As a marker for lipid oxidation serves a relatively stable dialdehyde ($OHCCH_2CHO$) – malonaldehyde (MDA) (Vattem and Shetty, 2007). The measurement of MDA has been performed by the thiobarbituric acid (TBA) test. Methods involving TBA as an analytical reagent are widely used to follow the progress of oxidation in dairy products. These methods are based on the condensation of two molecules of TBA with one molecule of the lipid oxidation end-product, MDA, resulting in the formation of a red or pink-colored complex that can be determined spectrophotometrically with maximum absorbance at 532 nm (MacGibbon and Taylor, 2006). The reaction scheme is given in Figure 2.

MDA formation and accumulation in foodstuffs depend on the degree of unsaturation of polyunsaturated fatty acids, MDA precursors from non-lipid origin, types of oxidation catalysts, conditions, etc. Of these factors, the degree of fatty acid unsaturation markedly influences the extent and amount of MDA. With increasing level of unsaturation, more MDA will be generated during autoxidation. Consequently, TBA values cannot be used as an indicator of lipid oxidation when comparisons of oxidation are being made between foods with significantly different fatty acid compositions. Heat and/or strong acid are thought to be essential for the liberation of MDA from precursors or bound forms, for condensation with TBA, and for

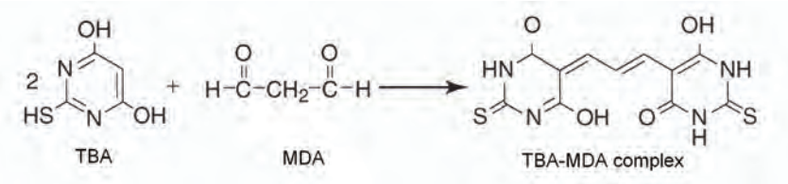


Figure 2. The reaction of the malonaldehyde with the thiobarbituric acid (Pegg, 2004).

maximal color development (Pegg, 2005; Seljeskog et al., 2006).

However, compounds other than MDA, such as alkanals, alkenals and dienals, conjugated aldehydes and hydroperoxydes, may react with TBA. Due to uncertainty concerning the exact identity of compounds that can react with TBA, the ambiguous term 'thiobarbituric acid reactive substances' (TBARS) is now commonly used in lieu of TBA number or value (Vattem and Shetty, 2007; Pegg, 2005). The kinds of TBARS produced depend on the substrate and oxidation conditions. Because these compounds may undergo extensive modification at advanced stages of oxidation, the TBA test is useful as a measure of lipid oxidation only during the initial stages of oxidation. The test is most useful for detecting incipient oxidation of lipids rich in methylene interrupted, three or more, double bonds. The color formation is empirical, as the color yield from each fatty acid varies (Pomeranz and Meloan, 1994). Probably this is the reason why different wavelengths are specified measuring TBARS: 532-535 nm (Vattem and Shetty, 2007), 536 nm (Инихов и Брио, 1971), and, as written by Miller and Miller (1998), the pink chromogen absorbs at 450, 530, and 538 nm.

Another important aspect is autoxidation of the sample during the analysis. As during the assay, especially the heating step, the autoxidation processes can be in progress consequently giving artifactually high analytical results, several scientists are advising the addition of antioxidants to the reaction mixture (Pegg, 2005). Still in biological samples, MDA can also react with proteins and give abnormally low values in an oxidizing system (Vattem and Shetty, 2007).

Because of many uncertainties, the appropriateness of the TBA assay for milk has been questioned and it needs empirical observations for each method and type of samples (MacGibbon and Taylor, 2006).

Many workers have attempted to make quantitative evaluations of rancidity through the use of carbonyl tests. They are based on the reaction of carbonyl moiety with 2, 4-dinitrophenylhydrazine (DNPH), followed by the reaction of the resulting hydrazones with alkali which are turned into quinoidals colored wine-red (see Fig. 3).

Variations in methodology for the choice of solvent, wavelength, or workup in which to analyze the quinoidal ion exist in literature. As with the TBA test, the carbonyl content in oils is usually monitored at discrete time intervals during storage and the contents of saturated, unsaturated and total carbonyls are compared against one another or with different chemical indices. The carbonyl compound method has been criticized because hydroperoxides decompose under the conditions of the test and thus possibly interfere with the quantitative determination of the carbonyls. Several modifications of this procedure have been offered to overcome this problem, but no ideal solution has been found (Pegg, 2005).

