Monografie Politechniki Krakowskiej

SERIA INŻYNIERIA I TECHNOLOGIA CHEMICZNA

Elżbieta Skrzyńska

BIOFUELS AND BIORESOURCES

Laboratory handbook of selected analyses
for Innovative Chemical Technologies

BIOPALIWA I BIOSUROWCE

Zbiór wybranych analiz laboratoryjnych
dla Innowacyjnych Technologii Chemicznych

Politechnika Krakowska
im. Tadeusza Kościuszki

Kraków 2018

MONOGRAPH

MONOGRAFIA

Cracow University
of Technology

Elżbieta Skrzyńska

BIOFUELS AND BIORESOURCES

Laboratory handbook of selected analyses for Innovative Chemical Technologies

MONOGRAFIE POLITECHNIKI KRAKOWSKIEJ

ENGINEERING AND CHEMICAL TECHNOLOGY

CRACOW 2018

Politechnika Krakowska
im. Tadeusza Kościuszki

Elżbieta Skrzyńska

BIOPALIWA I BIOSUROWCE

Zbiór wybranych analiz laboratoryjnych dla Innowacyjnych Technologii Chemicznych

MONOGRAFIE POLITECHNIKI KRAKOWSKIEJ

SERIA INŻYNIERIA I TECHNOLOGIA CHEMICZNA

KRAKÓW 2018

Publikacja opracowana w ramach projektu POWR.03.03.00-00-M070/16

ISBN 978-83-65991-01-0

Wydawnictwo PK, ul. Skarżyńskiego 1, 31-866 Kraków; tel. 12 628 37 25, fax 12 628 37 60
e-mail: wydawnictwo@pk.edu.pl ☐ www.wydawnictwo.pk.edu.pl
Adres do korespondencji: ul. Warszawska 24, 31-155 Kraków

Table of contents

1. Introduction

A detailed assessment of the chemical compositions of bioresources and natural products usually follows a period of research, including critical visual and sensory examinations. Typically, the overall state of the raw material (seed and plant impairment) is analysed, including colour, smell, and, in some cases, taste, to identify any trace of deterioration or the presence of mineral or organic pollutants. Next, carefully selected physical and chemical properties are verified, enabling a relatively simple and effective preliminary quality assessment of the biomaterial. Of course, the type of analysis strictly depends on both the nature and the destination of the bioproduct. For example, for herbaceous plants most attention is given to a visual evaluation of physical condition, an analysis of the ash and moisture contents, as well as the identification of any organic or inorganic pollutants. This is sufficient to determine the industrial and Pharmacopoeia usefulness of the raw material. Detailed guidelines and methods for determining plant purity and quality (including relevant tables with acceptable moisture levels and ash contents in numerous species) are given in the National and European Pharmacopoeia Editions [1]. Analogous and detailed standards can also be found for raw materials intended for food and feed purposes, where the most important elements are the analyses of water, protein and fat contents, as well as further determination of fibres, mineral salts and nitrogen compounds. Based on that, it is possible to calculate the nutritional value of the product, which must be added to the labelling on each package according to the National and EU regulations [3].

In the case of biomass intended for energy purposes (*e.g*. peat, straw, wood chips, dedicated crops like *Rosa Multiflora*, *Miscantus Giant*, osier – *Salix Viminalis*, giant reed – *Arundo donax*, *etc*.), an analysis of the moisture and ash residues are first carried out, followed by an analysis of the actual fuel calorific value, presence of potential corrosive substances, *etc.* For oils and fats to be used in biodiesel synthesis, the key parameters are: humidity and the presence of impurities and free fatty acids, as these determine the selection of basic processing conditions, such as: type of catalyst, temperature and excess of alcohol. On the other hand, the content of simple and complex sugars is crucial for the fermentation industry, as they determine the usefulness of the raw material in the synthesis of bioethanol.

A separate issue concern the methods used to characterise and potentially modify the final product, *i.e.* ethanol, purified glycerine, refined oil, liquid or gaseous biofuels, *etc.*, in order to meet the rigorous quality standards set by the industry.

Thus the ability to perform numerous analyses correctly is crucial for final product valorisation, including the evaluation of its market value and realistic application.

This handbook of manuals comprises a set of simplified preparatory and analytical instructions, intended for the synthesis and characterisation of selected bio-raw materials and biofuels. More detailed procedures and quality guidelines can be found in the corresponding standards and legal regulations. Some examples of National and European standards for each group of products are listed below:

- biodiesel (ester biofuel): PN-EN 14214 [4] and ASTM D6751 [5],
- biogas: PN-EN 16723-1: 2016-12 [6] and PN-C-04753: 2011 [7],
- solid biofuels: PN-EN ISO 17225-1: 2014-07 [8],
- spirits: PN-A-79522: 2001 [9],
- bioethanol fuel: EN 15376: 2007 [10] and ASTM D5798 [11],
- refined fats: PN-A-86908: 2000P [12].

The majority of the listed documents and related regulatory acts have been published on the Internet by the Polish Committee for Standardisation (https://pzn.pkn.pl/kt/work-program/own), the European Commission (http://eur-lex.europa.eu/oj/direct-access.html), and the ASTM International group (https://www.astm.org/).

2. Manuals for selected processes and syntheses

2.1. Biogas production

The modification of legal regulations in EU countries and the intense promotion of renewable energies in recent years have led to a significant diversification in the biogas market and a visible increase in its overall production. The processes are based on the anaerobic conversion of biomass, leading to a mixture of gases consisting primarily of methane and carbon dioxide, with small amounts of other components like hydrogen sulphide, ammonia, air, *etc.* An average biogas composition has been presented in Table 2.1.1.

Table 2.1.1

Typical biogas composition. Based on [13, 14]

Component	Content, % vol.
CH_4	50–75
CO_2	24–45
H_2O	2–7
H_2S	20–2000 ppm
N_2	< 2
O_2	< 2
H_2	< 1

The process itself is well known from the natural world, appearing spontaneously in the seabed, freshwater reservoirs, slurry and landfills. All stages of biogas production are enzymatic processes involving anaerobic bacteria (Fig. 2.1.1). The first stage involves hydrolysis, resulting in the breakdown of complex compounds into simple constituents, such as amino acids, sugars and fatty acids [13, 14]. Acidification is the second stage, where the intermediate components further degrade into low molecular weight carboxylic acids (butyric, propionic, acetic, *etc*.), carbon dioxide and hydrogen, while small amounts of lactic acid and alcohol may also be generated. All these products convert in the next phase to acetic acid, hydrogen and carbon dioxide. Finally, the process of methanogenesis to methane proceeds with the assistance of appropriate autotrophic and heterotrophic bacteria.

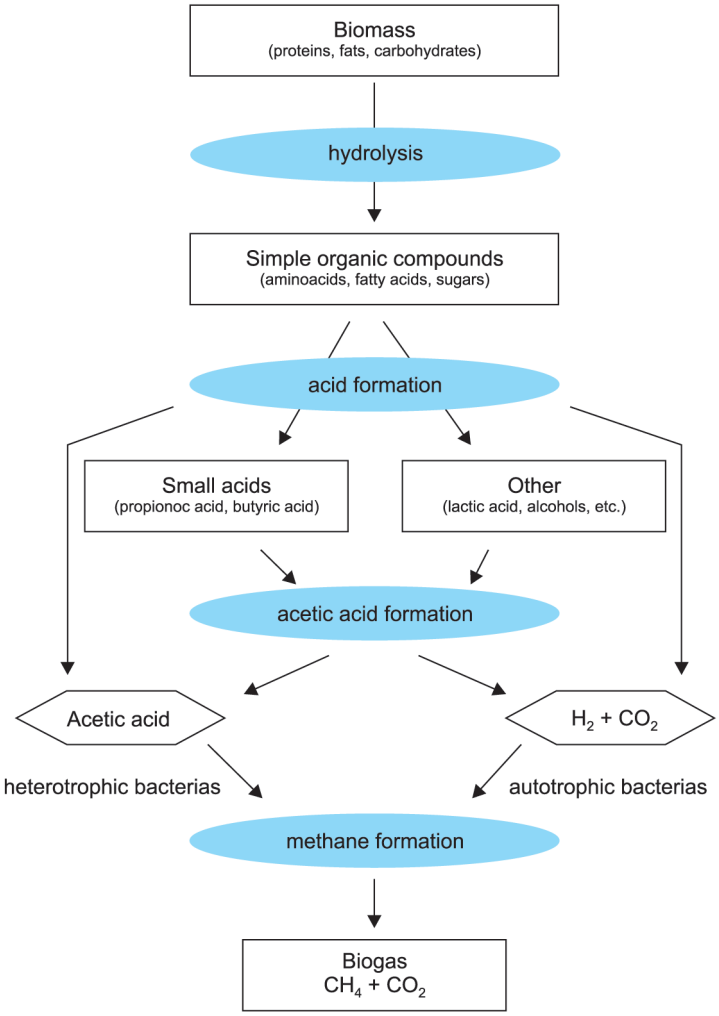


Figure 2.1.1. Processes occurring during biogas production and its average composition. Based on [13, 14]

All of these processes require the presence of water, and is essential for the successful activity of bacteria. In general, it is assumed that the yield of biogas and selectivity to methane depends on the properties of the biomass input (Table 2.1.2).

Table 2.1.2

Typical yields of biogas and methane content for different biomass inputs [13]

Dominating biomass composition*	Biogas yield, dm^3/kg DM**	CH_4 content, % vol.
Protein	600–700	70–75
Fat	1000–1250	68–73
Carbohydrates	700–800	50–55

* Only dominating available ingredients.
** DM – dry mass after subtracting the ash content.

Furthermore, the most influential parameters are: temperature, which depending on the stage and type of bacteria should be 32–42°C or 50–57°C; time, which usually varies between 18 and 78 days, although for landfills it may last several years; pH value, ranging from 4.5–6.3 for first steps of hydrolysis and acid production, up to 6.8–7.5 for final methane formation; nutrient balance (C : N : P : S ratio should be about 600 : 15 : 5 : 1) [13, 14]. Also important is the lack of fermentation inhibitors, such as elevated concentrations of alkali metal cations, heavy metals and branched fatty acids [13, 14].

It should be also underlined that the average composition of biogas significantly changes during subsequent stages of the fermentation process (Fig. 2.1.2).

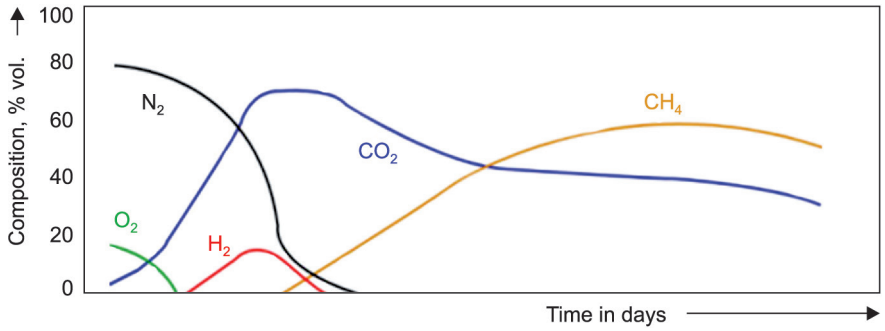


Figure 2.1.2. Change in biogas composition during anaerobic digestion. Based on real experiments and the literature [13]

2.1.1. Experimental section

Objectives:

The goals of the exercise are the anaerobic digestion of biomass, analyses of volumetric yield and composition of produced gases over time, and calculation of biogas yield with respect to dry mass of the raw material used for anaerobic fermentation.

Reagents and apparatus:

- 20–50 g of biomass (dry duckweed, fresh and green leaves or grass, hay, fruit or potatoes peels, fallen leaves ... *etc*.),
- dark glass bottle and hermetic rubber stopper, with a hose connector,
- measuring cylinder, 1–2 litre, filled with brine (receiver for gas samples),
- laboratory drier or any type of thermostat,
- sodium chloride salt for brine preparation,
- mortar, blender, scissors, knife, rasp or other type of tool for grinding the biomass,
- small glass vials with rubber seals for gas sampling,
- gas chromatograph, equipped with a TCD detector and 1 m Separon SDA packed column.

Characterisation and preparation of raw material for anaerobic digestion:

Before application, the raw material selected for anaerobic digestion should be carefully homogenised (reduced in size if needed) and characterised by the measurement of:

- dry matter content – Section 3.1.1,
- ash content – Section 3.1.2,
- crude fat content (optional) – Section 3.1.3,
- available protein content (optional) – Section 3.1.5,
- sugar content (optional) – Section 3.1.6.

Anaerobic digestion:

Weight the glass bottle (fermentation tank) and fill with homogenised biomass (dry or fresh) to about half its volume. After that, add a fresh water (from sink) in quantity to cover the surface of the raw material. Check the balance before and after water addition. In case of dry biomass, shake carefully the fermenter content and left for 30 minutes to absorb water. Re-adjust the water amount to required level and thoroughly mix again. Use inert gas (gentle nitrogen bubbling) to remove air from the cylinder and plug it with a rubber stopper equipped with a hose connector and place in a thermostat (dryer). Slowly heat up to 40°C (increase in temperature should be 2–5°/h in order to prevent deactivation of microorganisms). In case of faster heating add inoculum (active culture of bacteria) to fermenter and connect the outlet (hose connector) with a rubber tube and place its end inside a glass cylinder filled with brine to collect the gases evolved (see Fig. 2.1.3). Periodically mix (without opening) the content of the bottle, replace or fill-up the brine solution, record the volume of the evolved gases, and take small gas samples for chromatographic analysis (details in Section 3.3).

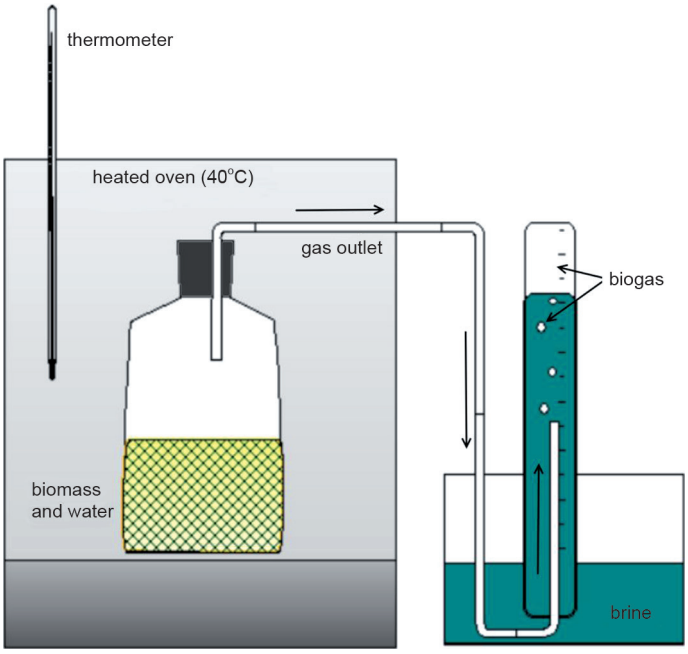


Figure 2.1.3. Scheme of apparatus for biogas production*

WORNING! After the initial incubation stage, the amount of evolved gases can increase rapidly, so after each gas sampling the cylinder should be emptied from the gas (the easiest way is to suck-out the gas using a plastic syringe with rubber hose) and refill it with brine.

Fermentation and heating should be carried out continuously without brakes, until it stops completely (the production of biogas diminish).

Biogas composition analysis:

The biogas composition should be determined by gas chromatograph (apparatus equipped with TCD detector, 1 m Separon SDA filled column) operating at room temperature. The volume of gases injected directly on column should be between 0.2–0.5 cm^3 (see Section 3.3.2).

Periodically verify the detector signal response for pure nitrogen or air injections (the detector response for both components is almost identical). Namely: inject onto column several volumes of selected gas (from 50 to 500 µl). Calculate the area under each peak ("*y*", $Area_{N_2}$ in a.u.) and plot it against the corresponding volume

* Figures and tables in this monograph without the sources mentioned were prepared by author.

of gas introduced into the column ("x", V_{N_2} in μl). Make the linear fit ($y = a \cdot x$) and compare the "a" coefficient with a value shown in Fig. 3.3.3 ($y = 2.3059x$). In case of significant difference, repeat the calibration of all components, or calculate the corrected coefficients based on the results of biogas analysis.

Namely:

a) for nitrogen use new value of the coefficient "a_{N_2}", a number taken directly from prepared calibration curve;
b) for CO_2, inject on chromatographic column several doses (volumes from 50 to 500 μl) of biogas evolved during the early fermentation stage. These samples should contain only two components, *i.e.* nitrogen (air) and carbon dioxide. Knowing the total volume of the gas sample injected and the actual volume of nitrogen (calculated from corresponding peak area and new calibration factor), calculate the volume occupied by carbon dioxide. Repeat the procedure for each injection volume and calculate the corresponding calibration curve for carbon dioxide. Read the "a_{CO_2}" coefficient in the same way as it has been done for nitrogen;
c) based on new calibration factors for nitrogen and carbon dioxide (two from total three components in biogas) calculate the actual methane volume in remaining biogas samples. The volume occupied by methane is equal total injection volume ($V_{injected}$) minus the volume occupied by CO_2 (V_{CO_2}) and N_2 or air (V_{N_2}):

$$V_{CH_4} = V_{injected} - V_{N_2} - V_{CO_2} = V_{injected} - \frac{\text{Area}_{N_2}}{a_{N_2}} - \frac{\text{Area}_{CO_2}}{a_{CO_2}} \qquad (2.1.1)$$

Report details:

The report should include:

1) Short Introduction based on the actual literature.
2) Detailed description of the experiment.
3) The results of the raw material used for fermentation characterisation.
4) An illustration of the changes in evolved biogas volume during fermentation (total change of biogas volume over time in respect to biomass dry mass, cm^3/g).
5) Results of chromatographic analyses – calibration of nitrogen, optional correction of other calibration factors, discussion of changes in biogas percentage composition during aerobic fermentation time.
6) Conclusions, including a comparison of the biogas composition with typical, commercially available biogas (see Fig. 2.1.1).
7) List of the literature.

2.2. Biodiesel synthesis from natural oils and fats

According to EU accepted standards and regulations, a biodiesel is a complex mixture of higher fatty acid methyl (or ethyl) esters. It is used as an additive for mineral diesel fuel or individual 100% fuel (B100) dedicated for supplying the diesel engines (compression-ignition engines, CI), and it has to fulfil numerous utility parameters listed in Table 2.2.1.

Table 2.2.1

Basic requirements for biodiesel B100. Based on [4]

Parameter	Unit	Range
Ester content	% (m/m)	min 96.5
Density at 15°C	kg/m^3	860–900
Viscosity at 40°C	mm^2/s	3.5–5.0
Flash Point	°C	above 101
Sulfur Content	mg/kg	max 10
Cetane Number		min 51
Sulphated Ash Content	% (m/m)	max 0.02
Water Content	mg/kg	max 500
Oxidation Stability, 110°C	hours	min 6
Acid Value	mg KOH/g	max 0.5
Iodine Value		max 120
Linolenic acid methyl ester	% (m/m)	max 12
Polyunsaturated (≥ 4 double bonds) methyl esters	% (m/m)	max 1
Methanol Content	% (m/m)	max 0.2
Monoglyceride Content	% (m/m)	max 0.8
Diglyceride Content	% (m/m)	max 0.2
Triglyceride Content	% (m/m)	max 0.2
Free Glycerol	% (m/m)	max 0.02
Total Glycerol	mg/kg	max 0.25
Phosphorus Content	mg/kg	max 10
Cold filter plugging point	°C	0 (summer) –10 (spring/autumn) –20 (winter)

The biodiesel in Europe is usually produced from rapeseed oil by alkali catalysed methanolysis, *i.e.* a homogeneous process characterised by mild synthesis conditions, considerable simplicity and fast reaction rates. Unfortunately, the use of alkaline

catalysts force further multistage purification of final biofuel, and also requires the usage of raw material free from water and free fatty acids (the possibility of soaps formation as a by-products). In the case of low-quality raw materials, a reasonable biodiesel yields requires operating at higher temperatures with a significant excess of alcohol and mineral acid as a catalyst. Other options are: a multistage synthesis process with the use of both the acidic and the basic catalysts; a heterogeneous process with solid bases or acids; an enzymatic transesterification or the synthesis without any catalyst under tough conditions [14–16]. Most typical process conditions, depending on the catalyst selected, have been compared in Table 2.2.2.

Table 2.2.2

Comparison of main conditions for biodiesel synthesis process. Based on [15, 16]

Conditions	Catalyst used			
	base	acid	enzymes	none
Temperature, °C	25–65	65–120	30–40	200–250
Pressure, MPa	0.1	0.1–1	0.1	6–9
Time	fast (< 1 h)	slow (5–12 h)	v. slow (24 h)	slow (> 3 h)
Alcohol/oil molar ratio	6–12	12–24	3	3–6
Restrictions: – presence of water – presence of FFA	 not allowed not allowed*	 limited allowed	 allowed allowed	 allowed allowed
Costs of production (most expensive part)	very low (oil)	low (oil)	very high (catalyst)	high (energy)
Glycerol recovery	hard	hard	easy	easy
Catalyst recovery	very hard	hard	hard**	n.a.***
Fuel purification	washing, distillation	washing, distillation	easy – distillation	easy – distillation

* Possible unfavourable soap formation, the acidic value for used raw oil (fat) should be as low as possible, preferably below 2 mg KOH/g.
** Separation is possible only for immobilised enzymes.
*** Not applicable.

2.2.1. Experimental section

Objectives:

The goal of the exercise is to synthesise the biodiesel by transesterification of vegetable oil, animal oil or waste fat; select the reagents amount based on raw material acid number measurement; purify the final ester and analyse its basic properties (determination of density, viscosity, flash point in open crucible, cloud point, freezing point and other tests listed by tutor, like: fractional composition,

cold filter blocking temperature, oxidation stability, determination of characteristic values, chromatographic distribution of fatty acids, *etc*.).

Reagents and apparatus:

- anhydrous methanol or ethanol,
- any type of animal or vegetable oil – fresh or waste fat from the gastronomy,
- KOH p.a.,
- 0.1 M KOH solution,
- hexane or heptane,
- phenolphthalein 1 mass% alcohol solution,
- mechanical glass stirrer,
- round-bottomed 3-neck flask (500 cm^3) with reflux,
- dropping funnel and 500 cm^3 separatory funnel,
- burette and pipette,
- filtering paper and big glass funnel,
- glassware and apparatus necessary to analyse selected properties of biofuel.

Characterisation and preparation of fatty raw material for biodiesel synthesis:

When a waste frying oil is used as a raw material for biodiesel synthesis, first it should be hot-filtered in order to remove any solid impurities and traces of moisture. Animal and solid vegetable fats should be melted and in case of foaming – carefully heated above 100°C (with mixing) to evaporate water. Other fats can be used directly. The optimum amount of fatty raw material is 200–250 g.

To properly select the reaction conditions that depends on fat properties, it is necessary to:

- analyse the acid number and saponification number of fat – Section 3.2.7.1;
- calculate the average molar mass of the fat on the basis of saponification number. Alternatively, it can be assumed that the whole fat consists of glycerol trioleate (for vegetable oils) or glycerol tristearate (for animal fat). In case of waste gastronomic fat, assume that it is a mixture of 30% glycerol tripalmitate, 30% glycerol tristearate and 40% glycerol trioleate;
- for raw oil with an acid value less than 4, the biodiesel synthesis should be performed by pathway A; for fats with an acid number between 4 and 8 – proceed according to pathway B; in case of higher acid value – choose the option C.

Biodiesel synthesis:

Pathway A:

1) Calculate the required amount of KOH for transesterification assuming that the mass of the catalyst should be 1% of the mass of the oil, plus the

amount of KOH necessary to neutralise all free fatty acids present in the raw material;
2) Dissolve KOH in methanol (or ethanol), adjusting a molar ratio of alcohol to oil to the value given by tutor (optimum molar ratio is 6–12 : 1);
3) Slowly (15–20 minutes) drop in an alcohol solution of KOH to heated (60°C) and intensively mixed oil. Continue stirring for another 30–60 minutes at the same temperature;
4) Transfer the warm mixture to the separatory funnel and wait for separation to take place. Remove the darker bottom phase of glycerol and unreacted alcohol;
5) Thoroughly wash the ester layer with cold water (to disappearance of turbidity), than continue washing with portions of saturated NaCl solution and slightly heated distilled water;
6) Dry the ester phase over anhydrous silica or $MgSO_4$ and separate by filtration or decantation (alternatively, the esters can be dried by simple filtration on a double filter paper).

Pathway B:

1) Heat the oil to 30°C (or slightly above the cloud point – see Section 3.2.4) and mix with such amount of 5% $CaCl_2$ alcoholic solution to convert all free fatty acids into insoluble calcium salts (calculate on the basis of measured acid number value). Stir the mixture for 15 minutes and cool. Separate the precipitate on a Buchner funnel under reduced pressure. Remove oil layer from excessive alcohol, wash it with saturated NaCl and examine the acid number again (it should decrease below 4). Proceed as in pathway A.

Pathway C:

1) Add selected oil to the methanol mixed with 0.5 cm^3 H_2SO_4 (alternatively you can use 10% alcohol BF_3 solution). To calculate the optimum amount of methanol solution, assume a molar ratio of alcohol to oil of 12–24: 1 (according to the guidelines);
2) Heat the mixture with vigorous stirring to a temperature of 60°C and maintain these conditions for 2 hours. Then, take a sample of the hydrophobic oil--ester phase, wash it several times with water in a small separatory funnel, and analyse the acid number and perform the test for unreacted glycerides: add 2 droplets of oil to small amount of solid $KHSO_4$ in a dry tube. Heat for several minutes. If an irritating acrolein odour is released and the acid number is less than 4 – remove the fatty layer, wash it with 5% Na_2CO_3 solution, dry on a filter paper and proceed as in pathway A. If the acid number is between 5 and 10 mg KOH/g, wash and dry the oil phase, and proceed according to pathway B.

Comparative characterisation of biodiesel and oil:

Analyse (according to the standards or instructions given in Section 3):

- density by pycnometric method at room temperature and reference 15°C – Section 3.2.2;
- selected low temperature properties – Section 3.2.4;
- open cup ignition temperature by Marcusson method – Section 3.2.5.1;
- viscosity at 40°C by Ubbelhold method – Section 3.2.3.1;
- fractional distillation (optional) – Section 3.2.6.2;
- oxidative stability (optional) – Section 3.2.8.4;
- selected characteristic numbers – Section 3.2.7;
- chromatographic fatty acid composition (optional) – Sections 3.2.9.1 and 3.2.9.2.

