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Analytical Method Development to Determine the Oxidative Status of Crude Algal Oil

Metropolia University of Applied Sciences

Bachelor of Laboratory Services

Laboratory Sciences

Bachelor's Thesis

23 May 2024

Abstract

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Title:	Analytical Method Development to Determine the Oxidative Status of Crude Algal Oil
Number of Pages:	34 pages + 5 appendices
Date:	23 May 2024
Degree:	Bachelor of Laboratory Services
Degree Programme:	Laboratory Sciences
Supervisors:	Minna Pietilä, Senior Researcher Mia Ruismäki, Senior Lecturer

This Bachelor's Thesis study was executed in collaboration with Neste Oyj and the practical work was carried out in the department of Research Analytics' laboratory of inorganic chemistry in Kilpilahti, Porvoo.

The objective of this study was to develop an analytical method based on ISO 27107:2010 to determine the peroxide value (PV) of crude algal oil. The process of method development has been carried out in two parts: first by evaluating the method performance, then by testing the method for crude algal oil samples. The method performance was evaluated by determining the within-laboratory reproducibility, for which vegetable oil samples of former proficiency tests were used, as they had previously assigned PVs. Additionally, the method performance was assessed through spiking experiments, where known amounts of a hydroperoxide compound were added to rapeseed oil. For testing the method with crude algal oil, similar spiking experiments were carried out.

The evaluation of the method performance was successful and the method was observed to be effective for measuring the PV of vegetable oils. However, the addition of hydroperoxides to the crude algal oil resulted in poor recoveries, likely due to either the viscosity of the sample or its possible antioxidant properties. Since the spiking experiments with crude algal oil were unsuccessful and there was no suitable reference material for the sample, a method for crude algal oil could not be developed during this study.

Although the main objective of the thesis study was not achieved, the method could possibly be brought into use as a new analytical method for the determination of PV of vegetable oils in the inorganic chemistry laboratory of Neste Oyj's Research Analytics department.

Keywords: peroxide value, crude algal oil, ISO 27107, oxidative status, hydroperoxides, lipid oxidation, potentiometric titration

Tiivistelmä

Tekijä:	Susanna Viukari
Otsikko:	Analyysimenetelmän kehittäminen raakaleväöljyn hapettumisasteen määrittämiseksi
Sivumäärä:	34 sivua + 5 liitettä
Aika:	23.5.2024
Tutkinto:	Laboratorioanalyytikko (AMK)
Tutkinto-ohjelma:	Laboratorioanalytiikka
Ohjaajat:	Tutkija Minna Pietilä Lehtori Mia Ruismäki

Opinnäytetyö toteutettiin yhteistyössä Neste Oyj:n kanssa, yrityksen Tutkimusanalytiikka-osaston Porvoon Kilpilahdessa sijaitsevassa epäorgaanisen kemian laboratoriossa.

Opinnäytetyön tavoitteena oli kehittää ISO 27107:2010 -standardiin pohjautuva analyysimenetelmä raakaleväöljyn peroksidiluvun määrittämiseksi. Menetelmän kehitys on toteutettu kahdessa osassa: ensin arvioimalla menetelmän suorituskykyä, sitten testaamalla menetelmää raakaleväöljynäytteille. Suorituskykyä arvioitiin ensiksi määrittämällä laboratorion sisäinen uusittavuus, hyödyntäen aikaisempien pätevyyskokeiden kasviöljynäytteitä, joilla oli ennestään määritetyt vertailuarvot peroksidiluvulle. Lisäksi suorituskykyä arvioitiin saantokokeilla, joissa rypsiöljyyn lisättiin tunnettuja määriä hydroperoksidiyhdistettä. Menetelmää testattiin raakaleväöljylle vastaavanlaisten saantokokeiden avulla.

Menetelmän suorituskyvyn arviointi oli onnistunut ja menetelmä havaittiin soveltuvaksi kasviöljyjen peroksidiluvun määrittämiseen. Hydroperoksidien lisääminen raakaleväöljyyn tuotti kuitenkin alhaisia saantoja, johtuen mahdollisesti joko näytteen viskoosisuudesta tai mahdollisista antioksidanttisista ominaisuuksista. Koska raakaleväöljyn saantokokeiden tulokset olivat huonoja, eikä näytteelle ollut saatavilla sopivaa vertailumateriaalia, toimivaa menetelmää leväöljynäytteelle ei onnistuttu kehittämään opinnäytetyön aikana.

Vaikka opinnäytetyön varsinaista tavoitetta ei saavutettu, menetelmää voidaan kuitenkin mahdollisesti hyödyntää uutena analyysimenetelmänä kasviöljyjen peroksidiluvun määrittämiseen Neste Oyj:n Tutkimusanalytiikka-osaston epäorgaanisen kemian laboratoriossa.

Avainsanat: peroksidiluku, raakaleväöljy, ISO 27107, hapettumisaste, hydroperoksidit, rasvojen hapettuminen, potentiometrinen titraus

Contents

List of Abbreviations

AnV: Anisidine value. An analytical method for the determination of secondary oxidation products.

ASTM: American Standard Test Method

CV: Coefficient of variation

CV(R): Coefficient of variation of reproducibility

FAME: Fatty acid methyl esters

ISO: International Standard

NEXBTL: Next Generation Biomass to Liquid. A technology that is used by Neste for the production of renewable fuels.

OSI: Oxidative stability index

PV: Peroxide value. An analytical method for the determination of primary oxidation products.

%R: Recovery rate

RT: Room temperature

SD: Standard deviation

TBHP: Tert-butyl hydroperoxide. An organic compound that is used as a model hydroperoxide for spiking experiments in this study.

wt%: Weight percent

1 Introduction

This Bachelor's Thesis study was executed in collaboration with Neste Oyj and the practical work was carried out in the department of Research Analytics' (part of Neste's Core R&D) laboratory of inorganic chemistry in Kilpilahti, Porvoo. Neste Oyj is a Finnish oil refining company established in 1948 and nowadays a leading producer of renewable transport fuels [1]. With the goal to combat climate change and accelerate circular economy, Neste's research focuses on new future raw materials for the feedstocks of renewable fuels and also for the sustainable production of chemicals and polymers, in order to find solutions that reduce the use of crude oil [2; 3]. Neste has committed to having carbon-neutral production by 2035 and for that, the company has begun the gradual transformation of the Porvoo oil refinery into a refinery of renewable and circular economy solutions [4; 5].

The objective of this study was to assess whether ISO (International Standard) 27107:2010, the potentiometric end-point determination of the peroxide value (PV) of animal and vegetable fats and oils, would be fit for determining the PV of crude algal oil. PV is a commonly used method for the quantitative measurement of primary oxidation products, mainly hydroperoxides, in fats and oils [6, p. 16–17]. The determination of the oxidative status of oils is an important tool in the assessment of their quality and storability, as the gradual and uncontrollable process of lipid oxidation can occur even in stable storage settings, causing degradation of oils [6, p. 3–4; 7]. With fuels, this quality deterioration caused by lipid oxidation can lead to problems in fuel distribution systems, including e.g. corrosion of the transfer pipelines and plugging of filters [7].

Crude algal oil is unrefined oil that has been extracted from microalgae, and that could be refined into renewable fuels. Although at the moment fuels based on algal oil cannot be produced on an industrial scale, there are several ideal properties, such as the efficient production of lipids and fatty acids with high energy densities, that make microalgae valuable to be researched as a potential

future feedstock. Therefore, in Neste, algal oils have been researched for over 15 years, in order to possibly apply them as a new raw material for the production of renewable fuels. [8; 9.]

2 Lipid Oxidation

Lipid oxidation is a complex series of reactions, caused by atmospheric oxygen, that can lead to the deterioration of oils' quality and storability [6, p. 3–4].

2.1 Autoxidation

The oxidation of lipids generally happens through an autocatalytic process called autoxidation, that occurs through a free radical mechanism. This mechanism consists of the stages of initiation, propagation and termination. For the most part, autoxidation takes place in unsaturated fatty acids, due to some of the hydrogens being in allylic and double allylic positions in the unsaturated and polyunsaturated alkyl chains. [6, p. 5–6; 10; 11.]

The initiation stage creates an alkyl radical (R•) by removing a hydrogen radical (H•) from an allylic position in a lipid molecule, as shown in Equation (1). An initiator, such as light, elevated temperature or a catalyst, is needed to release the hydrogen radical from the lipid molecule. [6, p. 5–6; 7; 10.]

$$RH + iniator\ (e.g. temperature, catalyst, light) \rightarrow R\bullet + H\bullet \quad (1)$$

Instantaneously, the formed alkyl radical reacts with molecular oxygen, first forming peroxyl radicals (ROO•), as shown in Equation (2). The peroxyl radicals then react with new lipid molecules to form both hydroperoxides (ROOH) and new alkyl radicals (Equation (3)), which will then propagate the reaction chain by reacting with another oxygen molecules (Equation (2)). The hydroperoxides formed in this stage are referred to as primary oxidation products. [6, p. 5–6; 7.

$$R\bullet + O_2 \rightarrow ROO\bullet \quad (2)$$

$$ROO\bullet + RH \rightarrow ROOH + R\bullet \quad (3)$$

In the termination stage, relatively stable non-radical products, e.g. ketones, alcohols, acids and aldehydes, are formed by a great variety of reaction pathways, including recombination between the radicals (Equations (4) – (6)) and decomposition of the hydroperoxides (Equation (7)). The variety of non-radical products that are formed in this stage are known as secondary oxidation products, and their amounts and types (monomeric, oligomeric or short chain) depend on multiple factors (such as the substrate, as well as light and temperature etc.) that affect the complex autoxidation process. [6, p. 5–6; 7; 10; 12.]

$$2\,ROO\bullet \rightarrow non-radical\ products \quad (4)$$

$$ROO\bullet + R\bullet \rightarrow non-radical\ products \quad (5)$$

$$2\,R\bullet \rightarrow non-radical\ products \quad (6)$$

The decomposition of hydroperoxides (Equation (7)) first produces radicals, such as alkoxyl and hydroxyl radicals, that then form different secondary oxidation products through various pathways [6, p. 6; 7].

$$ROOH \rightarrow radicals \rightarrow non-radical\ products \quad (7)$$

Once the first radicals have been formed, these three stages occur simultaneously, meaning the concurrent formation and decomposition of hydroperoxides. The rate in which the reactions take place is determined by the propagation stage, but varies throughout the process. The rate of hydroperoxide formation is greater at the first stages of the autoxidation process, meaning a lower number of free radicals in the system and therefore an overall slower oxidation rate. [6, p. 6; 7.]

The rate of oxidation becomes constant at a point known as the induction period, during which the hydroperoxides accumulate at high levels in the oil,

which results into their decomposition becoming faster than their formation. This causes a rapid increase in the overall oxidation rate, as the decomposition of hydroperoxides creates more radicals, which in turn catalyse the process. At the end of the induction period, secondary oxidation products increase in abundance, eventually leading to polymerization and thereby visibly detectable rancidity of the oil. [6, p. 6; 7.]