In this investigation three spectrophotometric methods were compared to choose the most appropriate for the assessment of oxidation of milk fats:

- the AOCS (American Oil Chemists' Society) Official Method Cd 19-90 (reapproved 2009) – 2-Thiobarbituric Acid Value Direct Method for the determination of TBA reactive substances (TBARS) in oils or lipid extracts (Pegg, 2005);
- the method of Aristova that uses TBA for the determination of oxidation degree of milk lipids (Инихов и Брио, 1971); and
- the method of Endo et al. (2003).

The method of AOCS is based on the protocol reported by Pokorny and Dieffenbacher (1989) and permits the direct determination of TBARS in oils and fats without preliminary isolation of secondary oxidation products. It is applicable to animal and vegetable fats and oils. The method of Endo et al. is used for the determination of total carbonyl compounds in frying oils and was derived from the Japan Oil Chemist's Society Standard Method and other similar conventional methods as of Henick et al. Japan scientists Endo et al. modified this method using less toxic 1-butanol as a solvent in place of benzene and reduced the volumes of reagents (Endo et al., 2003).

The aim of this study was to compare the above mentioned spectrophotometric methods to clarify their suitability for the assessment of oxidation processes of milk fat.

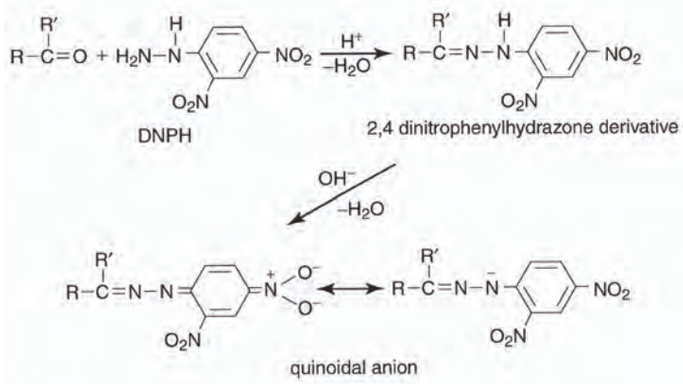


Figure 3. The reaction of a carbonyl with the DNPH to yield quinoidals colored wine-red (Pegg, 2005).

Materials and Methods

The experiments were carried out in the Research Laboratory of Biochemistry and Microbiology of the Research Institute of Biotechnology and Veterinary Medicine 'Sigra' of the Latvia University of Agriculture in winter 2010.

Two samples of milk fat were used in this investigation.

Fat sample preparation. The first sample was made from fresh cow milk obtained from a conventional dairy farm. At first milk was centrifuged in a separator, and cream was obtained. The second sample was commercial butter with expired shelf-life (approximately 50 days old, stored in a refrigerator) and had rancid flavor. The pure fat part for experiments was isolated by the centrifugation from cream and butter in a centrifuge (15.871 × *g*, 30 min. and 40 °C). Fat samples were divided into small portions (1 or 3 g) in Eppendorf or glass tubes (semi-filled, not special atmosphere was applied) for each day and type of analysis and were stored refrigerated at temperature of 4-6 °C. Samples were analyzed 6-7 times within a 22-day period after sample preparation (by the methods of AOCS and Aristova – in days 5, 7, 9, 12, 15, 17, and 22; by the method of Endo et al. – in days 5, 8, 11, 14, 16, 17, and 22). Before testing, fat was heated till 40 °C and mixed thoroughly.

Analytical reagents. 2-tiobarbituric acid (TBA) (purity >=98%), 1,1,3,3 – tetramethoxypropane (TMP) (purity 99%), 2,4-dinitrophenylhydrazine (DNPH), and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich; 1-butanol was of analytical grade containing water < 0.5% from Lab-Scan Analytical Sciences, Poland; isooctane was of pestician grade from Lab-Scan Analytical Sciences, Ireland. All other reagents were of analytical grade: glacial acetic acid was from Stanchem, Poland; potassium hydroxide was from Lachema, Czech Republic. Absolute ethanol was from Lako, Latvia.