Report details:

The report should include:

1) Short Introduction based on the actual literature.
2) Detailed description of the experiment with necessary calculations.
3) Calculated yield of biodiesel synthesis process.
4) Performed analysis results referred to the guidelines of EN 14214 standard [4].
5) Conclusions.
6) List of the literature.

2.3. Bioethanol synthesis from starch

A fermentation processes are widely known and used since antique times, when they were used for alcohol fermentation, bread leavening and milk fermentation. Nowadays, apart from food purposes, a fermentation processes are also used in the production of acetic, lactic and citric acids, as well as in biogas aerobic fermentation. The most simple version of ethanol synthesis is based on aqueous solution of sugar fermentation which *Saccharomyces cerevisiae* or *Saccharomyces uvarum* yeasts, used respectively in top or bottom fermentation (both strains and methods differs with respect to operating conditions, especially the temperature). Irrespective of the method selected, the fermentation processes can be divided into three main stages: raw material preparation (*e.g.* preparation of mash), main fermentation and finishing processes (clarification, aging, purification, distillation, *etc*.) [17]. Under best conditions, the main products of alcoholic fermentation are ethanol and carbon dioxide. Small amounts of organic acids, light alcohols, esters, aldehydes, and glycerine are also produced (yield up to 3%). However, this process can be

altered to obtain glycerol (up to 23% in yield) by simple adding of sodium or calcium sulphate (IV), *i.e.* changing the pH from slightly acidic to slightly alkaline [18]. On the other hand, in the presence of oxygen the reaction pathway changes to acetic acid formation.

Apart from production of wines and beers, and also an agricultural distilleries, all based on cereals (mainly the rye) and potatoes, the modern spirit production is carried out in industrial distilleries (including ethanol for fuel purposes) and use in majority the sugar cane, molasses and maize [17]. In the technological process based on starch as a raw material, the most important stages of production are: (I) liquefaction of starch into form soluble in water; (II) enzymatic hydrolysis to reducing sugars (so called saccharification); (III) fermentation with yeast; and (IV) distillation of raw spirit on packed column (so called rectification) in order to obtain azeotropic concentration of ethanol (96%) from diluted raw solution (initial concentration of ethanol does not exceed 10–15%, depending on the type of yeast used for fermentation).

It has been found that ethanol can be also produced from a lignocellulosic biomass, but important difficulties of cellulose hydrolysis and purification caused that this technology has not been fully commercialised yet [19].

As mentioned in Section 3.2.10, synthesis of ethanol for fuel industry requires a careful purification and additional dehydration to obtain min. 99.6% of the alcohol. The basic requirements set for fuel ethanol are summarised in Table 2.3.1.

Table 2.3.1

Requirements for bioethanol fuel [11]

Parameter	Unit	Range
Ethanol	Vol. %	99.6–99.8
Water	Mass %	0.3–0.4
Density at 20°C	g/cm^3	0.79–0.791
Colour Hazen	–	5
Refractive Index	–	1.3618–1.3626
Chloride	mg/l	max 20–40
Methanol	Mass %	0.1–0.2
Higher alcohols	Mass %	max 2
Acids as acetic acid	g/dm^3	max 0.03
Aldehydes & ketones	g/dm^3	max 0.2
Fusel oil/Amyl Alcohol	Vol. %	max 2
Phosphor	mg/kg	max 50

2.3.1. Experimental section

Objectives:

Transformation of starch hydrolysate to bioethanol by alcohol fermentation with the baker's yeast or distillery yeast. Distillation of the fermented solution on an efficient vigroux column or packed column. Dehydration of ethanol over calcined 3A molecular sieves or by reaction with magnesium fillings (optional). Analysis of alcohol concentration in collected distillate fractions and dehydrated samples by various available methods (measurement of density by pycnometric method, analysis of refractive index, gas chromatography).

Reagents and apparatus:

- potato or maize starch,
- bakery yeast or distillery yeast,
- *molecular sieves 3A dried at 350–375°C for 3 hours or magnesium fillings and iodine,
- vigroux or packed column for alcohol distillation,
- gas chromatograph equipped with FID detector, 1 m column packed with Chromosorb 101 heated to 130°C. Pure Helium under 1.1 bar pressure was used as a carrier gas.

Hydrolysis and fermentation of potato (corn) starch:

Weigh out about 75 g of potato starch (or respectively 100 g of maize starch) and thoroughly mix with cold water (100–150 cm^3). Pour the suspension into boiling water (about 0.7 dm^3) and boil with intensive stirring to obtain a homogenous mixture with the consistency of dense jelly. Take a 0.5 cm^3 sample, cool-down and test with drop of iodine solution (reference sample). After 5 minutes at 95°C, add dry or liquid alpha-amylase (1.3 g per kg of starch). Maintain the temperature for at least 1 h, stirring vigorously to avoid scorching. To observe the starch hydrolysis process, every 10–15 minutes take 0.5 cm^3 samples for analysis with iodine. At the end of the liquefaction process, *i.e.* with considerable starch degradation (colour change in the iodine test – see Section 3.1.6.2), decrease the temperature to 70°C and add the required amount of glucoamylase (1 g per kg of starch) to initiate the saccharification process. Continue heating and stirring until the degree of depolymerisation (DP) is max. 10–20 (at least 1 h). After this, slowly cool the mixture to 30°C, take a sample for analysis of the reducing sugars (Section 3.1.6.1) and inoculate the remaining mixture with dry spirit yeast (use 12.5 g per kg of starch). In the case of bakery yeast, use 35 g of dry yeast or 50 g of fresh yeast for each 3.5 kg of starch. Mix everything carefully, weigh, seal against water and ferment at 25–28°C until the end of CO_2 evolution (6–9 days). The outlet of the fermentation bottle should be connected with

a bubbler filled with a known amount of sodium or potassium hydroxide aqueous solution to allow the released CO_2 to be quantified (Section 3.3).

Deep hydrolysis of starch can also be achieved using concentrated H_2SO_4. For this purpose, make a paste of the starch and water (10 g per 50 cm^3) and heat to 80°C in a conical flask immersed in a water bath. Cool the mixture to about 50°C and carefully mix in 5 cm^3 of concentrated sulfuric acid (VI) up to reaching optimal pH 2. Place the flask in a water bath at 80°C and mix thoroughly to increase the temperature in the flask to 68–71°C within 2–3 minutes. Continue mixing and heating for a further 5 minutes, periodically sampling the solution for an iodine test. Cool the flask and neutralise with a 10% NaOH solution using a methyl orange indicator. The whole process should be undertaken very carefully, as a decrease in the amount of H_2SO_4 may result in the incomplete decomposition of the polysaccharides and oligosaccharides (mainly sucrose) into glucose and fructose. Prolonging the reaction time or raising the temperature may result in degradation of the hydrolysis products.

Alternatively, 10 cm^3 of 50% sulfuric acid solution can be taken for hydrolysis. This may require a prolonged process time, so the progress of the reaction should be checked using the iodine test. Solid calcium carbonate can be used for acid neutralisation – calculate the stoichiometric amount with respect to sulfuric acid (**CAUTION**! The solution may foam!). Then heat the solution to 80°C, filter and dilute to the desired volume (0.75 dm^3). Finally, inoculate with distillery or baker's yeast (see the proportions given above), weigh and complete the fermentation process.

Distillation and determination of alcohol concentration:

Prior to the distillation, weigh the flask with the digested mixture and determine the amount of CO_2 released by mass loss and titration of the alkali solution in the bubbler (see Section 3.3). Calculate the theoretical yield of fermentation on the basis of the released CO_2 and the amount of reductive sugars in the hydrolysed starch solution. Filter a small sample of the final solution and use to measure the ethanol concentration. Distil the remaining solution (packed column, vigroux or straight distillation), collecting four fractions: I – below 70°C; II – up to 78–80°C, III – up to maximum 90°C and IV – the residue. Allow the residue to cool, then weigh and filter a small sample for ethanol analysis.

Examine the density and refractive index of each fraction, and compare the results using GC chromatography (see Section 3.2.10).

Ethanol dehydration (optional):

Use two different types of sorbent for the dehydration of ethanol samples (3A, 4A, 5A or 13X molecular sieves, activated carbon) previously calcined

at 350–375°C for 3 hours (in the case of carbon samples, drying should take at least 5 hours at 150°C). Mix the alcohol samples of 20–50 cm^3 and 5–10 g of dried sorbent, seal and leave for a minimum of 24 hours. Verify the change in alcohol concentration by density analysis, refractive index measurement and gas chromatography. Determine the efficiency of water adsorption by each of the sorbents.

Alternatively, boil 200–500 cm^3 of alcohol with Mg filings (about 5 g of filings purified by rinsing with anhydrous acetone) and 0.5 g of iodine. Boil the mixture under reflux for 1 hour or longer (*i.e.* until the yellow colour disappears). Next change the reflux condenser for a straight one, and carry out a classical distillation process. Evaluate dehydration efficiency by the pycnometric method, chromatography and refractive index measurement.

Report details:

The report should include:

1) Short Introduction based on the actual literature.
2) Detailed description of the experiment with the results of the iodine tests.
3) Results of the reductive sugar concentration analysis in hydrolysate.
4) Calculation of the theoretical bioethanol yield assuming 100% starch hydrolysis and 100% sugar conversion.
5) Real mass balance on the basis of the released CO_2 amount.
6) Mass balance of the fermentation and distillation processes, calculated on the basis of the real ethanol concentrations in the final fractions.
7) Conclusions.
8) List of the literature.

2.4. Biomass pyrolysis and gasification

A high temperature conversion of biomass to gaseous and liquid chemicals can be achieved in two different ways. The first one is by gasification of the biomass in the presence of water vapour or air ($\lambda < 1$) at a temperature of about 600°C. The main product is a gas mixture, analogous to synthesis gas (Table 2.4.1). A similar gas composition, but with much lower yield, can be obtained during fast biomass pyrolysis, *i.e.* rapid conversion without oxidising agents ($\lambda = 0$). In this case the temperature is considerably higher (600–1000°C), promoting the formation of industrially valuable, high calorific liquid products (known as bio-oil) and some solid coke [20, 21].

Table 2.4.1

Comparison of typical gas composition obtained from different types of gasification and pyrolysis of biomass. Based on [20–23]

Process	Gasification			Pyrolysis
	with air	water	oxygen	(gas yield 10–30%)
Calorific value, MJ/m^3	3–6	12–14	13–20	5–11*
H_2, %	11–16	35–40	32	13–20
CO, %	13–18	25–30	48	37–38
CO_2, %	12–16	20–25	15	23–25
CH_4, %	3–6	9–11	2	7–14
CH, %	< 2	< 2	traces	19–25
N_2, %	45–60	< 1	3	< 1

* Unit MJ/kg.

On a laboratory-scale, both processes can be carried out periodically in a quartz tubular reactor. After initial outgassing in the inert gas flow, the temperature of the biomass fed is slowly or quickly raised to the selected temperature. The gasification reaction starts when water vapour or air under small partial pressure is introduced into the reaction zone, while pyrolysis occurs in the absence of oxidising agents.

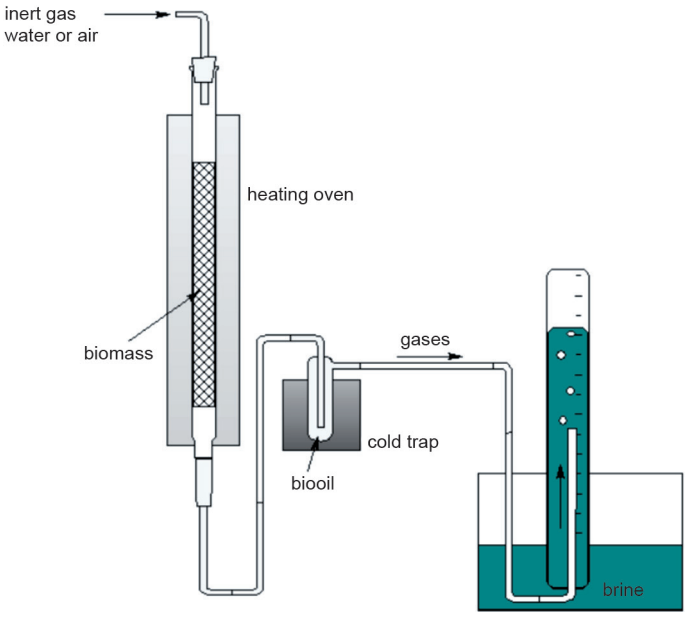


Figure 2.4.1. Schematic diagram of the biomass pyrolysis process or biomass gasification using laboratory-scale apparatus

Joining the reactor outlet to an external cooler (cold trap) enables the condensation of any liquid products, while the gases can be collected in a cylinder filled with brine. The solids remain in the quart reactor and can be weighed after cooling. A typical scheme for such apparatus is shown in Fig. 2.4.1.

2.4.1. Experimental section

Objectives:

Comparison of two processes carried out for the same biomass samples: fast or slow pyrolysis and gasification in the presence of water vapour. Calculation of each fraction yields. Qualitative and quantitative analysis of produced gas composition.

Reagents and apparatus:

- selected dried biomass: dry duckweed, hay, wood cuttings (sawdust), fallen leaves, *etc.*,
- heated quartz tube reactor connected with an inert gas source (nitrogen), cold trap (outlet) and measuring cylinder (250–500 cm^3) filled with brine (see Fig. 2.4.1),
- vials with a rubber stopper for collecting the gas samples,
- chromatograph equipped with TCD and FID detectors and a 2 m column packed with Porapak Q.

Characterisation and preparation of raw biomass material for pyrolysis or gasification:

Before using the biomass in the pyrolysis or gasification process, about 10–20 g should be carefully crushed, dried and tested for:

- dry matter content – see Section 3.1.1,
- ash content – see Section 3.1.2,
- fibre content – see Section 3.1.6.3.

Biomass pyrolysis and gasification:

Weigh a quartz tube having a 2 cm internal diameter and fill with a known amount of dried biomass that has been cut into small pieces. Install the reactor in the reactor furnace, in a vertical position, unless the biomass is powdered. In the latter case, the heating furnace and quartz tube should be positioned on a slope of approx. 30° (avoids the loss of solid matter during the reaction and allows the gravitational outflow of liquid products from the reaction zone). Degas the reactor feed in the inert gas flow (30 cm^3/min) for 30 minutes at 150°C. Then close the gas supply and connect the reactor outlet to the cold trap and gas cylinder filled with brine. Start the slow pyrolysis process by increasing the reactor temperature to 300–500°C

(temperature selected by the tutor). When the evolution of gasses has finished, note the volume collected in the cylinder, take the sample for analysis and begin cooling the reactor under the flow of nitrogen gas (10 cm^3/min). The reactor should only be opened with care, at a temperature below 100–120°C (**WORNING!** The solid residue remaining in the reactor zone should not contact air at higher temperatures! It could ignite!).

For rapid pyrolysis, after degassing the reactor feed, close the inert gas flow and carefully slide the quartz tube that removes the biomass above the furnace heating zone. Once the desired temperature is stabilised in the furnace, quickly slide the reactor tube with the biomass back into the heating zone. Ensure the correct connection of the outlet to the quenching bath and the brine cylinder. The other parts of the process are the same as for the slow pyrolysis process.

To carry out the biomass gasification process with water vapour, degas the reactor feed at 150°C, than reduce the inert gas flow to 10 cm^3/min and pass it through a heated bottle (40–80°C) with 50 cm^3 of water before introduction into the reaction zone (a bubbler placed before the reactor). The process should be carried out for at least one hour at a selected temperature in the range 250–600°C. Additionally, a reference test with an empty reactor should be carried out to determine the amount of excess water trapped in the cold trap and the volume of inert gas collected in the cylinder. These values should be excluded from the gasification process mass balance and taken into account during the qualitative and quantitative analyses of the gas.

Analysis of products yields and composition of produced gas:

Determine the yields of gas, liquid and coke remaining after each process. Analyse the gas composition by chromatography using both TCD and FID detection (Section 3.2.3).

Report details:

The report should include:

1) Short Introduction based on the actual literature.
2) Detailed description of each experiment.
3) Discussion and comparison of each process (yields of liquid, solid and solid products in respect to dry biomass feed, g/g and cm^3/g).
4) Results of chromatographic analyses (gas composition).
5) Conclusions.
6) List of literature.

2.5. Characterisation of biomass

From a chemical point of view, biomass is rich source of carbon, hydrogen and oxygen, accompanied by smaller amounts of nitrogen, sulphur and a number of other micro- and macroelements. Depending on its origin, biomass contains varying amounts of water as an inherent part of all living organisms, and also the proteins, fats and carbohydrates that are vital from a nutritional point of view. Moreover, smaller amounts exist of fibres (lignin), dyes, essential oils, active substances, vitamins and many other valuable compounds.

The preliminary characterisation of biomass usually starts from a visual assessment of its purity and quality, and also a percentage composition analysis of the water, ash, protein, sugars and fats. Then, each of the individual components are characterised and selected physical and chemical properties analysed. The type and number of tests depends on the intended use of the material, *e.g.* feed, food, pharmacy, cosmetics, chemical or energy, as well as technical purposes (building materials).

2.5.1. Experimental section

Objectives:

Determination of selected, basic properties of the biomass, especially fresh biomass intended for the biofuel or energy industry, as well as waste biomass.

Reagents and apparatus:

- any type of biomass with potential for biofuel application or as a waste material:
  a) fresh seeds or nuts of oil-rich crops (rape, colza, hemp, linseed, white or black mustard, coconut, sunflower seeds, pumpkin seeds, *etc.*),
  b) bagasse,
  c) starch-rich products,
  d) herbs,
  e) green biomass (*e.g.* grass),
  f) lignocellulosic biomass;
- set of basic laboratory glassware for filtering, titration, heating, *etc*.,
- dedicated apparatus for specific types of analyses.

Biomass characterisation:

For a chosen biomass analyse involving selected properties (according to the instructions in the guides):

- dry matter – see Section 3.1.1,
- ash content – see Section 3.1.2,
- optional analysis of selected macro-elements: phosphorus, calcium and magnesium (to analyse ash and phospholipids, see Sections 3.1.2 and 3.2.9.3 respectively),
- fats – see Section 3.1.3,
- volatiles (optional) – see Section 3.1.4,
- digestible proteins – see Section 3.1.5,,
- reducing sugars, starch and fibres – see Section 3.1.6,
- natural pigments – see Section 3.2.1.

Report details:

The report should include:

1) Short Introduction based on the actual literature.
2) Detailed description of completed experiments.
3) Analysis results – discussion of the qualitative and quantitative compositions.
4) Conclusions.
5) List of literature.

2.6. Natural oils and fats – obtaining and characterising

Among all the available oilseed crops, the most important are: rapeseed, soybean and sunflower, all harvested widely in EU, as well as the cotton, arachide, oil palm and coconut palm more abundant in the Americas and Oceania. Every year a list of traditional oils are cultivated for technical purposes, such as olive, sesame, rice or grape seed oils, which are enriched with less common, edible species (like cannabis or camelina) and numerous non-edible oil-plants, such as jatropha, karanja, algae or castor oil plant.

Other than speciality fats used for specific purposes irrespective of their price and yield, it is believed that it is only cost-effective to grow those plants with more than 15 wt.% oil content in the seeds (fruits). Moreover, in selecting the raw material for extrusion the moisture content should also be considered, with the ideal value lying between 5–7%. To increase the oil yield the preliminary drying, hydrating or preheating of the seeds might be required, or post-pressing oil-cake extraction with a non-polar solvent (extraction naphtha, hexane, *etc*.).

Crude oils contain a certain amount of free fatty acids and numerous non-glyceride impurities (dyes, proteins, phospholipids, *etc*.) which affect their taste, colour and oxidation stability. Most of these components are removed during refining, where the fat is treated with selected temperatures, water, sorbents and chemicals [24].

Similar stages also occur in the refining of some animal fats and waste fats from cooking, which from a chemical point of view may form a valuable raw material for the chemical industry, including biodiesel synthesis. See Fig. 2.6.1 for the most important steps in the processing of crude natural fats.

2.6.1. Experimental section

Objectives:

The extrusion and extraction of fat from selected oilseeds and the comparative characteristics of oils. Alternatively, comparison of different types of natural fats: vegetable oil, animal oil, fish, cooking fat (*e.g.* margarine), and waste oil.

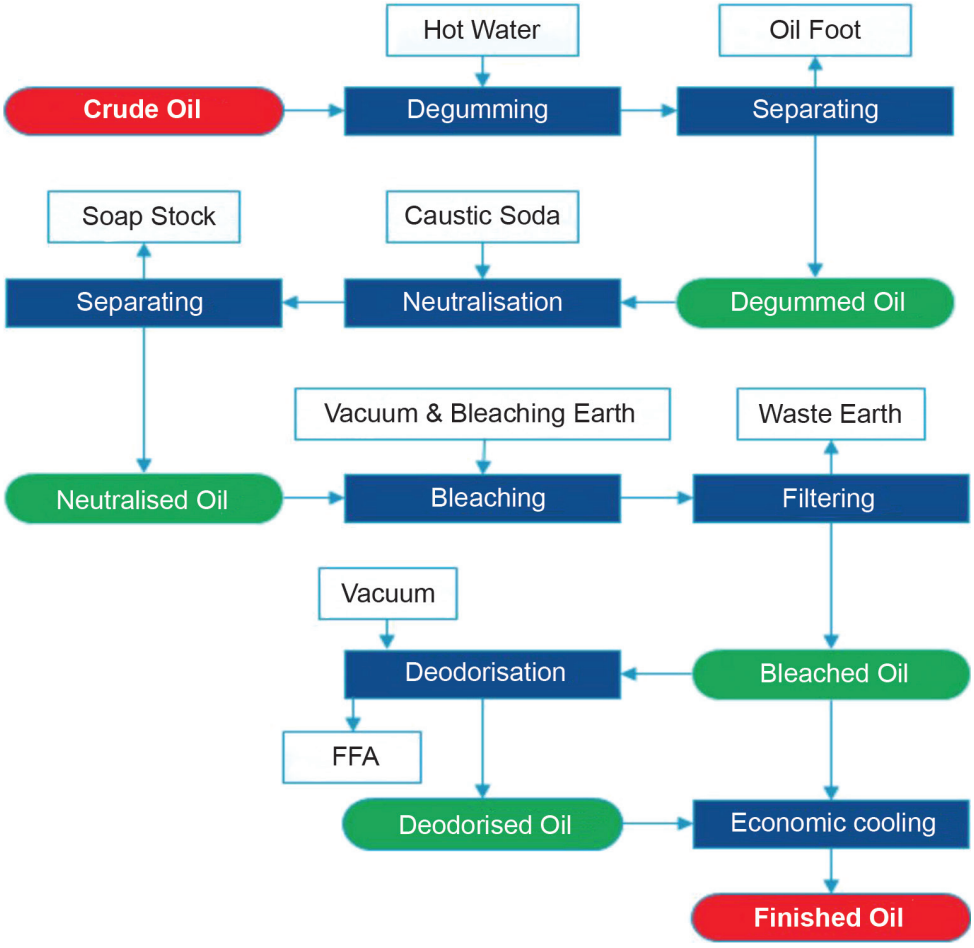


Figure 2.6.1. Main steps in natural fat refining [25]

Reagents and apparatus:

- selected oilseeds (rape, colza, hemp, linseed, white or black mustard, coconut, sunflower, pumpkin seeds, *etc*.) or any other fatty material indicated by the tutor,
- piteba manual extruder or automatic PS-10 apparatus (Fig. 2.6.2),
- a set of basic laboratory glass for extraction, filtration, titration, heating, *etc*.,
- a gas chromatograph equipped with an FID detector and a capillary column dedicated for fatty acid compositions and analysis of glycerides,
- apparatus for low and high temperature properties,
- equipment for density and viscosity measurement,
- spectrophotometer with glass and quartz cuvettes.

Figure 2.6.2. Two types of laboratory-scale oil extruders – from the left: Piteba hand--press, mechanical PS-10 extruder

Obtaining vegetable oil by pressing and extraction:

For the selected raw material, determine the moisture (Section 3.1.1) and fat contents by the Soxhlet extraction method (Section 3.1.3). Adjust the dimensions and the humidity of the raw material as required, and use one of the two extruders to press out the fat.

WORNING! For fine, hard material with a relatively low fat content, use the PS-10 automatic press. For very soft grains (sunflower, pumpkin, peanut, etc.) use the hand press. Evaluate the yields on the basis of the mass balance of the initial material, pomace (oil-cake), and oil amount (after filtering).

Crush and place the material in the extraction thimble, add solvent and allow to stand for several hours. Now run the Soxhlet extraction process for at least 6 hours. Distil the solvent using a rotary evaporator and leave the open extraction

thimble under a fume hood. Calculate the extraction efficiency based on the mass balance.

Animal raw material should be cut into small pieces, rendered and decanted (or hot filtered) to remove any tissue residues. Calculate the yield based on the mass balance.

Waste cooking fat should be subjected to a careful visual assessment. If turbidity is observed, increase the temperature to 60–80°C. If it foams (indicating the presence of water), gently warm it to 110–120°C. Maintain the temperature until all the water has evaporated. Filter while hot if necessary.

Comparative characterisation of fats:

After the preliminary visual and sensory evaluations, perform further analysis:

- total colour by Hazen or iodine scale and spectrophotometric evaluation of natural pigments (chlorophyll and carotenoids) – see Section 3.2.1 for a full description,
- density measurement – see Section 3.2.2,
- viscosity measurement – see Section 3.2.3,
- cloud point and freezing temperature – see Section 3.2.4.1,
- smoke point and flash point in the open crucible – see Section 3.2.5.1,
- selected characteristic numbers – see Section 3.2.7,
- oxidative stability – see Section 3.2.8.4 (if rancid fat is used, identify the oxidised derivatives – see Section 3.2.8),
- fatty acid composition by chromatography – see Section 3.2.9.1,
- optionally examine the phospholipids, sterols and partially hydrolysed glyceride content – see Section 3.2.9.

Report details:

The report should include:

1) Short Introduction based on the actual literature.
2) Detailed description of the experiment with necessary calculations.
3) Comparison of the results in respect to the literature and official restrictions for edible oils.
4) Conclusions.
5) List of the literature.

3. Manuals for selected analyses

3.1. Selected analyses for solid samples

3.1.1. Dry mass (humidity)

Water content in materials not containing essential oils or volatile substances is usually determined using the gravimetric method, by analysing the loss of mass in material dried under specific circumstances, usually at a temperature of about 100°C, until a constant weight of the sample is reached. Depending on the sample mass, units of g or kg are used, or the result is given in % m/m.