2.2 Photo-oxidation

Besides autoxidation, another mechanism called photo-oxidation can form hydroperoxides in oils in the presence of both light and photosensitizers, such as chlorophyll. By absorption of light, the photosensitizers are activated. These activated species can act through two ways, either as a single free radical initiator or by producing singlet oxygen through energy transfer. In the first way, the activated photosensitizers transfer electrons to lipids, forming radicals that react with oxygen, similarly to the autoxidation process – and thus forming the same hydroperoxides as in autoxidation. In the second way, reactive singlet oxygen reacts directly with unsaturated lipids, creating specific hydroperoxides that have an allylic double bond that is unique to this mechanism. [6, p. 11.]

The process of photo-oxidation with singlet oxygen occurs at a faster pace than the process of autoxidation, however, autoxidation is generally accepted to be the predominant mechanism, once initial hydroperoxides have been formed in oils [6, p. 11; 12]. Unless oils are exposed to either direct sunlight or fluorescent light without a protective container, direct photochemical oxidation of lipids is not very likely to occur [13].

2.3 Affecting Factors

Several factors play a part in the rate of lipid oxidation, many of which can also act simultaneously. These factors include temperature, presence of light, the concentration of oxygen, the fatty acid composition of the oil (particularly the degree of unsaturation), the physical characteristics and the composition of the

oil matrix, traces of metals and the oil's content of antioxidants – to name a few. [6 p. 11–15; 12.]

2.3.1 Fatty Acid Composition

The fatty acid composition, especially the degree of unsaturation, is one of the most determining factors in lipid oxidation. The increase of the unsaturation of fatty acids generally leads to an increase in the formation of fatty acid radicals, which in turn increases the rate of autoxidation. As an example, linolenic acid, a fatty acid which has three double bonds, has a higher degree of unsaturation and therefore relatively a much higher rate of autoxidation when compared to oleic acid, which has only a single double bond. In mixtures of fatty acids, which oils usually are, the rate of oxidation is determined by the most unsaturated fatty acid present. [6, p. 13; 14.]

Generally, oils with a low composition of unsaturated fatty acids have been observed to be more resistant to oxidation [14]. Besides the number of double bonds, their positions and geometrical configurations, as well as the fatty acid chain lengths, affect the rate of oxidation [6, p. 13].

2.3.2 Accelerating Factors

Elevated temperature increases the rate of oxidation exponentially by reducing the length of the induction period, as well as acting as the initiator of the autoxidation process. Temperature also affects the compounds that are formed as secondary oxidation products during the termination stage. [6 p. 12; 7.]

Light is another accelerating factor, primarily by being an initiator in the initial stage of autoxidation. However, it should be noted that this effect of light as an accelerating factor in autoxidation is different than the process of photo-oxidation. [6, p. 13; 12.]

Besides temperature and light, the presence of metals also increases the rate of free radical process by being pro-oxidants, mostly through catalysing the decomposition of hydroperoxides [6, p. 13; 7].

2.3.3 Inhibiting Factors

As oxygen is essential to both autoxidation and photo-oxidation, the complete absence of it in the oil (or its container) would effectively prevent lipid oxidation [6, p. 12]. Antioxidants, on the other hand, do not prevent lipid oxidation, but they can protect oils by delaying it through different mechanisms. One of the main ways is the interruption of the propagation reaction chain. This is common for phenolic antioxidants, like tocopherols, which react with peroxyl radicals leading to the formation of less reactive products – this extends the induction period and thereby slows down the rate of autoxidation. As another example of a different mechanism, carotenoids, such a β-carotene, can inhibit photo-oxidation by the deactivation of singlet oxygen. [6, p. 13; 12; 15.]

3 Evaluation of Oxidation in Oils

Numerous analytical methods have been developed for evaluating the oxidation of oils, through which the quality and storability of oils can be assessed. However, there is no single comprehensive method for the objective analysis of the degree of oxidation, meaning that it is most beneficial to use a combination of different methods. Many of the methods for assessing lipid oxidation in e.g. vegetable oil-based biodiesels have been adapted from the food industry. Besides the degree of oxidation, oils' susceptibility to it can also be assessed by evaluating their oxidative stability. [6, p. 16; 11.]

3.1 Oxidative Status

The oxidative status of oils generally refers to the extent of their oxidation at a current point in time. It can be evaluated by determining the primary and

secondary oxidation products that are present in the oil sample at hand. [6, p. 16–17.]

Primary oxidation products can be quantitively measured with e.g. peroxide value (PV), as in this study. The determination of PV is discussed in more detail in the Chapter 4 Potentiometric Titration of Peroxide Value. By itself, PV would be an insufficient indicator of lipid oxidation, since it only measures the primary oxidation products, which eventually decompose into secondary oxidation products as the oxidation processes. Therefore, it would be necessary for PV to be paired with another method that measures the secondary oxidation, in order to evaluate the total state of the lipid oxidation. One of the most commonly used methods for this is the anisidine value (AnV), which measures the level of aldehydes that react with p-anisidine, forming products that can be detected with a spectrometer at the wavelength of 350 nm. [6, p. 16–17; 11.] The determination of AnV can be carried out with e.g. ISO 6885:2016 (*Animal and vegetable fats and oils. Determination of anisidine value*).

Several other methods also exist for the evaluation of primary and secondary oxidation products. Namely, these can include the measurement of conjucated dienes for evaluating primary oxidation, as well as the thiobarbituric acid (TBA) test and the analysis of volatile oxidation products, by e.g. gas chromatography, for measuring secondary oxidation products. [6, p. 16–19; 15.]

3.2 Oxidative Stability

The evaluation of oxidative stability gives insight of the oxidative behaviour of oils, rather than just the amount of oxidation products that have been formed in the oil sample prior to its measurement, as in the determination of oxidative status. As lipid oxidation is a relatively slow process that can take several months to occur under stable storage conditions, the oxidative stability is measured with dynamic methods that apply conditions, such as elevated temperatures and the presence of oxygen in high concentration, for accelerating the rate of oxidation. [6, p. 23–25.]

The oxidative stability can be measured with e.g. the Rancimat test or oxidative stability index (OSI). The Rancimat method measures the duration of the induction period at 110 °C, expressed in hours, and is used in various European standards as a parameter of the oxidative stability in fuels. OSI is a similar method, developed by the AOCS (American Oil Chemists' Society). [12.]

3.3 Regulations for Fuels

The oxidation of lipids causes undesired oxidation products that can then deteriorate the quality of fuels, leading to problems in the fuel distribution system, as well as negatively impact their storability by reducing shelf life. The changes of the fuel's chemical and physical properties, caused by lipid oxidation, lead to the deterioration of the fuel's quality and therefore cause it to no longer fit the respective quality parameters addressed for it in required standards. The compounds that are formed during oxidation, e.g. acid products, can lead to the corrosion of metal systems in engines, besides other various negative effects, such as formation of precipitates. [7; 12; 16.]

For biodiesels, there is no required analytical quality parameters for the determination of the oxidative status, including the measurement of PV. The assessment of oxidative stability, however, has been addressed in some standards. For example, the European biodiesel standard (EN 14214) and the European paraffinic diesel standard (EN 15940) both include specifications for oxidative stability. [12; 16.] EN 14214 requires the determination to be done according to EN 14112 (*Fat and oil derivatives - Fatty Acid Methyl Esters (FAME) - Determination of oxidation stability (accelerated oxidation test)*) and EN 15940 according to EN ISO 12205 (*Petroleum products. Determination of the oxidation stability of middle-distillate fuels*), regardless of the fuel's FAME content [12; 17; 18].

Renewable fuels in Neste are produced with their NEXBTL (Next Generation Biomass to Liquid) technology, that has also been applied to algal oils in Neste's research regarding the use of microalgae as a feedstock [9]. The

renewable fuels produced with the NEXBTL technology are classified as paraffinic diesel and meet the qualifications of the aforementioned EN 15940 [19].

4 Potentiometric Titration of Peroxide Value

In this study, ISO 27107 [20] was chosen for the determination of PV of crude algal oil. As previously stated, ISO 27107 is the potentiometric end-point determination of the PV of animal and vegetable fats and oils, that quantitatively measures the primary oxidation products, mainly hydroperoxides, present in an oil sample. The general principle of the standard is that in an acidic solution, in this case, a solvent mixture of acetic acid and isooctane, the peroxides in the sample will liberate iodine from potassium iodide due to an oxidation-reduction reaction, as presented in Equation (8):

$$ROOH + 2\,KI + 2\,H^{+} \rightarrow ROH + I_2 + H_2O + 2\,K^{+} \quad (8)$$

The amount of the liberated iodine is then volumetrically determined by a titration with a sodium thiosulfate standard solution, as presented in Equation (9):

$$I_2 + 2\,Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2\,NaI \quad (9)$$

[11; 20.]

To validify analytical results in titrations, the amount of one of the reactants used must be known. Primary standards are usually used as titrants for this reason, as they are pure and stable enough that their concentrations can be calculated accurately from their weighted mass. However, sodium thiosulfate, which is commonly in the form of $Na_2S_2O_3 \cdot 5\,H_2O$, is too impure to be a primary standard. Therefore, prior to the PV determination, sodium thiosulfate is first standardized by using it to titrate a primary standard, in this case, potassium iodate. [21, p. 154, p. 394.] This correction of the sodium thiosulfate titrant's

concentration, which has been done according to ISO 27107 [20], is further described in Chapter 6.2 Factor Determination.

The amount of liberated iodine is calculated based on the used volume of the sodium thiosulfate standard solution. This is determined by the equivalence point of the titration, in other terms, the point where the quantity of sodium thiosulfate is exactly what is required for the stoichiometric reaction with iodine. Since the equivalence point is theoretical, it is measured by the end-point of the titration where a rapid shift of a physical property occurs. With ISO 27107, the titration end-point is detected by electrochemical potential that, in response to a redox reaction, develops on the metal surface of an inert platinum electrode. The electrochemical potential is monitored throughout the titration, plotting out a titration curve, from which the end-point can be observed (Figure 1). The difference between the equivalence point and the end-point of a titration is called a titration error, which is determined by a blank titration. [20; 21, p. 154, p. 346.]