The method of AOCS. A fat sample of 10-40 mg was put into a 20 ml test tube, recording accurate weight, and dissolved in 4.95 ml of 1-butanol (with 50 mg of BHT in 100 ml). Blank trial was made taking 5 ml of 1-butanol. Five ml of TBA reagent (prepared by dissolving 0.2 g of TBA in 1-butanol in a 100-ml volumetric flask by means of careful heating till 75 °C) were added. Tubes were sealed, mixed by vortex mixer and heated in 95 °C water bath for 2 h, then cooled till room temperature. Absorbance readings at 532 nm were taken, using plastic cuvettes (path lengths – 1.00 ± 0.01 cm). Calibration curves were made with series of 0.02 m*M* 1,1,3,3-tetramethoxypropane (TMP) solution aliquots (0.2 to 2.0 ml), adding 1-butanol to each tube till 5 ml total. Addition of TBA and further steps were performed as described previously.

The method of Aristova. A fat sample of 3 g was put into a 20-ml test tube and dissolved in 10 ml of isooctane. Blank trial was made taking 3 ml of distilled water instead of fat. 10 ml of TBA reagent (prepared by dissolving 0.67 g of TBA in distilled water in a 100 ml volumetric flask by means of careful heating till 60 °C and US bath to succeed the dissolution of TBA; after cooling till room temperature, TBA solution was mixed with glacial acetic acid in ratio 1:1) were added. Tubes were sealed, mixed for 4 min. The lower layer was separated and heated in boiling water bath for 30 min. Then tubes were cooled till room temperature, and the solution was filtered through a paper filter. Absorbance was read at 536 nm using glass cuvettes (path lengths – 1.00 ± 0.01 cm). Calibration curve was made with series of 0.02 m*M* TMP solution aliquots (0.02 to 0.2 ml). To each tube with TMP solution, distilled water was added till 3 ml total. Addition of TBA and further steps were performed as described previously.

The method of Endo et al. A fat sample of 5-20 mg (10µl) was put into a 10-ml test tube recording accurate weight and was dissolved in 990 µl of 1-butanol. One ml of 2,4-DNPH solution (prepared by dissolving 50 mg of 2,4-DNPH in 100 ml of 1-butanol containing 3.5 ml of concentrated HCl) was added, the tube was sealed and mixed by vortex mixer, then heated in 40 °C water bath for 20 min., and cooled till room temperature. Then 8 ml of KOH solution in 1-butanol (0.8 g l^{-1}) were added, the tubes were shaken and centrifuged at 845 x *g*, 5 min. at 20 °C. The absorbance of the upper layer was measured at 420 nm, using glass cuvettes (path lengths – 1.00 ± 0.01 cm).

In the methods of AOCS and Endo et al., modifications were made to reduce the amounts of reagents. In the method of Aristova, filtration was applied to get transparent solution, suitable for absorbance readings, because there were droplets of isooctane and fat remained into the reaction solution after the first separation.

All analyses were made in triplicate employing the same apparatus – spectrophotometer Spectro 2000 RSP from Labomed, Inc., USA, and taking at least four readings of absorbance for each sample.

The results were calculated, and analyzed, and graphs were made using MS Office program Excel.

Results and Discussion

The first principal difference of the three spectrophotometric methods chosen for the comparison is the spectrum of the secondary oxidation products detected and revealing the processes of fat oxidation. The methods of AOCS and Aristova are based on the reactions of TBA reactive substances, while the method of Endo et al. is based on the reactions of a larger spectrum of substances – total carbonyl compounds. The different chemicals used as reagents and solvents yielding different colour nuances are the basic reason why different wave lengths are used for the measurements.

The second principal difference is in the expression and interpretation of the results obtained by measurements. In the practice there are two ways for the assessment of lipid oxidation. The first approach is the exact determination of analyte concentration in the product, by making stationary measurements reflecting its quality in one exact

point of time. The second practice is to observe the dynamics of the products' quality in longer period of time, by reporting absorbance values from the assay directly, as do some researchers to avoid having to construct a standard curve and to perform recovery experiments, if it is not necessary to know the exact concentrations of oxidation products (Pegg, 2005; Pokorny and Dieffenbacher, 1989). In the first case, the results, calculated as concentration in grams, moles and like, can be used to compare the quantities of the oxidation products, estimated by different methods or to compare accuracies of different methods. In the second case it is possible to observe the progress and intensity of oxidation processes in the limits of one separate method, furthermore comparisons can be made between the results if equal conditions (analytical reagents, types of the samples, etc.) are used.