Determining the mass of dry matter is often equivalent to determining water (moisture) content in solid samples, *e.g.* seeds, plant parts, peats, *etc*. In this case, moisture content is given as the complement of the dry matter assay result to 100%. Dry matter assay is of great importance especially in biomass combustion and co--combustion, where it is one of the basic parameters determining a fuel's suitability for power generation purposes. Too much water content lowers the fuel's calorific value, increases the exhaust gas volume, and contributes to lowering the process temperature, thereby hindering complete fuel burning [26].

Drying temperature is selected depending on the nature of the substance tested to eliminate any potentially adverse changes in the material. For example: if there are no volatile substances (*e.g.* essential oils or decomposing chlorides) other than moisture, drying is carried out at a temperature of 100–105°C. For resistant materials, temperatures up to 200°C can be utilised, while drying delicate materials is best done with forced air circulation and at a reduced temperature [27].

Remember that long drying of plant samples at elevated temperatures may cause adverse oxidative changes, *e.g.* the mass of oil plant seeds may rapidly increase following an initial reduction, due to oxidation of fatty components, while drying material rich in vitamins and other active ingredients may substantially reduce the active ingredient content. Thus in many cases, the most beneficial is to remove water through lyophilisation, which prevents fats and most proteins from decomposing (so called sublimation drying [27]). For materials containing highly volatile substances, *e.g.* essential oils, water content is assayed using the volumetric method, by azeotropic distillation with organic solvents, such as toluene or xylene. The assay is carried out in a typical azeotropic distillation apparatus, fitted with a calibrated receiver, used for reading the volume of water distilled from a weighed amount of material [28].

Performing the assay – samples without volatile component content:

Pour a thin layer of fragmented material (about 1–2 g) into a shallow weighing vessel, weigh on an analytical balance and place in the laboratory dryer chamber (105°C) for approx. 3–4 hours. To accelerate drying, shake the vessel's content periodically or use forced air circulation in the dryer. After this duration, move the vessel with its content to a desiccator, cool, and weigh again.

ATTENTION! Do not place hot objects on the balance.

Write down the mass and place the vessel with its content in the dryer again. After another hour, cool and weigh the sample again. Repeat the above steps until so called constant mass is achieved, *i.e.* until the difference in mass between two successive weighings is no greater than 0.0005 g.

Calculate dry matter content (*DM*) using formula (3.1.1.1), while moisture content (*H*) using formula (3.1.1.2):

$$DM,\ \%\ \text{mas.} = 100 \cdot \frac{m_1}{m_0} \tag{3.1.1.1}$$

$$H,\ \%\ \text{mas.} = 100 - DM = 100 \cdot \frac{m_0 - m_1}{m_0} \tag{3.1.1.2}$$

where:

m_0 – mass of fragmented material before drying (without mass of vessel), g,
m_1 – mass of material after drying – constant mass (without mass of vessel), g.

Performing the assay – samples containing essential oils or volatile substances:

Place the fragmented material (approx. 10–20 g) in a 500 cm^3 round-bottom flask, forming part of an azeotropic distillation set, Deryng apparatus, or Aufhauser apparatus (Figure 3.1.1.1). Add 250 cm^3 toluene or xylene to the flask and maintain boiling until water is no longer being released.

Read the amount of water released on the graduation and calculate to mass using formula (3.1.1.3), and calculate water content in the material using equation (3.1.1.4):

$$m_{\text{water}},\ \text{g} = V_{\text{water}} \cdot \rho_{\text{water}} \tag{3.1.1.3}$$

$$H,\ \%\ \text{mas.} = 100 \cdot \frac{m_{\text{water}}}{m_{\text{sample}}} \tag{3.1.1.4}$$

where:

V_{water} – water volume read from graduation, cm^3,
ρ_{water} – water density at the ambient temperature, g/cm^3 (find the value in the tables at the end of the handbook),
m_{sample} – mass of the material, g.

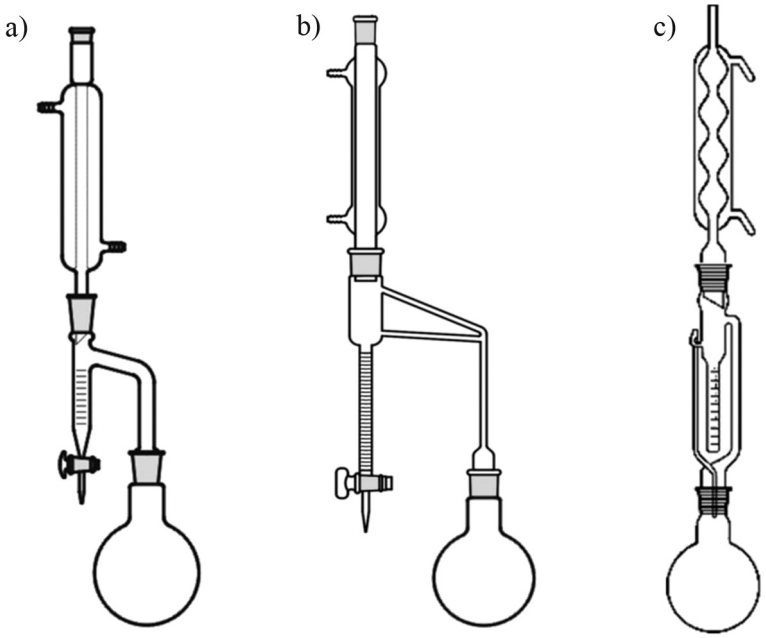


Figure 3.1.1.1. Azeotropic distillation equipment set according to Dean-Stark (a), Deryng apparatus (b), Aufhauser apparatus (c)

3.1.2. Ash (crude, water and acid insoluble, sulfated, analysis of composition)

One indicator of the quality of energy resources, both traditional (coal) and bioresources (brown coal, peat, lignocellulose biomass), is ash content and element composition assay. It must be stressed that the nature of ash from biomass combustion and co-combustion is radically different from coal combustion ash. The presence of significant amounts of alkali metals (mainly sodium and potassium) reduces ash melting point, which fosters quick growth of ash deposits on boiler walls and reduces its efficiency [26].

In the case of food and pharmacopoeian materials, this assay enables determining the content of mineral compounds in plant material, which depends on the concentration of these substances in the soil, groundwater, fertilisers and plant protection products, and – if heavy metals are present – is an indication of environmental pollution in the area where the plant was grown [28, 29]. Furthermore, differences in ash content and composition enable determining the quality of the material (*i.e.* detecting accidental mineral pollutants, e.g. sand, that get into the material during harvest and drying), as well as revealing intentional counterfeiting of herbal products with materials of different origin or nature (varieties, leaves,

stems) [30]. It bears stressing that the composition and quantities of mineral substances in materials of animal origin are variable to a much lesser degree than in plant material and are mainly dependant on the availability of these elements in the feed, as well as on the species, physiological condition and age of the animal. A number of elements present in food may also originate from the equipment used during the production process and from food packaging, or may be intentionally added to the end product (preservatives, pigments, *etc*.).

Complete characteristics of ash are gathered in multiple stages by assaying:

a) general (total) ash content remaining after sample mineralisation under such conditions that chloride decomposition and chlorine oxidation do not occur – dry (incineration) or wet (mineralisation using mineral acids with oxidants in special microwave ovens or Kjeldahl flasks) method;
b) physical ash content, *i.e.* ash soluble in 10% hydrochloric acid, recommended for testing of herbs, cereals, cereal preserves, teas, fruits, vegetables;
c) water-soluble ash content, which is done by extracting raw ash with hot water and assaying the loss of mass. Sodium and potassium salts, chlorides, and carbonates are dissolved under such conditions, and the assay is an indicator of, for example, fruit content in preserved jams and marmalades;
d) ash reaction, which is determined based on the amount of volumetric acid or base solution necessary to neutralise the ash dissolved in a known volume of acid. The ash reaction is important mainly in testing food counterfeiting with mineral compounds;
e) content of individual mineral components – titration, precipitation, or spectrophotometric methods.

Certain sources (*e.g.* Pharmacopoeias) recommend sulfated ash assay instead of raw ash assay, *i.e.* residue from roasting a material after adding concentrated sulfuric acid. Alkali metal sulfates formed under such conditions possess much greater thermal stability compared to their corresponding carbonate analogues [28].

Performing the assay – dry assay of total (raw) ash:

Pour a thin layer of fragmented material in a porcelain crucible or a porcelain evaporating dish, weigh it on an analytical balance and place it in the muffle furnace chamber for 5–6 hours. For materials rich in moisture and volatile substances, pre-dry the material at 105°C to constant mass. To ensure complete incineration of the material, the organic material can be pre-charred by holding the open crucible on a heating machine or burner (**CAREFUL!** Work under a fume hood, do not allow the material to ignite), or by using gentle forced circulation of air in the furnace while gradually increasing the temperature. The recommended calcination conditions and the amount of sample for analysis are summarised

in Table 3.1.2.1. In extreme situations, mineralisation with selected mineral acid can be applied (only with your tutor's assistance).

Once incineration is complete, carefully move the crucible or evaporation dish onto a wire mesh and after cooling slightly (about 10 minutes), place it in a desiccator until it cools completely. Weight it on an analytical balance. The end of incineration is most commonly recognised by the white or grey colour of ash. If the ash contains visible non-incinerated sections of the sample or lumps, stir the ash lightly with a wire, then place it again in the furnace (650°C) for about 0.5–1 h. After cooling, weight it again to determine the constant mass.

Calculate total ash content in solid materials (Ash_C) is using formula (3.1.2.1), or formula (3.1.2.2) for moist materials and materials dried before incineration:

$$\mathrm{Ash}_C,\ \%\ \mathrm{mas.} = 100 \cdot \frac{m_2}{m_1} \tag{3.1.2.1}$$

$$\mathrm{Ash}_C,\ \%\ \mathrm{mas.} = 100 \cdot \frac{m_2}{m_0} \cdot \frac{100}{DM} \tag{3.1.2.2}$$

where:

m_1 – mass of dry matter of the material before incineration (without mass of vessel), g,
m_0 – mass of fragmented material before drying (without mass of vessel), g,
m_2 – raw ash mass (without mass of vessel), g,
DM – dry matter, % (as per formula 3.1.1.1).

Table 3.1.2.1

Recommended incineration conditions for selected biomaterials and solid fuels*

Sample	Sample mass	Incineration temperature, °C
Carbon black, black coal, graphite	2 g	500°C 30 min, 925°C 2 h
Brown coal, peat, pellet	1–2 g	815°C 1 h
Dry lignocelulose (wood)	5 g	300°C 1 h with air, 600–650°C 1 h
Dry plants (herbs)	5 g	525–550°C 2–3 h
Crushed oilseeds	3–5 g	105°C for 4–6h, 300°C 1 h with air, 650°C 4 h with air
Plants with high water content	5–10	drying at 105°C to obtain a dry mass, 300°C 1–2 h with air, 600–650°C 4 h

* All materials should be adequately fragmented before they are placed in the muffle furnace. Temperatures and weighed amounts selected based on [31, 32] and own research.

As stated in European Union Directive [33], ash of substances difficult to incinerate should be pre-incinerated for at least 3 hours, then cooled, and a few

drops of 20% ammonium nitrate should be added, while avoiding any scattering or agglomerating of the sample. After drying in a furnace, continue calcination until dry matter is obtained. If required, repeat the operation to effect complete incineration. In the case of oils and fats, accurately weigh a sample of approx. 25 g and incinerate it, igniting it together with a strip of ashless filter paper. Once burned down, moisten with a small amount of water and calcinate.

Water-soluble and -insoluble ash assay:

For this assay, use a weighed amount of ash obtained in accordance with the method above. Add 20 cm^3 of distilled water to a crucible and heat almost until boiling (15 min), then filter through medium ashless filter paper. Wash bot the crucible and the filter paper containing the precipitate with hot distilled water (40–50 cm^3). Move the filter paper to a crucible, carefully evaporate on water bath, then dry and roast at 525°C (1–2 h). After cooling, weigh the crucible and place it in the furnace for another 0.5 h. Repeat the operation until constant mass is achieved (difference between consecutive weighings no greater than 0.001 g). Keep the filtrate for ash alkalinity determination. Calculate water-insoluble ash content (Ash_{WI}) in relation to the dry matter mass of the material using formula (3.1.2.3), while water-soluble ash content (Ash_{WS}) is calculated using formula (3.1.2.4):

$$\mathrm{Ash}_{WI},\ \%\ \mathrm{mas.} = 100 \cdot \frac{m_3}{m_0} \cdot \frac{100}{DM} \tag{3.1.2.3}$$

$$\mathrm{Ash}_{WS},\ \%\ \mathrm{mas.} = 100 \cdot \frac{m_2 - m_3}{m_0} \cdot \frac{100}{DM} \tag{3.1.2.4}$$

where:

m_3 – water-insoluble ash mass (without mass of vessel), g,
m_0, m_1, m_2, DM – as in formulae (3.1.2.1 and 3.1.2.2).

Water-soluble ash alkalinity assay:

Cool the filtrate and titrate with a solution of approx. 0.1 N hydrochloric acid in the presence of methyl orange as an indicator. Alkalinity expressed in milliequivalents per 100 g of sample, calculated to dry matter, is calculated using formula (3.1.2.5), while alkalinity expressed in weight percentage, calculated to potassium hydroxide, is calculated using formula (3.1.2.6):

$$Y_{\mathrm{alk1}},\ \mathrm{meq/100\ g} = V \cdot C \cdot \frac{100}{m_0} \cdot \frac{100}{DM} \tag{3.1.2.5}$$

$$Y_{\text{alk2}}, \%\ \text{mas. KOH/g} = \frac{56 \cdot V \cdot C}{1000} \cdot \frac{100}{m_0} \cdot \frac{100}{DM} \tag{3.1.2.6}$$

where:

C – actual concentration of acid used for titration, mol/dm^3,
V – volume of acid used for titration, cm^3,
m_0, DM – as in formulae (3.1.2.1 and 3.1.2.2).

Hydrochloric acid-soluble and -insoluble ash assay:

Use raw or water-insoluble ash for the assay. Add 20 cm^3 of concentrated hydrochloric acid, diluted beforehand with water (1 : 9 v/v), to a crucible with ash, cover with a clock glass to avoid splashes, and heat to boil for 10 minutes. Leave to cool, filter through medium ashless filter paper, then wash the crucible and filter paper with precipitate using hot distilled water until reaction to chloride ions ceases (silver nitrate test). Move the filter paper with residue to a crucible, carefully evaporate on water bath, then dry and roast at 525°C (1–2 h). Subsequent steps are identical as in the water-insoluble ash assay. Calculate the acid-insoluble ash content (Ash_{AI}) relative to dry matter mass of the material using formula (3.1.2.7):

$$\text{Ash}_{AI}, \%\ \text{mas.} = 100 \cdot \frac{m_4}{m_0} \cdot \frac{100}{DM} \tag{3.1.2.7}$$

where:

m_4 – acid-insoluble ash mass (without mass of vessel), g,
m_0, DM – as in formulae (3.1.2.1 and 3.1.2.2).

Sulfated ash assay:

Dry the fragmented material in a crucible at 105°C to constant mass, then moisten with a small amount of concentrated sulfuric acid (usually 1 cm^3) and heat until complete charring of organic substances. After cooling, moisten the residue again with sulfuric acid (1 cm^3) and heat until white fumes are no longer produced. Roast the residue at a temperature of 550–650°C until the substance is incinerated. After cooling, weigh it and place for 0.5 h into a furnace. Repeat the heating and cooling steps until constant mass is achieved. Sulfated ash is calculated relative to dry matter using the following formula:

$$\text{Ash}_{S}, \%\ \text{mas.} = 100 \frac{m_5}{m_0} \cdot \frac{100}{DM} \tag{3.1.2.8}$$

where:

m_5 – sulfated ash mass (without mass of vessel), g,
m_0, DM – as in formulae (3.1.2.1 and 3.1.2.2).

Assay of Ca and Mg content in ash:

Quantitative and qualitative assay of ash components can be done both for raw ash (*e.g.* XPS), ash obtained through "wet" mineralisation (ICP), and for water or acid extracts from raw ash (ASA and other spectroscopic, gravimetric, or titrating methods).

The most important agents used in "wet" mineralisation include aqua regia, individual concentrated acids or mixtures thereof, and hydrogen peroxide solution. Papers by [34, 35] include a review of methods dedicated for various sample types, while the most extensive collection of procedures has been published as industry standards [36] and on the EPA website (United States Environmental Protection Agency) [37].

To prepare an ash extract, moisten the evaporation dish with ash using distilled water, then add 10 cm^3 of 10% hydrochloric acid and cover it with a clock glass. When decomposition of carbonates, observed as foaming of the mixture, is over, remove the clock glass, washing it with distilled water, and evaporate the content of the dish until dry. Next, add 15 cm^3 of 1 M HNO_3 to the residue, heat on water bath until the precipitate dissolves, make up to 100 cm^3 with distilled water and filter to a fresh flask.

For small extract samples, perform qualitative analysis following the methodology familiar from analytical chemistry – for example:

- solution containing K^+ – in the presence of sodium nitrite cobaltate, turbidity or yellow precipitate appears;
- Ca^{2+} cations – white precipitate or turbidity appears when solution alkalised with ammonia is heated in the presence of ammonium oxalate;
- Fe^{3+} – red colour appears in the presence of ammonium thiocyanate;
- PO_4^{3-} – in the presence of ammonium molybdate, yellow turbidity appears in a hot solution of phosphates acidified with nitric acid;
- SO_4^{2-} – white precipitate (turbidity) appears after adding a barium chloride solution;

If required, perform quantitative analysis of calcium ions (method I), or calcium and magnesium ions (method II).

Method I – to 25 cm^3 of clear ash extract, add 15 cm^3 of 30% ammonium citrate, which forms soluble complexes with iron and aluminium and prevents their precipitation together with calcium. Next, alkalise the solution with 2–4 cm^3 of concentrated ammonia, heat to boil, remove the heat source and precipitate calcium by adding 20 cm^3 of saturated ammonium oxalate solution. Leave the contents of the beaker until the precipitate settles, then filter the precipitate, wash it using water with added ammonium oxalate and subsequently with just water, move the precipitate to a beaker with 20 cm^3 of water and decompose it, adding

concentrated sulfuric acid by the drop (until the precipitate dissolves completely). After heating, titrate the hot solution with 0.1 N $KMnO_4$ until pink colour remains for about 1 minute. When doing calculations, take into account that 1 cm^3 of 0.1 N $KMnO_4$ corresponds to 2 mg Ca or 2.8 mg CaO. Give the result in percent by mass.

Method II – the assay is done in two steps. During step one, the total content of calcium and magnesium is determined by using an EDTA solution to titrate about 20–25 cm^3 of ash extract (V_{sample}) mixed with 25 cm^3 of ammonium buffer and 50 cm^3 of water, heated to 60°C, and with a pinch of eriochrome black T ground with NaCl (red/violet colour changes to blue). The total number of moles of calcium and magnesium cations in the sample volume is calculated using the following formula:

$$n_{EDTA(1)},\ \text{mmol} = n_{Ca} + n_{Mg} = V_{EDTA(1)} \cdot C_{EDTA} \tag{3.1.2.9}$$

where:

$V_{EDTA(1)}$, C_{EDTA} – volume (cm^3) and concentration (mol/dm^3) of the EDTA solution, respectively.

During the second assay, only calcium is determined by titrating the same volume of extract (V_{sample}) with a volumetric solution of EDTA in the presence of 10 cm^3 of 2 M NaOH, 60–70 cm^3 of distilled water, and a pinch of sodium salt of calcocarboxylic acid (ground with NaCl). Under such conditions, the red and violet colour turns in the presence of calcium into a pink-violet and blue one, and magnesium hydroxides present at high pH do not affect the assay result. Alternatively, murexide can be used, in which case the colour will turn from pink to violet. The number of mmoles of Mg^{2+} is determined as a difference between the results of the two titrations, according to the following equations:

$$n_{EDTA(2)} = n_{Ca},\ \text{mmol} = V_{EDTA(2)} \cdot C_{EDTA} \tag{3.1.2.10}$$

$$n_{Mg},\ \text{mmol} = n_{EDTA(1)} - n_{EDTA(2)} \tag{3.1.2.11}$$

while the percentage content of calcium and magnesium (Ash_{CaO} and Ash_{MgO}) in raw ash is calculated using formulae (3.1.2.12) and (3.1.2.13), with the result calculated to calcium and magnesium cations or CaO and MgO:

$$Ash_{CaO},\ \%\ \text{mas.} = 100\frac{10\,000\ n_{Ca}}{V_{sample}} \cdot \frac{M_{CaO}}{m_2} \tag{3.1.2.12}$$

$$Ash_{MgO},\ \%\ \text{mas.} = 100\frac{10\,000\ n_{Mg}}{V_{sample}} \cdot \frac{M_{MgO}}{m_2} \tag{3.1.2.13}$$

where:

n_{Mg}, n_{Ca} – number of mmoles of calcium and magnesium from formulae (3.1.2.10) and (3.1.2.11),

V_{sample} – sample volume (cm^3) taken from 100 cm^3 of ash extract for the titration,

m_2 – mass of ash used to prepare the extract,

M_{MgO}, M_{CaO} – mole mass of calcium and magnesium oxides.

3.1.3. Fats

Lipids are among the basic construction blocks of living organisms, and also constitute a basic nutrient, providing energy and participating in the synthesis of many other important compounds. It bears stressing that under current legal regulations, food producers are obligated to state the content of fat per food portion [2, 3], and raw fat content determination in oil plant seeds is one of the most essential assays in the cooking oil industry. High quality oils for nutrition purposes are most commonly produced by pressing (cold or hot) and rendering, applied mainly to oilseeds and animal fats, respectively. Both these methods enable obtaining high quality products valued by consumers, but they do not allow for complete removal of the lipophilic fraction from the material, and neither are they suitable for emulsions (*e.g.* dairy products) or materials with low fat content and sensitive to high temperatures. In such cases, extraction, centrifuging, or a combination thereof is performed. It must be stressed that in addition to glycerol and fatty acid esters (proper fats), a lipid fraction obtained this way also contains free fatty acids, waxes, phospholipids, cerebrosides, sulfolipids, steroids, higher alcohols, and many other lipophilic organic substances, including oil-soluble vitamins and essential oils. For example, fat from carrot seeds contains up to 47% m/m of essential oil [38].

Most fat content assays are based on extraction (*e.g.* by Soxhlet method) of fragmented material using low-boiling organic solvents, such as hexane, petroleum ether, ethyl ether, toluene, petroleum, *etc.* [39–43]. Acid or base is usually added to liquid materials and emulsions (*e.g.* dairy products), which are then extracted using two solvents of different chemical natures and limited miscibility [44]. The result is read gravimetrically after distilling the solvent away. A similar method of assaying fat content in emulsive or high-protein food products (milk, cheese, meat, powdered milk) is Gerber method, employing calibrated butyrometers. Fat content determination by chromatographic methods [45] and non-destructive methods, such as NMR [46], FT-IR [47] or NIR [48], is also suggested in literature, but these methods are usually recommended for routine tests and require appropriate calibration.

Raw fat assay by Soxhlet method:

Place fragmented and dried material of known mass in the extraction thimble of the Soxhlet extractor, filling it to 2/3 of its height. Very moist materials can be mixed with anhydrous sodium sulfate at a mass ratio of 1 : 1. Weigh a round-bottom flask with several boiling chips and fill with hexane or low-boiling petroleum ether. The solvent amount must be selected so it suffices to fill the Soxhlet chamber and 1/3 of the round-bottom flask volume at the same time. After connecting water to the condenser, begin heating. Adjust the heating mantle setting so that one overflow occurs every 5–10 minutes. When extraction is complete (3–6 hours, depending on the amount and fragmentation of the material), cool the system and evaporate most of the solvent away from the flask, minding not to burn the fat. Including an additional adapter to the system, a Soxhlet adapter with tap or a separatory funnel with ground glass joint, allows for the solvent to be distilled away almost completely without the need to disassemble the set (Figure 3.1.3.1).

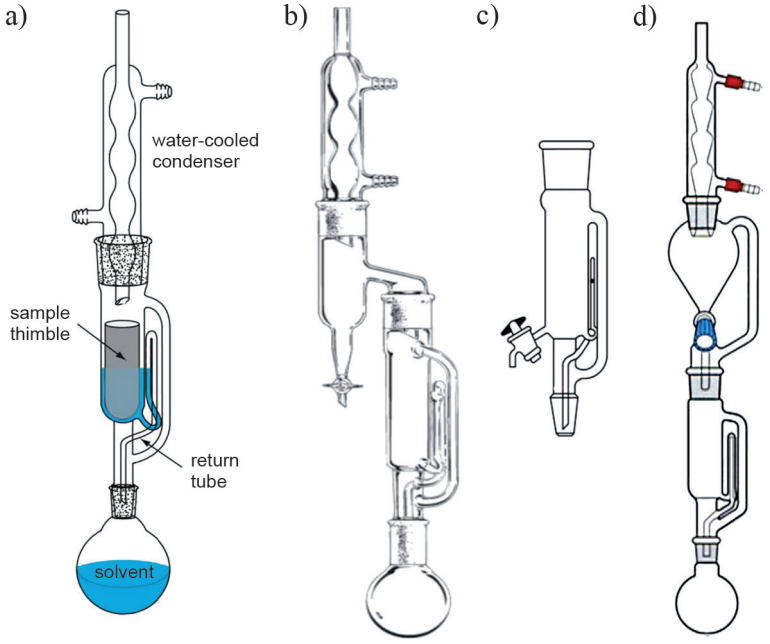


Figure 3.1.3.1. Soxhlet distillation equipment set. Standard apparatus (a), apparatus with a Dean--Stark adapter (b), Soxhlet apparatus with a tap (c), apparatus with a separatory funnel (d)

Evaporate away the remaining solvent on a rotary evaporator. Cool and weigh the flask with an accuracy to 1 mg. Alternatively, cool and weigh the thimble with the defatted material. Calculate fat content in the material using formula (3.1.3.1) or (3.1.3.2):

$$\text{Fat, \% mas.} = 100\frac{m_6}{m_0} \tag{3.1.3.1}$$

$$\text{Fat, \% mas.} = 100\frac{m_0 - m_7}{m_0} \tag{3.1.3.2}$$

where:

m_6 – mass of fat in the flask (without mass of flask and chips), g,
m_7 – mass of material after extraction (without mass of thimble), g,
m_0 – mass of material used for extraction, g.

