Electrochemical Method for the Assay of Organic Peroxides Directly in Acetonitrile

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Article

Electrochemical Method for the Assay of Organic Peroxides Directly in Acetonitrile

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Abstract: Lipid peroxidation is a major process that determines the quality of various oil samples during their use and storage, in which the primary products are hydroperoxides (HP's). HP's are very stable compounds at ambient conditions and are harmful to human health. Therefore, the evaluation of the degree of oil oxidation is an excellent tool for ensuring food safety. The peroxide value (PV) is the main parameter for quality control of oils. Herein, we propose an alternative electrochemical method to the most widely used classical iodometric titration for determining the PV. Our approach is based on the electrochemical quantification of hydroperoxides/peroxides in an organic solvent medium (acetonitrile and organic ammonium salt) using a composite electrocatalyst-glassy carbon electrode modified with 2D-nanomaterial graphitic carbon nitride doped with Co_3O_4. Calibration was made by standard addition method using benzoyl peroxide (BPO) as a model peroxide compound, dissolved in chloroform and added to fresh Rivana brand anti-cellulite oil used as a model oil sample. Calibration plots showed a linear response and very good reproducibility of the analytical result (R^2>0.99). Further, in term of accuracy, the method showed good results, since the BPO quantitative analysis was close to the theoretical response. In addition, the accuracy of the electrochemical method was compared with that of the standard iodometric titration method for determining the PV of vegetable fats (according to Bulgarian State Standard, BSS EN ISO 3960:2017). Finally, using the electrochemical method, the concentration of peroxides was determined in a real sample - an anti-cellulite oil of the trademark Rivana with an expired shelf life.

Keywords: composite catalyst; 2D nanomaterial; non-aqueous electroanalysis; organic hydroperoxides; peroxide value (PV)

1. Introduction

Organic peroxides are considered very harmful compounds because when they come into contact with bodily tissues such as the gastrointestinal tract, epidermis or mucous membranes, they tend to form free radicals responsible for the development of severe health problems such as chronic inflammation, ulcers or proliferating tumors. Therefore, the precise control of peroxides content in commercial products containing lipids - pharmaceutical, cosmetic or food products - is of key importance [1]. The generation of organic peroxides *ex vivo* is due to the processes of lipid peroxidation of oils, which are often also components of drugs, cosmetics or foods, as they are oxidized in the air in the presence of daylight [2]. This process of autooxidation is a free radical chain reaction and takes place in three steps- initiation, propagation and termination. The primary products of this process are hydroperoxides, which are relatively stable at room temperature and can be used to estimate the degree of lipid oxidation of oil samples [3]. The main indicator of primary oxidation processes in oils and for the amount of peroxides in oil-containing samples is the peroxide value (PV). The amount of peroxides indirectly serves to determine the degree of rancidity of the oils due to their autooxidation [4].

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There are different methods for quantitative determination of PV like instrumental and titrimetric approaches. Various rapid, selective and accurate instrumental techniques have been developed for estimating PV in oil samples, such as: spectroscopic, chromatographic and electrochemical methods [5]. The most widely used technique for quantification of PV is based on infrared radiation. Fourier transform infrared spectroscopy (FTIR) has attracted increasing interest in the last few years as it is non-destructive and requires minimal sample preparation [6]. FTIR is an excellent tool for the determination of peroxides in various oils, because the intensity of the bands in the infrared spectrum is directly proportional to the concentration of peroxides in the sample [7]. The powerful combination of FTIR and chemometric methods is often used for estimation of olive oil freshness and oil oxidation, as well as for monitoring of the fatty acid content in virgin olive oil [8–10]. Determination of PV by FTIR uses different methodologies such as near-infrared spectroscopy region (4000-1200 cm^{-1}) and FTIR with Attenuated Total Reflection (ATR) equipment [11]. Direct quantification of peroxides in oil samples by FTIR in NIR is difficult due to low sensitivity and complex calibration using multivariate approach. However, FTIR with an ATR mode gives accurate results with a low signal-to-noise ratio, requires small sample volume and is excellent tool for viscous oil samples analyses [11]. Whereas the FTIR-ATR method may not be suitable for very viscous oils because high viscosity can lead to difficulties in obtaining a reproducible sample thickness, which can affect the accuracy of the measurements [12]. In addition, one of the main disadvantages of FTIR-ATR approach is its low sensitivity. This drawback can be overcame using multispectral identification methods, which are increasingly used in food analysis, e.g. the combination of FTIR and Raman spectroscopy [13]. FTIR spectra are generated by molecular vibrations and rotational frequencies, while Raman spectra are a result from molecular scattering, which is inelastic scattering of incident IR light from the sample [17–19]. Thus, multispectral FTIR-Raman identification technique could provide rich information about the content of oil samples, but the problem with overlapping of analyte bands with those of other substances in the sample cannot be ignored. Therefore, it is necessary to combine FTIR and Raman spectroscopy with chemometric methods [16].

Among the instrumental techniques, UV-Vis spectroscopic approaches are widely used for determining PV in edible oils. These approaches are frequently based on the oxidation of Fe (II) to Fe (III) by hydroperoxides in acidic medium, which then reacts with different reagents, resulting in color complexes such as Fe(III)- thiocyanate and Fe(III)- xylenol orange, absorbing light in the visible region of the electromagnetic spectrum [14]. These methods are simple and low cost, but their accuracy is easily affected by the content of carotenoids in oils. Hence, it is necessary to extract carotenoids from the solution containing Fe(III)-thiocyanate or Fe(III)- xylenol orange complexes using organic solvents before PV determination [15,16].

Fluorescence and chemiluminescence are emerging spectroscopic techniques for qualitative and quantitative determination of organic peroxides in oil samples due to their high sensitivity, rapidness and robust information content [5]. In addition, fluorescence spectroscopy (FS) has significant advantages in terms of wide dynamic range, selective detection and non-destructiveness. However, several shortcomings of FS limit its practical applications, such as specific sample preparation through extraction or derivatization and the need for specific excitation sources [17]. In contrast, in the chemiluminescense method, no external light source for excication is used, which simplifies the instrumentation. Additionally, sample preparation in chemiluminescense spectroscopy (CS) is not so complex and this technique allows real time monitoring of changes in peroxide levels during reactions. The main drawback of CS is the need to carry out specific chemical reactions that produce light, which also limits the application of the method to different oil samples.

More recent research has focused on applying ^{1}H nuclear magnetic resonance (NMR) spectroscopy for PV determination in edible oils, because of its simplicity and time efficiency [5]. The chemical specificity of the NMR spectrum is the major advantage of this technique over other spectroscopic methods [17] as the NMR (^{1}H) spectra of fatty acids and their derivatives can be fully assigned [18–20]. However, all currently available instruments based on the NMR technique use expensive high-field super-conducting magnets, as well as probes requiring cryogenic cooling and specially trained personnel to operate the instrument. Hence, none of the above mentioned

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spectroscopy-based instrumental methods seems to be a suitable candidate for routine and low-cost analysis in industrial settings [17].