In this investigation, the determination of secondary oxidation products could be made in the method of Aristova. The exact concentrations of TBARS expressed as μg per 1 g of fat were obtained by means of the calibration curve. It was found that in the rancid fat sample it was 13.8–27.0 μg, but in the fresh lipid sample - 0.1–7.5 μg per 1 g of fat.

In the method of AOCS, the calibration curve was not obtained although trials were repeated several times. Commonly 1,1,3,3-tetramethoxipropane (TMP) or its ethyl analog is used as a standard. In the presence of an acid solution, these chemicals are hydrolyzed to MDA and therefore can be used to obtain TBARS calibration curve; one mole of MDA is released for each mole of TMP (Pegg, 2005). In the method of AOCS, acid is not added, but in the method of Aristova the medium of reaction is more acidic due to the glacial acetic acid. Another probable factor can be the presence of water. It is known that MDA has hydrophilic nature. In the method of Aristova there is the presence of the water, while in the method AOCS – is not. So, probably MDA can be liberated from TMP reagent and reacted with TBA easier in environment, which is in the method Aristova.

The results obtained from all the three methods are shown as absorbance readings in Figs 4 - 6.

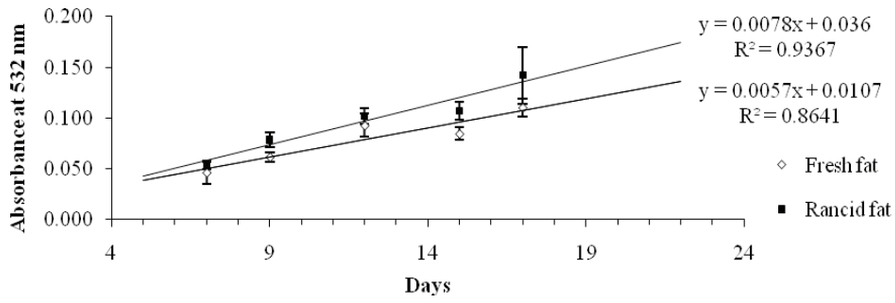


Figure 4. Results of the method of AOCS.

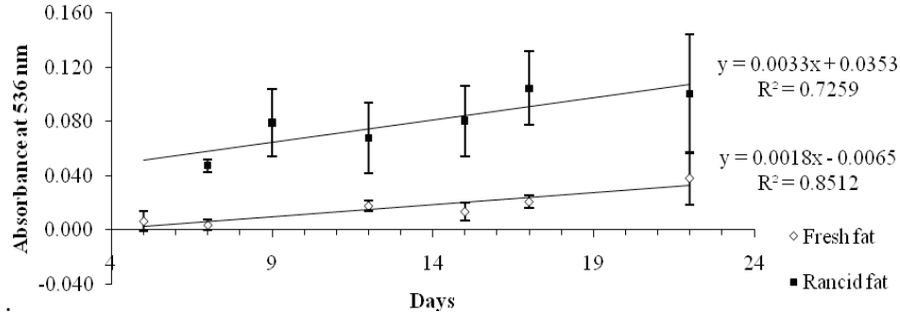


Figure 5. Results of the method of Aristova.

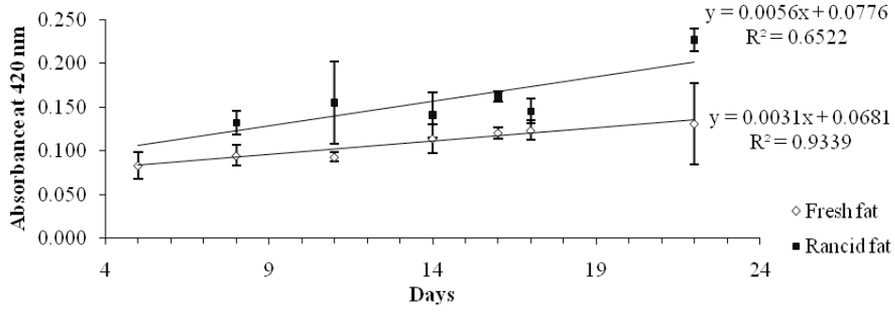


Figure 6. Results of the method of Endo et al.