Fat content assay – simplified method:

If we do not have a sufficiently large sample for testing (approx. 1–2 g), the fat content assay can be performed in a simplified manner: place the weighed amount in a pre-weighed tea bag and carefully tie it up with a thread (Figure 3.1.3.2). Bags can be placed in a Soxhlet apparatus, to be subjected to standard extraction, or fully immersed in a selected solvent in a round-bottom flask and kept boiling gently under a reflux condenser for about 2–2.5 h. After this time, carefully pour away the solvent, pour a fresh portion of the solvent to immerse the samples and boil again for 1–1.5 h. When the flask contents cool, wash the bags with acetone and dry them (under a fume hood or in a dryer with forced air circulation at 60–70°C) to constant mass. Weigh the dried bags again and calculate the fat content using formula (3.1.3.2), where m_0 and m_7 correspond to the mass of material before and after extraction, after subtracting the mass of bag and thread.

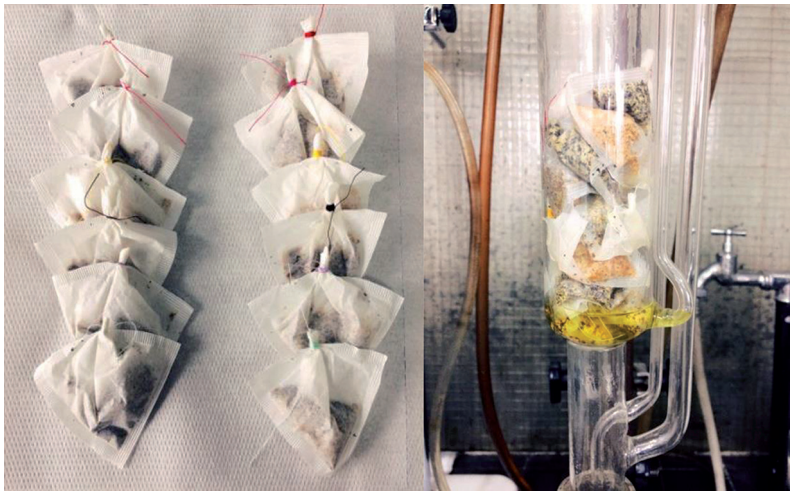


Figure 3.1.3.2. Tea bags with ground seeds of oil plants, intended for extraction in Soxhlet apparatus. Image by Noemie Brun

3.1.4. Volatile components and essential oils

Determining the content of volatile components, *i.e.* ones decomposing at high temperatures without air access, is one of the more important assays in heat generation industry, counted among essential technical assays of solid biofuels. Together with moisture, ash, combustion heat and calorific value assays, it provides basic information about the structure and functional properties of the given biofuel. Unlike moisture and ash content values, which measure the amount of dead weight in a fuel sample, volatile component assay determines the degree of coalification, which the functional value of the given material depends on [49]. Moreover, it provides information about the potential yield from coking, and determines the change of combustion conditions in furnaces and boilers for maximising the thermal energy yield. As is known, fuels with high volatiles content require feeding additional amounts of air into the combustion chamber to ensure smokeless and complete combustion of the entire charge [49]. For most natural biofuels with low coalification levels (wood, straw, etc.), the volatiles content reaches 70–80%, while the lowest volatiles content is found in coke coal and anthracite (13–35% and 2–10%, respectively) [50].

According to national standards, the volatile part is defined as percentage loss of mass by an analytical sample of solid fuel during heating without air access (porcelain crucible with cover) for exactly 7 minutes at a temperature of 850°C in a vessel of standardised shape. The mass loss value is reduced by the percentage content of moisture, according to the following formula [49]:

$$V_{\text{volatile}},\ \%\ \text{mas.} = 100\frac{\Delta m}{m_0} - H \tag{3.1.4.1}$$

where:

V_{volatile} – amount of volatiles in the analytical sample, %,
Δm – loss of mass during roasting, g,
m_0 – mass of weighed amount of sample, g,
H – moisture content, %.

The volatiles content, calculated to dry and ashless matter (V_{DAF}, %), is an approximate indicator of the level of coalification of the given natural fuel:

$$V_{DAF},\ \%\ \text{mas.} = V_{\text{volatile}} \cdot \frac{100}{100 - (H + \text{Ash})} \tag{3.1.4.2}$$

where:

Ash, % – ash content in the sample.

For plant materials, a major part of volatiles are essential oils, which are obtained from plant material primarily through extraction with low-boiling organic

compounds, such as ethyl ether, methylene chloride, acetone, *etc*. After stripping the solvent, a solid or semi-liquid residue (concrete), which – after purification with cooled ethanol (removal of precipitated resins, fats, *etc*.) – is used directly in cosmetic products as so called absolute. A much simpler method is expression of natural materials, mainly citruses, as well as maceration with solid or semi-solid fats, which enables obtaining so called enfleurage from very delicate material with low oil content. A good quality and efficiency of essential oil production is achieved through steam distillation (Figure 3.1.4.1), frequently supplemented with additional extraction of the resulting distillate using volatile solvents. Decidedly the purest oils

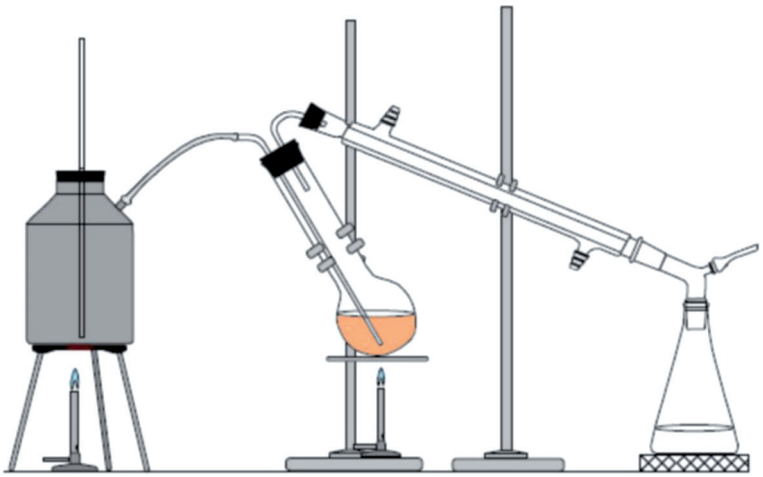


Figure 3.1.4.1. Typical equipment set for steam distillation, comprising from the left: steam generator, heated test tube with the material, standard condenser, and receiver

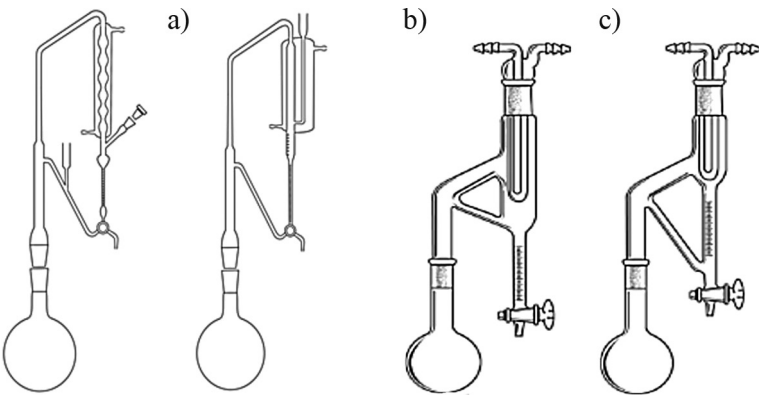


Figure 3.1.4.2. Sample Deryng apparatus with various condenser types (a) and Clevenger apparatus designed for distillation of oils heavier (b) and lighter (c) than water

are obtained through hydrodistillation in Deryng or Clevenger apparatus, whose special design enables obtaining oils with densities both higher and lower than water (Figure 3.1.4.2).

A separate and broad subject is qualitative analysis and investigation of essential oil identity, as they can contain several tens or hundreds of different chemical compounds. The simplest methods are based on measuring the refractive index of light, while chromatographic analysis can provide more detailed information.

Isolating of essential oils using the hydrodistillation method:

Place fragmented material of known mass in a round-bottom flask of a Deryng of Clevenger apparatus and immerse it in such amount of distilled water so as to cover the top of the material (about 2/3 of height). After putting a few boiling chips in the flask and connecting water to the condenser (or the cold finger of a Clevenger apparatus), begin heating. Adjust the heating mantle setting so that the mixture is kept in a gentle boil for at least 2–3 hours or until oil is no longer released, read the amount of oil from the graduation on the equipment and move it to a dry bottle of known mass and weigh it. Hydrodistillation efficiency, equivalent to oil content in the material, can be calculated using equation (3.1.4.3) or formula (3.1.4.4), if oil density at ambient (room) temperature T is known.

$$\text{Essential Oil, \% mas.} = 100\frac{m_8}{m_0} \tag{3.1.4.3}$$

$$\text{Essential Oil, \% mas.} = 100\frac{V_8 \cdot \rho}{m_0} \tag{3.1.4.4}$$

where:

m_8 – distilled oil mass (without mass of vessel), g,
V_8 – volume of distilled essential oil at temperature T, cm^3,
ρ – density of the essential oil at temperature T, g/cm^3,
m_0 – mass of material used for hydrodistillation, g.

3.1.5. Proteins

Proteins belong to another, after fats, group of components of great nutritional importance, and information about their content in food products must also be given on the packaging. Protein determination is primarily done using the Kjeldahl method, the oldest indirect method of protein determination in liquid and solid products; it is also a recognised reference method, recommended in national and international standards [51]. In this method, protein material is subjected to hot mineralisation using sulfuric acid, frequently in the presence of a catalytic amount

of Cu_2SO_4, agents that raise mineralisation temperature (Na_2SO_4), and oxidants (H_2O_2). The ammonia released during mineralisation is fixed by the sulfuric acid, forming ammonium sulfate, which is subsequently decomposed using sodium hydroxide, in accordance with the following reactions:

$$2NH_3 + H_2SO_4 \rightarrow (NH_4)_2SO_4 \quad (3.1.5.1)$$

$$(NH_4)_2SO_4 + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O \quad (3.1.5.2)$$

The released ammonia is distilled into a receiver containing the weak boric acid, then the mixture is titrated with a strong mineral acid (sulfuric or hydrochloric acid), quantitatively determining the fixed ammonia and calculating it using an appropriate conversion coefficient (depending on the type of the sample analysed) [52–54] to raw protein:

$$NH_3 + H_3BO_3 \rightarrow NH_4H_2BO_3 \quad (3.1.5.3)$$

$$NH_4H_2BO_3 + HCl \rightarrow NH_4Cl + H_3BO_3 \quad (3.1.5.4)$$

$$2NH_4H_2BO_3 + H_2SO_4 \rightarrow (NH_4)_2SO_4 + 2H_3BO_3 \quad (3.1.5.5)$$

Aside from nitrogen contained in proteins, the Kjeldahl method is used to assay nitrogen originating from ammonium ions, amide, amine and imine groups, but not one from nitrates and nitrites, or nitrogen contained in heterocyclic aromatic compounds [55].

Other than mineralisation, fragmented solid material can be subjected to the effects of a buffer (*e.g.* Tris-HCl, pH 8, or pH 7.4 phosphate buffer), followed by determination of soluble proteins using spectrophotometric methods (direct absorbance measurement methods and measurements using dyes such as amido black 10B, orange G, Coomassie blue G-250), or using titration methods (*e.g.* formaldehyde titration). Both method groups are characterised by significant simplicity of execution and are commonly used in liquid material analysis.

Most of the popular methods of protein determination are compared in paper [56] and scripts [29, 57].

Soluble protein assay using the direct absorbance measurement method:

Absorbance measurement at ultraviolet wavelengths is a quick and non--destructive method of determining protein content in solutions. The assay is based on absorption of near ultraviolet light (280 nm) by the main components of proteins, *i.e.* aromatic amino acids (mainly tryptophan and tirosine, and to a lesser degree phenylalanine) and cystines, which contain a disulfide bond in their structure [58].

To perform the assay, placed fragmented material of known mass (1–5 g) in a conical flask and add pH 7.4 phosphate buffer (dissolve 0.272 g KH_2PO_4 and 0.284 g Na_2HPO_4 in 100 cm^3 of distilled water and titrate with 0.2 M NaOH

until pH 7.4 is reached). Adjust the amount of buffer so that the expected protein concentration does not exceed 10 mg/cm^3. Stir the sample intensively at room temperature for 2 hours to extract so called soluble protein. Before measuring, centrifuge the solution at maximum speed or filter it. Measure absorbance in quartz cells at 280 nm wavelength, using water as the reference solution.

Read the protein concentration from the calibration curve (Figure 3.1.5.1), prepared for casein solutions of known protein concentrations.

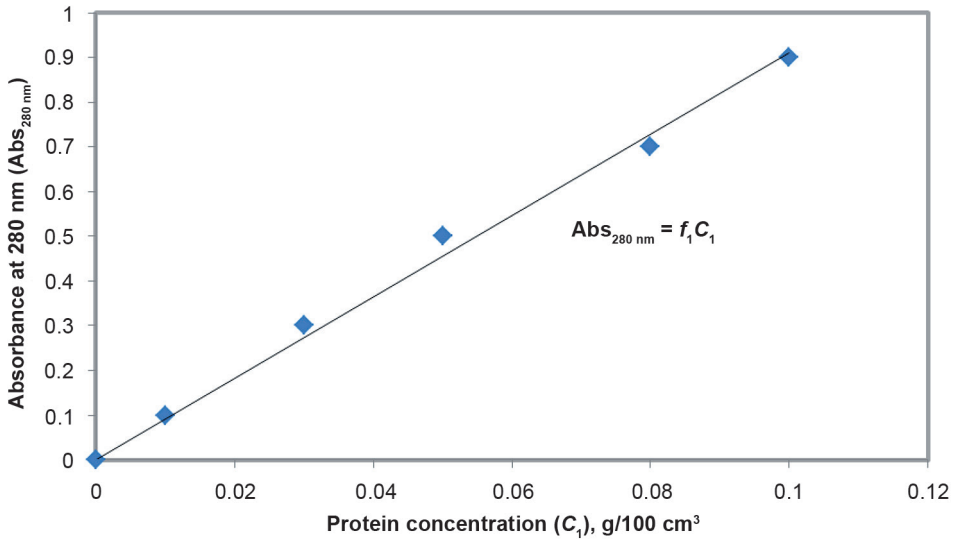


Figure 3.1.5.1. Calibration curve for determining the concentration of a protein (casein) dissolved in a buffer solution using direct absorbance measurement at a wavelength of 280 nm

If the concentration is too high – additionally dilute the sample. Calculate the amount of dissolved protein using the following formula:

$$\text{Protein, \% mas.} = 100\frac{C_1 \cdot V}{m_0} \tag{3.1.5.6}$$

$$C_1,\ \text{g/100 cm}^3 = \frac{\text{Abs}_{280\,\text{nm}}}{f_1} \tag{3.1.5.7}$$

where:

C_1 – protein concentration read from the calibration curve, g/100 cm^3,
m_0 – mass of material sample used for the assay, g,
V – total volume of buffer used to dissolve the sample,
f_1 – slope of the calibration curve line (Figure 3.1.5.1).

The above method is reliable for samples that do not contain nucleic acids, whose absorbance maximum occurs at λ = 260 nm. In samples where both substances are expected, the Warburg and Christian method is applied, whereby measurements are performed both at 280 nm and 260 nm, and the proportion of both absorbance values is designated with the letter *F*, which is a multiplier for the actual absorbance value at 280 nm. The *F* values are constant and can be read from the relevant charts – more on this subject can be found in literature [59].

Preparation of standard protein solutions:

Using small scraps of aluminium foil as weighing vessels, weigh portions of approx. 1 g, 0.5 g, 0.1 g, 0.08 g, 0.05 g, 0.03 g and 0.01 g of casein on an analytical balance. Move the portions quantitatively to conical flasks with ground glass joints (100 cm^3) and add 100 cm^3 of phosphate buffer, resulting in standard solutions of differing protein concentrations. Before use, store the solutions for 30 minutes at room temperature to form colloidal protein solutions.

Protein assay using the biuret method:

The assay is based on the formation of a blue-violet colour complex between peptide bonds and Cu^{2+} ions in an alkaline environment (Figure 3.1.5.2). Additionally, cooper is reduced to Cu^{+} by the peptide bonds to a degree proportional to the protein

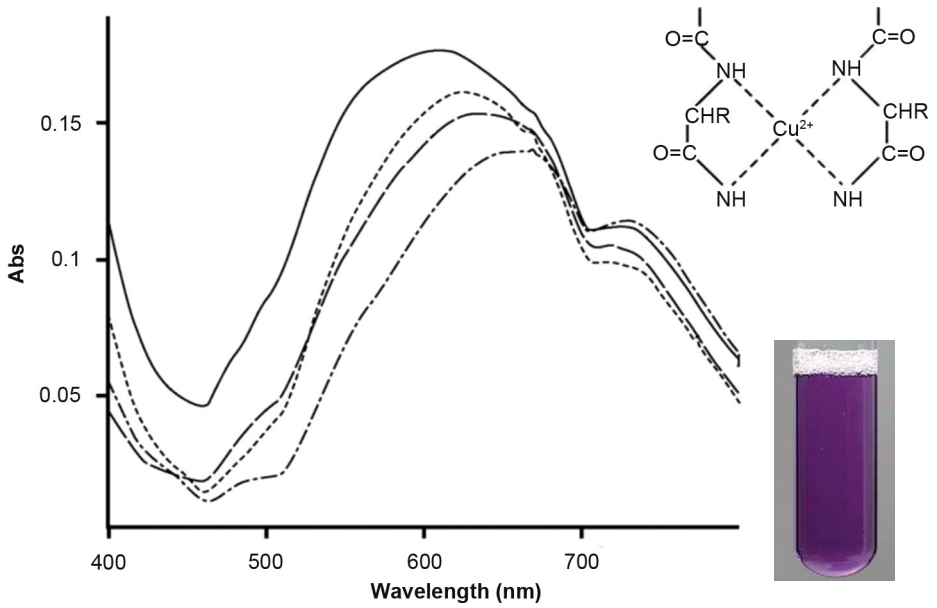


Figure 3.1.5.2. Copper complex with peptide bonds in proteins, with its adsorption maximum read at 540 or 650 nm. Based on [61, 62]

concentration. The condition for forming the complex is the presence of at least to -NH-CO- groups, and a positive result is also achieved with oxamid, biuret, and tripeptide. The test is hampered by the presence of magnesium sulfate, which precipitates in an alkaline environment as insoluble $Mg(OH)_2$, masking the solution's colour, and the presence of ammonium salts, which form colourful combinations with copper ions [45, 60].

To perform the assay, dilute a standard or test protein solution (preparation of samples in a buffer as in the direct method) with 0.9% NaCl at a 1 : 1 volume ratio, then add 4 cm^3 of the copper reagent to 1 cm^3 of the solution, stir, and leave at room temperature for 30 minutes. Measure absorbance at a wavelength of 540 or 650 nm with 0.9% NaCl as the reference sample. Prepare a calibration curve (similar to than in Figure 3.1.5.1) using the absorbance of standard solutions, then read the protein concentration from the curve. Perform calculations using formulae similar to those shown in the direct absorbance measurement method (formulae 3.1.5.6 and 3.1.5.7).

Prepare the copper solution by dissolving 1.5 g of copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and 6 g of sodium potassium tartrate ($NaKC_4H_4O_6 \cdot H_2O$) in 500 cm^3 of distilled water. After dissolving the salts, add 300 cm^3 of 10% NaOH solution free of sodium carbonates, and 1–2 g of KJ, which protects copper from self-reduction. Make up with water to 1 dm^3.

Protein assay using the Bradford method:

The assay is based on the formation of a colourful complex between the protein and Coomassie Brilliant Blue G-250 indicator. In an acidic solution, the indicator solution takes on a brown colour with its absorption maximum at about 465 nm and 650 nm (depending on pH), while in the presence of a protein, the absorption maximum shifts to 595 nm (Figure 3.1.5.3), and the pronounced blue colour is proportional to the protein's concentration in the solution.

To perform the assay, dilute a standard or test protein solution (preparation of samples in a buffer as in the direct method) with 0.9% NaCl at a 1 : 1 volume ratio, then add 0.5 cm^3 of the Bradford reagent to 0.5 cm^3 of the solution, stir, and leave at room temperature for 5 minutes. Measure absorbance at a wavelength of 595 nm with 0.9% NaCl as the reference sample. Perform calculations and calibration as in the direct absorbance measurement method (formulae 3.1.5.6 and 3.1.5.7).

Prepare the Bradford reagent by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 cm^3 of 95% ethanol and 100 cm^3 of 85% phosphoric acid. The next day, after the dye dissolves completely, make up to 1 litre with cold water.

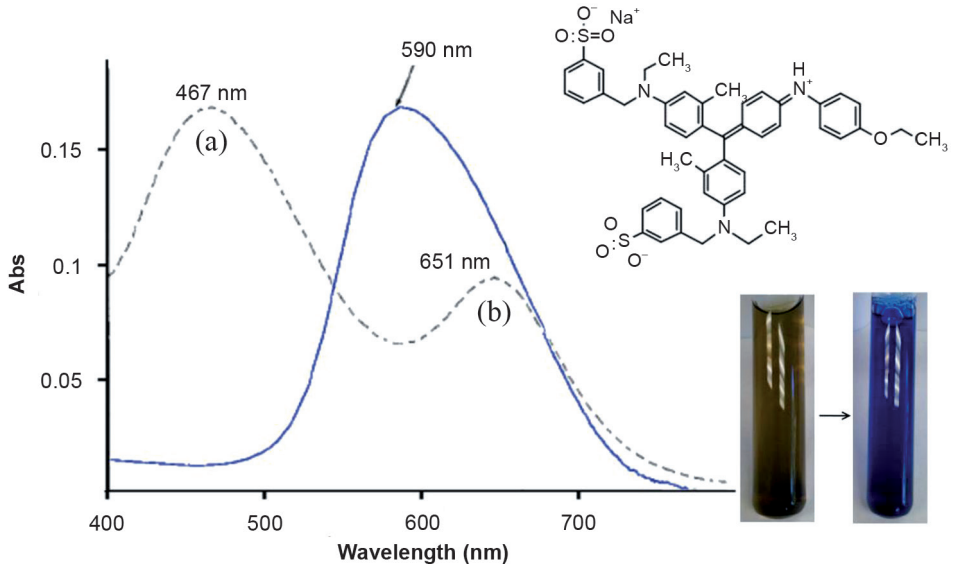


Figure 3.1.5.3. Absorption maxima for the Coomassie Brilliant Blue G-250 dye solution at low pH in an aqueous solution (cation form – a, and anion form – b), and absorption maximum shift to 590 nm for the protein complex. Based on [63–65]

Soluble protein assay using the spectrophotometric method with amido black 10B:

Amido black 10B is a diazo dye, a derivative of 8-amino-1-naphtholo-3,6-disulfonic acid, p-nitroaniline and aniline (Figure 3.1.5.4). In a weakly acidic environment, usually below the isoelectric point of proteins, it bonds with cation groups of proteins using dissociated sulfonic groups, creating insoluble complexes that can be removed from the solution by centrifuging or filtering. The resulting solution's colour intensity depends on the amount of dye not fixed by proteins (an excess of the dye is added), *i.e.* it is inversely proportional to the amount of protein in the analysed sample. Importantly, the blue-coloured amido black 10B does not bond with non-protein nitrogen compounds, enabling one to discern these derivatives (unlike the Kjeldahl method).

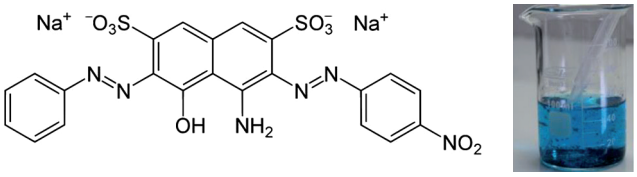


Figure 3.1.5.4. Formula of amido black 10B and image of residue precipitated in a reaction with proteins

To perform the assay, prepare standard casein solutions and a solution of the solid sample following the procedure described in the direct spectrophotometric measurement method. Prepare the primary black solution separately: Dissolve 3.17 g of citric acid ($C_6H_8O_7$), 0.4156 g of disodium phosphate ($Na_2HPO_4 \cdot 2\ H_2O$) and 0.088 g of amido black in 1 dm^3 of distilled water. Introduce 1 cm^3 of the liquid sample or buffer extract from the solid sample to be analysed (prepared as in the direct spectrophotometric assay method) and 8 cm^3 of the amido black 10B solution to each centrifuge tube. Plug the tubes, shake for 5 minutes, then centrifuge for 5–10 minutes at maximum centrifuge speed (at least 400 RPM). Transfer the supernatant to a measurement cell and measure absorbance at a wavelength λ = 590 nm relative to distilled water. If the solution lacks colour, i.e. protein content is too high, dilute the test solution twice and repeat the assay. Follow the same steps for the standard protein (casein) solutions.

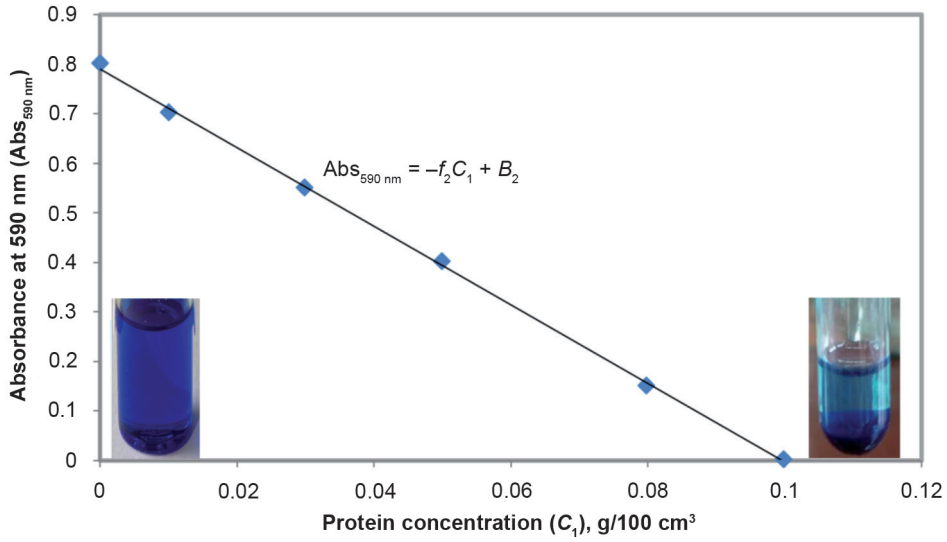


Figure 3.1.5.5. Calibration curve for determining the concentration of a protein (casein) in a solution with amido black 10B added – absorbance measurement at a wavelength of 590 nm

Read the protein concentration in the solution directly from the calibration chart (Figure 3.1.5.5) or calculate using the curve equation, taking into account the dilution ratio (3.1.5.8). Calculate the protein content in the solid sample using equation (3.1.5.6).

$$C_1,\ \mathrm{g/100\ cm^3} = \frac{B_2 - \mathrm{Abs}_{590\ \mathrm{nm}}}{f_2} \qquad (3.1.5.8)$$

where:

C_1 – protein concentration read from the calibration curve, g/100 cm^3,
B_2, f_2 – parameters from the calibration curve equation (Figure 3.1.5.5).