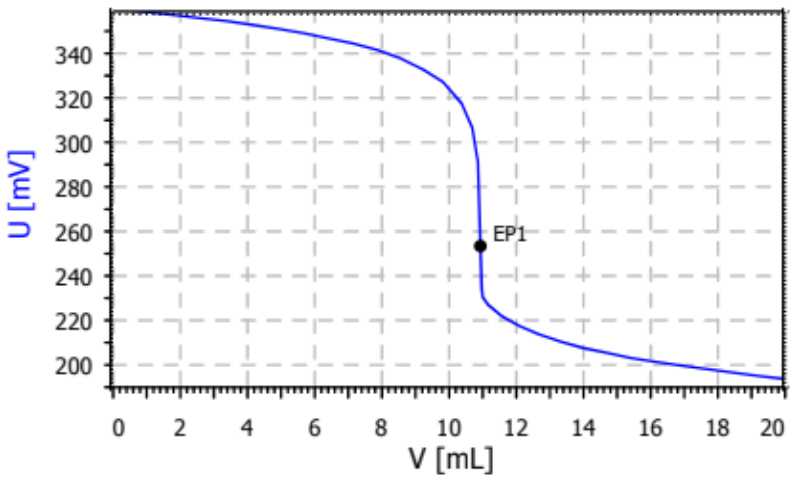

Figure 1. Titration curve of an olive oil sample, measured with a potentiometric titration according to ISO 27107. EP1 represents the detected end-point. The x-axis represents the used volume of the 0.01 N sodium thiosulfate titrant in milliliters (ml) and the y-axis the change of the voltage in millivolts (mV).

In this study, PV is expressed according to ISO 27107 in milliequivalents of active oxygen per kilogram of oil (meqO_2/kg) [20]. As previously mentioned in

Chapter 3.3 Regulations for Fuels, PV is not used as an analytical parameter according to the European fuel standards. Therefore, PV has no reference value for fuels. However, in the food industry, oils are considered to be fresh with a PV below 10 meqO_2/kg, and rancid, if the PV exceeds 30 meqO_2/kg [22]. Generally, a low obtained PV can be an indicator that the oil is of good quality, and its oxidative status is sufficient. However, due to the decomposition of hydroperoxides during the autoxidation process, especially at the later stages of it, a measurement of PV could give a relatively low result, even if the lipid oxidation would be far processed. Thus, the total oxidation of oils should always be determined by pairing the PV method with another that measures secondary oxidation products, in order to reliably assess the state of the lipid oxidation as a whole. [6, p. 6, p. 17.]

Besides the potentiometric end-point titration described in this chapter, PV could also be determined by an iodometric titration, with e.g. ISO 3960:2017 (*Animal and vegetable fats and oils, Determination of peroxide value, Iodometric endpoint determination*). However, due to the strong coloration of crude algal oil, an iodometric method would have been unsuitable for the sample matrix, as iodometry is based on a visual end-point determination of the reaction between iodine and starch [21, p. 392]. Thus, ISO 27107 was chosen for this study.

5 Crude Algal Oil

The composition of crude algal oil is defined by many factors. One of the biggest affecting factors is the algae species used for the oil's production. Thousands of different species of microalgae exist, resulting in a lot of diversity in the chemical compositions of algal oils. Other important determining factors are the conditions, in which the algal oil has been cultivated in, and the methods applied for the processing and the extraction of the oil from the algae biomass. [23; 24; 25.]

Of their dry weight, microalgae can have an oil content of 20–50 % (some species even 80 %), most of which consists of triglycerides. The compositions of lipids vary for the different strains of microalgae. The fatty acids that are accumulated by many algae species have typically a length of 16 to 20 carbons, and commonly not more than five sites of unsaturation. [15; 24; 26.] The most common fatty acids that have been detected in many strains of microalgae include oleic acid, linoleic acid, linolenic acid, palmitic acid, myristic acid and palmitoleic acid, to name a few. Palmitic acid is generally the most common saturated fatty acid in microalgae. Several other types of fatty acids can also be present, depending on the algal species. [26; 27.]

Microalgae, and therefore algal oils, generally contain natural antioxidants, such as tocopherols, carotenoids and phenolic compounds [15; 28]. Besides the aforementioned fatty acids and antioxidants, compositions of algal oils commonly also contain, for example, hydrocarbons, pigments (carotenoids and chlorophylls), sterols, waxes, phospholipids and glycolipids [15; 26].

6 Materials and Methods

The practical work of this study was executed in the laboratory of inorganic chemistry, in the department of Research Analytics (a part of Core R&D) of Neste Oyj. The following chapters describe how the study was carried out.

6.1 Reagents and Chemicals

All of the reagents that were used in this study are listed on Table 1. Besides these, gaseous nitrogen (Linde, purity unknown), ultrapure water (Milli-Q® IQ 7000, 18.2 MΩ·cm) and a starch solution, which was prepared according to the instructions in ASTM D 3703 (*Standard Test Method for Hydroperoxide Number of Aviation Turbine Fuels, Gasoline and Diesel Fuels*), were used.

Table 1. Reagents and chemicals used in the study.

Product name	Assay (%)	Manufacturer	CAS number
Isooctane for spectroscopy Uvasol®	≥ 99.8	Sigma-Aldrich	540-84-1
Acetic acid (glacial) 100 % anhydrous for analysis EMSURE® ACS, ISO, reag. Ph Eur	≥ 99.8	Sigma-Aldrich	64-19-7
Sodium thiosulfate 0.1 N	-	FF-chemicals	10102-17-7
Potassium iodide AnalaR NORMAPUR	100.4	VWR	7681-11-0
Potassium iodate Certipur® (volumetric standard, traceable to NIST SRM)	99.74 ± 0.10	Sigma-Aldrich	7758-05-6
Hydrochloric acid 30 % Suprapur®	-	Sigma-Aldrich	7647-01-0
Tert-butyl hydroperoxide solution - 5.0–6.0 M in decane Tert-butyl hydroperoxide Decane	 ≥50 – <70 ≥30 – <50	Sigma-Aldrich	 75-91-2 124-18-5
Xylene (mixture of isomers) AnalaR NORMAPUR ACS, Reag. Ph. Eur	≥ 98.5%	VWR	1330-20-7
Toluene for analysis EMSURE® ACS, ISO, reag. Ph Eur	≥ 99.9	Sigma-Aldrich	108-88-3

As instructed in ISO 27107 [20], the titrant was prepared by diluting 0.1 N sodium thiosulfate to 0.01 N. Each day, fresh solvent was made by mixing 3 parts of acetic acid and 2 parts of isooctane. Then, the solvent mixture was degassed in an ultrasonic bath for 45–60 minutes, depending on the volume of the prepared mixture. For the determination of the correction factor F, a solution of potassium iodate was prepared according to ISO 27107 [20] by weighing 0.3009 g of the potassium iodate into a 250 ml volumetric flask, which was then filled to the mark with ultrapure water.

Fresh saturated potassium iodide solution was prepared daily by weighing approx. 14 g of the potassium iodide in approx. 8 g of ultrapure water, then the flask of the solution was wrapped with tinfoil. Before use, the saturated

potassium iodide solution was tested by adding 0.5 ml of the solution and 2 drops of starch solution into 30 ml of solvent mixture. If a blue colour would have formed and it would have required more than one drop of 0.01 N sodium thiosulfate solution to remove it, the saturated potassium iodide solution would have been discarded. This is because the blue colour would have been formed by a reaction between starch and iodine, indicating the presence of liberated iodine in the potassium iodide solution [21, p. 392].

6.2 Factor Determination

To correct the concentration of the 0.01 N sodium thiosulfate titrant, the correction factor F was determined. For this, the titre of the titrant was determined by titration according to ISO 27107 [20]. Into a beaker, 5 ml of potassium iodate solution, 60 ml of ultrapure water, 5 ml of 4 M HCl solution, and 0.5 ml of saturated potassium iodide solution were added and then titrated with the 0.01 N sodium thiosulfate titrant. The factor (F) was calculated by Equation (10):

$$F = \frac{m_{KIO_3} * V_1 * 6 * 1000 * W_{KIO_3}}{M_{KIO_3} * V_2 * V_3 * c_{thio} * 100} \quad (10)$$

Where:

- m_{KIO3} is the mass of potassium iodate (0.3009 g)
- 6 is the equivalent mass for the titre (1 mol KIO_3 ⇔ 3 mol I_2)
- V_1 is the volume of potassium iodate solution used for the titration (5 ml)
- V_2 is the total volume of the potassium iodate solution (250 ml)
- V_3 is the volume of 0.01 N sodium thiosulfate solution used for the titration
- W_{KIO3} is the purity of potassium iodate (99.74)
- M_{KIO3} is the molecular weight of potassium iodate (214 g/mol)
- c_{thio} is the concentration of the sodium thiosulfate solution (0.01 mol/l).

The factor was determined daily and prior to the determination of PV.

6.3 Purging the Reaction Vessels with Gaseous Nitrogen

In order to remove the atmospheric oxygen (O_2) from the reaction vessels prior to the measurements of the blank and the sample, the reaction vessels were purged. Two different ways of purging were tested: one according to a modified method based on ASTM D3703 (American Standard Test Method), in which a three-neck round-bottom flask is used with a constant stream of gaseous nitrogen throughout the titration, and the other following ISO 27107 [20], where, prior to the titration, an erlenmeyer flask is purged with gaseous nitrogen for a minute. The different ways of purging were tested with measuring the PV of reference material vegetable oils (Appendices 1 and 2). Based on the obtained results, the purging of ISO 27107 was chosen, as the purging based on the modified ASTM D3703 method caused undesired disturbances to the titration curves.

6.4 Measurement of the Peroxide Value

PV was determined according to ISO 27107 [20] by using a titrator (888 Titrando, Metrohm) equipped with a combined Pt ring electrode (6.0451.100, Metrohm) and a stirrer (801, Metrohm). The titration was controlled by Tiamo 2.5 software.

Blank measurements were done by stirring 50 ml of solvent mixture at a medium speed and then adding 0.5 ml of saturated potassium iodide solution. Exactly 60 seconds after adding the potassium iodide, 100 ml of ultrapure water was added, the combined Pt electrode was immersed into the solution and the mixture was titrated with the 0.01 N sodium thiosulfate standard solution. The stirring was continued throughout the measurement.

Samples were measured similarly to blanks, with the exception that here, the solvent mixture has been combined with samples. Samples were weighed into

the reaction vessel with an analytical balance prior to the addition of the solvent mixture. However, due to the highly viscous nature of the crude algal oil, these samples were prepared by first adding the solvent mixture into the reaction vessel before the weighing of the algal oil. Based on ISO 27107 [20], the recommended sample masses are 5.0 ± 0.1 g for a predicted PV of 1–30 meqO_2/kg and 10.0 ± 0.1 g for a predicted PV of ≤1 meqO_2/kg. In the standard [20], it is stated that the method is applicable for samples whose PVs are between 0 and 30 meqO_2/kg.

Between each measurement, the electrode and the buret tip of the titrator were rinsed with ultrapure water and toluene.

The PV was calculated by Equation (11):

$$PV = \frac{(V - V_0) * c_{thio} * F * 1\,000}{m} \quad (11)$$

Where:

- V is the volume of 0.01 N sodium thiosulfate solution used for the titration of the sample (ml)
- V_0 the volume of 0.01 N sodium thiosulfate solution used for the titration of the blank measurement (ml)
- c_{thio} is the concentration of the sodium thiosulfate solution (0.01 mol/l)
- F is the correction factor F
- m is the weighted mass of the sample (g).