Table 1

Comparison of the principal parameters of the methods

Parameters for method comparison	Symbol, units	Method					
		AOCS		Aristova		Endo et al.	
		fresh fat	rancid fat	fresh fat	rancid fat	fresh fat	rancid fat
Coefficient of determination	R^2	**0.86**	0.94	**0.85**	0.73	**0.93**	0.65
Coefficient of variation [a]	%	**10.13**	9.76	**45.78**	26.60	**11.12**	9.72
Time consumption [b]	h: min.	3:00		1:45		1:30	
Expenses of chemicals [b]	LVL	0.56		1.08		0.91	
Sample amount [b]	g	0.03 - 0.12		9.00		0.02 - 0.06	

[a] Calculated within parallel analyses.
[b] Calculated for 3 parallel analyses of 1 sample (not included time or expenses for making calibration curve).

It is important to remember that all the three test procedures differ in sample amounts, used solvents and reagents, heating temperatures, wavelengths of spectrophotometrical measurements, and lead to different levels of secondary oxidation products recovered from an identical sample (Pegg, 2005). In this case, when the exact concentrations of the secondary oxidation products are not determined in all the methods, its accuracy cannot be compared directly by absorbance readings, carbonyl or TBARS values.

However, graphical images can serve to compare how similarly all methods are representing the development of oxidation processes in lipid samples.

All methods used in this experiment show that the absorbance of the rancid fat sample is significantly higher ($p = 0.05$) than that of the fresh fat. Concentrations of secondary oxidation products are increasing linearly in both fat samples; however, the slopes of the trendlines show that the speed of the secondary oxidation products development is higher in the rancid fat sample. The summary of the results is given in Table 1.

Further only the results of fresh fat were analyzed for method comparison, as in practice the monitoring of the oxidation processes is more important for the fresh fat.

As seen from Table 1, the R^2 values of the results obtained by all three methods are rather high (AOCS – 0.86, Aristova – 0.85, and Endo et al. – 0.93). Deviations within parallel analyses of each sample were compared by the coefficient of variation, expressed as percentage, also called 'the relative standard deviation', which is often preferred since it generally has the advantage of being constant at every level of concentration of the quantity to be measured (Grappin, 1996). The methods of AOCS and Endo et al. show rather good coefficients of variation (10.13%, 11.12%), while the method of Aristova has the greatest variation (45.78%). Possibly it can be explained with the fact that absorbance readings for fresh fat in the method of Aristova are very low, near zero. As known, low absorbance values give particularly high errors. Smaller errors could be obtained, if the absorbance of the measured light is within the interval of 0.2-1.4. It should be considered that this method originally was prescribed for the measurements on photo-electro colorimeter using cuvette with longer path length, yet in this experiment measurements were made on the spectrophotometer. The readings on both apparatus can be different. If readings are taken on spectrophotometer, greater absorbance values can be obtained by preparing the solution with higher analyte concentration.

Time consumption, expenses of chemicals and necessary sample amounts also were calculated to compare the methods and, as seen in Table 1, the fastest is the method of Endo et al., while the method of AOCS is the most time consuming. Regarding analytical reagent expenses, the last method is the cheapest while other two methods are in the same level and, in general, are more cheaper, compared to sophisticated analytical techniques. The method of Aristova needs the biggest amount of the sample (9 g), though that is not always easy obtainable. Other two methods need considerably smaller amounts of the sample (< 0.2 g), but care must be taken to the very accurate weighting technique.

Conclusions

1. The positive linear correlation between absorbance and storage time was established in all used methods.
2. The methods of AOCS and Endo et al. are suitable for the assessment of the dynamics of fat oxidation by reporting absorbance values from the assay directly.
3. The Aristova method can be used for exact determination of oxidation product concentrations.
4. The methods of AOCS and Endo et al. are cheaper and require smaller sample amounts (< 0.2 g) compared with Aristova method (9 g), while the method of AOCS is the most time-consuming method.

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