Among the methods for PV determining of oil samples, chromatographic techniques such as gas (GC) and high pressure liquid chromatography (HPLC) are characterized by a number of advantages- excellent selectivity, low LOD's, wide detection range and less sample consumption [21]. However, some major shortcomings limit the practical applications of chromatographic techniques for real-time oil oxidation monitoring such as expensive instruments, complex and time-consuming sample preparation before GC and HPLC, the high cost for analysis of a single sample, as well as long expensive work and complex data processing after HPLC [22].

All considered instrumental techniques are accurate, rapid and sensitive, but require relatively expensive instrumentation and highly trained personnel.

The most frequently used method in laboratory practice in Bulgaria for PV determination is the conventional method of iodometric titration in a mixed aqueous-non-aqueous medium. This approach is widely applied as a classical analytical method due to its simplified operation and low-hardware requirements [23]. The method is based on the reaction between peroxides in sample with KI resulting in the formation of molecular iodine, which is titrated using sodium thiosulfate solution. Starch is used as an indicator, which changes the color of the solution from colorless to blue-violet in the presence of iodine released from the sample. When titrating the water sample with sodium thiosulphate, the sample is discolored when the equivalence point is reached \BSS EN ISO 3960-2017\. Although, the titrimetric method is simple, low-cost and routine, several shortcomings restrict its application, such as the need for special sample preparation (dissolving oil samples in organic toxic solvents - trichloromethane, toluene and ether, extraction, and titration in mixed medium), the process is affected by a number of factors such as the nature of the reagents, temperature, extraction time, etc. In the titration of crude oils, the equivalence point is difficult to distinguish because of the dark color of the oil samples [23,24]. Other disadvantages are the high LODs and narrow detection range [23].

Recently, electrochemical techniques belonging to the group of instrumental approaches, have attracted increasing interest due to a number of advantages such as simplicity, low-cost, high sensitivity, selectivity and portability [25–29]. A relatively new electrochemical method for PV determination in edible oils is based on a change in electrical conductivity in an aqueous medium during a reaction between KI and hydroperoxides in the oil sample [6]. This approach has high reproducibility, but its accuracy is low. In the pursuit of *in situ* monitoring of lipid oxidation and miniaturization of the instrumentation, electrochemical portable sensors like electronic nose (E-nose) and electronic tongue (E-tongue) have been developed. The latter represent simple, rapid, inexpensive and highly efficient portable miniaturized devices for lipid oxidation monitoring [30]. The E-nose method was used to study the flavor qualities of oils, while the E-tongue approach was applied to monitor the PV of olive oil during storage [31–33]. However, such portable miniature devices are still in the early stages of research. Further research is needed on their long-term storage stability and biocompatibility [23].

In the recent decades, there has been an intensive development of electrode modification techniques for various applications, such as solar energy conversion and storage [34], selective electroorganic synthesis [35], molecular electronics [36], electrochromic display devices [37], protection from corrosion [38] and electroanalysis [39]. The rapid development of experimental chemistry and the desire of scientists to develop fast and accurate methods for the determination of the oils oxidation extent, led to the idea of emerging materials such as 2D disulfides of transition metals, metal-organic frameworks, covalent organic frameworks, halide perovskites, etc., to be introduced into the basis of various analytical techniques, including in the modification of electrode surfaces [40].

After the discovery of monolayer graphene in 2004 [41], 2D carbon-based nanomaterials have attracted the attention of many researchers in various fields ranging from electronics to biomedicine [42]. Their unique catalytic properties and high surface-to-volume ratio make them suitable for a wide range of applications, such as environmental catalysis [43], drug delivery carriers [44], sensing

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and energy applications [45]. Often, carbon-based nanomaterials are combined with other materials, resulting in nanocomposites that significantly improve the sensitivity and selectivity of modified surfaces, as a result of the synergistic effect between them. 2D nanomaterials provide features such as increased electroactive surface area, additional catalytic centers, and tunable structure [46]. It was found that the addition of nitrogen atoms is the most effective approach to improve the electrocatalytic activity of carbon materials [47]. Carbon nitride-based compounds with a high N-to-C ratio are new generation materials that are used to develop electrocatalysts [48]. Graphitic carbon nitride (g-C_3N_4) is a non-metallic, graphene-like 2D material composed of covalent bonds (C-N), forming a π-conjugated polymer with a high molecular weight and unique electronic structure determining its semiconductor properties [49]. g-C_3N_4 is a multifunctional, heterogeneous, non-metallic catalyst [50], the main drawback of which is its low specific surface area. A known approach to increase the specific surface area of g-C_3N_4 is its doping with transition metal oxides in the form of ultrafine dispersion, which distribute between g-C_3N_4 nanosheets and prevent their agglomeration [51,52]. Transition metal oxides (TMOs) possess good electrical and photocatalytic properties due to their size, shape and larger surface area [52].

An earlier study of our group demonstrated that a composite comprising Co_3O_4-g-C_3N_4 and Nafion used as a modifying phase of glassy carbon surfaces possesses excellent electrocatalytic activity in the reduction of water-soluble hydroperoxides [53]. Unfortunately, most naturally formed hydroperoxides are insoluble in water and their direct evaluation has to be performed under non-aqueous conditions. Therefore, the aim of this study is to explore the electrochemical activity of composite-modified glassy carbon electrode in acetonitrile with respect to water-insoluble peroxides and hydroperoxides and to develop on its basis a simple method with rapid response for the quantification of organic peroxides directly in a non-aqueous medium. In characterisation of the analytical performance of the discussed electrocatalytic method classical iodometric titration was used as a reference.

2. Results

2.1. Optimisation of the Operating Conditions

Electrochemical studies in a non-aqueous medium have a number of limitations such as: low conductivity, limited solubility of electrolyte salts, high noise and the impossibility of using conventional reference electrodes, however, non-aqueous electrochemistry allows the redox properties of a number of insoluble in water organic compounds to be studied. Keeping this in mind, our further electrochemical studies were performed in the aprotic organic solvent acetonitrile containing 0.1 M of the electrochemically inert electrolyte tetrabutyl ammonium perchlorate (TBAP) to increase the electrical conductivity of the medium.

The electrochemical behavior of the Co_3O_4 -doped g-C_3N_4/Nafion modified electrode (Co-g-C_3N_4/Naf) was investigated by cyclic voltammetry. Figure 1 compares the cyclic voltammograms (CVs) of the electrode - catalyst recorded in the absence and presence of benzoyl peroxide (BPO). This peroxide was chosen as a model analyte because it is non-hygroscopic solid and its solutions in organic solvents can be prepared with exact concentrations, and hence – to be used as analytical standards. The background CV – i.e. the voltammogram recorded in the absence of peroxides (black solid line) suggests that the catalytic layer possess catalytic activity towards oxygen, which is dissolved in the acetonitrile, as the I-E curve possesses an "oxygen tail" appearing over the potential region between -0.4 and -0.6 V vs. $Ag|Ag^+$.