3.1.6. Carbohydrates

Carbohydrates occurring in natural materials include both simple and complex sugars. The former include glucose, ribose, and other sugars that do not undergo hydrolysis, while complex sugars (oligosaccharides) include all those containing between 2 and 10 monosaccharide radicals (*e.g.* lactose, cellobiose, saccharose, raffinose), while larger molecules are called polysaccharides. These include, for example, starch, dextran, glycogen, gum arabic, and galactans, whose hydrolysis involves multiple stages, proceeding through oligosaccharides to the simplest sugars of the pentose (*e.g.* D-ribose, D-xylose, L-arabinose) or hexose groups (*e.g.* D-glucose, D-fructose, D-mannose).

The most common monosugars include D-glucose and D-fructose, which occur mainly in honey, fruits, and vegetables. Lactose (a dimer of D-glucose and D-galactose) is present in mammal milk, while saccharose – in sugar beets and sugar cane. On the other hand, starch dominates in cereal seeds, processed cereals, and potatoes, while the most common complex polysaccharide is cellulose. In nature, saccharides do not exist only in pure forms; their derivatives containing amine groups (*e.g.* glucosamine) – particularly frequent in mucus – ester and ether derivatives, sugars in oxidised or reduced forms, bound with simple and complex proteins (glycoproteins) or lipids are equally common. As stated in literature [53], more than 100 different types of sugars have been identified in food products.

Because saccharides present in food include both digestible and indigestible (so called dietary fibre) compounds, it is recommended to use digestible saccharides, which are the difference between total saccharide and dietary fibre content, instead of total saccharides for calculating the energy values of products [53].

Sometimes the content of total saccharides (CH_{total}) and digestible sugars ($CH_{available}$) is calculated in a simplified manner and given as [52, 54]:

$$CH_{total}, \% = 100 - (\text{water} + \text{ash} + \text{protein} + \text{fat} + \text{alcohol}) \quad (3.1.6.1)$$

$$CH_{available}, \% = 100 - (\text{water} + \text{ash} + \text{protein} + \text{fat} + \text{alcohol} + \text{dietary fibre}) \quad (3.1.6.2)$$

where the content of individual food components (water, ash, protein, fat, and fibre) are determined using the chemical methods discussed in previous sections.

3.1.6.1. Total available carbohydrates and reducing sugars

Digestible saccharides are an important source of energy in food, and information about their content must be placed on product labels in the same way as information about fats or protein [3]. These include mainly simple sugars, although oligosaccharides and polysaccharides that are not hydrolysed during the digestion process are included as well. Digestible sugars can be determined using chromatographic (especially HPLC), enzymatic, physical, chemical, and spectrophotometric methods [53]. The simplest include physical methods, among which the following are notable:

- measurement of aqueous sugar solution density using a pycnometer and appropriate conversion tables or a properly graded areometer (Figure 3.1.6.1) – a method commonly used in the wine industry, but only providing approximate results (due to possible non-sugar substances dissolved in the solution);
- measurement of the refractive index of light using a refractometer, with sugar concentration read using the Brix (Figure 3.1.6.1), Plato, Balling, or another scale accepted in the given country for standardised mustmeters [66] – similarly to the densimetric method, it only provides approximate results, reliable exclusively for single substance solutions;
- measurement of specific optical rotation of a sugar solution, enabling the saccharide to be identified (Table 3.1.6.1), although the test solution must be colourless, clear, and without colloidal suspensions.

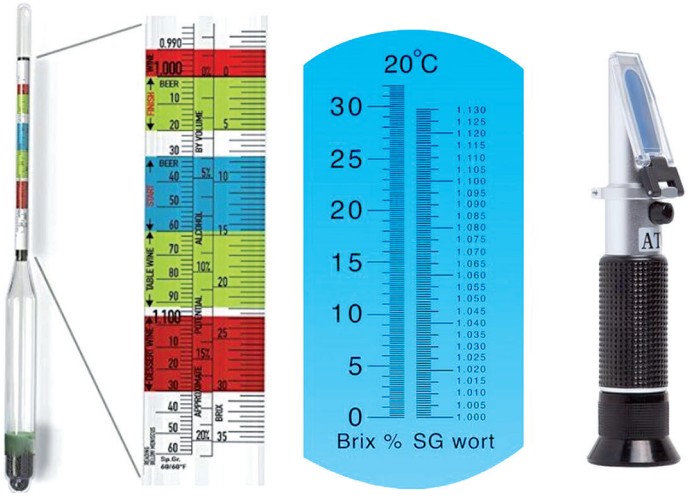


Figure 3.1.6.1. Areometer with a sugar-alcohol scale, and a refractometer with a typical Brix scale (from the left) [67, 68]

Table 3.1.6.1

Comparison of specific optical rotation for selected sugars. Based on [69]

Sugar	$[\alpha]_D^{20}$	Cukier	$[\alpha]_D^{20}$
D-arabinose	–105°	Lactose	+52°
D-fructose	–93°	Sucrose	+66°
D-deoxyribose	–56°	D-galactose	+82°
D-ribose	–24°	L-arabinose	+105°
Inverted sugar	–20°	α-D-glucose	+109.1°
D-mannose	+15°	Maltose	+130°
β-D-glucose	+19.8°	Starch	+196°
D-glucose	+52.7°	Glycogen	+198°

In polarimetry, the determination is based on measuring the rotation angle of the plane of polarised light at a temperature of 20°C and in sodium light with a wavelength of 589.3 nm [69]. For a solution of a sugar or another compound containing at least one asymmetric carbon atom in its molecule, the characteristic value is specific rotation (α), calculated using equation (3.1.6.3) for pure substance solutions with a concentration of 1 g/cm^3.

$$[\alpha] = 100\frac{\alpha}{c \cdot l} \tag{3.1.6.3}$$

where:

$[\alpha]$ – specific rotation expressed in degrees and determined for 1% solutions,
α – measured angle of plane rotation of polarised light, in degrees,
l – polarimetric tube length (thickness of solution layer in dm),
c – solution concentration, g/cm^3.

This equation also enables calculating the substance concentration in the solution, although this method is limited to single-component solutions.

Chemical sugar assay methods utilise the reductive properties of the free carbonyl group occurring in monosaccharides and some disaccharides. Saccharide concentration determined this way is referred to as “reducing sugar content”. In the case of carbohydrates with higher molecular masses and without reductive properties, it is necessary to hydrolyse (inverse) them to monosaccharides and determine them as “total sugars”. The Clerget-Herzfeld method is most commonly used in this case, involving acidic hydrolysis at an elevated temperature.

The majority of natural materials require initial defatting, deproteinisation, and clarification of sugar solution in addition to hydrolysis. The purpose of these actions is to remove substances that can disrupt the determination of sugar content or inflate its result. It must be remembered that other than sugars, also certain organic acids

(ascorbic acid), purine bases, some aldehydes and amino acids exhibit reductive properties, which are the basis of sugar determination. Defatting can be done through extraction using a non-polar solvent, similarly as in the methodology used for fat analysis (see section 3.1.3), while deproteinisation and clarification are usually done using the **Carrez method** [53]. The essence of this method is the formation of a colloidal precipitate of zinc(II) hexacyanoferrate(II) – reaction 3.1.6.4 – which, when settling, co-precipitates large-molecule compounds, such as proteins, pectins, and tannins.

$$2ZnSO_4 + K_4Fe(CN)_6 \rightarrow Zn_2Fe(CN)_6\downarrow + 2K_2SO_4 \qquad (3.1.6.4)$$

Copper(II) hydroxide, lead(II) acetate with sodium sulfate, mercury(II) nitrate with sodium hydroxide, chloroacetic acid, 70% ethanol or acetone are also used for clarification, while selection of the reagent is dictated by the nature of the product to be analysed [45].

Performing sugar inversion using the Clerget-Herzfeld method [45]:

Introduce 50 cm^3 of the primary solution to a 250 cm^3 conical flask, then use a pipette to **CAREFULLY!** Add by the drop 5 cm^3 of concentrated sulfuric acid while stirring. Mix everything thoroughly and put the flask in a water bath at a temperature of 80°C, bring the temperature of the solution in the flask to 68–71°C within 2–3 minutes and maintain it for another 5 minutes. Cool the flask and neutralise the solution with a 10% NaOH solution in the presence of methyl orange. Transfer the contents of the flask quantitatively to a 250 ml volumetric flask and make up to the mark with distilled water. Perform the assay maintaining the correct temperature and amount of acid, as any change may result in incomplete decomposition of polysaccharides and monosaccharides (too short duration, too low temperature of not enough acid), or the opposite – lead to decomposition of the products of hydrolysis.

Alternatively, hydrolysis can be carried out under milder conditions, *i.e.* introduce 0.1 M H_2SO_4 to the saccharide solution (1/1 volume ratio compared to the sample) and heat it to boil for an hour. Neutralise the solution as in the method above.

Deproteinisation and clarification using the Carrez method [45]:

Introduce 1.25 cm^3 250 mM zinc sulfate solution (dissolve 7.2 g $ZnSO_4 \cdot 7H_2O$ in 100 cm^3 water) and 1.25 cm^3 85 mM potassium hexacyanoferrate (3.6 g $K_4[Fe(CN)_6] \cdot 3H_2O$ in 100 cm^3) into 25 cm^3 of test sugar solution. Stir thoroughly and bring to pH approx. 8 using 3M NaOH and litmus paper. Filter the solution and make up with distilled water to 50 cm^3.

Total sugar assay using the phenol method [70, 71]:

Under the effects of concentrated sulfuric acid, simple sugars, *i.e.* pentoses and hexoses, are dehydrated to furfural and oxymethylenefurfural, respectively. In an acidic environment, these compounds form orange-coloured permanent complexes with phenol, which are spectrophotometrically determined using a reagent sample as the reference solution. To perform the assay, add 0.2 cm^3 5% phenol solution and 1 cm^3 concentrated sulfuric acid to 0.2 cm^3 diluted post-inversion solution (**WORNING!** Sugar content should not exceed 0.08 g/cm^3), standard solution (0.02–0.08 g/cm^3) or water (reagent sample). Leave the samples for 30 minutes at room temperature, then read absorbance at a wavelength of 490 nm. Read the sugar concentration from a calibration curve prepared for multiple solutions with different sugar concentrations (g/cm^3), or calculate it using the following formula:

$$C = \frac{C_{wz} \cdot A}{A_{wz}} \tag{3.1.6.5}$$

where:

C – sugar concentration in the sample, g/cm^3,
C_{wz} – sugar concentration in the standard solution, g/cm^3,
A – absorbance of the test solution,
A_{wz} – absorbance of the standard solution.

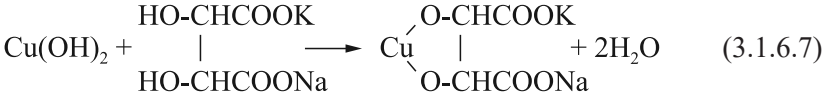
Total sugar determination using the 3,5-dinitrosalicylic acid method [45]:

In an acidic environment, simple sugars reduce 3,5-dinitrosalicylic acid to a red--brown 3-amino-5-nitrosalicylic acid, for which the absorbance maximum is read at 540 nm with the reagent sample with water as the reference solution. To perform the assay, add 5 cm^3 water and 1 cm^3 1% 3,5-dinitrosalicylic acid solution (dissolve 1 g acid in 50 cm^3 water in a 100 cm^3 volumetric flask, then add 20 cm^3 2 M NaOH, 30 g sodium potassium tartrate, and when they dissolve, make up to the mark with water) to 1 cm^3 reducing sugars (in multiple dilutions). Secure the samples with aluminium foil and heat on water bath for 5 minutes. Cool under a cold water stream and measure absorbance and 540 nm wavelength. Read the sugar concentration from a calibration curve prepared for multiple solutions with different glucose concentrations (g/cm^3), or calculate it as per the phenol method.

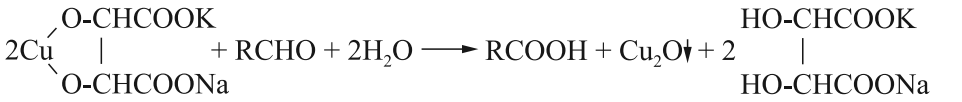
Reducing sugar or total sugar assay using the Bertrand method [70, 71]:

The Bertrand method is used for strongly coloured solutions. The reducing sugar assay involves quantitative reduction of Cu^{2+} ions to Cu^{+} by saccharides at the boiling point and in an alkaline environment. Under such conditions, the following reactions occur between the components of Bertrand I and Bertrand II solutions:

$$CuSO_4 + 2NaOH \rightarrow Na_2SO_4 + Cu(OH)_2 \qquad (3.1.6.6)$$



(3.1.6.7)



(3.1.6.8)

Next, Cu^+ ions participate in a redox reaction with iron(III) sulfate, and the resulting Fe^{2+} ions are titrated with a volumetric solution of $KMnO_4$:

$$Cu_2O + Fe_2(SO_4)_3 + H_2SO_4 \rightarrow 2CuSO_4 + 2FeSO_4 + H_2O \qquad (3.1.6.9)$$

$$2KMnO_4 + 10FeSO_4 + 8H_2SO_4 \rightarrow K_2SO_4 + 5Fe_2(SO_4)_3 + 8H_2O + 2MnSO_4 \qquad (3.1.6.10)$$

Before the assay is performed, prepare:

Bertrand solution I: dissolve 4 g copper(II) sulfate ($CuSO_4 \cdot 5H_2O$) in distilled water and make up to 100 cm^3 with water;

Bertrand solution II: dissolve 20 g sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$) in about 50 cm^3 distilled water, add 15 g NaOH dissolved in 25 cm^3 distilled water, mix, then make up with distilled water to 100 cm^3;

Bertrand solution III: dissolve 5 g anhydrous iron(III) sulfate ($Fe_2(SO_4)_3$) solution in 50 cm^3 hot distilled water, cool the solution, then carefully add 20 cm^3 concentrated sulfuric acid (d = 1.84 g/cm^3). Make up with distilled water to 100 cm^3. Before use, check if the solution reduces potassium permanganate(VII) solution, if so – add enough $KMnO_4$ solution to achieve slightly pink colour, which disappears by itself.

Performing the assay: introduce 5 cm^3 of the saccharide solution (initial solution in the "reducing sugars" assay, or post-inversion solution when determining "total sugars") and 15 cm^3 distilled water into a conical flask. Add 20 cm^3 each of Bertrand I and Bertrand II solutions, mix, and place on a heating mantle. Bring the solution to boil and maintain this temperature for 3 minutes, then leave to cool (**CAUTION!** avoid exposing the precipitate beneath the solution). The liquid over the precipitate should have a distinct blue colour, indicating an excess of reduced copper salt. If the solution changes colour to dirty green, the amount of saccharide exceeds the reduction capabilities of Bertrand I and II reagents. In such an event, dilute the sample and repeat the assay.

Decant the supernatant to a Schott funnel in such a way that the surface of the Cu_2O precipitate is not exposed, then transfer the precipitate quantitatively from

the flask to the funnel using hot water. Wash the precipitate in the funnel with how water, until blue colour in the filtrate disappears. Perform all actions briskly, so no portion of the precipitate is oxidised. Next, place the Schott funnel in a clean flask and dissolve the precipitate with three or four 5 cm^3 portions of Bertrand III solution. Finally, wash the funnel with hot water, collecting the filtrate in the flask. Titrate the collected solution with diluted potassium permanganate(VII) solution (about 0.005 M) until the colour changes from green to pink, which remains for 30 seconds.

Based on reaction stoichiometry (3.1.6.9) and (3.1.6.10) and the actual concentration of the potassium permanganate solution (C_{KMnO_4}, mol/dm^3), calculate so called copper titre of the potassium permanganate solution ($T_{KMnO_4/Cu}$, mg Cu · cm^3 $KMnO_4$), then determine the number of mg of copper (m_{Cu}) corresponding to the volume of $KMnO_4$ (V_{KMnO_4}, cm^3) used for titration:

$$T_{KMnO_4/Cu} = 5 \cdot C_{KMnO_4} \cdot M_{Cu} \tag{3.1.6.11}$$

$$m_{Cu} = T_{KMnO_4/Cu} / V_{KMnO_4} \tag{3.1.6.12}$$

where:

M_{Cu} – molar mass of copper (63.57 g/mol).

Calculate the equivalent number of mg of reducible (or inverted) sugar contained in the sample taken for the assay (5 cm^3) using the trend line equation of the curve in Figure 3.1.6.2.

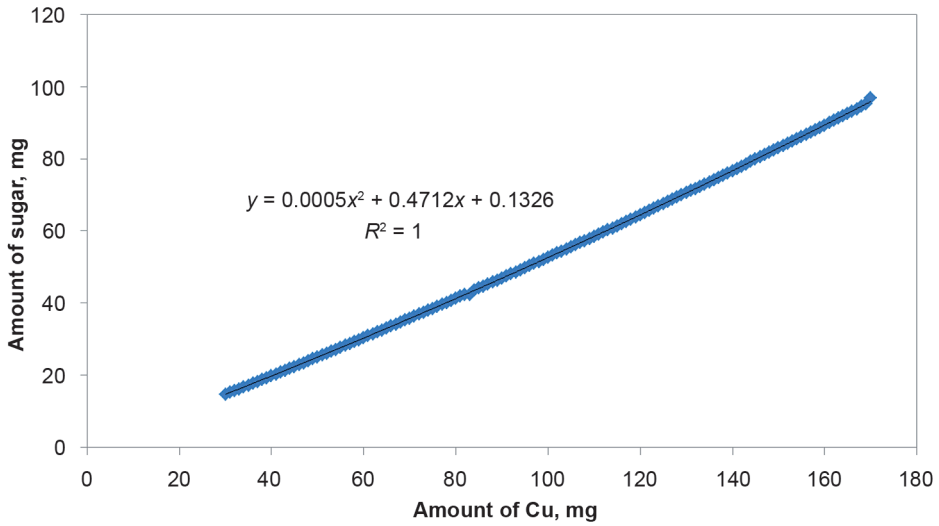


Figure 3.1.6.2. Relation between the amount of reducible sugar and the number of Cu^{2+} copper ions reduced by the sugar. Based on [45, 72]

Finally, calculate the percentage content of "reducible sugars" or "total sugars" in the material, taking into account the dilution ratios.

Reducing sugar assay using the Luff-Schoorl method:

This method is recommended by European Union Directives for feed analysis [73] and is suitable for determining lactose, maltose, glucose and inverted sugars (reducing sugars) in colourless solutions.

The assay is again based on reduction of Cu^{2+} cations present in Luff's reagent by simple sugars at the boiling point and in an alkaline environment (pH about 9.5). The composition of Luff's reagent is similar to Bertrand solution, although sodium hydroxide is replaced with its carbonate, while sodium potassium tartrate is replaced with citric acid. Introduction of potassium iodide and sulfuric acid into the mixture enables reaction with the copper sulfate unreduced by sugars, and precipitation if copper(I) iodide and iodine, which are titrated with sodium thiosulfate:

$$2CuSO_4 + 4KI \rightarrow 2K_2SO_4 + Cu_2I_2 + I_2 \qquad (3.1.6.13)$$

$$I_2 + 2Na_2S_2O_3 \rightarrow 2NaI + Na_2S_4O_6 \qquad (3.1.6.14)$$

The results are calculated to sugars using an appropriate conversion table [73] and taking into account a blank reagent sample, in which sodium thiosulfate(VI) used to titrate the iodine released by the total amount of copper present in Luff's reagent is determined.

Before starting the analysis, prepare a 30% KI solution, 6 N sulfuric acid, 0.1 N sodium thiosulfate solution, a starch solution, and the Luff-Schoorl reagent: Mix 50 g citric acid in 50 cm^3 water with cooled sodium carbonate solution (dissolve 143.8 g anhydrous carbonate in 300 cm^3 hot water) and 100 cm^3 of a solution containing 25 g $CuSO_4 \cdot 5H_2O$. Make up to 1 dm^3 and filter after the solution settles. The pH of the solution should be approx. 9.4.

Add exactly 25 cm^3 Luff-Schoorl reagent to 25 cm^3 sugar or lactose solution. Heat it to boil for 10 minutes, then quickly cool in cold water, add 10 cm^3 potassium iodide solution and titrate with 0.1 N sodium thiosulfate in the presence of starch until blue colour disappears. In a similar way, titrate 25 cm^3 Luff-Schoorl reagent and 25 cm^3 water after adding 20 cm^3 KI solution and 25 cm^3 6 N sulfuric acid (do not boil).

Using the calibration curves shown in Figure 3.1.6.3 determine the mass of the carbohydrate, which corresponds to the difference in volumes of 0.1 N sodium thiosulfate solution (cm^3) between the reagent and proper samples.

Regardless of the assay method used, when analysing carbohydrates in a sample directly after its dissolving and after inversion, saccharose content (m_{sach}) in grams is calculated using the following formula:

$$m_{sach} = 0.95(m_{inw} - m_{red}) \quad (3.1.6.15)$$

where:

m_{inv} – the content of reducing sugars after inversion, g,
m_{red} – the content of reducing sugars, g.

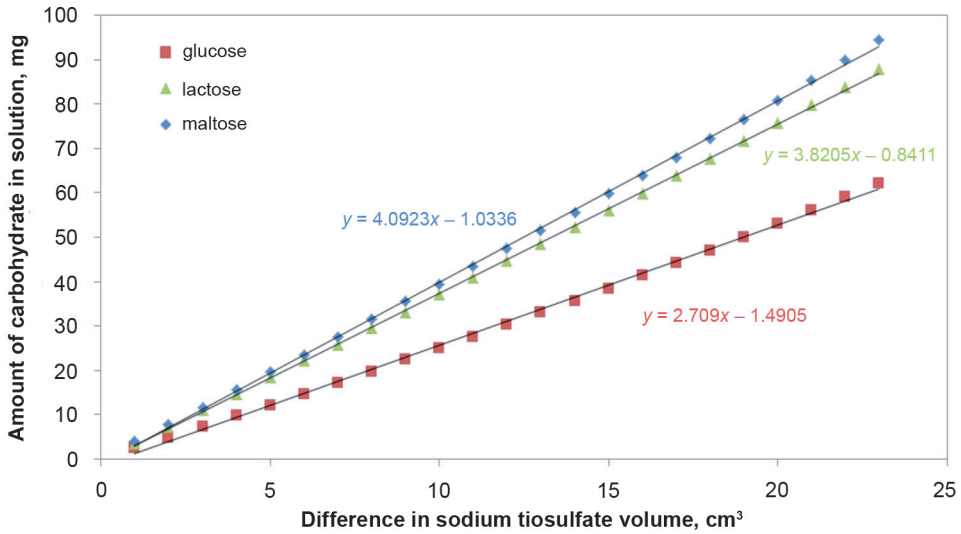


Figure 3.1.6.3. Curves showing the carbohydrate values corresponding to titration with 25 cm³ Luff--Schoorl reagent (maintaining 10 minutes heating to boil and using 0.1 N $Na_2S_2O_3$). Based on [73–76]

3.1.6.2. Starch and starch hydrolysis products

Determination of starch undergoing hydrolysis during enzymatic processes and so called resistant starch, not absorbed in the intestines, requires using different assay methods than other digestible sugars. Polarimetric methods are usually employed, as they are characterised by quickness and simplicity. They involve measuring the rotation angle of polarised light by the starch solution to be tested, which must, however, be colourless, clear, and free of colloidal suspensions. Starch can also be determined using gravimetric methods, *e.g.* Rask method, where a sample is successively extracted with: diethyl ether, 10% ethanol, hydrochloric acid solution, 70% ethanol, and finally 96% ethanol. Starch content is determined based on the mass of the remaining precipitate. However, gravimetric methods are relatively inaccurate, time-consuming, and can not be applied to many food products. The most accurate results are provided by chemical methods, where starch is hydrolysed to glucose in an acidic environment or using amylolytic enzymes, and the content of the simple sugar is then determined using one of the methods

employed for reducing sugars. The amount of starch is calculated by multiplying the determined amount of glucose by a coefficient of 0.9 [77].

Starch content assay using the polarimetric method:

To determine the amount of starch in a material (*e.g.* potato pulp from which water-soluble substances have been removed [78]), prepare a calcium chloride solution with a density of 1.3 g/cm^3 and pH 2.5 (dissolve 913 g $CaCl_2 \cdot 6H_2O$ in 760 cm^3 distilled water, bring to density of 1.3 g/cm^3 at a temperature of 20°C and adjust pH to 2.5 using glacial acetic acid). Next, grind the test material (2–15 g) in a mortar with 5 g of fine-grained sand (0.3–0.5 mm fraction) roasted at 130°C, transfer everything quantitatively to a flask with ground glass joint and suspend in 70 cm^3 of the $CaCl_2$ solution. Bring the suspension to boil within 5 minutes. After 15 minutes of boiling under an air condenser, quickly cool it to a temperature of about 20°C. Transfer the solution quantitatively to a 100 cm^3 volumetric flask, washing the original flask with a small amount of the calcium chloride solution. Add 3 cm^3 of Carrez I solution and Carrez II solution each (see sugar clarification, section 3.1.6.1), stir, make up with calcium chloride solution to 100 cm^3, stir again and leave for 5 minutes. Filter the solution through corrugated filter paper to a dry, clean flask, discarding the first few drops of filtrate. After washing the polarimetric tube, fill it with the test solution, place it in a polarimeter and read the polarisation plane rotation angle. Calculate starch content using the following formula:

$$\text{Starch, } \% = 100 \cdot \frac{\alpha \cdot (100 - p) \cdot V}{[\alpha] \cdot I \cdot m} \tag{3.1.6.16}$$

where:

- α – rotation angle of light polarisation plane, circle degrees,
- $[\alpha]$ – specific rotation of starch in a calcium chloride solution, equalling 203° for measurements in sodium light at 589 nm wavelength, or 240° for measurements in an automatic polarimeter in mercury light at 546 nm wavelength,
- p – volume of 5 g sand (3 cm^3),
- V – volume of solution (100 cm^3),
- I – length of polarimetric tube, dm,
- m – weighed amount of test substance, g.