The determination and the calculation of the correction factor F was described in Chapter 6.2 Factor Determination.

6.5 Samples and Sample Preparation

Besides crude algal oil, different vegetable oils, including reference material from previous proficiency tests, were also used for e.g. the evaluation of the method performance. The following samples were measured in this study:

- sample A (reference material from a proficiency test)
  - A mix of refined and crude vegetable oils (sunflower and others).
  - Reference value: 9.77 ± 1.51 meqO_2/kg (assigned value ± standard deviation (SD) for proficiency assessment, n = 105).
- sample B (reference material from a proficiency test)
  - Olive oil (mix of extra virgin, virgin, pomace oil and a lower quantity of other vegetable oils).
  - Reference value: 17.81 ± 3.68 meqO_2/kg (assigned value ± SD for proficiency assessment, n = 107).
- sample C (reference material from a proficiency test)
  - Edible palm oil.
  - Reference value: 1.10 ± 0.084 meqO_2/kg (assigned value ± uncertainty of assigned value, n = 46).
- rapeseed oil (Pirkka Rypsiöljy)
- olive oil (Xtra Oliiviöljy)
- crude algal oil.

Sample C was heated in the oven at 60 °C for approx. 30 minutes prior to its measurement, as it was solid at room temperature (RT). Other vegetable oils used in this study were measured without heating or other preparations.

The crude algal oil was divided into three aliquots and it was stored in a nitrogen atmosphere at -20 °C, sheltered from light. Before use, the samples were thawed at RT for 1–2 hours. The composition of the crude algal oil was heterogeneous and highly viscous. Therefore, prior to the measurements, the samples were heated in an oven at 40 °C for 20 minutes. The samples were mixed by vigorous shaking approximately every 5 minutes during the heating.

6.6 Spiking Samples with Model Hydroperoxide

Tert-butyl hydroperoxide (TBHP, Sigma-Aldrich, 5.3 M, approx. 13 100 meqO_2/kg) was chosen for introducing known amounts of hydroperoxide to the samples. A stock solution of approx. 328 meqO_2/kg was prepared by diluting the commercial solution 40-fold in xylene.

The volume (V) of the TBHP solution to be added in the spiking experiments were calculated by Equation (12):

$$V(spike) = \frac{Desired\ peroxide\ value\ of\ the\ spike\ *\ V(sample)}{328 \frac{meqO_2}{kg}} \quad (12)$$

All of the recovery rates (%R) were calculated by Equation (13):

$$\%R = \frac{(PV\ of\ spiked\ sample - PV\ of\ unspiked\ sample)\ *\ 100\ \%}{Known\ peroxide\ value\ of\ the\ addition} \quad (13)$$

The acceptable %R in this study was chosen to be between 80 and 120 %, which is suggested in the Analytical Method Validation Procedure at Neste.

7 Results

The method development was started by evaluating the method performance with vegetable oils, for which the within-laboratory reproducibility was also determined. Vegetable oils were chosen for the evaluation of the method performance due to the limited amount of the crude algal oil samples that could be analysed in this study, and also because of the lack of suitable reference materials for crude algal oil.

Validation parameters, such as trueness and uncertainty, were intended to be applied for testing the suitability of the method for crude algal oil. Since there were no suitable reference material available for crude algal oil, which could have been used for determining trueness and uncertainty, TBHP was used to introduce known amounts of hydroperoxides into samples, so recovery rates could be measured with a spiking experiment.

7.1 Evaluation of the Method Performance

The performance of ISO 27107 was evaluated by determining the within-laboratory reproducibility of vegetable oils with samples of former proficiency tests, and then by recovery rates of spiking experiments with rapeseed oil. The spiking of rapeseed oil was also executed in order to see the suitability of the TBHP solution for acquiring satisfactory recoveries with the method.

7.1.1 Measuring Reference Material

Three samples (A, B and C) of former proficiency tests were applied as reference material. The PVs of these samples were determined in quadrupoles and compared to those reported in the proficiency tests, respectively. The reference values of each sample are shown in Table 2. Uncertainty of the assigned reference value has only been given to sample C, but not for samples A or B in their respective reports regarding the proficiency tests. Therefore, in this study, the SD for proficiency assessment has been referred to instead of the uncertainty.

Table 2. Assigned reference values of each sample, the SD for the proficiency assessment and the number of laboratories that participated in the proficiency test.

Sample	Reference Value ($meqO_2/kg$)	SD for Proficiency Assessment ($meqO_2/kg$)	Number (No.) of Laboratories
A	9.77	1.51	105
B	17.81	3.68	107
C	1.10	0.22	46

The measured PVs of each sample are shown in Table 3. The titration curves are presented in Appendix 1 and the full data of the measurements in Appendix 2.

Table 3. Measured PVs of the samples (n = 4).

Sample	Mean ($meqO_2/kg$)	SD ($meqO_2/kg$)	Difference to ref. value ($meqO_2/kg$)
A	9.44	0.68	- 0.33
B	23.45	1.11	+ 5.64
C	3.90	0.20	+ 2.77

The measured and the reference values of the samples have been compared in two ways: with Figures 2–4 that visualise the differences between the values, and by performing a one-sample, two-tailed t test against a known value (the sample's reference value) for each sample respectively.

Based on the t test, the PV of sample A measured in this study (9.44 $meqO_2/kg$) does not differ statistically significantly from the sample's known value (9.77 $meqO_2/kg$), with a confidence level of 95 %. On alliance with that, Figure 2 shows that the measured value is very close to the reference value of sample A. Thus, the values of sample A were concluded to be comparable.

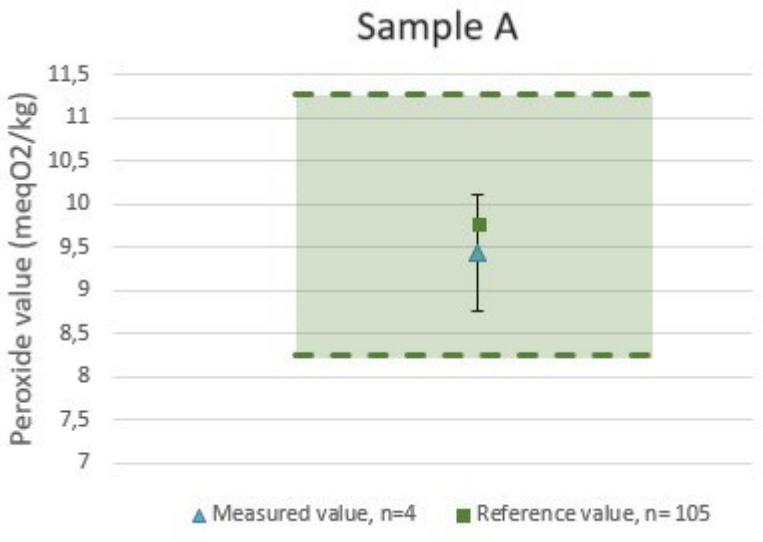

Figure 2. The measured PV (blue point ± black lines; mean ± SD, n = 4) of sample A has been compared to its reference value (green point ± green dashed lines; assigned value ± SD for proficiency assessment, n = 105). The green area between the two dashed lines indicates where the values are comparable with one another.

The measured value of sample B was observed to be higher than the reference PV value assigned in the proficiency test, which can be observed from Figure 3. The result of the t test supports this by confirming that the measured value of sample B (23.45 meqO_2/kg) differs statistically significantly from the sample's known value (17.81 meqO_2/kg) with a confidence level of 95 %.

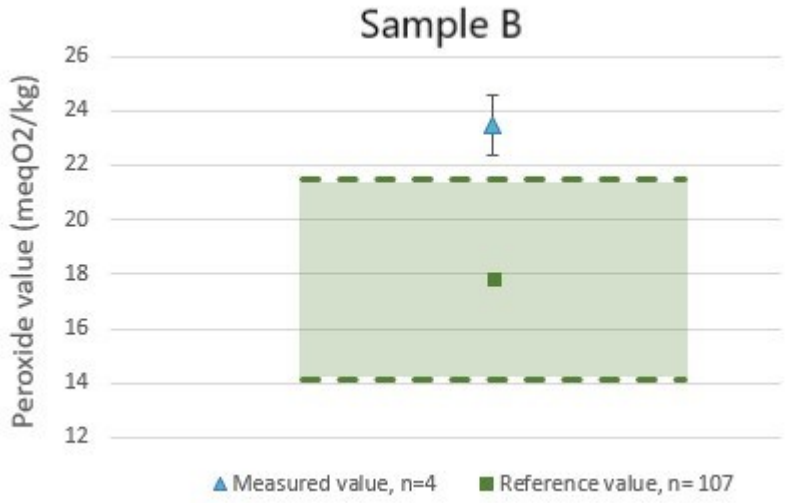

Figure 3. The measured PV (blue point ± black lines; mean ± SD, n = 4) of sample B has been compared to its reference value (green point ± green dashed lines; assigned value ± SD for proficiency assessment, n = 107). The green area between the two dashed lines indicates where the values are comparable with one another.

Similarly to sample B, the result of the t test for sample C concluded that the measured value (3.90 meqO_2/kg) and the reference value (1.10 meqO_2/kg) differ statistically significantly with a confidence level of 95 %. Besides that, it can also be observed from Figure 4 that the measured value is notably higher than the reference value.

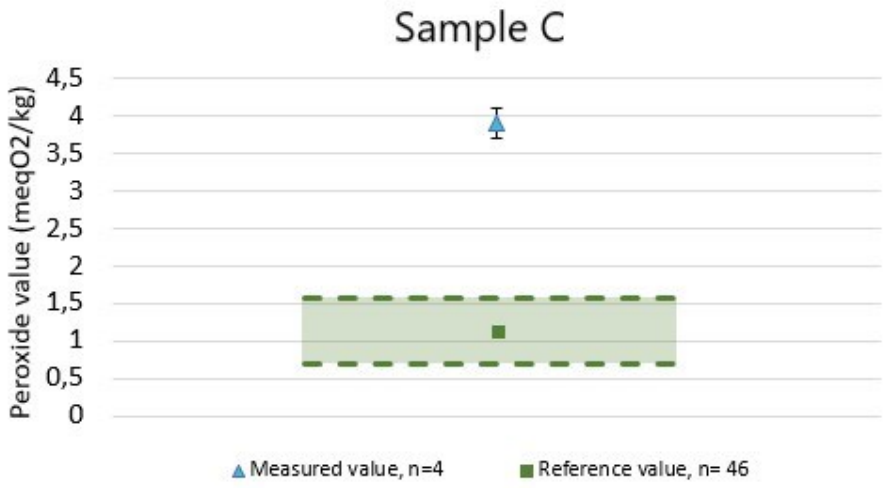

Figure 4. The measured PV (blue point ± black lines; mean ± SD, n = 4) of sample C has been compared to its reference value (green point ± green dashed lines; assigned value ± SD for proficiency assessment, n = 46). The green area between the two dashed lines indicates where the values are comparable with one another.