When portions of BPO standard solution are added to the electrolyte in the cell, an electrochemical reduction of the peroxide occurs (red, blue and purple solid lines). Upon scanning in the negative direction in BPO present, a reduction wave is observed, starting at a potential of 0.3 V (vs. $Ag|Ag^+$ pseudo-reference electrode) and reaching a broad peak at -0.2 V, which upon further change of the potential passes into a second wave of reduction. As the concentration of the model peroxide increases, the reduction maximum gradually shifts to more negative potentials.

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The polarization dependences suggest the possibility for quantification of the selected organic peroxide by means of chronoamperometry at a constant working potential of -0.2 V that can be done on the basis of a calibration plot. Chronoamperometric experiments performed at a constant potential of -0.2 V (vs. Ag|Ag$^+$) showed a stepwise change in the reduction current upon addition of portions of peroxide, reaching steady-state values within 10 seconds (Figure 2, left). The calibration graph obtained on the basis of the chronoamperometric record shows a linear dependence of the signal versus the peroxide concentration up to 50 μM. It should be noted that at low concentrations (up to about 2 μM) a deviation from linearity is observed, which is probably due to the weak electrode signal over this concentration range, which in turn leads to considerable errors of the determination.

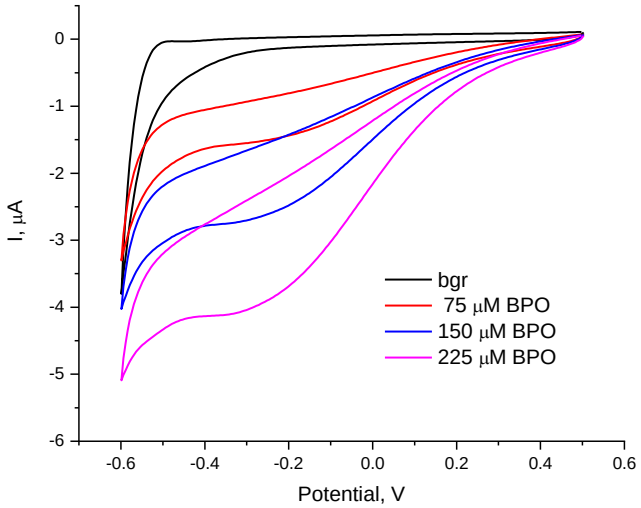

Figure 1. Cyclic voltammograms of Co-g-C_3N_4/Nafion/GCE in the absence and presence of BPO/ACN; background electrolyte 0.1 M TBAP in acetonitrile, reference electrode Ag|Ag$^+$; scan rate 20 mV/s.

The calibration equation obtained from the linear regression analysis is:
$I_S - I_0 = (-3{,}962.10^{-9} \pm 4{,}9483.10^{-10}) + (3{,}59.10^{-3} \pm 2{,}12994.10^{-5}) * C_{BPO}$
where: I_S - steady state signal in the presence of the peroxide, μA;
I_0 – background current, μA;
C_{BPO} – concentration of BPO, μM.

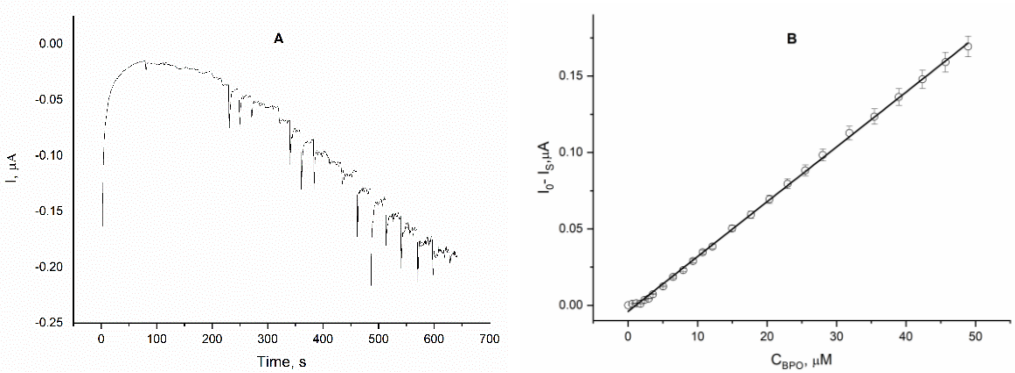

Figure 2. Chronoamperometric record (left) and calibration plot (right) of an electrode modified with Co-g-C_3N_4/Naf. upon addition of aliquots of 0.003M BPO; background electrolyte: 0.1 M solution of TBAP in acetonitrile, reference electrode Ag|Ag$^+$; working potential: - 0.2 V.

Keeping in mind that the designed peroxide electrode is to be used for assaying peroxide value (PV) in vegetable oils, several other peroxides have been tested as possible standards for quantification of peroxides under non-aqueous medium by applying the same approach. In Figure 3 are depicted the cyclic voltammograms of the catalytic peroxide electrode in the absence and presence

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of varying concentrations of dilauroyl peroxide – a solid aliphatic peroxide compound. The CVs recorded manifest similar behavior as observed under equivalent conditions in the presence of BPO as a model peroxide. However, the electrochemical reduction of the aliphatic peroxide compound took place with much lower rate at the chosen operating potential of –0.2 V, which indicates that the reduction proceeds with a considerable overpotential as compared with the electrochemical reduction of benzoyl peroxide. It is plausible, that the bulkier molecule of dilauroyl peroxide adsorbs stronger on the electrode surface thus blocking it to a great extent. These results indicate that the choice of BPO as a standard for calibration is reasonable and further studies were implemented using BPO standard solutions.