Determination of enzymatically digested starch and insoluble non-starch substances:

The assay involves isolating water-soluble substances and enzymatically saccharificated starch from plant pulp and gravimetrically determining the remainder [78]. Carefully filter off fresh plant pulp (5–20 g) on a suction funnel, then wash with

small portions (approx. 20 cm^3) of water to wash away water-soluble substances (in total, use about 200–400 cm^3, collecting all filtrate for soluble sugar analysis). Transfer the remainder to a mortar and grind with 5 g of roasted sand. Grind the sample for 8 minutes and transfer quantitatively using distilled water (30 cm^3) to a conical flask, bring to pH 6–6.4 and place in a water bath at a temperature of 85°C. Introduce 2 cm^3 1% Novo enzyme solution and maintain the temperature for 15 minutes. Next, bring the water bath to boil and heat the sample in the bath for another 15 minutes. After cooling to 70°C, adjust pH to a value of 6-6.4 using 0.2 N NaOH, add 4 cm^3 of bacterial amylase solution and maintain at a temperature of 85°C for another 30 minutes. After starch saccharification, filter the sample, collecting the filtrate for sugar analysis, then move the remainder to large centrifuge tubes with water and centrifuge them (4 minutes at 4000 RPM). Filtrate the supernatant through a tared quantitative filter paper again. Immerse the precipitate in the tube with 80 cm^3 distilled water at a temperature of 80°C, stir and centrifuge again. Filter the liquid again (the same filter paper), adding the filtrate to that collected previously. Repeat these steps three times, combining the filtrate in a volumetric flask and keeping it for a reducing sugar assay. After the fourth washing, collect the filtrate separately, make up with water to a known volume, take a 5 cm^3 sample and test it for presence of sugars (α-naphthol test). If the result is positive, keep this filtrate for sugar content determination and repeat the washing of precipitate.

Transfer all of the washed precipitate to a filter paper, wash with 96% ethanol and dry at 130°C for 2 hours. Cool in desiccator and weigh.

Determine the amount of reducing sugars in the filtrate after clarifying the solutions with Carrez reagents, using any quantitative method described in the previous section. Calculate to starch using a calculation coefficient of 0.9.

Calculate the content of insoluble non-starch substances using the following formula:

$$m_{ns},\ \% = 100 \cdot \frac{a-(b-c-d)}{m} \tag{3.1.6.17}$$

where:

a – mass of vessel with dried precipitate, sand, and filter paper, g,
b – mass of weighing vessel, g
c – mass of sand, g
d – mass of filter paper dried to constant mass, g,
m – weighed amount of test substance, g.

The reduction sugar presence test mentioned above is based on a colourful condensation reaction of two α-naphthol molecules and one molecule of furfural or 5-(hydroxymethyl) furfural (Figure 3.1.6.4).

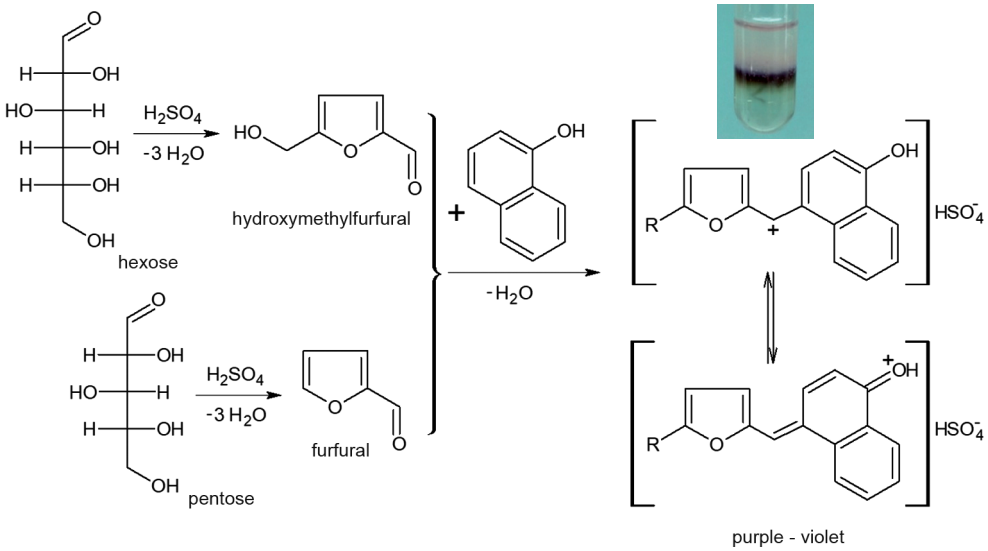


Figure 3.1.6.4. Diagram of reaction occurring in the Molisch test

Both furfural and hydroxymethylfurfural are formed *in situ* in a concentrated mineral acid environment through dehydration of a pentose or glucose, respectively. This reaction, known as the Molisch test, is not specific for simple sugars. Under this assay's condition, polysaccharides that undergo hydrolysis, and certain acids, *e.g.* citric acid, will cause a positive response. Lack of positive reaction definitely rules out that sugars are present in the solution or product being tested.

Performing the Molisch test for sugar presence:

Add 2 drops of alcohol solution of α-naphthol with 0,1 g/cm^3 concentration to 5 cm^3 of filtrate, the carefully pour 2 cm^3 concentrated sulfuric acid so it flows on the wall of the tube and to its bottom without mixing with the liquid. The red and violet ring forming after a minute at the interface between the liquids indicates that sugars are present.

The **fluoroglucine test** is performed in a similar manner; about 0.01 g of carbonate is dissolved in 5 cm^3 6M HCl, 10 mg fluoroglucine is added, and the solution is heated until boiling for a minute. A clear red colour appearing indicates the presence of a pentose, while hexoses react with the appearance of yellow, orange, or brown colour [79]. The **orcinol reaction** (Bial test) also enables discerning between pentoses and hexoses; following dehydration in a hydrochloric acid environment, sugars react with orcinol in the presence of iron(II) chloride, with hexoses forming yellow and green complexes, and pentoses – brown and grey (Figure 3.1.6.5).

To perform the assay, mix 2 cm^3 of the test solution with 2 cm^3 of Bial solution (dissolve 0.4 g orcinol in 200 cm^3 concentrated HCl and add 0.5 cm^3 10% $FeCl_3$ solution) in a test tube. Heat the contents to boil for a minute, observing colour changes [80].

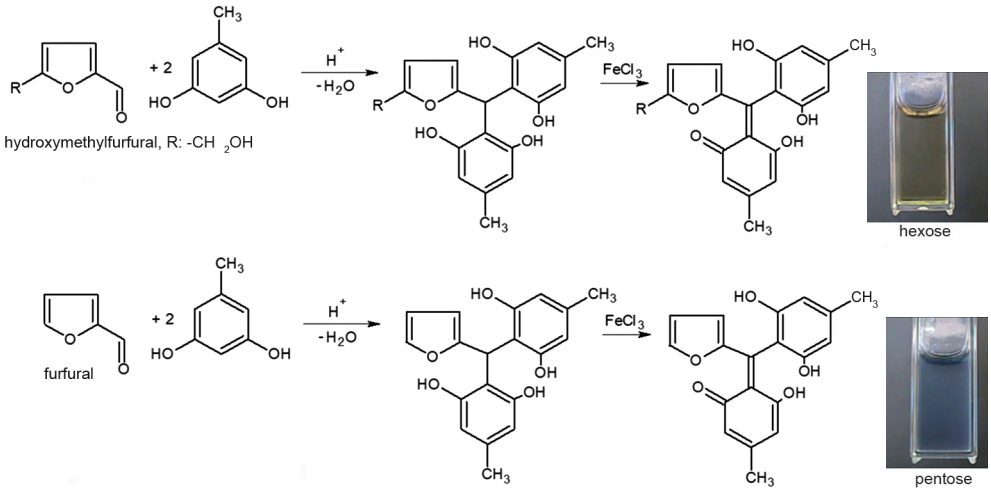


Figure 3.1.6.5. Reaction in the Bial test and image of tests with pentoses and hexoses. Image downloaded from [81] website

Another available test is the **Benedict test**, where the presence of reducing sugars is determined on the basis of changes in colour of an alkaline copper solution. In this reaction, the clear blue solution containing copper (II) ions changes its colour with rising concentration of reducing sugars from greenish (about 1% sugar concentration), to orange (2–3% concentration), to brick red (reducing sugar concentration above 4%) as a result of copper oxide Cu_2O precipitating.

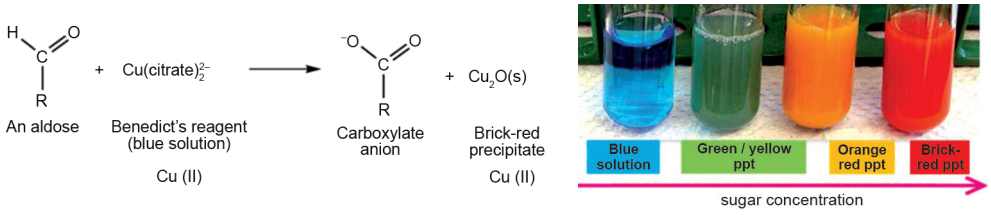


Figure 3.1.6.6. Reaction in the Benedict test and image of samples with different sugar concentrations. Image downloaded from [82] website

Performing the Benedict test for sugar presence:

Measure 1 cm^3 2 M NaOH solution and Benedict reagent each to a test tube. Add 0.5 cm^3 of the test solution of sugars (filtrate or starch hydrolysate) and place the tube in a boiling water bath for 3 minutes. Observe the colour.

Benedict reagent: Dissolve 173 g anhydrous trisodium citrate and 100 g anhydrous sodium carbonate in 500 cm^3 hot water, filter, cool, and add 17.3 g $CuSO_4 \cdot 5H_2O$. Make up the mixture with water to 1 dm^3.

The last of the proposed tests (**iodine test**) is the most commonly known and easiest to perform. It forms the basis of all analytical tests based on iodine analysis, which takes on an intensive, black-navy blue colour in the presence of starch. Tests conducted more than half a century ago [83, 84] clearly demonstrate that carbohydrates of different molecular masses produce colours with absorption maxima at different wavelengths, and consequently – the colours themselves differ. Amylose is observed to form a dark blue colour, amylopectins give purple colour, glycogen – red and brown, while dextrins – depending on molecule size – from violet (amylodextrins), to red (erythrodextrins), to yellowish, typical for iodine itself (archodextrins). No reaction is also observed for solutions of simple sugars and disaccharides.

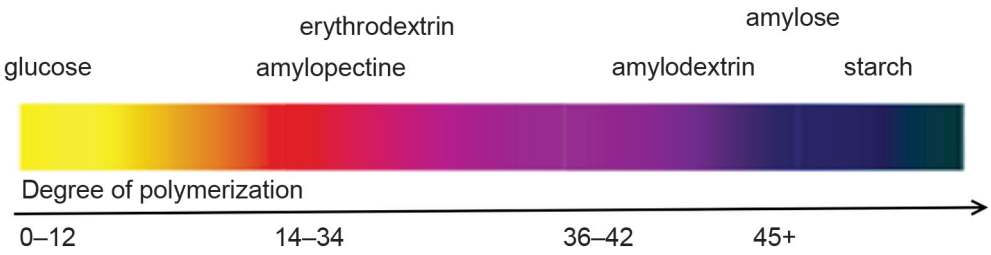


Figure 3.1.6.7. Sample change in Lugol's iodine colour in the presence of starch degradation products

Prepare the iodine solution in potassium iodide (Lugol's iodine) by dissolving about 0.1 g iodine in a solution containing 2 g potassium iodide in 50 cm^3 water. Make up with water to 100 cm^3.

3.1.6.3. Dietary fibre – raw fibre, ADF and NDF fibre, pectins

Determining insoluble non-starch substances in natural materials is equivalent to determining so called dietary fibre, which contains lignins, cellulose, hemicellulose, pectins, soluble non-pectin substances, resistant starch, and other residues [52]. The Regulation of the European Parliament and of the Council (EU) no. 1169/2011 of 25 October 2011 includes fibre content in the list of detailed information that are

elective to declare (Article 30 par. 2). Also national regulations [3] specify the rules for declaring nutritional value information on food labelling, including dietary fibre content. One of the best methods of determination is to perform enzymatic hydrolysis of insoluble, defatted biomass, then analyse the resulting sugar solution using HPLC. This enables estimating the content of individual dietary fibre fractions, *i.e.* glucose content is proportional to cellulose content, pentoses correspond to hemicellulose, glucuronic acid corresponds to pectins, while phenylpropene alcohols – to lignin [45]. In practice, it is necessary to carry out a multi-stage extraction and hydrolysis with the use of various enzymes (including thermally stable α-amylase, pepsin, and pancreatin [85]), which enable the hydrolysis of all the components mentioned above. The Codex Alimentarius Commission has published the [86] guidelines, which includes various methods for assessing dietary fibre content in different product types. The most widespread are methods AOAC 2001.03 for food not containing resistant starch, and AOAC 2009.01, applied also when resistant starch is present in the test sample. On the other hand, method AOAC 991.43:1994, while it cannot always be used for inulin-rich functional food, enables determining total fibre content broken down into water-soluble and -insoluble.

The most commonly used are much simpler gravimetric methods, where water-soluble substances, then components hydrolysed in acids and in alkali are successively removed from a defatted sample.

Gravimetric assay of raw fibre [87]:

In this assay, a defatted sample of 1 g is subjected for 30 minutes to the effects of, successively, sulfuric acid solution (150 cm^3 0.13 mol/dm^3 acid) and potassium hydroxide (150 cm^3 0.23 mol/dm^3), adding a few drops of *n*-octanol as an anti-foaming agent to the boiling solution if needed. Wash the residue on a glass (or quartz) sintered disc funnel, let it drain, wash it with three 25 cm^3 portions of acetone, then dry to constant mass at 130°C. Roast the residue at 475–500°C to constant mass (heating cycles lasting at least 30 minutes). Calculate raw fibre content using the following formula:

$$m_{ns},\ \% = 100 \cdot \frac{\Delta m}{m} \tag{3.1.6.18}$$

where:

Δm – loss of mass after incineration during sample assay, g,

m – weighed amount of test substance, g.

The above analysis is part of so called Weende (basic) analysis, under which five primary fractions are isolated from feed: water, total protein, raw fibre, nitrogen-free extracts, and raw ash. This method, developed in the 19th century by Henneberg and Stohmann, forms the basis of contemporary feed value assessment systems

to this day. Unfortunately, the method of raw fibre determination only reflects the content of cellulose and lignin. A newer methodology proposed in laboratories is based on the Van Soest method, which determines neutral detergent fibre (NDF), comprising cellulose, hemicellulose and lignin, and ADF (acidic detergent fibre), enabling cellulose and lignin content in the material to be assessed. Pectins contained in the soluble fibre fraction (*i.e.* dietary fibre) must be determined by other methods.

Gravimetric assay of neutral (NDF) and acidic (ADF) detergent fibre [87]:

Today, detergent fibre assays are done using automatic Floss Tecator devices and closed Fibertec capsules. Conventional methods and glass crucibles with P-2 sinters can also be used. To determine NDF, weigh about 0.5 g of the fragmented plant material on an analytical balance and heat it to boil (60 minutes) in a solution of stabilised sodium-lauryl sulfate. After this time, filter the undissolved precipitate, wash several times with water, then dry at 105°C (4 hours). Weigh all precipitate and transfer to an acidic detergent solution for ADF. Heat in the same way as in NDF assay. Wash with water and subsequently with acetone (two portions of 10–15 cm^3), and dry precipitate at 105°C to constant mass (4 hours). Finally, roast the reminder at 500°C for 1 hour.

Calculate neutral (m_{NDF}) and acidic (m_{ADF}) detergent fibre content in dry matter using the following formulae:

$$m_{NDF},\ \% = 100 \cdot \frac{m_1 - m_3}{m \cdot DM} \tag{3.1.6.19}$$

$$m_{ADF},\ \% = 100 \cdot \frac{m_2 - m_3}{m \cdot DM} \tag{3.1.6.20}$$

where:

m_1 – mass of crucible with precipitate in NDF method, g,
m_2 – mass of crucible with precipitate in ADF method, g,
m_3 – mass of crucible after roasting, g,
m – weighed amount of test substance, g,
DM – dry matter content in sample.

Prepare the solution for NDF, *i.e.* sodium lauryl sulfate solution, by dissolving 18.61 g disodium EDTA and 6.81 g borax ($Na_2B_4O_7 \cdot 10H_2O$) in 500 cm^3 distilled water, then add 30 g sodium lauryl sulfate to the solution. Separately dissolve 4.56 g sodium hydrophosphate (Na_2HPO_4) in hot distilled water. After cooling, mix both reagents and make up with distilled water to 1 dm^3.

Prepare the ADF solution by diluting 27 cm^3 concentrated sulfuric acid with water to a volume of 0.5 dm^3 and dissolve 20 g CTABr (cetyltrimethylammonium bromide) in it. After dissolving the reagent in warm solution, make up with water to 1 dm^3.

Gravimetric assay of pectins [88]:

One of the simplest pectin determination methods is the gravimetric method. For pure pectin-rich substances (*e.g.* marmalades and jams), extract a fragmented solid sample for 10 minutes with hot diluted nitric acid (100 nM) at a 1 : 40 sample : liquid ratio. Filter off insoluble substances, cool the liquid to 4°C and add cold 66% ethanol or acetone. After an hour in cold storage conditions, separate the precipitated pectins and dry at 50°C to constant mass.

For dried and fragmented non-processed materials (e.g. plant parts), suspend a 2–10 g sample in water (100 cm^3), bring pH to 4–4.5 (1 N HCl), add 0.5 g sodium hexamytaphosphorate and heat in water bath to 80°C for an hour. Filter off insoluble substances, add an amount of cooled 95% ethanol double twice the volume of filtrate to the solution and put in a refrigerator for 1 hour. Filter off the pectin gel and wash it three times with 30 cm^3 of an ethanol : water mixture (1 : 1 v/v) mixed with 5 cm^3 2 N HCl. Write the result relative to dry matter [89].

If precipitating the pectinates in solid form proves difficult, a precipitation method can be used [90]: suspend a 5 g sample in hot water for 15 minutes, the filter to a 100 cm^3 flask, washing the reside on filter paper with water. Add 20 cm^3 0.25 M NaOH to 50 cm^3 of filtrate, mix thoroughly and leave for 15 minutes. Next, add 10 cm^3 1.5 M acetic acid and after 5 minutes, 20 cm^3 1.5 M $CaCl_2$ solution. Mist thoroughly and leave for 90 minutes. Filter the precipitated calcium pectinate and wash with cold water until reaction to chlorides disappears. Dry the precipitate at 105°C until constant mass and calculate to pectin.

Titration assay of pectins [91]:

Carefully moisten 20 g plant material of known moisture content or 0.2–0.5 g pectins with 2 cm^3 ethanol and dissolve (or suspend) in 20 cm^3 distilled water, and add 2 cm^3 1 N NaOH. Mix and leave for 30 minutes at room temperature. Acidify the solutions with 1 N HCl (3 cm^3), mix thoroughly and precipitate pectins using 53 cm^3 0.1 N HCl. Leave for 20 minutes, then filtrate through wide-pore filtration paper. Transfer exactly 10 cm^3 filtrate to a separate flask, and combine the rest of the filtrate with the precipitate in the original flask, together with 10 cm^3 water used to wash the funnel. Titrate the contents of both flasks with 0.1 N NaOH in the presence of Hilton indicator.

Calculate the amount of pectins using the following formula:

$$m_{\text{pect}},\ \% = 100 \cdot \frac{(V_2 - V_1 \cdot f) \cdot 176 \cdot C}{1000\, m \cdot DM} \tag{3.1.6.21}$$

where:

V_1 – volume of 0.1 N NaOH in cm^3, used to titrate the HCl in 10 cm^3 of filtrate,

f – if the volumes from the procedure are maintained, the conversion factor for calculating the NaOH volume used to titrate the HCl in 10 cm^3 of filtrate and HCl present in the remainder of the mixture equals 9,
V_2 – volume used to titrate the combined filtrate, precipitate, and washing water,
C – concentration of KOH used for titration, mol/dm^3
176 – molar mass of polygalacturonic acid,
V – weighed amount of test substance, g,
DM – dry matter mass of sample, %.

To create the Hinton indicator, mix 20 cm^3 0.4% bromothymol blue solution with 60 cm^3 0.4% phenol red, 20 cm^3 0.4% cresol red, and 20 cm^3 water. Use their corresponding sodium salts to prepare the individual indicators [92].

Colorimetric assay of pectins [92]:

Carefully moisten 20 g plant material of known moisture content or 0.2–0.5 g pectins with 2 cm^3 ethanol and dissolve (or suspend) in 20 cm^3 distilled water, and add 2 cm^3 1 N NaOH. Mix and leave for 30 minutes at room temperature. Make up to 100 cm^3 with distilled water and stir. Take 2 cm^3 of the solution for the test and add 1 cm^3 of carbazole solution. Dissolve the white precipitate by adding 12 cm^3 concentrated sulfuric acid. In the same manner, prepare the reference sample, where the carbazole reagent is replaced with 1 cm^3 anhydrous ethanol. After exactly 15 minutes, measure absorbance at 525 nm wavelength. Read the result from a calibration curve prepared for standard solutions of polygalacturonic acid monohydrate dissolved in 0.05 N NaOH (the solution should be prepared a day earlier). Calculate pectin content in the sample using the formula:

$$m_{\text{pect}},\ \% = 100 \cdot \frac{V \cdot C}{1000\, m \cdot DM} \tag{3.1.6.22}$$

where:

V – volume of sample solution (100 cm^3),
C – concentration read from the calibration curve, mg/cm^3,
m – weighed amount of test substance, g,
DM – dry matter mass of sample, %.

3.2. Selected analyses for liquid samples

3.2.1. Colour analyses

The colour of a plant oil stems from its origin, variety, and method of processing, and together with *e.g.* measurements of characteristic numbers, enables determining the degree of fat refinement (purification). The deacidifaction, degumming, and bleaching processes not only remove solid impurities, free fatty acids, phospholipids, proteins, *etc.*, but also natural vitamins and dyes that significantly affect the hue and intensity of oil colour. Similarly to mineral oils, visual assessment enables determining the approximate degree of lubricant wear.

The most common methods of colour assessment include comparative colorimetric (iodine and Hazen scales) and spectrophotometric methods, frequently combined with chromatographic methods to separate individual dyes into fractions.

3.2.1.1. Colour analyses by Iodine and Hazen scale

Among simplest methods of colour analysis is visual comparison of samples with I_2 solutions in KI at different dilution ratios (so called iodine scale for colours from yellow to orange) or with platinum and cobalt solutions in the case of the Hanzen scale (colour from yellow to brown).

Colour assessment in iodine scale:

Colour is estimated by visually comparing the colour of a sample with the colour of an iodine solution of known concentration, diluted in 10% potassium iodide. To make visual comparison of colour intensity easier, solution volumes should be identical (*e.g.* 1 cm^3). Examples of samples are shown in Figure 3.2.1.1.

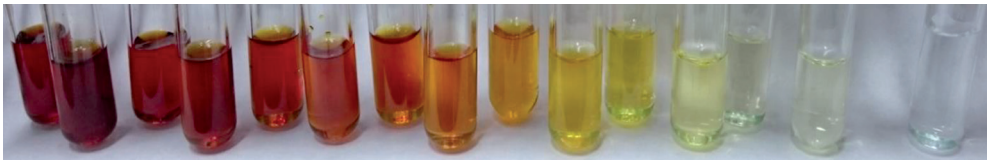


Figure 3.2.1.1. Presentation of solutions prepared for determination of colour in the iodine scale. Concentrations from 38.6 to 0.2 mmol I_2/dm^3 and water on the right for comparison

When preparing the solutions, remember that iodine must be poured directly into a weighed flask with a known volume of 10% KI. When the solution is weighed on an analytical balance again, the exact mass of iodine in the solution can be calculated. In this way, iodine sublimation in the weighing vessel is avoided.

Determination of colour in APHA scale (Hazen):

According to the standard PN-81/C-04534.01 [93], the colour of liquid chemical products not containing mechanical purities can be expressed using a platinum and cobalt scale, the so called Hazen scale, which at its maximum value of H500 corresponds to the colour of a solution containing exactly 1 mg of platinum in the form of potassium chloroplatinate and 0.5 mg of cobalt in the form of cobalt chloride in 1 dm^3. The primary H500 solution is produced by dissolving 1.245 g K_2PtCl_6 and 1.00 g $CoCl_2 \cdot 6H_2O$ in water with 100 cm^3 concentrated hydrochloric acid added, then making up to 1 dm^3 with distilled water. Absorbance of the primary solution prepared this way (measured in relation to water as the standard), measured at wavelengths: 430, 455, 480 and 510 nm should fall within the ranges: 0.11–0.12; 0.13–0.145; 0.105–0.12 and 0.055–0.065, respectively. Using an appropriate scale (following the relation shown in Figure 3.2.1.2), prepare a series of standard solutions, diluting the primary solution with distilled water to a volume of 100 cm^3. Introduce samples of clear test solutions and standard solutions to Nessler cylinders and perform a visual determination or use a colorimeter.