It should be noted that sample C had arrived half a year ago, while samples A and B a year prior to this study, meaning that autoxidation of some of the samples could be a possible explanation for the increase in their PVs. Besides autoxidation, a possible reason for the notable increase in the PV of sample C could be the heating of the sample in the oven at 60 °C, which, due to the sample being solid in RT, was done multiple times during this study and possibly during the proficiency testing as well.

Since two out of the three measured reference materials did not give comparable results for PV, and there were no additional suitable samples from previous proficiency tests, spiking tests with rapeseed oil (Pirkka Rypsiöljy) were executed to further evaluate the method performance, and also test the suitability of TBHP for the use of spiking experiments with ISO 27107 (Chapter 7.1.3 Spiking of Rapeseed Oil).

7.1.2 Within-Laboratory Reproducibility

The within-laboratory reproducibility was determined as the coefficients of variation (CV) of the measured PVs of samples A, B, and C. The measurements

of the samples were done with the same method, by the same person and the same apparatus, however, on different days and with different solutions that had to be prepared fresh each day. The CVs were calculated by Equation (14), based on the results of Table 3, and then compared to the coefficients of variation of reproducibility (CV(R)) of the interlaboratory test published in Annex B of ISO 27107:2010 [20].

$$CV = \frac{Standard\ deviation}{Mean} * 100\ \% \qquad (14)$$

The calculated CVs of each sample are presented in Table 4.

Table 4. The CVs of samples A, B and C.

Sample	CV (%)
A	7.2
B	4.7
C	5.1

The CVs of both samples A and B were compared to the CV(R) of vegetable oil mixture, and the CV of sample C to the CV(R) of raw palm oil. The CV(R)s, and other relevant parameters of these two oils of the ISO's interlaboratory test, are presented in Table 5.

Table 5. The CV(R)s, and the data related to those values, given in the interlaboratory test published in Annex B of ISO 27107:2010 [20].

Matrix	CV(R) (%)	Mean ($meqO_2/kg$)	SD ($meqO_2/kg$)	Number of Laboratories
Vegetable oil mixture	10.6	17.92	1.90	22
Raw palm oil	5.6	7.52	0.42	20

All of the CVs of the measured samples A, B and C were found to be lower than the respective CV(R)s. With these results, the within-laboratory reproducibility of the method was deemed to be adequate, at least for vegetable oils.

7.1.3 Spiking of Rapeseed Oil

First, prior to the spiking, the PV of unspiked rapeseed oil was determined with 8 parallel measurements. Then, spiking has been carried out in duplicates by adding 102 µl of TBHP, equivalent to 6 meqO_2/kg, to the rapeseed oil, which had a volume of approximately 5.6 ml. The volume of the TBHP-addition was calculated by Equation (12).

The results of both the unspiked and spiked determinations are presented in Table 6, and in full with the titration curves of the measurements in Appendix 3.

Table 6. The measured PV of both unspiked and spiked rapeseed oil.

Rapeseed Oil	Mean (meqO_2/kg)	SD (meqO_2/kg)	No. of Measurements
Unspiked	2.64	0.35	8
Spiked	8.27	0.16	2

The average %R of the spiking was calculated by Equation (13), based on the results of Table 6 and Appendix 3, equalling to 94 %. According to the Analytical Method Validation Procedure at Neste, an acceptable %R would be 80–120 %. With the %R acquired by the spiking of rapeseed oil, the method performance of ISO 27107 was concluded to be sufficient with the current setup in the inorganic chemistry laboratory of Neste Oyj's department of Research Analytics. The TBHP solution was also concluded to be adequate for the use of a model hydroperoxide in spiking experiments with ISO 27107.

7.2 Testing with Crude Algal Oil

To test whether ISO 27107 would be suitable for crude algal oil, a spiking experiment was carried out similarly to the spiking of rapeseed oil. The use of validation parameters, such as trueness and uncertainty, was intended to be applied for assessing the quality of the measurements and thus the suitability of the method. The spiking experiment was chosen since there was no suitable reference material available for crude algal oil. The aim was to measure the PV of both unspiked and spiked crude algal oil samples 10 times, as how trueness and uncertainty would be determined according to the Laboratory Guide to Method Validation by Eurachem [29].

7.2.1 PV Determination of Crude Algal Oil

First, the PV of crude algal oil was measured. As the crude algal oil was expected to have a strong matrix effect, the first measurements were done with blended oils. For the blends, the crude algal oil was mixed with olive oil. The PV of olive oil was determined before its use for the blending, the results of which are presented in Table 7 and in full in Appendix 4.

Table 7. PV of the olive oil that was used for the blending with crude algal oil.

Sample	Mean ($meqO_2/kg$)	SD ($meqO_2/kg$)	No. of Measurements
Olive oil	1.83	0.10	8

The blends were done at a ratio of 33 weight percent (wt%), 42 wt% and 50 wt% crude algal oil, each of the three blends measured once. The mass ratios of the blends were factored in, and the PVs were calculated for the algal oil fractions by Equation (15):

$$PV\ (crude\ algal\ oil) = \frac{Algal\ oil\ wt\% \ * \ PV(total)}{100\ \%} \qquad (15)$$

The results of these measurements are shown in Table 8 and in full in Appendix 5.

Table 8. PV of crude algal oil that was blended at different ratios with olive oil.

Crude algal oil (wt%)	PV, total ($meqO_2/kg$)	PV, Algal Oil ($meqO_2/kg$)
33	0.72	0.24
42	0.98	0.41
50	0.66	0.33

The measured PVs for blended crude algal oil were observed to be very low. The different ratios of the added olive oil did not seem to have observable effects on the measured values. Therefore, 100 wt% crude algal oil was tested in quadrupoles. The results of these measurements are presented in Table 9 and in Appendix 5.

Table 9. PVs of 100 wt% unspiked crude algal oil.

Crude Algal Oil, 100 wt%	PV ($meqO_2/kg$)
I	0.23
II	0.22
III	0.20
IV	0.28
Mean	0.23
SD	0.03

Surprisingly, the PVs of 100 wt% crude algal oil were observed to be very low as well, comparable to those of the blends. Titration curve of the 100 wt% crude algal oil sample II is presented in Figure 5. The titration curves of the blended samples (presented in Appendix 5) were similar to the ones of 100 wt% crude algal oil.

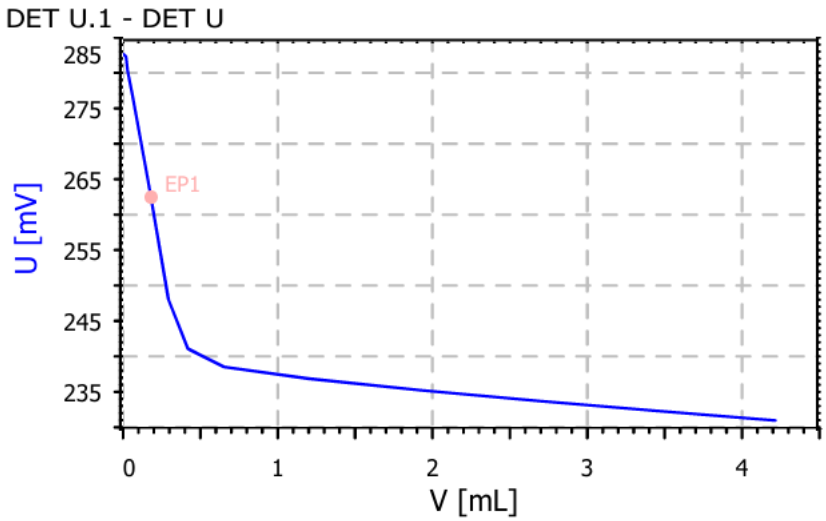

Figure 5. Titration curve of the unspiked, 100 wt% crude algal oil sample II.

It should be noted that due to the heterogeneous and highly viscous nature of crude algal oil, the weighing of the samples took a relatively long time, during which the sample vessels were unsealed and therefore exposed to atmospheric oxygen. This could potentially have affected the measurements, which in turn could affect the reliability of the results.

As the sample matrix was challenging to handle, alternative ways of measuring were also tested for crude algal oil. Sample I (Table 9) was measured with a decreased sample mass (2.55 g instead of 5 g) and an increased volume of solvent (80 ml instead of 50 ml). Additionally, sample III (Table 9) was measured with an increased sample mass (10 g instead of 5 g), since it is suggested in ISO 27107 that 10 g sample mass should be used for samples that have a PV below 1 meqO_2/kg. However, none of these changes seemed to have any observable effects regarding the measured PVs of crude algal oil, as all of the results were relatively uniform. Thus, the measurements were continued according to ISO 21707. The handling of the samples was improved by heating them, as described in Chapter 6.5 Samples and Sample Preparation, prior to their measuring.

7.2.2 Spiking of Crude Algal Oil

The spiking was carried out in triplicates by adding TBHP, equivalent to 15 meqO_2/kg, to crude algal oil. The titration curve of the spiked crude algal oil sample I is presented in Figure 6.

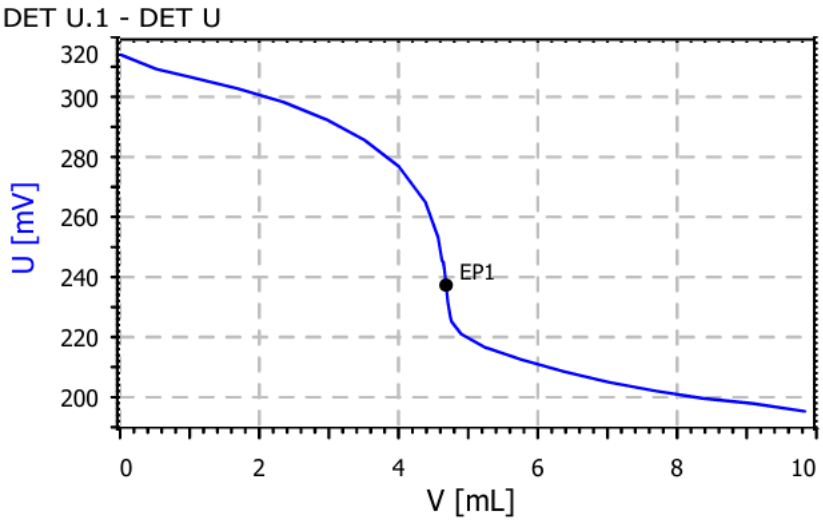

Figure 6. Titration curve of spiked sample I.

The results of the spiking are presented in Table 10 and in full in Appendix 5. The mean of the measured PVs of 100 wt% unspiked crude algal oil (Table 9) was applied for the calculations of the %Rs.

Table 10. PVs and %Rs of the spiked crude algal oil.