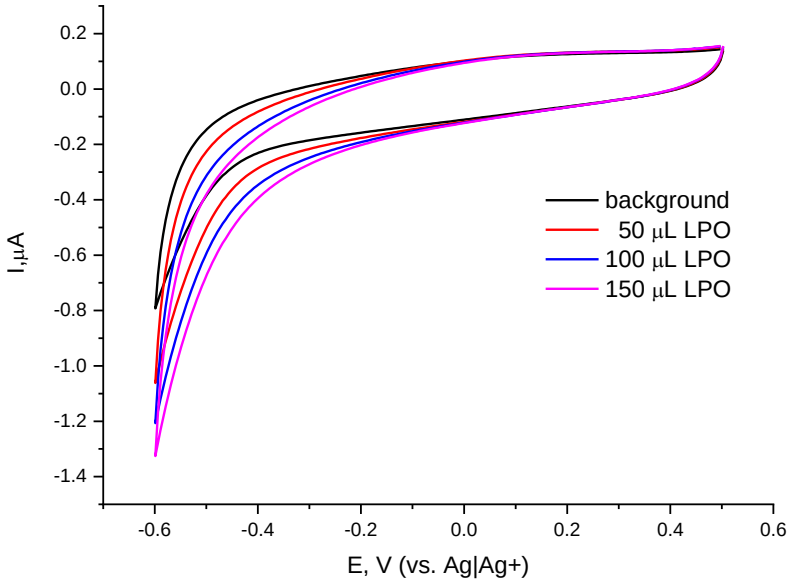

Figure 3. Cyclic voltammograms of Co-g-C_3N_4/Nafion/GCE in the absence and presence of lauroyl peroxide (LPO) in ACN; background electrolyte 0.1 M TBAP in acetonitrile, reference electrode $Ag|Ag^+$; scan rate 20 mV/s.

Furthermore, the catalytic peroxide electrode was used to determine the concentration of peroxides in a real sample – a mixture of vegetable and essential oils with expired shelflife (anti-cellulite massage oil Rivana, Bulgaria). For this purpose, the electrochemical behavior of the electrode - catalyst upon addition of anti-cellulite oil dissolved in chloroform ($CHCl_3$) was investigated by cyclic voltammetry run over the range of potentials from -0.6 to 0.6 V (vs. $Ag|Ag^+$). Addition of aliquots of the real sample solution to the working medium resulted in a pronounced reduction wave (visible in the reverse scan) starting at a potential of 0.18 V and reaching a plateau between -0.2 V and -0.4 V (Figure 4). The presence of this reduction wave gave us a reason to further explore the opportunity to determining the amount of organic peroxides in the real sample by means of chronoamperometry at constant potential.

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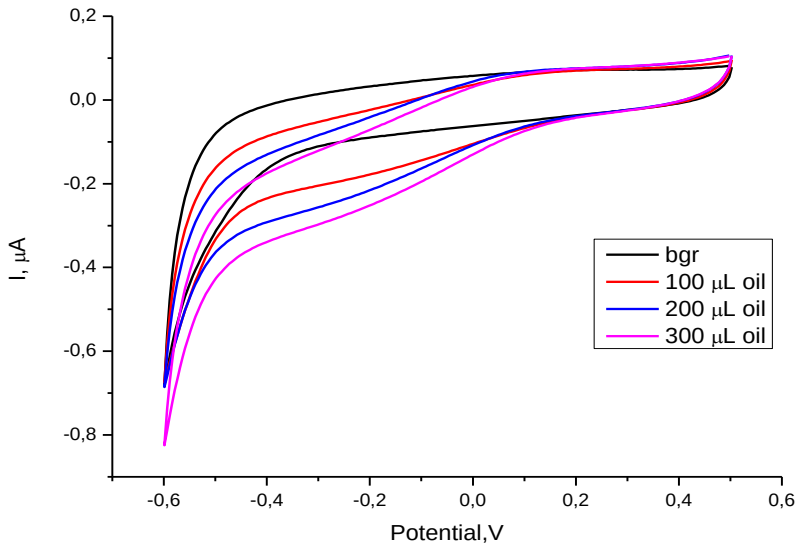

Figure 4. Cyclic voltammograms of Co-g-C_3N_4/Nafion/GCE in the absence and presence of oil (dissolved in $CHCl_3$); background electrolyte 0.1 M TBAP in ACN, reference electrode $Ag|Ag^+$; scan rate 20 $mV.s^{-1}$.

Chronoamperometric measurements were performed (Figure 5, inset) at a constant potential of -0.2V (vs. $Ag|Ag^+$) while adding aliquots of the standard peroxide (BPO) solution to the operating medium. The calibration plot shows a linear dependence of the electrode response versus BPO concentration (correlation coefficient of 0.993). To account for the effect of the real sample constituents, the standard 3 mM BPO solution was dissolved in chloroform to which 0.4 g of fresh non-peroxidized oil was added.

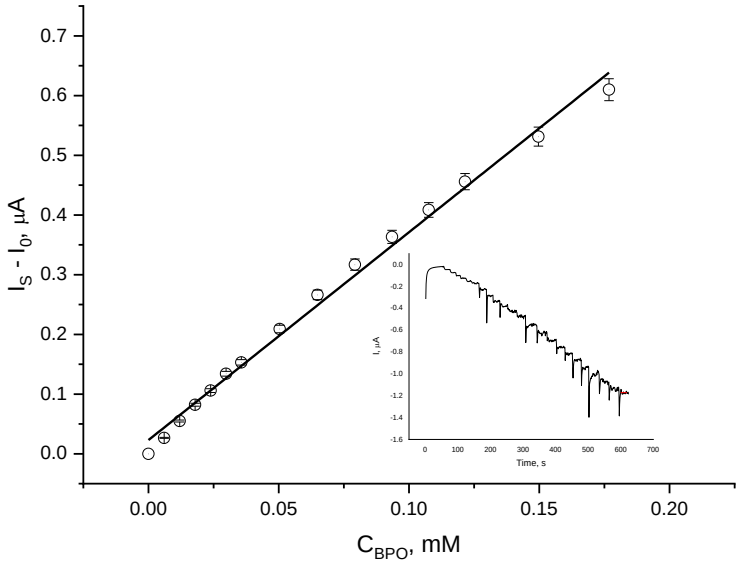

Figure 5. Chronoamperometric record (inset) and calibration plot of a Co-g-C_3N_4/Nafion modified electrode upon addition of aliquots of 0.003M BPO (dissolved in $CHCl_3$ /oil); background electrolyte: 0.1 M TBAP in ACN, reference electrode $Ag|Ag^+$; potential: - 0.2 V.

2.2. Analytical Performance of the Catalytic Peroxide Electrode

In order to evaluate the accuracy of the electrochemical method for the determination of PV, calibration standard solutions of BPO as a model peroxide were prepared in an interval of low (1, 2, 3, 6, 10 µM) and high (50,100, 200, 300, 400 µM) peroxide concentrations. The signals of all standard solutions of BPO in $CHCl_3$ were measured. For this purpose, 0.5 ml are taken from each standard

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solution and nearly 0.4 g of fresh anti-cellulite oil was dissolved in them. The results obtained (Figure 6) show a good agreement of the experimentally obtained values with the theoretically calculated ones, with high correlation coefficients (R^2 = 0.995 and R^2 = 0.994, respectively).