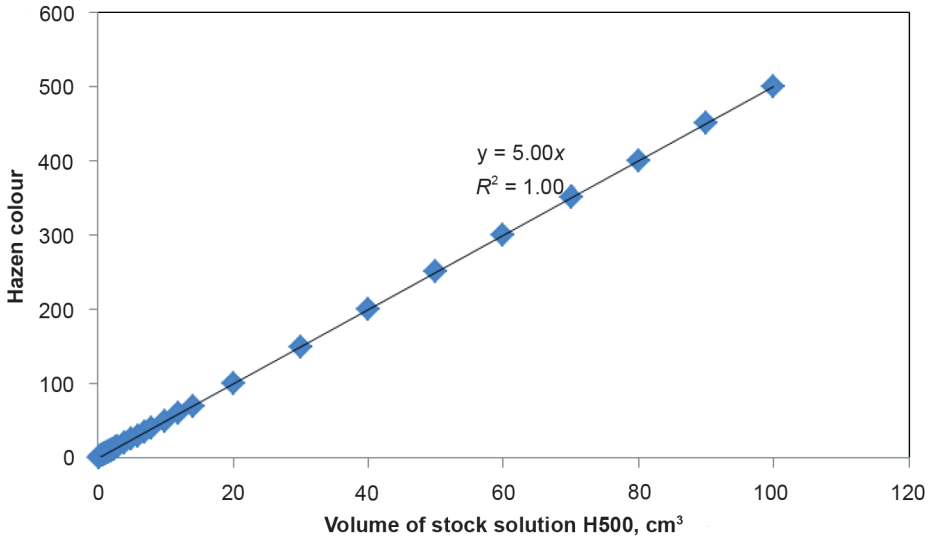
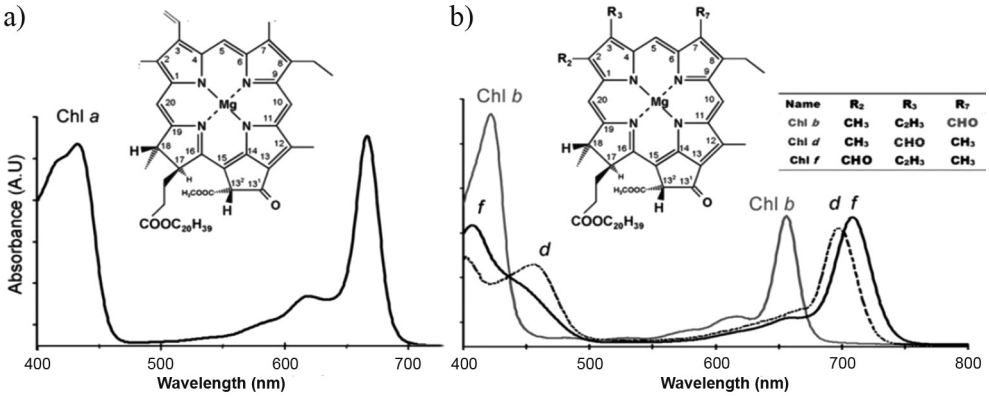


Figure 3.2.1.2. Template for H500 primary solution dilutions to 100 cm^3 for the Hazen colour scale. Based on [93]

3.2.1.2. Spectrofotometric quantitative analyses of natural pigments

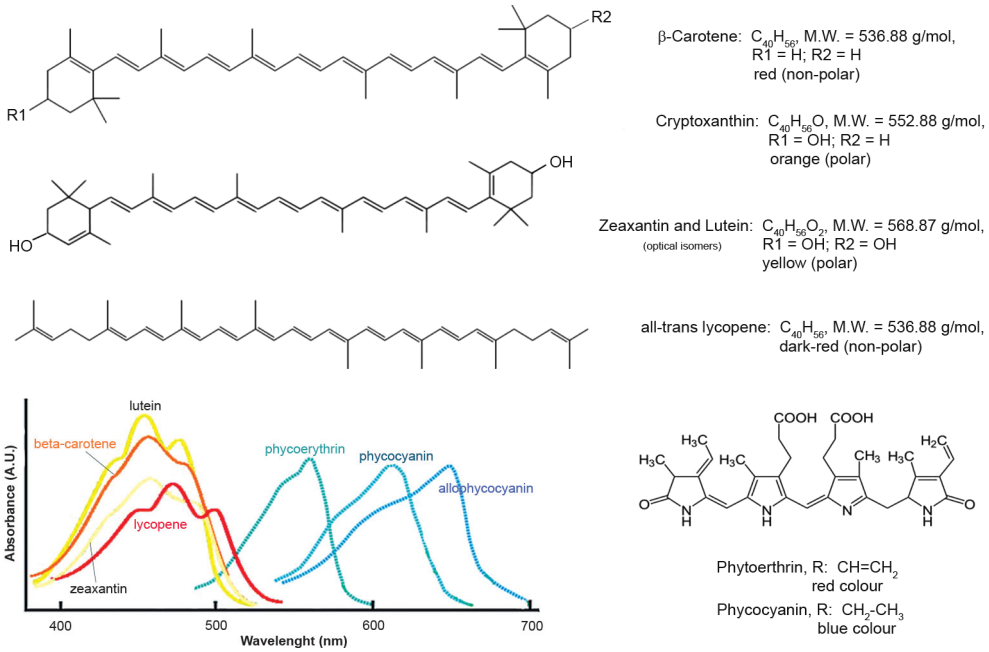
Spectrophotometric methods enable assessment of colours by direct determination of the actual content of chlorophyll and carotenoid dyes. The assay is performed on liquids, by measuring absorbance at appropriately selected



Chlorophyll *a*: $C_{55}H_{72}MgN_4O_5$, M.W. = 893.50 g/mol, blue-green, polar

Chlorophyll *b*: $C_{55}H_{70}MgN_4O_6$, M.W. = 907.49.50 g/mol, green, polar

Figure 3.2.1.3. Chemical structure and spectra of methanol solutions of the individual chlorophyll types (a) and (b) [94]



β-Carotene: $C_{40}H_{56}$, M.W. = 536.88 g/mol, R1 = H; R2 = H, red (non-polar)

Cryptoxanthin: $C_{40}H_{56}O$, M.W. = 552.88 g/mol, R1 = OH; R2 = H, orange (polar)

Zeaxantin and Lutein (optical isomers): $C_{40}H_{56}O_2$, M.W. = 568.87 g/mol, R1 = OH; R2 = OH, yellow (polar)

all-trans lycopene: $C_{40}H_{56}$, M.W. = 536.88 g/mol, dark-red (non-polar)

Phytoerthrin, R: $CH{=}CH_2$, red colour

Phycocyanin, R: $CH_2{-}CH_3$, blue colour

Figure 3.2.1.4. Chemical structures and spectra of selected non-chlorophyll plant dyes. Based on [95, 96]

wavelengths. For example – for chlorophyll, it is approx. 660 nm, while for carotenoids – 440 nm. While literature mentions four types of chlorophyll (*a*, *b*, *d*, and *f* – Figure 3.2.1.3), the most widespread are chlorophyll *a* and *b*, which – together with carotenoid dyes – are responsible for the observed plant colours.

Chlorophylls, as primary dyes, and carotenoids (auxiliary dyes) are referred to as assimilation dyes and are responsible for the correct functioning of photosynthesis in plants. In nature, they are frequently accompanied by other dyes, whose structural formulas and corresponding IV–VIS spectra are shown in Figure 3.2.1.4.

Aside from determination of natural dye content, spectrophotometric methods also enable assessing the content of proteins (*e.g.* direct method – section 3.1.5) and unsaturated derivatives (see section 3.2.8.3).

Assessment of general colour using the spectrophotometric method:

The colour assessment involves measuring the absorbance of samples of fat or plant extracts after they are dissolved in *n*-hexane at two wavelengths within the visible light spectrum [97]. To measure absorbance within the range characteristic to carotenoids, dissolve 1 cm^3 oil in 10 cm^3 *n*-hexane, mix thoroughly, then measure absorbance (A_{442}) at a wavelength λ = 442 nm with pure solvent as the standard. Absorbance within the range characteristic to chlorophylls (A_{668}) is measured at a wavelength λ = 668 nm with pure *n*-hexane as standard, and the sample must be diluted with the solvent at a volume ratio 1 : 1.

Sum the absorbance values read and express then as an integer:

$$B = 1000 \cdot (A_{422} + A_{668}) \qquad (3.2.1.1)$$

Thus method is highly simplified, as it does not take into account the differences in the positions of absorbance maxima for different chlorophyll dye types (Figure 3.2.1.3).

Quantitative assay of assimilation dye concentration:

According to literature [98] it is possible to determine the content of chlorophyll *a*, chlorophyll *b*, and carotenoid dyes for acetone (80%), methanol, ethanol (95%) and ether plant extracts. Absorbance determination at appropriate wavelengths (Table 3.2.1.1) is employed here, with the use of pure solvent as the standard sample. Regardless of the solvent selected, grind an approx. 0.5 g sample in a mortar with a bit of sand and $CaCO_3$, centrifuge or filter the mixture, and make up with solvent to known volume. Dye concentration is calculated using formulae shown in the table.

Table 3.2.1.1

Equations for calculating g/cm^3 concentration of chlorophyll *a* (C_a), chlorophyll *b* (C_b) and carotenoids (C_c) in various solvents [98]

Solvent	λ, nm	Equation
80% acetone	663.2 nm 646.8 nm 470 nm	$C_a = 12.25A_{663.2} - 279A_{646.8}$ $C_b = 21.5A_{646.8} - 5.1A_{663.2}$ $C_c = (1000A_{470} - 1.82C_a - 85.02C_b)/198$
95% ethanol	664 nm 649 nm 470 nm	$C_a = 13.36A_{664} - 5.19\,A_{649}$ $C_b = 27.43A_{649} - 8.12\,A_{664}$ $C_c = (1000A_{470} - 2.13C_a - 97.63C_b)/209$
Diethyl-ether (DEE)	660.6 nm 642.2 nm 470 nm	$C_a = 10.05A_{660.6} - 0.97A_{642.2}$ $C_b = 16.36A_{642.2} - 2.43A_{660.6}$ $C_c = (1000A_{470} - 1.43C_a - 35.87C_b)/205$
Methanol	665.2 nm 652,4 nm 470 nm	$C_a = 16.72A_{665.2} - 9.16A_{652.4}$ $C_b = 34.09A_{652.4} - 15.28A_{665.2}$ $C_c = (1000A_{470} - 1.63C_a - 104.96C_b)/221$

* A_λ – absorbance read with the appropriate solvent as the standard at a wavelength λ, nm.

3.2.1.3. Chromatographic qualitative analyses

Qualitative assessment of the content of individual dyes can be performed using column chromatography or TLC, which enable separation of individual dyes present in plant samples. To this end, grind a sample of the natural material (*e.g.* levels, stems, or flowers) in a mortar with sand, 20 cm^3 hexane and 5 cm^3 methanol. Shake the mixture vigorously with water, then discard the cloudy water phase (bottom). For hexane extracts and solutions (*e.g.* plant oil), washing can be skipped. Carefully transfer the sample solution (1 cm^3) to a hexane-saturated chromatographic column filled with aluminium oxide (5 cm^3) and starch or silica gel (20 cm^3). Elute the pigments successively with pure hexane (about 20 cm^3), hexane mixed with acetone at volume ratios 9 : 1, 7 : 3, and 1 : 1, and finally with pure acetone. Collect the dyes eluted from the column to separate flasks in order to perform spectrophotometric identification. Adjust the rate of liquid flow from the column with a valve, not allowing air to get in the column bed. To accelerate separation, partial vacuum can be used at the column's outlet (suction flask) or overpressure at the column's inlet (pressurised gas).

If TLC is used, place the silica gel plate or Whatman 3 blotting paper strip with the sample applied into a chamber (beaker) with a mixture of solvents. Use petroleum ether 40–60°C, toluene and anhydrous ethanol at a volume ratio of 8 : 3 : 2 as the developing system [101]. After developing the chromatogram, mark the spots and calculate the R_f coefficient, i.e. the ratio of distance travelled by the analysed substance to the distance travelled by the solvent front. The R_f values are

strictly dependent on the conditions of the analysis, *i.e.* separating layer polarity and composition of the solvent mixture used. Under the above conditions, the R_f parameters are: β-carotene 0.8–0.95; pheophytin 0.65–0.7; chlorophyll *a* 0.6–0.7, chlorophyll *b* 0.5–0.6, xantofphylls 0.2–0.5.

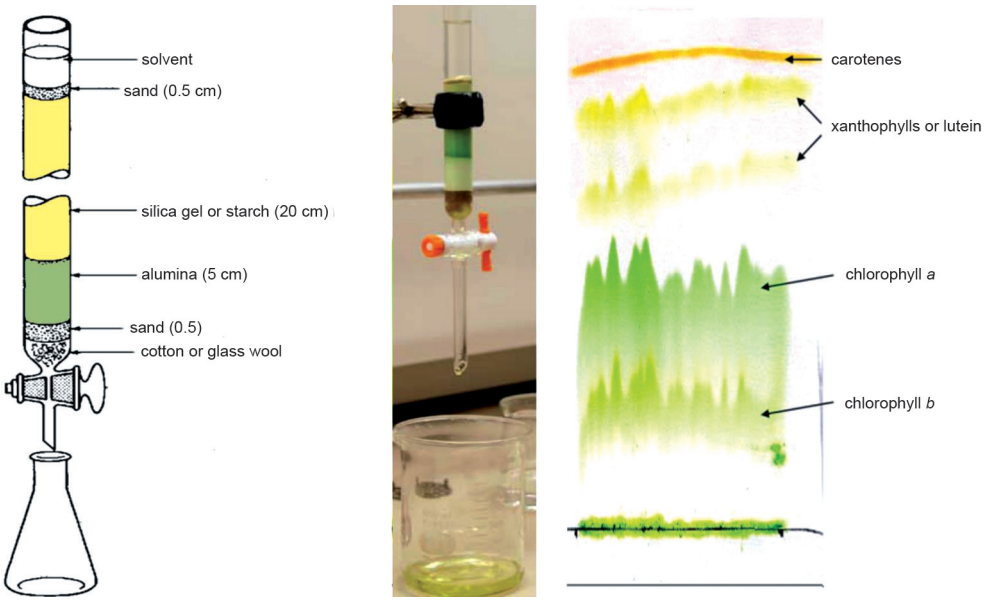


Figure 3.2.1.5. Dye separation on a chromatographic column and a TLC plate. Images source [99, 100].

When separating synthetic dyes added to food products, very good results are achieved using a developing system composed of 25%NH_3 : C_2H_5OH : H_2O at a volume ratio of 1 : 2 : 3 [102]. Sample R_f values for commercial dyes (BASF) are: yellow-coloured tetrazine 0.78; red-coloured cochineal red 0.85; amaranth 0.47 and erythrosine 0.56; sunset yellow 0.6; and blue-coloured indigotine 0.52 and brilliant black 0.21. A comprehensive review of solvent systems used for natural dye separation can be found in a Forgacs and Cserhati paper [103].

3.2.2. Density

Solution density can be determined using the areometric method, basing on the Archimedes' principle. In this method, a liquid at a stabilised temperature *T* is introduced into a cylinder where an areometer or thermoareometer is immersed (Figure 3.2.2.1). Density of the test liquid at temperature *T* is read from the float's

grade. A drawback of this method is the requirement to have a sufficient amount of the test liquid at one's disposal (100–150 cm^3).

For smaller sample volumes, the pycnometric method is more suitable [104], whereby the test liquid at temperature T is introduced to a weighed pycnometer, and after weighing, the pycnometer is filled with water at the same temperature and weighed again.

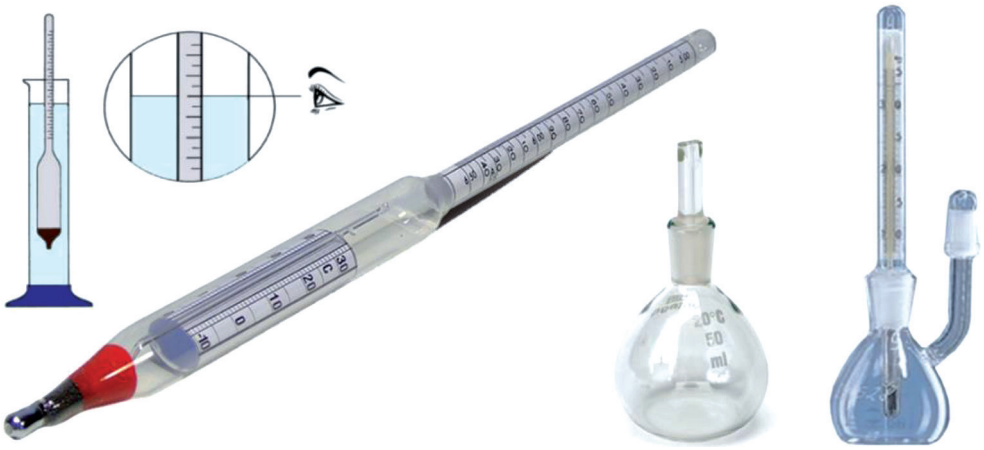


Figure 3.2.2.1. From the left: principle of measurement with areometer, thermoareometer, pycnometer with capillary plug and pycnometer with thermometer. Image compilation [105–108]

Test liquid density at temperature T (ρ_T, g/cm^3) is calculated using formula (3.2.2.1), where correction for weighing in air and for water density at temperature T (ρ_{wT}, kg/m^3) is read from the appropriate tables [104]. Because water density at temperatures between 15 and 55°C is characterised by a polynomial relation with temperature, it can also be calculated using the curve equation shown in Figure 3.2.2.2.

$$\rho_T = \frac{\rho_{wT} \cdot (m_T - m)}{1000 \cdot (m_{wT} - m)} - P \qquad (3.2.2.1)$$

where:

- ρ_T – density of test liquid at temperature T, g/cm^3,
- ρ_{wT} – density of water at temperature T, g/cm^3,
- m_T – mass of pycnometer with test liquid at temperature T, g,
- m_{wT} – mass of pycnometer with water at temperature T, g,
- m – mass of empty pycnometer, g
- P – correction for weighing in air, equal to: 0,0011 for densities 0,7600–0.7899 g/cm^3; 0.0012 for densities 0.7900–0.8199 g/cm^3;

0.0013 for densities 0.8200–0.8599 g/cm^3; 0.0014 for densities 0.8600––0.8899 g/cm^3; 0.0015 for densities 0.8900–0.9299 g/cm^3; 0.0016 for densities 0.9300–0.9599 g/cm^3; 0.0017 for densities 0.9600––0.9899 g/cm^3; 0.0018 for densities 0.9900–1 g/cm^3 [109].

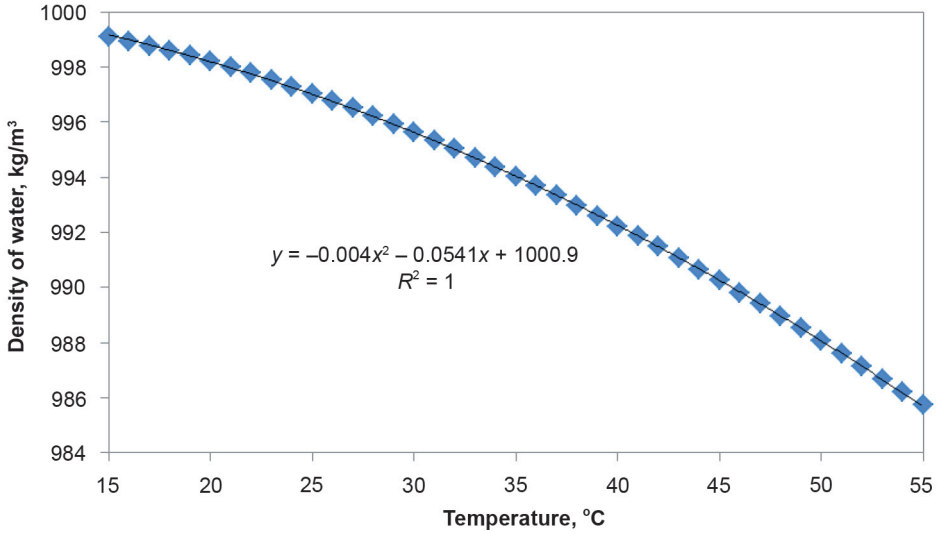


Figure 3.2.2.2. Water density as a function of temperature. Based on tables [110]

In order to enable comparing the densities of multiple liquids, the test must be performed at the same selected temperature T (pycnometers with their contents should be thermostated for 30 minutes), or use a calculation formula (3.2.2.2), which is useful for most oils and crude oil products [109].

When measuring the density of any liquid, regardless of method, special attention must be paid to its uniformity; it is also important that measurement is performed at a temperature above the cloud point of the given liquid.

$$\rho_{T'} = \rho_T + \alpha \cdot (T - T') \tag{3.2.2.2}$$

where:

$\rho_{T'}$ – density of test liquid at standard temperature T, kg/m^3,
ρ_T – density of test liquid at temperature T, kg/m^3,
T – measurement temperature T, °C,
T' – standard temperature, °C,
α – temperature factor of density, g/cm^3 · °C, read from the appropriate tables [x]. For the predicted density from the 0.88–0.89 g/cm^3 range the value of the factor α is 0.000647; for densities between 0.89–0.9 g/cm^3 it is 0.000633; for 0.9–0.91 g/cm^3 0.00062; while for densities from further

density ranges, from 0.92 to 1 g/cm^3 (with a step every 0.01 g/cm^3), the values of α are 0.000607; 0.000594; 0.000581; 0.000567; 0.000554; 0.000554; 0.000541; 0.000528 and 0.000515, respectively.

With a good approximation (with minor differences between the measurement temperature and standard temperature), the following formula can be used to calculate the calculation factor value:

$$\alpha = -0.0013 \cdot \rho_T + 0.0018 \tag{3.2.2.3}$$

According to the standard for biodiesel fuel [4], the standard temperature is recommended as 15°C, while the density correction for the mixture of methyl esters of fatty acids is calculated on the basis of formula 3.2.2.2, where the correction factor α value is 0.723.

For crude oil products, global petroleum companies often use density determinations in arbitrary, dimensionless units, so called °API (American Petroleum Institute), which can be calculated knowing the dimensionless relative density of the liquid at a temperature of 60°F (15.6°C), using the formula [111]:

$$^{\circ}\text{API} = \frac{141.5}{SG_{60\,^{\circ}\text{F}}} - 131.5 \tag{3.2.2.4}$$

where relative liquid density (*SG*) is the proportion of its density at temperature *T* (usually 15.6°C), given in kg/m^3, to water density under identical conditions. Appropriate calculators enabling automatic conversion between the above units are also available in the Internet [112].

3.2.3. Viscosity and viscosity index

Viscosity, as one of the most important properties of fluids (liquids and gases), is related to intermolecular interactions and constitutes the resistance that occurs during movement of layers of medium relative to each other. The magnitude of internal friction forces in liquids is therefore decided by the energy of molecule vibrations, which strongly depends on temperature. Dynamic viscosity expresses the ratio of shearing stresses to shearing rate, and its SI unit is kg/m · s (in the CGS system it is poise, 1 P = 1 g/cm · s). On the other hand, kinematic viscosity expresses the ratio of dynamic viscosity to fluid viscosity, and its unit is m^2/s (in the CGS system, it is stokes, 1 St = 1 cm^2/s). It bears stressing that with rising temperature, gas viscosity rises, while it drops for all liquids. An exception here is water in the 2–4°C range.

The most common measurements methods are based on measuring the rate of liquid flow through a capillary tube (kinematic viscosity measurement), and methods based on measuring the rate of ball fall in the test liquid (dynamic viscosity measurement).

Conversion of dynamic viscosity η (mPa · s) to kinematic ν (mm²/s) is based on the relation (3.2.3.1); mind that the temperature of liquid viscosity and density measurements (ρ_c, g/cm³) should usually be the same:

$$\eta = \rho_c \cdot \nu \tag{3.2.3.1}$$

3.2.3.1. Kinematic viscosity

Kinematic viscosity measurement is based on the Hagen-Poiseuille law [113], under which the equation (3.2.3.2), after a transformation, enables calculating the density of liquids based on the measurement of flow time *t* (sec) of a known liquid volume *V* (cm³) under its own weight through a capillary of diameter *r* (cm) and length *l* (cm):

$$\eta = \frac{\pi \cdot r^4 \cdot \Delta p}{8 \cdot l \cdot V} \cdot t \tag{3.2.3.2}$$

$$\Delta p = \frac{\Delta h}{2} \cdot g \cdot \rho \tag{3.2.3.3}$$

$$\nu = \left(\frac{\pi \cdot r^4 \cdot \Delta h \cdot g}{16 \cdot l \cdot V} \right) \cdot t = k \cdot t \tag{3.2.3.4}$$

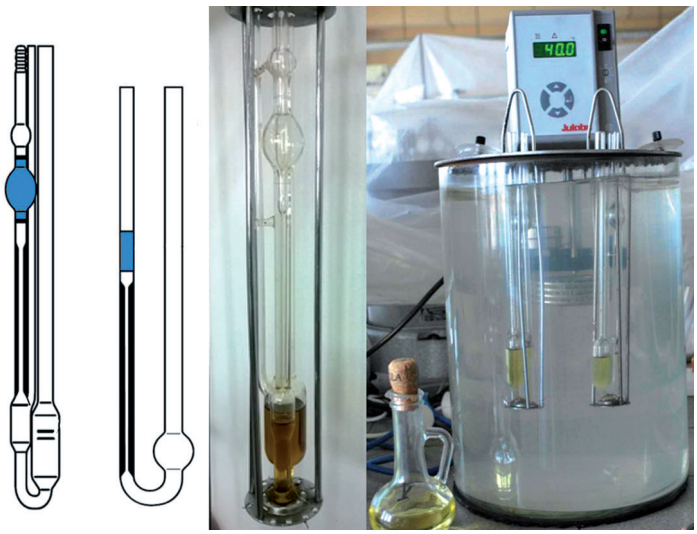


Figure 3.2.3.1. From the left: Ubbelohde and Ostwald capillaries, Ubbelholde capillary in a protective basket, and sample placed in a thermostated water bath. Blue marks the measurement bubble, where the time of liquid flow is measured

where:

Δp – difference of pressures at capillary ends, Pa,
Δh – difference in liquid levels, cm,
g – gravitational acceleration, cm/s^2,
ρ – liquid density, g/cm^3,
k – so called capillary constant, determined by measuring the time it takes an exactly known volume of standard oil to flow through a capillary.

Sample Ubbelohde and Ostwald capillaries are shown in Figure 3.2.3.1, the most important elements of their structure are marked. These capillaries are placed in appropriately designed baskets and in a water or oil bath to stabilise the temperature of samples.

Performing the viscosity determination:

Using an Ubbelohde capillary, place it in a basket and fill it to the indicated level (about 3/4 of the bottom container) with the test liquid, pouring it through the widest tube of the viscosimeter. Place it in the bath and thermostat it at 40°C for 30 minutes before performing the determination. Using a pump or a filler, suction the liquid to the measurement capillary above the measurement bubble level (to do this, plug the outlet of the most narrow tube of the viscosimeter with a finger at the same time). Measure the time of free liquid flow through the measurement bubble (between the two marked thresholds). Make the measurement in three repetitions, and substitute the time measured in seconds into equation (3.2.3.4). The measurement is done correctly if the liquid flow time is within the 2–30 minutes range. If the time is shorter, repeat the analysis using a capillary with a lower constant, while if it is longer – the capillary should have a lower value of the constant k.