Spiked sample	PV (meqO_2/kg)	%R
I	7.93	51
II	7.96	51
III	9.27	55

The average %R of the measurements was 52 %, which is well below the desired %R interval of 80–120%, as is suggested in the Analytical Method Validation Procedure at Neste. The spiking experiment was discontinued as the

obtained %Rs were insufficient for determining neither the trueness or the uncertainty of the method with crude algal oil as the sample matrix, suggesting that the method is not suitable for crude algal oil at its current form.

8 Discussion

With crude algal oil, the low recoveries of TBHP could indicate that something in the sample matrix is interfering with the spiking, since both the method performance and the spiking with TBHP had already been determined to be sufficient with vegetable oils earlier in this study. The interferences with crude algal oil could potentially be due to the high viscosity of the sample, which, for example, could lead to the TBHP not being properly mixed in the sample at the time of the measurement. Additionally, the sample matrix could possibly contain some compounds that are antioxidant and could therefore neutralise the added hydroperoxides. As discussed in Chapter 5 Crude Algal Oil, it is common for microalgae, and therefore algal oils, to contain natural antioxidant compounds, such as tocopherols and carotenoids.

Although the low PVs and the possible antioxidant properties of the crude algal oil are ideal for its storability and oxidative stability, these properties do, however, disturb the method development in this study. As there was only a limited amount of both time and the crude algal oil, the method development was unable to be completed during this study. A few feasible options can be tried for the further development of the method, one of which could be to try and separate the antioxidant compounds from the crude algal oil samples with e.g. solid phase extraction, prior to their analysis. Another option to try could be to intentionally induce crude algal oil to oxidation with e.g. elevated temperatures and high concentrations of oxygen, and then to perform a spiking experiment for the oil.

Besides the aforementioned suggestions, it could also be studied whether there is some other property than the possible antioxidants and the viscosity of the sample that could interfere with the method chemistry, precluding the spiking of

crude algal oil. Additionally, an alternative way altogether to the spiking could be studied for testing the method suitability.

9 Conclusion

The objective of this study was to assess whether ISO 27107 could be implemented for the measurement of PV of crude algal oil, in order to determine the oxidative status of algal oils and thus assess their storability and quality. The method performance of ISO 27107, in the inorganic chemistry laboratory of the Research Analytics department of Neste Oyj, was first evaluated with vegetable oils, before the method was tested for crude algal oil.

The measuring of the vegetable oil reference material gave comparable results for one of the three measured proficiency test samples (Table 3), the PVs of the other two samples were observed to be higher than their assigned values. The within-laboratory reproducibility was then determined for the vegetable oils by comparing their CVs (Table 4) to the CV(R)s of similar samples used in the interlaboratory test published in Annex B of ISO 27107:2010 (Table 5). All of the CVs were found to be lower than the respective CV(R)s, suggesting that the within-laboratory reproducibility is sufficient.

When spiking the rapeseed oil, an adequate %R of 94 % (mean, n = 2) was observed. With the acquired %R, the TBHP solution was concluded to be suitable for the use of a model hydroperoxide in spiking experiments with ISO 27107.

Based on the aforementioned results, the method performance of ISO 27107 was evaluated to be sufficient. Additionally, the standard could potentially be utilized as a new analytical method for determining PVs of vegetable oils in the inorganic chemistry laboratory of the Research Analytics department of Neste Oyj, as there currently is no similar method in use.

The average measured PV of the crude algal oil (Table 9) was observed to be very low (0.23 meqO_2/kg, n = 4). The spiking of the crude algal oil resulted in a %R of 52 % (mean, n = 3), from which trueness nor uncertainty, i.e. the validation parameters that would have been used for assessing the quality of the measurements, could not be determined. These results could indicate that either the crude algal oil effectively neutralises hydroperoxides or its matrix effects interfere with the measurements. Due to this, spiking experiments (which would have been used for determining the trueness and uncertainty) are currently not feasible for crude algal oil, without possibly some kind of sample preparation prior to the measurement of PV. In conclusion, further studies are needed to continue the method development.

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Titration Curves with Different Ways of Purging with N_2

The proficiency test samples were measured with two different ways of purging, one according to ISO 27107:2010 and the other according to a modified method based on ASTM D3703. Each sample was measured 4 times with the ISO way of purging. With the ASTM way of purging, samples A and C were measured twice and sample B three times.

Sample A

The measurement of sample A with ASTM D3703 way of purging is shown in Figure 7.

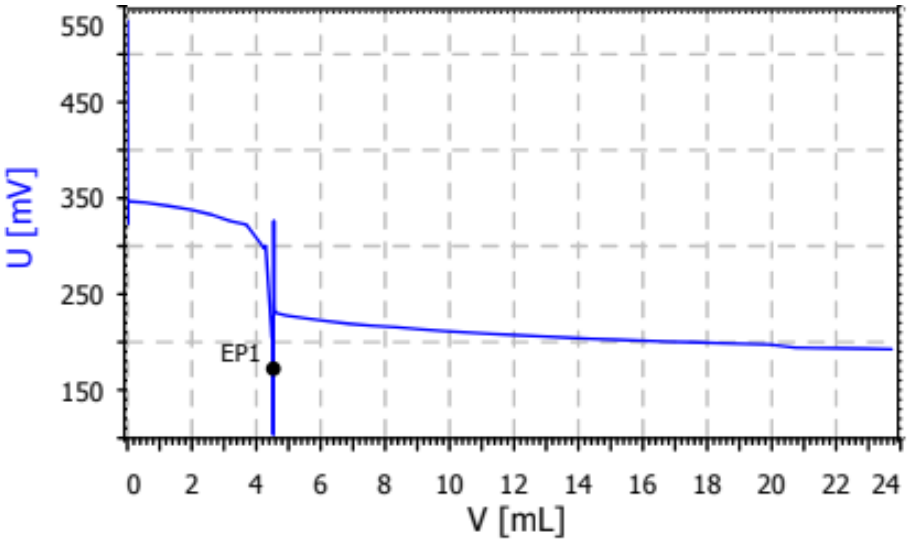

Figure 7. Titration curve of sample A measured with ASTM D3703.

The purging according to ASTM D3703 caused disturbances for the titration curve, which in turn made the measuring of PV more difficult. Figure 8 shows the same sample measured with ISO 27107 way of purging.

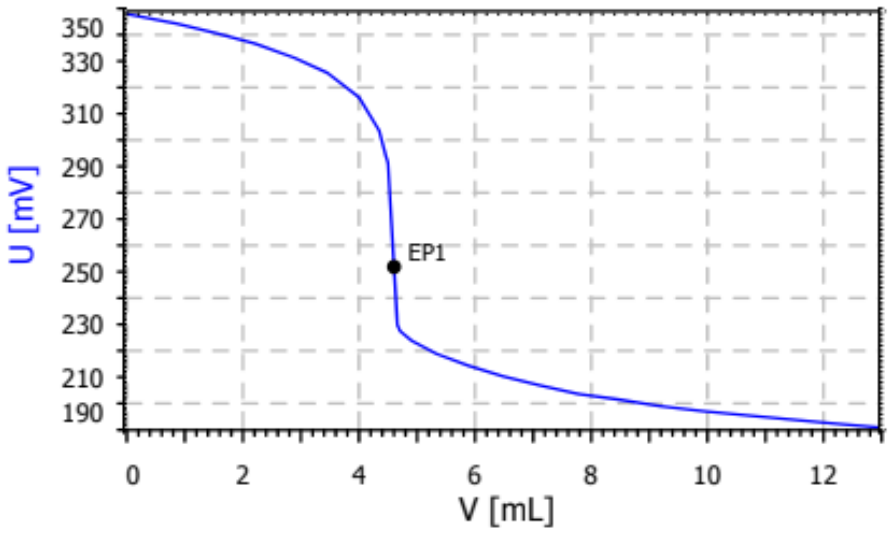

Figure 8. Titration curve of sample A with ISO 27107.

Based on these observations, the purging according to ISO 27107 was chosen as it is more suitable than that based on ASTM D3703.

Sample B

The measurement of sample B with ASTM D3703 way of purging is shown in Figure 9.

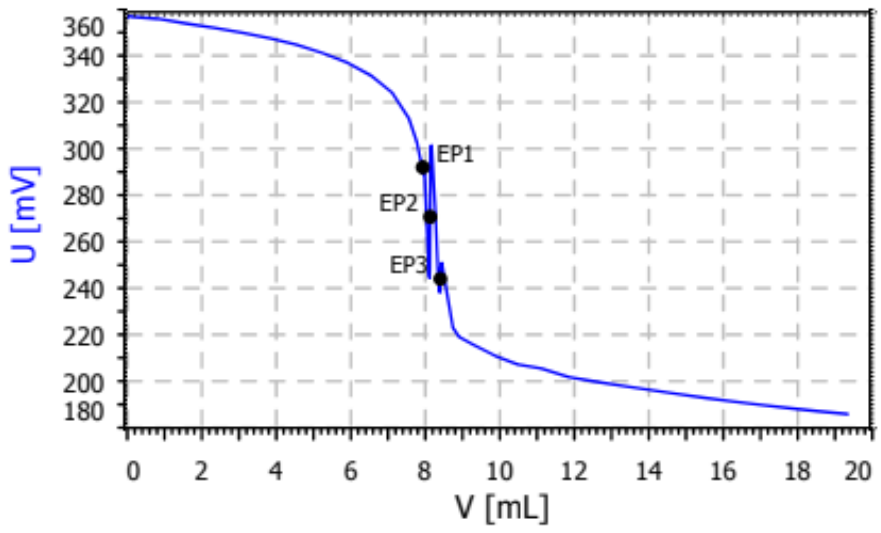

Figure 9. Titration curve of sample B with ASTM D3703.

The purging according to ASTM D3703 caused disturbances for the titration curve, which in turn made the measuring of PV more difficult. Figure 10 shows the same sample measured with ISO 27107 way of purging.

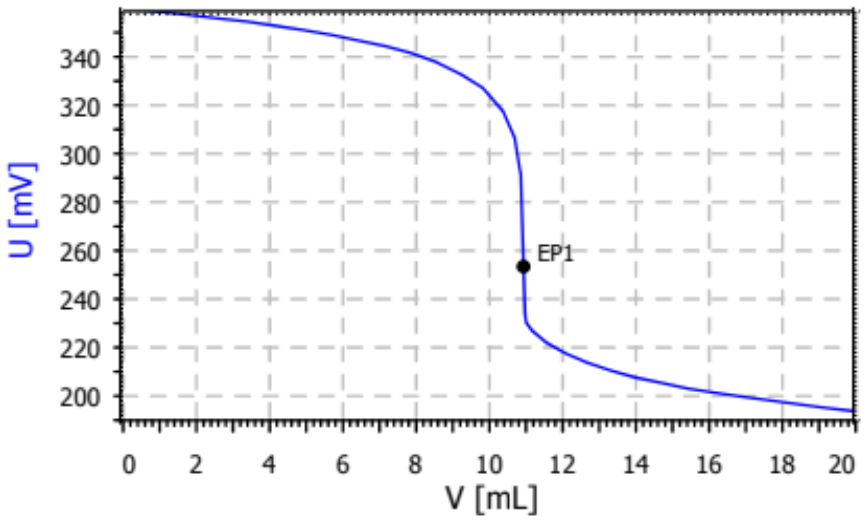

Figure 10. Titration curve of sample B with ISO 27107.