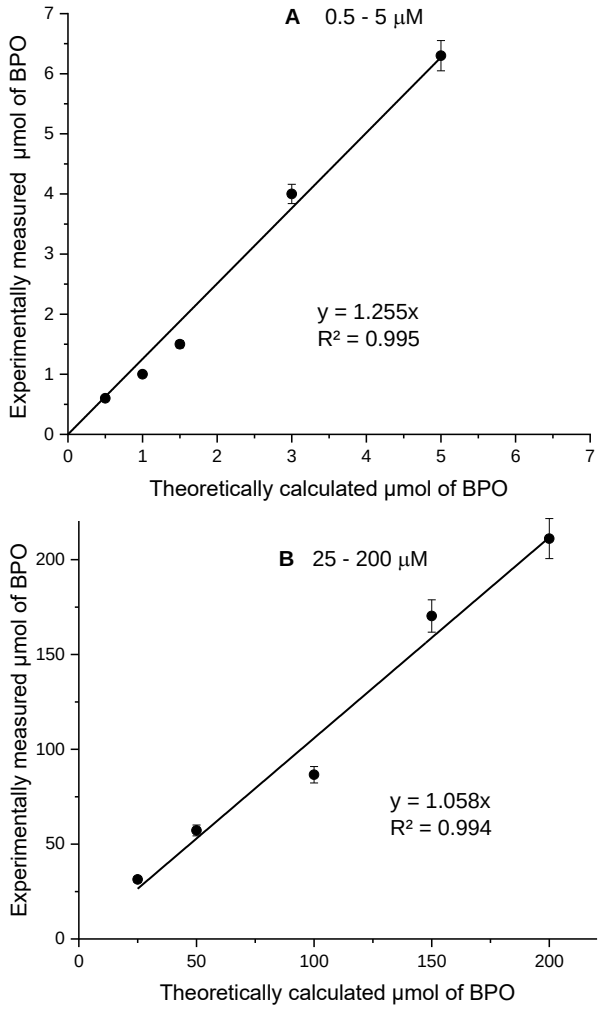

Figure 6. Correlation dependences of measured versus theoretical amounts of peroxide (BPO) in a real oil sample enriched with peroxides in a narrow (0.5-5 µM) and in a wider (25-200 µM) range of peroxide concentrations (in µmol l^{-1}).

The slopes of the linear dependencies (1.255 and 1.059, respectively) indicate a deviation of the measured values from the expected ones with 25.5% for the low concentrations (from 0.5 to 5 µM) and 5.9% for the range from 25 to 200 µM. These results indicate that if the oil contains 2.0 mmol/kg peroxides, 2.51 mmol/kg will be detected in the low concentration range (< 5 µM). Similarly, if the oil contains 20 mmol/kg peroxides, 21.2 mmol/kg will be detected in the concentration range 25 -200 µM. On the other hand, the response is linear and shows good reproducibility (R^2>0.99) of the analytical result.

Further, expected and measured µmol BPO levels were converted into expected and measured peroxide values (PVs), respectively, which are compared in Fig.7.

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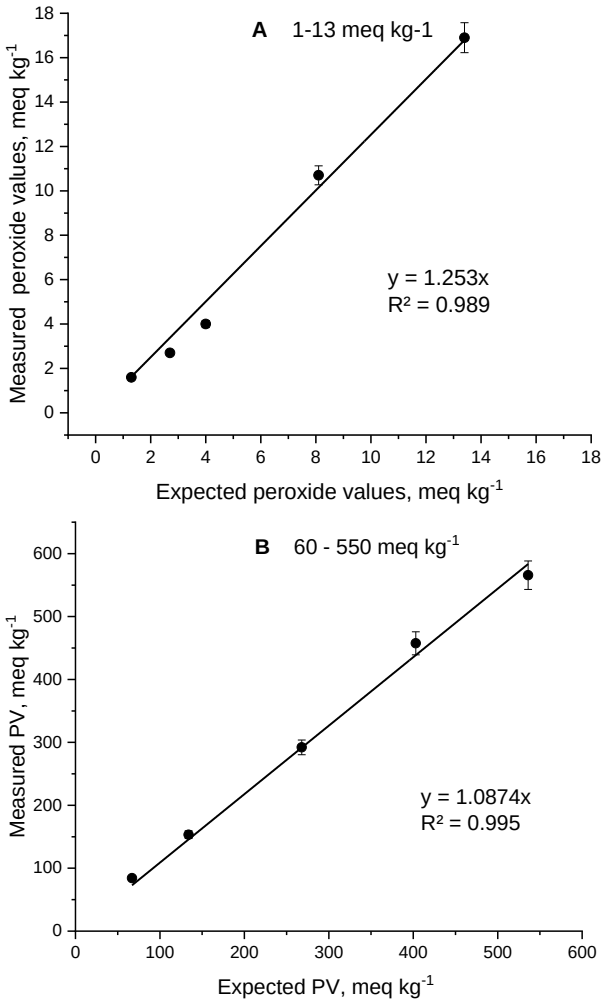

Figure 7. Correlation dependences of measured versus expected amounts of O_2 in a real oil sample enriched with benzoyl peroxide in a low (1 – 13 meq kg^{-1}) and in high (60- 550 meq kg^{-1}) range of peroxide concentrations, expressed in meq/kg.

A similar positive deviation of the measured from the expected values for PV is observed, which for the low concentration interval is 25.3 %, and for the wide one is 8.7 %.

The accuracy of the electrochemical method in a wide concentration range was compared with that of the conventional titrimetric approach for the determination of PV. For this purpose, known amounts of the BPO spiked at different concentrations to fresh anti-cellulite oil was measured. Regardless of the concentration of BPO, the electrochemical response was linear and showed good reproducibility (R^2>0.99). In contrast, the iodometric titration for all BPO amounts demonstrated significant deviations between measured and expected peroxide values (0.7795 was the line's slope in the iodometric titration, in comparison with 1.0874 in the electrochemical method) (Figure 8) (Table 1). These results imply that if the oil sample contains 20 mmol/kg peroxides, 15.6 mmol/kg peroxides will be detected by iodometric titration and 21.7 mmol/kg peroxides – by electrochemical method. Probably, the low reproducibility of the titrimetric method compared to the electrochemical one is due to the extraction step of the sample pretreatment that is a part of the titrimetric method.

Table 1. Comparison of the accuracy of the electrochemical method and the classical titrimetric method for the determination of PV.

Expected PV, meq O_2/kg (standard solution)	Measured PV, meq O_2/kg by titrimetric method	Deviation of the titrimetric value from the expected, in %	Measured PV, meq O_2/kg by electrochemical method	Deviation of the electrochemically measured PV from the expected, in %

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67	97.0	+44.78	84.2	+25.67
134	165.3	+23.36	153.3	+14.40
268	195.0	-27.24	292.1	+8.99
403	297.4	-26.20	457.5	+13.52
537	417.3	-22.29	565.8	+5.36

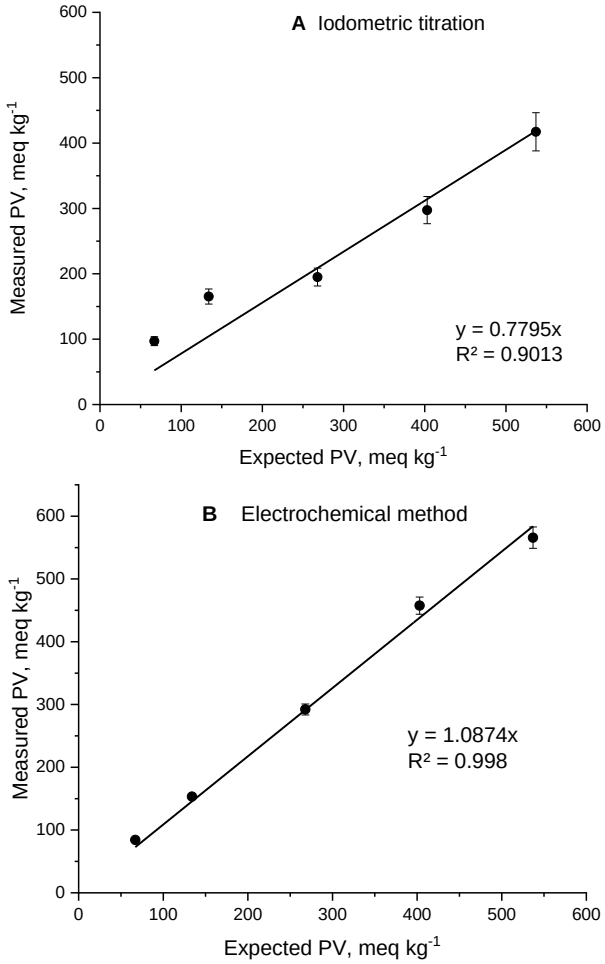

Figure 8. Measured vs. expected peroxide value of a real sample spiked with known amounts of BPO analyzed using the titrimetric method (BSS EN ISO 3960:2017) – left, and using the electrochemical method - right.

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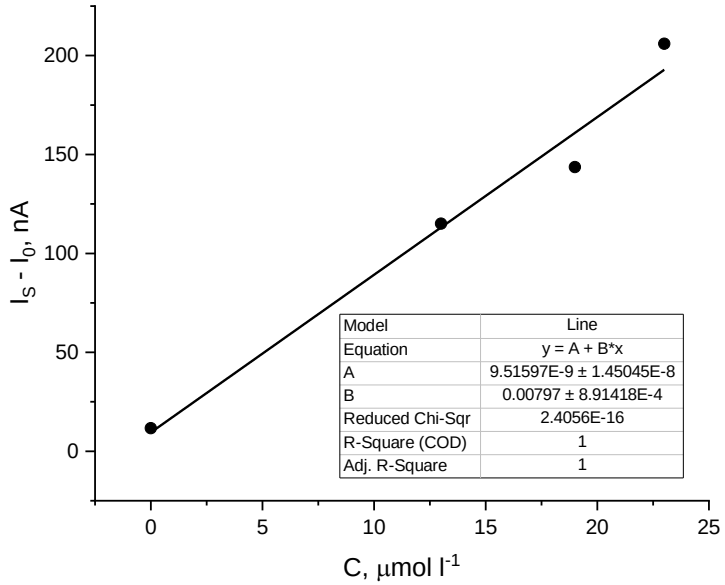

Figure 9. Determination of the real sample PV by the method of standard addition: after adding the sample, three more aliquots of the external standard solution were added (standard peroxide solution – BPO in chloroform to which the real sample was added) to the electrochemical cell.

Finally, the peroxide value of the real sample containing a mixture of easily oxidizable vegetable oils was determined by the standard addition method. According to this method, the intercept of the calibration line corresponds to the signal of the real sample (Figure 9). Dividing this signal by the slope of the line, one obtains the concentration of peroxides in the electrochemical cell of $1.25.10^{-6}$ M. Taking into account the dilution and mass of the real sample, we get that the real sample contains 276 µmol of peroxides, which corresponds to PV = 739.75 ± 64,36 meq O_2 kg^{-1}. Therefore, the vegetable oils contained in the real sample are highly oxidized.

3. Discussion

The iodometric titration is an easy conventional way to determine the PV. However, this method is labor-intensive, time-consuming, requires a lot of reagents and large amounts of sample and organic solvents. In addition, its accuracy and reproducibility strongly depend on reaction conditions –e.g. light, temperature, skills of operator, sodium thiosulfate decomposition, etc. Furthermore, the iodine released in the organic phase can participate in side reactions such as oxidation by dissolved in the solution oxygen or interaction with unsaturated lipids. These interfering processes can lead to overestimation or underestimation of PV.

Unlike iodometric titration, the electrochemical method, developed by our research group, allows the analysis of lipid samples directly in acetonitrile. Its accuracy and reproducibility far exceed the ones of iodometric titration, and skipping the extraction step considerably shorten the sample preparation. Due to the simplicity of operating with electrical signals and the compact sizes of the equipment, the electrochemical method represents a good alternative of traditional instrumental analysis (e.g. by HPLC or FTIR) in the same time providing a simple, accurate, rapid, and reproducible analysis of PV with very small amounts of sample required. In addition to that, our electrochemical approach ensures high accuracy over a wide range of PV (from 1 to 540 meq kg^{-1}). Hence, the analytical method developed on the basis of the catalytic peroxide electrode has a significant potential to be used as an alternative to most of the known methods for determining the peroxide values of in lipid-containing samples.

4. Materials and Methods

4.1. Materials

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The following reagents and chemicals were used in the present studies: dibenzoyl peroxide, p.a., dilauroyl peroxide, p.a., dicumyl peroxide, p.a., di-tertbutylperoxide, p.a., (Acros Organics) used in the form of solutions with an initial concentration of 0,3 M or 3 mM in chloroform; Nafion™ 117, a 5% suspension in water-alcohol mixture (Sigma-Aldrich, MI, USA); ultrapure solvents acetonitrile (CH_3CN) and chloroform ($CHCl_3$) with specification p.a. (Fisher Chemical, ThermoFisher Scientific, USA); quaternary ammonium salt (tetrabutylammonium perchlorate - TBAP, Acros Organics).

Anti-cellulite oil from the Rivana brand (Bulgaria) was tested as a real sample. Both fresh and with expired shelf life products were used. The mixture contains: *Vitis Vinifera* Seed Oil, *Citrus Paradisi* Peel oil, *Cimbopogon Schoenanthus* Oil, *Rosmarinus Officinalis* Oil, *Juniperus Communis* Oil, Linalool, Limonene, Geraniol, Citral.