Based on these observations, the purging according to ISO 27107 was chosen as it is more suitable than that based on ASTM D3703.

Sample C

The measurement of sample C with ASTM D3703 way of purging is shown in Figure 11.

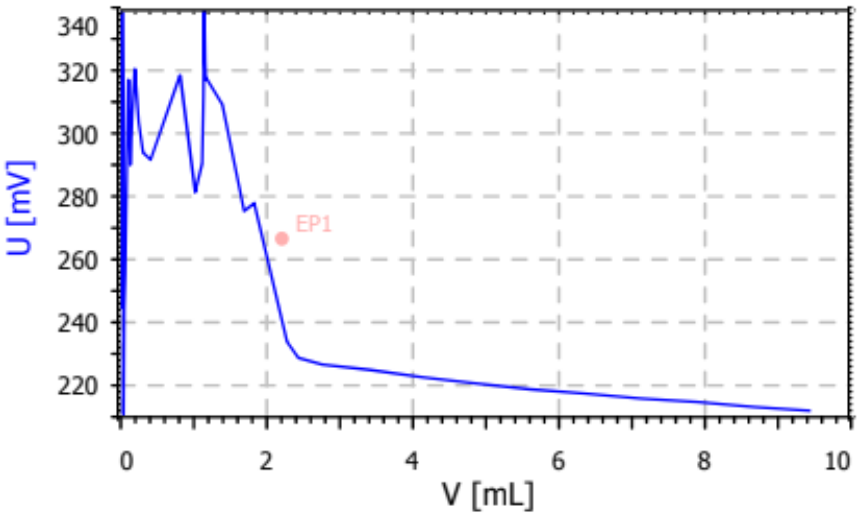

Figure 11. Titration curve of sample C with ASTM D3703.

The purging according to ASTM D3703 caused disturbances for the titration curve, which in turn made the measuring of PV more difficult. Figure 6 shows the same sample measured with ISO 27107 way of purging.

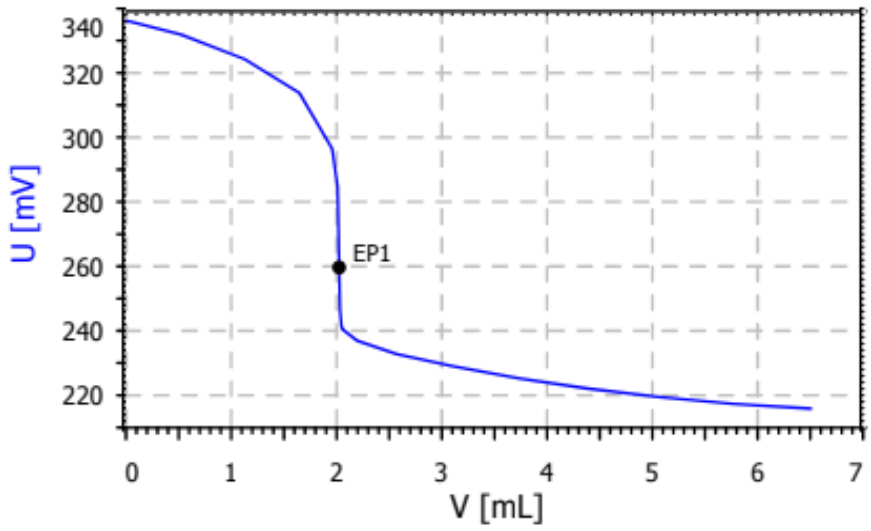

Figure 12. Titration curve of sample C with ISO 27107.

Based on these observations, the purging according to ISO 27107 was chosen as it is more suitable than that based on ASTM D3703.

Measured Data of the Proficiency Test Samples

All of the three proficiency test samples were measured on 22.9.2023, 26.9.2023 and 27.9.2023. Besides these, sample B was also measured on 19.9.2023 and 20.9.2023. The correction factor F and the titre of a blank measurement for each date is shown on Table 11.

Table 11. Date, correction factor F and titre of a blank measurement for each separate day the reference material was measured. In brackets besides the correction factor is the volume of 0.01 N sodium thiosulfate solution used for the factor's determination.

Date	Factor F	Blank (ml)
19.9.2023	1.00 (16.8106 ml)	0.0472
20.9.2023	1.00 (16.8954 ml)	0.0415
22.9.2023	1.01 (16.7422 ml)	0.0445
26.9.2023	1.02 (16.5776 ml)	0.0594
27.9.2023	1.01 (16.7229 ml)	0.0515

An example of the titration curve of a blank titration is presented in Figure 13.

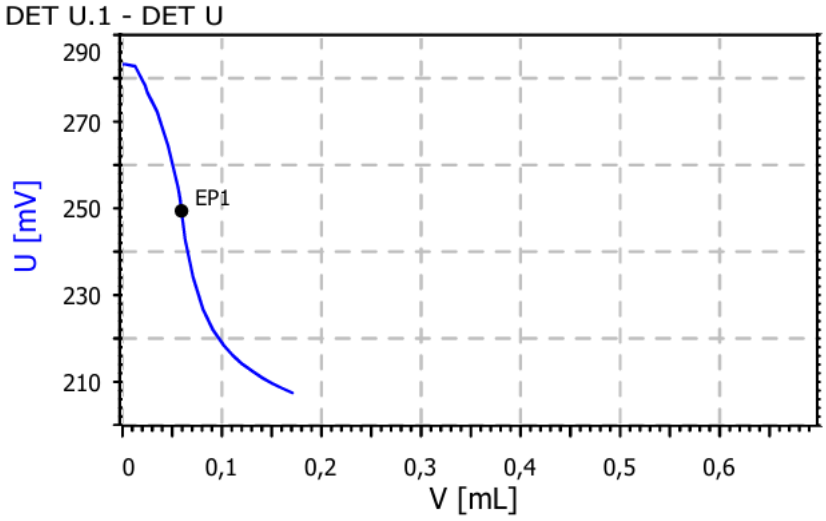

Figure 13. The titration curve of a blank titration with a titre of 0.0594 ml, measured on 26.9.2023.

Sample A

The reference value of sample A was 9.77 ± 1.51 meqO2/kg (assigned value ± SD for proficiency assessment, n = 105). The measured values are shown in Table 12.

Table 12. Measured sample volumes, end-points and PVs of sample A.

Date	Purging	m (g)	End-point (ml)	PV (meqO2/kg)
22.9.2023	ASTM D3703	5.00	4.5334	9.07
22.9.2023	ASTM D3703	5.00	4.0784	8.15
26.9.2023	ISO 27107	5.08	4.6025	9.12
26.9.2023	ISO 27107	5.04	4.3234	8.63
27.9.2023	ISO 27107	5.02	5.0438	10.04
27.9.2023	ISO 27107	5.00	4.9806	9.96

The mean ± SD of the measured PV of sample A is 9.44 ± 0.68 meqO2/kg and the CV is 7.21 % (n = 4). The first two of the obtained results were not taken into account for any of the calculations, as they were measured using another purging method.

Sample B

The reference value of sample B was 17.81 ± 3.68 meqO2/kg (assigned value ± SD for proficiency assessment, n = 107). The measured values are shown in Table 13.

Table 13. Measured sample volumes, end-points and PVs of sample B (DGF 2022).

Date	Purging	m (g)	End-point (ml)	PV (meqO2/kg)
19.9.2023	ASTM D3703	5.01	8.1338	16.16
19.9.2023	ASTM D3703	5.03	10.2119	20.23
20.9.2023	ASTM D3703	5.03	7.6506	15.12
26.9.2023	ISO 27107	5.02	11.8823	24.02
26.9.2023	ISO 27107	5.09	10.9319	21.79
27.9.2023	ISO 27107	5.02	11.9729	23.99
27.9.2023	ISO 27107	5.03	11.9988	23.99

The mean ± SD of the measured PV of sample B is 23.45 ± 1.11 meqO2/kg and the CV is 4.71 % (n = 4). The first three of the obtained results were not taken into account for any of the calculations, as they were measured using another purging method.

Sample C

The reference value of sample C was 1.10 ± 0.084 meqO_2/kg (assigned value ± uncertainty of assigned value, n = 46). The measured values are shown in Table 14.

Table 14. Measured sample volumes, end-points and PVs of sample C.

Date	Purging	m (g)	End-point (ml)	PV (meqO2/kg)
22.9.2023	ASTM D3703	5.07	1.6680	3.23
22.9.2023	ASTM D3703	5.04	2.2071	4.33
26.9.2023	ISO 27107	5.01	2.0251	4.00
26.9.2023	ISO 27107	5.02	2.0009	3.94
27.9.2023	ISO 27107	5.04	2.0742	4.05
27.9.2023	ISO 27107	7.93	2.8856	3.61

The mean ± SD of the measured PV of sample C is 3.90 ± 0.20 meqO_2/kg and the CV is 5.09 % (n = 4). The first two of the obtained results were not taken into account for any of the calculations, as they were measured using another purging method. Worth to note that one of the samples, the last one on Table 4, had a higher sample mass than recommended in ISO 27107, which in turn resulted in a higher titre.

Data of Unspiked and Spiked Rapeseed Oil Measurements

The PV of rapeseed oil (Pirkka rypsiöljy) was measured on three different days. The correction factor F and the titre of a blank measurement for each date is shown on Table 15.

Table 15. Date, correction factor F and titre of a blank measurement for each separate day the PV of rapeseed oil was measured. In brackets besides the correction factor is the volume of 0.01 N sodium thiosulfate solution used for the factor's determination.

Date	Factor F	Blank (ml)
2.10.2023	1.02 (16.5776 ml)	0.0440
4.10.2023	1.00 (16.7470 ml)	0.0881
5.10.2023	1.00 (16.8858 ml)	0.0748

Unspiked rapeseed oil samples I–III were measured on 2.10.2023, samples IV–VI on 4.10.2023, and samples VII and VIII on 5.10.2023 (Table 16).

Table 16. Measured sample volumes, end-points and PVs of unspiked rapeseed oil.

Sample	m (g)	End-point (ml)	PV (meqO2/kg)
I	5.10	1.2090	2.33
II	10.00	2.0845	2.08
III	5.08	1.2161	2.35
IV	5.04	1.4460	2.69
V	5.05	1.5922	2.98
VI	5.03	1.5056	2.82
VII	5.04	1.6044	3.03
VIII	5.09	1.5188	2.84

The mean ± SD of the measured PV of unspiked rapeseed oil is 2.64 ± 0.35 meqO_2/kg and the CV is 13.1 % (n = 8). A titration curve of an unspiked rapeseed oil measurement is presented in Figure 14.