Electrode material: Commercially available glassy carbon materials were used as working electrodes. The electrodes are cylinders of chemically resistant polymers with a length of 6 cm and an outer diameter of 6 mm, in which an embedded cylinder of glassy carbon with a diameter d = 3 mm (visible surface area 0.071 cm^2, manufactured by CHI, Austin, TX, USA) or with a diameter d = 2 mm (visible surface area 0.031 cm^2, manufactured by Metrohm, Utrecht, The Netherlands). The glassy carbon material is connected to a metal current lead, which serves to connect it to the electrochemical apparatus.

4.2. Apparatus and Measurments

All electrochemical measurements were performed in a conventional three – electrode cell (working volume 20-100 ml, Metrohm-Autolab), with catalyst – modified disc of glassy carbon (GCE) as working electrode, a $Ag|Ag^+$ reference electrode and a platinum foil as auxiliary electrode. An electrochemical workstation PGSTAT Autolab 302N (Metrohm, Switzerland) with Nova 2.1.6 software has been used in all experiments. Cyclic voltammograms (CVs) were recorded at a scan rate of 20 mV s^{-1}. Chronoamperometric measurements were performed under constant stirring.

4.3. Synthesis of Co-Doped g-C_3N_4

The synthesis of the Co-doped g-C_3N_4 was carried out in one step method by thermal polycondensation on protonated melamine in the presence of cobalt salts, which further are thermally decomposed to a spinel cobalt oxide (Co_3O_4) [54]. To reach 5% of Co by weight in the final catalyst, a certain amount of $Co(NO_3)_2 \cdot 6H_2O$ was dissolved in 50 cm^3 of methanol and added to the protonated melamine, under continuous stirring at 50 deg. until evaporation of the MeOH. The obtained solid was dried at 60°C for 10 h and calcined at 550°C for 2 h.

4.4. Modification of Working Electrode

The modifying phase was prepared as described previously [53], in brief: 1 – 2 mg of graphitic carbon nitride, doped with tricobalt tetroxide (with a Co_3O_4 content of 5 wt.% to 8 wt.%) were added to 1 ml of an aqueous suspension of the ionomer Nafion™ (with a concentration of 0.2%) and dispersed ultrasonically for 30 minutes. Then, a 5 µL drop of the resulting composite electrocatalyst is applied to the surface of the cleaned glassy carbon electrode and air-dried for 16 – 24 hours at room temperature.

The Co-g-C_3N_4/ Nafion catalytic electrode, can be used to perform analysis up to 10 times within 1 day and stored for one year or more. It is stored dried, after washing with ultrapure water, in air and at room temperature, capped with a plastic protector. Before use after long storage (more than 3 days), the catalytic electrode is reactivated by placing it in the electrolysis cell filled with background electrolyte (0.1 M tetrabutylammonium perchlorate dissolved in acetonitrile), connecting it in a three-electrode configuration together with reference and auxiliary electrodes to the electrochemical workstation and treating it by cyclic voltammetry over the potential range from -0.4 V to +0.6V for at least three cycles, after which it is calibrated twice according to the description (section 4.6.).The background electrolyte with a concentration of 0.1 M was prepared by dissolving tetrabutylammonium perchlorate in acetonitrile.

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4.5. Study of the Electrocatalytic Activity of Co-g-C_3N_4/Nafion – Modified GCE

The ***Co-g-C_3N_4/Nafion – modified GCE*** working electrode, the reference electrode Ag|Ag+, and the auxiliary electrode – platinum foil, were positioned in a standard single-compartment electrochemical cell with a working volume of 10-50 ml without, 10 ml of background electrolyte – 0.1 M tetrabutylammonium perchlorate, dissolved in acetonitrile is added and the electrodes are connected to a DC source - the electrochemical workstation, through which the set DC voltage is controlled and the current strength is measured. The working potential is set to -200 mV and the establishment of a stationary background current of about 0.2 - 0.5 μA is awaited. Then, 20 to 50 μl of the analyte are added to the background electrolyte in the cell under continuous stirring. The increase in the concentration of the analyte containing organic peroxide in the electrochemical cell is achieved by successive addition to the solution in the electrolysis cell of equal volumes of the analyte. After each increase in the concentration of the analyte, 5 to 10 seconds for the signal to reach a steady-state value are necessary to read the current strength. The peroxide concentration in the analyte is determined using a previously prepared calibration curve.

4.6. Preparation of the Calibration Curve

A calibration curve of BPO at different concentrations was made by preparing a stock solution with concentrations of 0,003 M or 0,3 M in chloroform. In order to cover the largest possible concentration range, several aliquots of 20 μl, and then several times of 50 μl of the standard solution were added to the background electrolyte in the cell under continuous stirring at 450-550 rpm. The linear range of the calibration graph is up to 0.2 mM and above, i.e. exceeding a concentration of 48.45 mg/l.

4.7. Assessment of the Accuracy of the Method Over the Range of Low Peroxide Concentrations

Initially, the catalytic electrode is calibrated with 0.003 M BPO/$CHCl_3$ added to fresh oil (anti-cellulite oil - Rivana, peroxide-free) according to the following procedure:

1. Preparation of a stock solution with a concentration of 0.03 M BPO in $CHCl_3$
2. Weighing of 0.4 g (0.5 ml) peroxide-free anti-cellulite oil and dissolving it into 0.03 M BPO/$CHCl_3$ solution. This solution is designated as BPO/$CHCl_3$/fresh oil.
3. Dilution of 0.03 M BPO/$CHCl_3$/fresh oil 10 times with $CHCl_3$ to a final concentration of 0.003 M BPO/$CHCl_3$/fresh oil.
4. Calibration is performed at a working potential of -0.2 V with the following portions of 0.003 M BPO/$CHCl_3$/fresh oil: 5 x 20 μl, then 6 x 50 μl are added.
5. All measurements were performed as described in section (4.5.), strictly following the procedure. The regression equation of the calibration plot is: $Y= -2.62.\ 10^{-9}+0.00272x$.

Further, standard solutions of benzoyl peroxide (BPO) in chloroform ($CHCl_3$) with concentrations: 1, 2, 3, 6, 10 mM were prepared by diluting 0.5 M BPO in $CHCl_3$. The standard solutions have a final volume of 10 ml. The signals of all listed standard solutions of BPO in $CHCl_3$ were measured. For this purpose, 0.5 ml of each standard solution is taken and 0.4 g of peroxide-free anti-cellulite oil (Rivana) is dissolved in them according to Table 2.

The aliquot portion taken for analysis from each standard solution was 0.1 ml.

Table 2. Standards used for calibration of the catalytic peroxide electrode.

Standard solution	Volume (BPO/$CHCl_3$), ml with the given concentration,	Mass of the weighed oil, g
St1	0.5 ml 1mM BPO/$CHCl_3$	0.4000 ±0.0075
St2	0.5 ml 2mM BPO/$CHCl_3$	0.4000 ±0.0075
St3	0.5 ml 3 mM BPO/$CHCl_3$	0.4000 ±0.0075
St4	0.5 ml 6 mM BPO/$CHCl_3$	0.4000 ±0.0075
St5	0.5 ml 10 mM BPO/$CHCl_3$	0.4000 ±0.0075

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4.8. Assessment of the Accuracy of the Electrochemical Method in a Wide Concentration Range

First, a calibration of the catalytic electrode was performed with 0.003 M BPO/$CHCl_3$ added to fresh oil (anti-cellulite oil - Rivana, peroxide-free) following the procedure described in section 4.6.

Then, standard solutions of BPO/$CHCl_3$ were prepared with concentrations: 50,100, 200, 300, 400 mM by dilution of 0.5 M BPO/$CHCl_3$. The standard solutions have a final volume of 2 ml. Their signals were measured, taking 0.5 ml of each standard solution and dissolving ~0.4 g of fresh anti-cellulite oil in them according to the Table 3.

Table 3. Scheme for preparing standard solutions.

Standard solution	Volume (BPO/$CHCl_3$) with certain concentration, ml	DF*	Mass of the weighed oil, g
St0	0.5 ml chloroform	1	0.4000 ±0.0075
St1	0.5 ml 50mM BPO/$CHCl_3$	4	0.4000 ±0.0075
St2	0.5 ml 100 mM BPO/$CHCl_3$	10	0.4000 ±0.0075
St3	0.5 ml 200 mM BPO/$CHCl_3$	20	0.4000 ±0.0075
St4	0.5 ml 300 mM BPO/$CHCl_3$	30	0.4000 ±0.0075
St5	0.5 ml 400 mM BPO/CHCl3	50	0.4000 ±0.0075

* DF -dilution factor.

The standard solutions from St1 to St5 were pre-diluted and then a 0.1 ml aliquot portion was taken from the already diluted solutions for analysis. The regression equation from the calibration graph is: $Y= 2{,}30.\ 10^{-8}+0{,}00348x$

4.9. Peroxide Value (PV) Measurement of the Anti-Cellulite Oil by Iodometric Titration

The PV measurement of oil samples of fresh anti-cellulite oil from the brand Rivana was done by iodometric titration according to the Bulgarian state standard (BSS EN ISO 3960:2017). Typically, 0.1000-0.2000 g of vegetable oil is weighed with an accuracy of 0.0002 in a 100 ml Erlenmeyer flask and 5 ml of chloroform and 2.5 ml of acetic acid are added. The flask is shaken until the oil is completely dissolved and 1 ml of 50% KI solution (freshly prepared) is added. The sample is left for 5 minutes in the dark, then 15-20 ml of distilled water and a few drops of 1% starch solution are added until a blue-purple color appears. Then, the sample is titrated with 0.002 N sodium thiosulfate solution slowly and with continuous homogenization by shaking until the color disappears in the aqueous (upper) layer.

For each sample tested, two parallel measurments were made and the arithmetic mean value is taken as the final result.

The same procedure is done for a blank sample, in which only the reagents without vegetable oil are present.

The peroxide value (PV) was calculated according to:

$$PV = \frac{(V - Vo).0{,}002.1000}{m}, meq.O_2/kg$$

where:

V- volume of 0,002 N $Na_2S_2O_3$ in cm^3, used to titrate the vegetable oil sample.

Vo- volume of 0,002 N $Na_2S_2O_3$ in cm^3, used to titrate the blank sample.

0,002- the concentration in normality of the $Na_2S_2O_3$ solution

m- the mass of vegetable oil

4.10. Determination of Peroxide Concentration in a Real Sample - Highly Rancid Anti-Cellulite Oil From the TRADEMARK RIVANA

The following procedure was applied:

Four 1ml samples are taken from the initial solution of rancid oil.

An initial standard solution of BPO/$CHCl_3$ with a concentration of 10 mM is prepared.

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Standard solutions were prepared according to Table 4 and the catalytic electrode was calibrated using the standard addition method.

Table 4. Standard solutions prepared for applying the standard addition method.

Standart solution	Volume of a highly rancid anti-cellulite oil- Rivana, ml	Volume of a standard 10 mM BPO/$CHCl_3$ solution, ml
St 0	0.1	0.000
St1	1.0	0.150
St2	1.0	0.200
St3	1.0	0.250
St4	1.0	0.300

The signals of all standard solutions were measured, with the aliquot portion for analysis being 0.1 ml.

4.11. Calculus

The intercept in the calibration graph corresponds to the signal of the real sample. Therefore, if we divide this signal by the sensitivity, we will get the concentration of peroxides in the sample: $10^{-8}/0.008=1.25\times10^{-6}$ M peroxides in the sample. Therefore, this signal must be divided by the sensitivity from the calibration plot, which gives the concentration of peroxides in the sample: $1.10^{-8}/0.008=1.25\times10^{-6}$ M peroxides in the sample. To account for the dilution of 0.1 ml of sample after its introduction into the cell containing 10 ml of electrolyte, the concentration of peroxides must be multiplied by the dilution factor (DF=100). Therefore: $1.25.10^{-6}$ M peroxides x $100=1.25\times10^{-4}$ M peroxides are present in 0.1 ml of sample. Since an injection of the rancid oil (1.300 g) is dissolved with chloroform to a final volume of 15 ml, the concentration of peroxides in these 15 ml of undiluted oil should be found the following way:

$1,25.10^{-4}$ M × 0.1 ml of the sample = x × 14,9 ml sample

$x=1,86\times10^{-2}$ M peroxides in 15 ml sample

The number of moles of peroxides in this 15 ml sample is calculated:

$n=M.V=1,86.10^{-2}$mol/l × 15.10^{-3} liters= 2.76×10^{-4} mol peroxides

The number of moles is converted to μmol:

1 mol→106 μmol

2.76×10^{-4} mol = 276 μmol

From the data obtained when studying the accuracy of the method in a wider range of PV we know that 200 μmol correspond to PV=536 meq/kg, then 276 μmol shall be equall to PV=739.75 ± 64.65 meq/kg. Therefore, the sample containing a mixture of vegetable oils is very rich in peroxides and is highly peroxidized (rancid).

5. Patents

This research resulted in a Bulgarian patent application No 113803 (BG/P/2023/113803).

Author Contributions: Conceptualization, N.D. and M.S.; methodology, M.P.; software, M.P.; validation, M.P., and V.I.; formal analysis, V.I.; investigation, M.P. and V.I.; resources, N.D.; data curation, M.P. and V.I.; writing—original draft preparation, V.I.; writing—review and editing, N.D..; visualization, V.I.; supervision, N.D.; project administration, N.D.; funding acquisition, N.D. All authors have read and agreed to the published version of the manuscript.

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