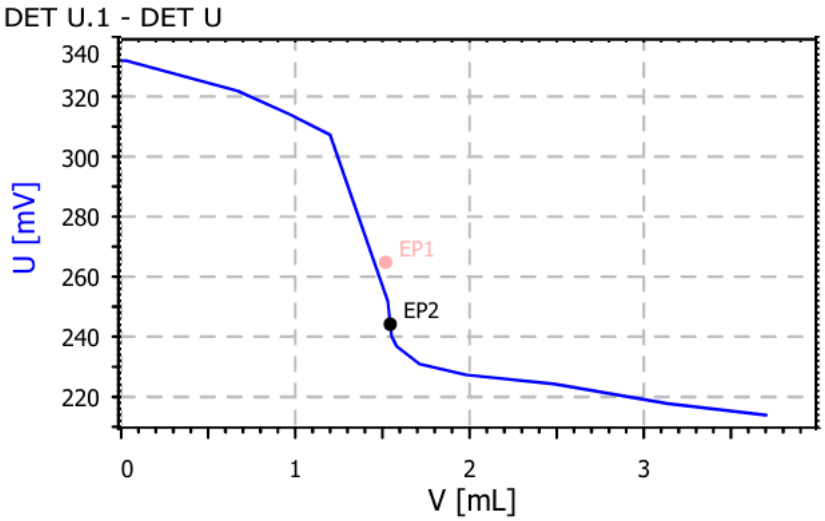

Figure 14. Titration curve of the unspiked rapeseed oil sample VIII. The end-point EP1 has been used for the results.

The spiking was carried out in duplicates by adding 102 µl of TBHP, equivalent to 6 meqO2/kg, to the rapeseed oil, which has a volume of approximately 5.6 ml. Spiked rapeseed oil samples I and II (Table 17) were both measured on 5.10.2023.

Table 17. Measured sample volumes, end-points, PVs and recovery rates of spiked rapeseed oil.

Sample	m (g)	End-point (ml)	PV (meqO2/kg)	%R
I	5.02	4.1701	8.16	92.4
II	5.06	4.3129	8.38	96.1

The mean ± SD of the measured PV of spiked rapeseed oil is 8.27 ± 0.16 meqO_2/kg, equivalent to %R of 94.2 ± 2.6% (n = 2). A titration curve of a spiked rapeseed oil measurement is presented in Figure 15.

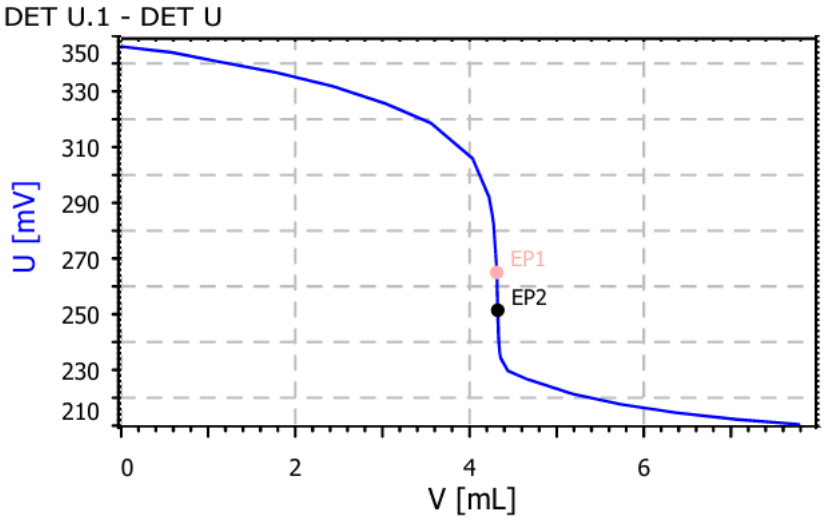

Figure 15. Titration curve of the spiked rapeseed oil sample II. The end-point EP1 has been used for the results.

Data of Olive Oil Measurements

The PV of olive oil (Xtra Oliiviöljy) was measured on three different days. The correction factor F and the titre of a blank measurement for each date is shown on Table 18.

Table 18. Date, correction factor F and titre of a blank measurement for each separate day the PV of olive oil was measured. In brackets besides the correction factor is the volume of 0.01 N sodium thiosulfate solution used for the factor's determination.

Date	Factor F	Blank (ml)
13.10.2023	1.00 (16.7912 ml)	0.0692
17.10.2023	1.00 (16.8731 ml)	0.0721
18.10.2023	1.00 (16.8676 ml)	0.0879

Samples I-VI were measured on 13.10.2023, VII-VIII on 17.10.2023 and IX on 18.10.2023 (Table 19).

Table 19. Measured sample volumes, end-points and PVs of olive oil.

Sample	m (g)	End-point (ml)	PV (meqO2/kg)
I	5.01	1.2179	2.29
II	5.01	0.9182	1.69
III	5.06	0.9259	1.69
IV	5.02	1.0394	1.93
V	5.05	0.9718	1.79
VI	5.02	0.9852	1.82
VII	5.04	1.0593	1.96
VIII	5.00	1.0016	1.86
IX	5.00	1.0477	1.92

The mean ± SD of the measured PV of olive oil is 1.83 ± 0.10 meqO_2/kg and the CV is 5.71 % (n = 8). It should be noted that the first result of the Table 1 was not taken into account for the calculation of the mean, SD and CV, as it was determined a significant outlier ($P < 0.05$) with a two-sided Grubb's test.

When compared to the CV(R) of olive oil (11.3%) in the interlaboratory test published in Annex B of ISO 27107:2010, the CV measured in this study is lower, which suggests an adequate within-laboratory reproducibility for olive oil.

Measured Data of Unspiked and Spiked Crude Algal Oil Samples

The PV of algal oil was measured on six different days. The correction factor F and the titre of a blank measurement for each date is shown on Table 20.

Table 20. Date, correction factor F and titre of a blank measurement for each separate day the PV of algal oil was measured. In brackets besides the correction factor is the volume of 0.01 N sodium thiosulfate solution used for the factor's determination.

Date	Factor F	Blank (ml)
17.10.2023	1.00 (16.8731 ml)	0.0721
18.10.2023	1.00 (16.8676 ml)	0.0879
20.10.2023	1.00 (16.8669 ml)	0.1247
23.10.2023	1.00 (16.8863 ml)	0.1327
1.11.2023	1.00 (16.8058 ml)	0.0700
2.11.2023	1.00 (16.8753 ml)	0.0774

From Table 20 it can be observed that on 20.10.2023 and 23.10.2023 the titres of blank measurements were higher. On these days, 80 ml of the solvent were used instead of the typical 50 ml. This was done as the solvent volume was also raised to 80 ml for the measurements of crude algal oil (done on 20.10.2023 and 23.10.2023) to test how it would affect the analysis.

Unspiked crude algal oil was mixed with olive oil (Xtra Oliiviöljy, 1.83 ± 0.10 meqO2/kg, mean ± SD, n = 8, Appendix 5) at a ratio of 33wt%, 42wt% and 50wt%. Each blend was measured once. Four measurements were done with different amounts of 100 wt% (unspiked) crude algal oil. The results obtained from these measurements are shown on Table 21. The mass ratios of the blends have been factored in, and the PVs have been calculated for the algal oil by Equation (15):

$$PV\ (crude\ algal\ oil) = \frac{Algal\ oil\ \%mass\ *\ PV(total)}{100\ \%} \quad (15)$$

Table 21. Measured sample volumes, end points and PVs of unspiked crude algal oil.

Date	Total mass (g)	Algal oil %mass	End-point (ml)	PV, total (meqO2/kg)	PV, algal oil (meqO2/kg)
17.10.2023	5.06	33	0.4389	0.72	0.24
18.10.2023	5.07	42	0.5866	0.98	0.41
20.10.2023	5.00	50	0.4534	0.66	0.33
23.10.2023	2.55	100	0.1918		0.23
1.11.2023	5.08	100	0.1827		0.22
2.11.2023	10.21	100	0.2771		0.20
2.11.2023	4.99	100	0.2183		0.28

The mean ± SD of the measured PV of unspiked, 100 wt%, crude algal oil is 0.23 ± 0.03 meqO2/kg and the CV is 13.0 % (n = 4).

The titration curve of the blend with 33 wt% ratio of crude algal oil is presented in Figure 16.

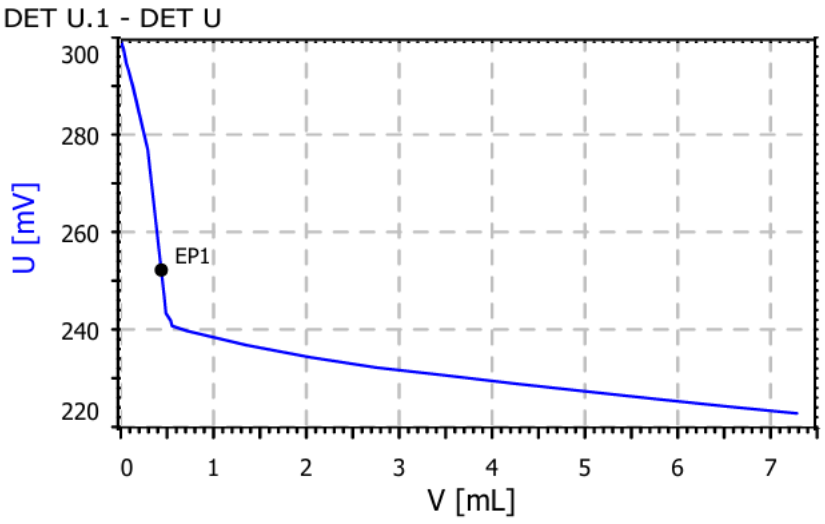

Figure 16. Titration curve of the blend with 33 wt% ratio of crude algal oil.

Spiking was carried out in triplicates by adding TBHP equivalent to 15 meqO_2/kg to crude algal oil. The average measured PV of unspiked, 100 wt%, crude algal oil (0.23 meqO_2/kg, n=4) was applied for the calculation of the %R. Spike I was measured on 1.11.2023, and spikes II and III on 2.11.2023 (Table 22).

Table 22. Measured sample volumes, end-points, PVs and %Rs of spiked crude algal oil.

Sample	m (g)	m, TBHP (g)	End-point (ml)	PV (meqO2/kg)	%R
I	4.97	0.24	4.2008	7.93	51.0
II	4.72	0.23	4.0188	7.96	50.7
III	4.75	0.25	4.7111	9.27	55.1

The mean ± SD of the measured PV of spiked crude algal oil is 8.39 ± 0.77 meqO2/kg and the CV is 9.12 % (n = 3). The calculated %R is 52.2 ± 2.5 % (mean ± SD, n = 3).