As stated in literature [114], for pure plant oil and biodiesel fuel, it is possible to estimate the kinematic viscosity (cSt) values when knowing the density (ρ, g/dm^3), biodiesel flash point (FP, °K), or the molar composition of methyl esters of fatty acids (y_i) and the cetane index of individual components (CN_i) as per the equations:

$$\nu_{\text{oil}} = -0.7328 \cdot \rho_{\text{oil}} + 938.78 \tag{3.2.3.5}$$

$$\nu_{\text{FAME}} = -16.155 \cdot \rho_{\text{FAME}} + 930.78 \tag{3.2.3.6}$$

$$\nu_{\text{FAME}} = 22.981 \cdot FP + 346.79 \tag{3.2.3.7}$$

$$\ln \nu_{\text{FAME}} = \sum_{i=1}^{n} y_i \cdot CN_i \tag{3.2.3.8}$$

A fairly good correlation is also achieved between viscosity at 40°C (mm^2/s) and saponification value (N_s) and FAME iodine value (N_I), although the fit (3.2.3.9) was done on the basis of only 19 samples of biodiesel fuel made of 10 different

plant oils [115]. Despite this, a clear rise in ester biofuel viscosity is observed with increasing chain length and degree of acid radical unsaturation [116].

$$\nu_{\mathrm{FAME}}(40\,^{\circ}\mathrm{C}) = 146 - 0.488N_s - 0.149N_I \tag{3.2.3.9}$$

From the standpoint of utility, knowledge of the relation between viscosity and temperature, which for most lubricant oils decreases hyperbolically, is fairly important. This, coupled with experimental data [117], enabled formulating the following correlation between viscosity expressed in cSt and temperature (°C) for individual methyl esters of fatty acids and for free fatty acids (FFA):

$$\ln \nu_{\mathrm{sat}\,C_6-C_{12}} = -2.915 - 0.158z + \frac{492.12}{T} + \frac{108.35z}{T} \tag{3.2.3.10}$$

$$\ln \nu_{\mathrm{sat}\,C_{12}-C_{18}} = -2.177 - 0.202z + \frac{403.66}{T} + \frac{109.77z}{T} \tag{3.2.3.11}$$

$$\ln \nu_{C_{18:1}} = -5.03 + \frac{2051.5}{T} \tag{3.2.3.12}$$

$$\ln \nu_{C_{18:2}} = -4.51 + \frac{1822.5}{T} \tag{3.2.3.13}$$

$$\ln \nu_{C_{18:3}} = -4.18 + \frac{1685.5}{T} \tag{3.2.3.14}$$

$$\ln \nu_{C_{22:1}} = -5.42 + \frac{2326.2}{T} \tag{3.2.3.15}$$

$$\ln \nu_{\mathrm{FFA}} = -2.496 - 0.326z + \frac{657.12}{T} + \frac{173.31z}{T} \tag{3.2.3.16}$$

where:

sat C_6–C_{12}, sat C_{12}–C_{18} – saturated esters of fatty acids with short and longer carbon chains,

z – number of carbon atoms in a molecule.

3.2.3.2. Dynamic viscosity

Measurement of dynamic viscosity is based on Stokes law [118], according to which a ball falling in a viscous medium is affected by internal friction force S, expressed by the equation (3.2.3.17) and equal to the force of gravity Q, reduced in accordance with Archimedes' principle by the buoyant force P (3.2.3.18):

$$S = 6 \cdot \pi \cdot \eta \cdot r \cdot v \tag{3.2.3.17}$$

$$Q - P = \frac{4}{3}\pi r^3(\rho_k - \rho_c)g \tag{3.2.3.18}$$

where:

r, v – radius and ball fall rate, respectively,
ρ_k, ρ_c – density of ball and liquid, respectively, g/cm^3,
g – gravitational acceleration,
η – dynamic viscosity of test liquid, mPa · s.

Ball density is calculated using the following formula:

$$\rho_k = \frac{3}{4} \cdot \frac{m_k}{\pi r^3} \tag{3.2.3.19}$$

where:

r, m_k – mean ball diameter and mass, respectively.

After transforming both equations, the following formula is obtained:

$$\eta = \frac{2}{9} \cdot \frac{r^2}{l} \cdot g \cdot (\rho_k - \rho_c)t = K(\rho_k - \rho_c)t \tag{3.2.3.20}$$

where:

K – ball constant, mPa · cm^3/g,
t – ball fall time in seconds along a way of length l, cm.

Measurements are performed in a thermostated Höppler viscometer, shown in Figure 3.2.3.2.

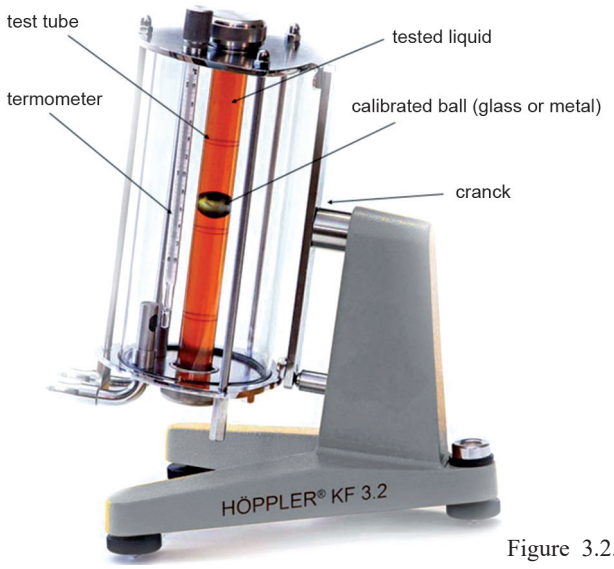


Figure 3.2.3.2. Höppler viscometer

Performing the viscosity determination:

For tests using a Höppler viscometer, fill the measurement tube with the test liquid, so that no air bubbles are trapped inside. Next, select one of the standard balls (glass or metal) and insert it in the cylinder containing the liquid. Close the tube inlet with a tight-fitting cap and observe the rate of ball fall – if it is too fast, change the ball to a lighter one (with lower density), while if the time is too long, select a heavier ball.

Before performing the measurement proper (time of ball fall between two extreme lines marked on the measurement tube), thermostat the system for 30 minutes. Substitute the average from three measurements in the formula (3.2.3.20) – the moving arm of the apparatus enables ball movement in both directions. Select the ball constant based on Table 3.2.3.1. When performing the measurement, pay attention to the uniformity of the solution, there shod be no visible mechanical impurities, phase separation, or turbidity characteristic for liquids below their cloud point.

Table 3.2.3.1

Measurement ball constants for Höppler viscometers [119]

Ball number	Type of ball	Measurement range, η, mPa · s	Ball density ρ_k, g/cm^3	Ball diameter *r*, mm	Constant *K*, mPa · cm^3/g
1	glass	0,6–10	2.2÷2.4	15.81 ±0 .01	0,007
2	glass	4–130	2.2÷2.4	15.6 ± 0.05	0,09
3a	metal	20–700	7.7÷8.1	15.6 ± 0.05	0,09
3b	glass		2.2÷2.4	15.15 ± 0.05	0,7
4a	metal	150–4 800	7.7÷8.1	15.2 ± 0.2	0,7
4b	glass		2.2÷2.4	14.2 ± 0.1	5.4
5	metal	1500–45 000	7.7÷8.1	14.0 ± 0.5	7
6	metal	7500–80 000	7.7÷8.1	11.0 ± 1	35

3.2.3.3. Viscosity index

Based on kinematic viscosity measurements at temperatures of 40°C and 100°C, so called viscosity indicator (*VI*) is calculated, which provides information about changes in viscosity of the given oil, depending on temperature. Essentially, the higher the *VI* value, the lower the temperature's impact on kinematic viscosity. This parameter is important primarily for mineral lubricant oils working under high loads. The viscosity indicator is calculated using formula (3.2.3.21), where results of viscosity determination for the given oil are compared with the results obtained for standard oils with viscosity indicators of 0 and 100:

$$VI = \frac{L - U_{40}}{L - H} \cdot 100 = \frac{L - U_{40}}{D} \cdot 100 \qquad (3.2.3.21)$$

where:

L – kinematic viscosity at 40°C of L-series standard oil (viscosity indicator 0), which has at 100°C an identical viscosity as the test oil,

H – kinematic viscosity at 40°C of H-series standard oil (viscosity indicator 100), which has at 100°C an identical viscosity as the test oil,

U_{40} – kinematic viscosity of the test oil at 40°C, mm²/s,

D – difference between L and H.

Attention must be paid to the stability of the test liquid (natural oil) at elevated temperatures, as the presence of volatile or low-boiling components (essential oils, short-chained free fatty acids, *etc.*) may make measuring viscosity at 100°C impossible due to intensive evaporation or boiling.

For the other oils with kinematic viscosity values at 100°C (U_{100}) fitting within the 2–70 mm²/s range (most natural fats), the L and D values are read from tables in the standard [120], using linear interpolation if necessary. These relations can also be read from chart 3.2.3.3, which presents this data in graphical form.

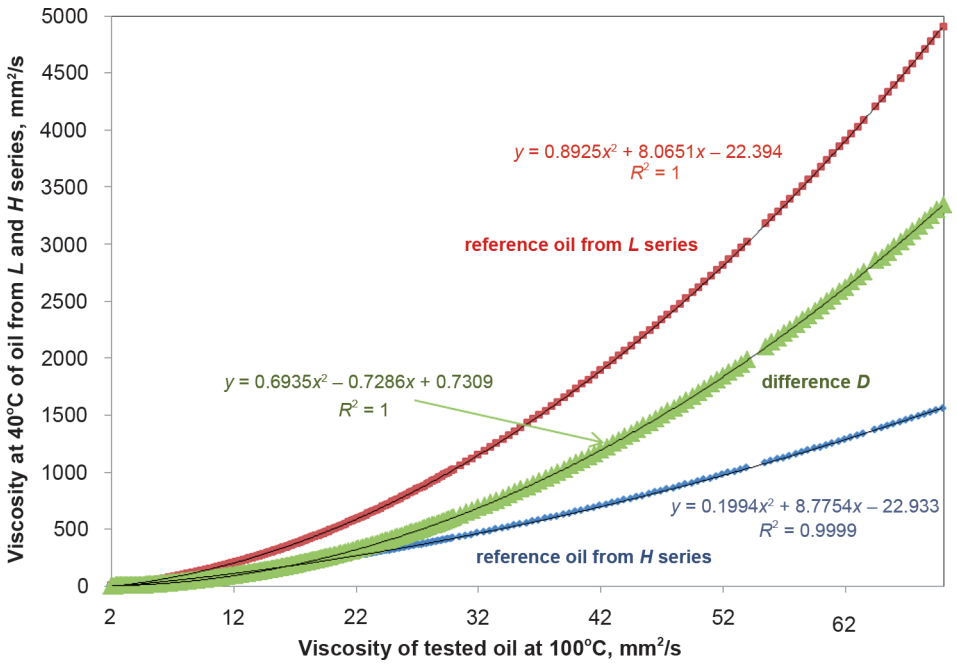


Figure 3.2.3.3. L, D and H values as a function of the test oil's viscosity at a temperature of 40°C. Based on tabular standard data [120]

However, for oils with viscosities U_{100} exceeding 70 mm²/s, but lower than 100 mm²/s, the L and H values are calculated using the following formulae:

$$L = 0.8353 \cdot U_{100}^2 + 14.67 \cdot U_{100} - 216 \tag{3.2.3.22}$$

$$D = 0.6669 \cdot U_{100}^2 + 2.82 \cdot U_{100} - 119 \tag{3.2.3.23}$$

For oils whose VI values, determined using the above method, exceed 100, the actual viscosity indicator is calculated using the following formula:

$$VI = \frac{\text{Anty}\log N - 1}{0.00715} + 100 \tag{3.2.3.24}$$

where:

$$N = \frac{\log H - \log U_{40}}{\log U_{100}} \tag{3.2.3.25}$$

H – read from tables in the standard (for values between 2–70 mm²/s) or calculated from formula (3.2.3.26) for values exceeding 70 mm²s:

$$H = 0.1684 \cdot U_{100}^2 + 11.85 \cdot U_{100} - 97 \tag{3.2.3.26}$$

3.2.4. Low temperatures properties

Low-temperature values are of great importance for motor fuels, as they limit the consumption of the given fuel under winter conditions. For pure substances, solidification temperature provides a method of identification, as do refractive index of light or boiling point. For mixtures of multiple components (*e.g.* plant oils, motor fuels and biodiesel fuel, pure or mixed with mineral fuel), gradual reduction of temperature results first in precipitating crystallisation nuclei, which manifests in a clearly visible turbidity in the sample (so called cloud point, CP). Further reduction of temperature fosters sedimentation of crystals and their growth, which leads to blocking fuel lines and filters in the engine supply system, and consequently to vehicle immobilisation. Such temperature is referred to as cold filter plugging point (CFPP), and according to standards, it should be lower than 0°C for the summer period, at least –1°C for the intermediate period, and at least –20°C for the winter period [4, 121]. Further cooling of the sample leads to solidification throughout its volume and is commonly referred to as the solidification point. It is equivalent to the pour point, which according to the standard [122], constitutes a threshold below which a fuel loses its liquid nature (PP).

3.2.4.1. Cloud and freezing point by probe method

The simplest method of determining cloud and solidification points is so called test tube method [123]. It involves placing an oil in an isolated test tube with a thermometer, which is thermostated in a cooling mixture at a temperature of –40°C (Figure 3.2.4.1). The use of insulation is intended to ensure an even reduction in temperature throughout the volume of the test sample. During cooling, sample clarity is inspected periodically, noting the temperature of first clouding in a part or in the whole volume of the fluid. Continue cooling the sample, inspecting its condition about every 1 minute, by tilting the tube by about 45°. Solidification

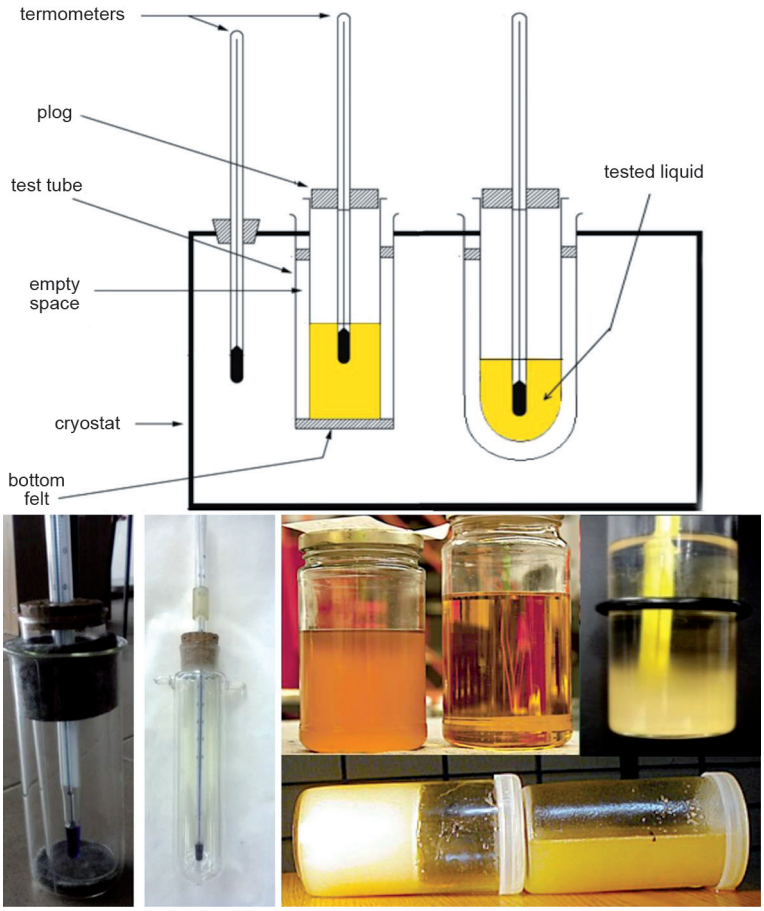


Figure 3.2.4.1. Diagram of the test station for measuring cloud and solidification points using the test tube method, and images of test tubes and samples. Compilation of own images and images available online [126–128]

temperature is identified as the highest temperature where no movement of the meniscus is observed within a minute of tilting the test tube.

According to literature, biodiesel clouding can be connected to fuel chemical composition (% m/m), in particular the presence of saturated derivatives and ones with long hydrocarbon chains [124, 125]:

$$CP = 299.6 \cdot y_{C_{16:0}} + 378.5 \cdot y_{C_{18:0}} + 266.5 \cdot y_{C_{18:1}} + 265.2 \cdot y_{C_{18:2}} \qquad (3.2.4.1)$$

3.2.4.2. Cold filter plugging point

According to current standard [129], the cold filter plugging point is the highest temperature at which a specific volume of fuel cooled under standardised conditions does not flow through a standardised filtration system. This determination is performed by suctioning the sample through standardised filters to a pipette under controlled vacuum conditions and at a temperature gradually reduced in 1°C steps. The measurement is complete when flow is stopped or slowed to such a degree that the time to fill a 20 cm^3 pipette with the cooled fuel exceeds 60 s or fuel does not flow completely back to the measurement vessel (Figure 3.2.4.2).

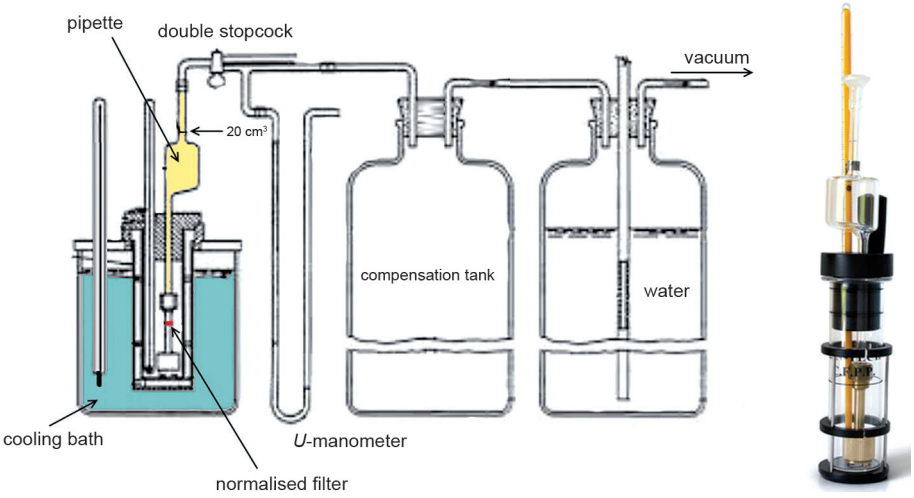


Figure 3.2.4.2. Diagram of apparatus for measuring cold filter plugging point, with an image showing the measurement pipette [130]

As stated in sources [131], there is a relation between the cloud point and CFPP that can be expressed with the following equations:

$$CFPP_{RME} = 1.0 \cdot CP - 4.5 \qquad (3.2.4.2)$$

$$CFPP_{RME+FAME} = 1.0 \cdot CP - 2.9 \quad (3.2.4.3)$$

$$CFPP_{RME+ON} = 0.95 \cdot CP - 4.3 \quad (3.2.4.4)$$

These equations apply to pure rapeseed biodiesel (RME), mixtures of rapeseed biodiesel with other fatty acid esters (RME + FAME), and to rapeseed biodiesel and diesel oil (RME + ON).

Correlation of low-temperature properties and chemical composition of fuel (% m/m) appears even more accurate, especially if saturated derivatives or ones with long hydrocarbon chains are present in its composition [124, 125]:

$$CFPP,\ ^{o}K = 3.1417(0.1y_{C_{16}} + 0.5y_{C_{18}} + y_{C_{20}} + 1.5y_{C_{22}} + 2y_{C_{24}}) - 16.477 \quad (3.2.4.5)$$

3.2.5. High temperature properties

High-temperature properties, which include smoke point, flash point, and autoignition temperature. All these parameters are of great importance from the perspective of user safety and storage of the given product. According to current regulations, flash point measurement is the basis for classifying the fire hazard posed by the given product (class I to III), while autoignition temperature is the basis for classifying explosive mixtures in six explosive hazard groups, specified by so called temperature classes (T1-T6) [132].

3.2.5.1. Smoke point, ignition point and fire point

The smoke point is a value characteristic for edible oils and fats, as well as some mineral oils. It shows in a simple manner up to what temperature it is possible to use oils (*e.g.* frying) without them degrading. This temperature is lower than flash point, at which liquid heated under standardised conditions (*i.e.* at an appropriate rate in a standardised apparatus) generates an amount of vapours sufficient to create a mixture with air that is flammable when a flame is placed nearby. The amount of generated vapours, however, is insufficient to sustain combustion and once they burn out, the flame extinguishes itself. Usually it is observed as a short flash of flame, accompanied by a loud crack. Further temperature rising leads to reaching the burning temperature, *i.e.* a condition where the amount of vapours generated in a unit of time is equal to the amount of vapours burned, and therefore sufficient to sustain combustion. All three analyses can be performed in an apparatus with so called open crucible, while in an apparatus with a closed crucible, given its specific structure, only flash point is analysed. Typical apparatus used in the above tests are shown in Figure 3.2.5.1.

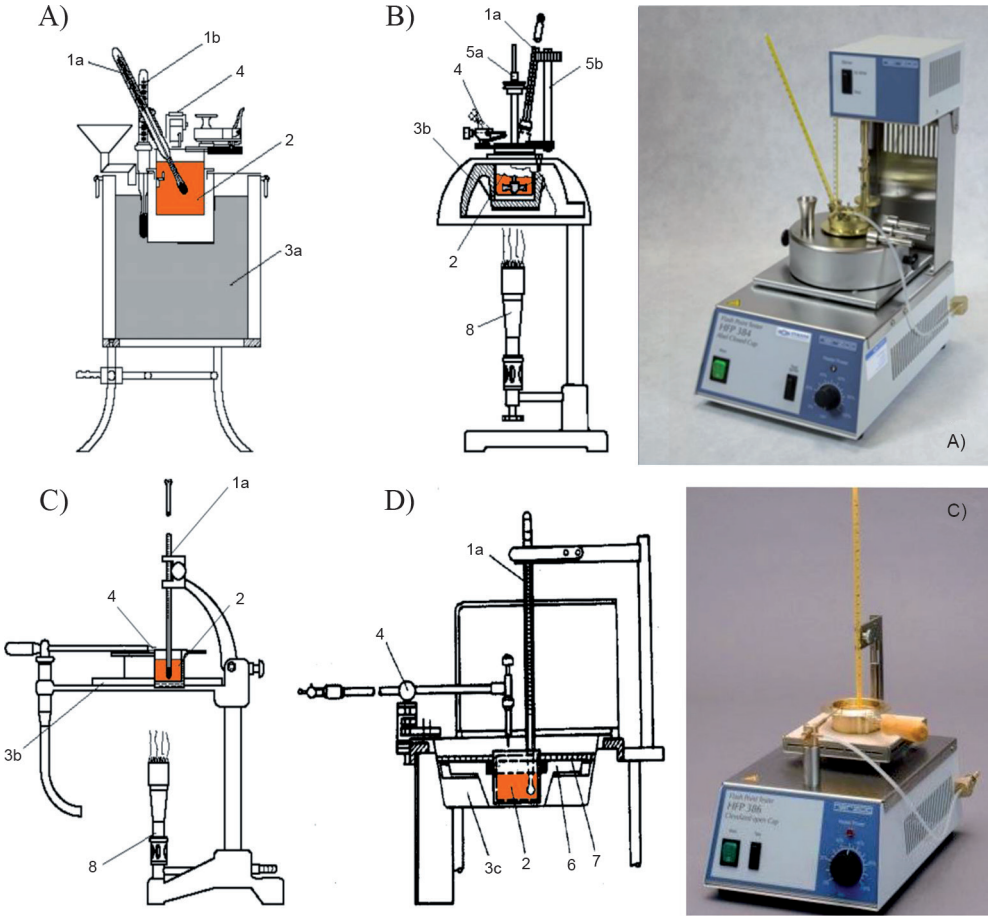


Figure 3.2.5.1. Schematic diagrams of the most common apparatus for measuring flash point and high-temperature properties. From the top, closed crucible apparatus according to Abel-Pensky (A) and Martens-Pensky (B); bottom – open crucible apparatus by Cleveland (C) and Marcusson (D). Key: 1 – thermometer for measuring sample (a) and thermostating water bath temperature (b); 2 – test sample; 3 – heating elements: a – water bath, b – metal block, c – vessel with sand; 4 – burner of ignition element; 5a – stirrer; 5b – knob that activates window opening and burner tilting; 6 – fastening ring; 7 – asbestos plate; 8 – burner. Based on [132] and own artwork

When performing measurements using Martens-Pensky or Cleveland apparatus, at a pressure of 101.3 kPa, a correction to the determined flash point must be taken into account, in accordance with the following equation:

$$\Delta t = \frac{101.3 - p}{3.3} \cdot 0.9 \qquad (3.2.5.1)$$

where:

p – barometric pressure at which the determination is performer, kPa.

ATTENTION! All the measurements mentioned must be performed under an inactive fume hood, minimising air motion above the surface of the liquid, as it changes vapour pressure above the solution and inflates the result of the assay. As high-temperature analyses destroy samples, once they are complete, pour the test liquid to an appropriate waste container and thoroughly wipe (**do not wash!**) the crucibles.

It bears stressing that the result read in a closed crucible apparatus, which is dedicated for liquids with higher volatility values, is always several degrees lower than the temperature read during measurements in an open apparatus, which is used for lubricant oils and other consumable liquids, operating under high loads and in open systems.

According to literature [133], it is possible to calculate an approximate flash point (FP) for pure substances based on known boiling point (T_b, °K), heat of evaporation ($\Delta_{vap}H°$, kJ/mol), and number of carbon atoms in the molecule (n):

$$\text{FP, } {}^{o}\text{K} = 1.477 \cdot T_b^{0.79686} \cdot \Delta_{vap}H^{o\,+0.16845} n^{-0.05948} \tag{3.2.5.2}$$

For biodiesel, this formula takes the following form [124]:

$$\text{FP, } {}^{o}\text{K} = 0.3544 \cdot T_b^{1.14711} \cdot n^{-0.07677} \tag{3.2.5.3}$$

Furthermore, based on analyses of several different types of biodiesel, certain authors have determined the empirical relations enabling fuel flash point (°C) to be calculated based on knowing its density at 15°C (kg/m^3) or viscosity at 40°C (cSt) [134]:

$$\text{FP, } {}^{o}\text{C} = 1.4601 \cdot \rho - 1099.9 \tag{3.2.5.4}$$

$$\text{FP, } {}^{o}\text{C} = 12.357 \cdot v - 176.3 \tag{3.2.5.5}$$

3.2.5.2. Autoignition point

Flash point and fire point must be clearly distinguished from autoignition temperature, which is the lowest temperature at which automatic ignition of vapours of a heated liquid in air atmosphere (without the use of a burner or another ignition system). This determination is performed using specially designed apparatus (Figure 3.2.5.2), where the liquid product is added by the drop (*e.g.* from a burette or an automatic feed) to a chamber heated with an aluminium block or hot air. A comparison of methods recommended by different standards can be found in literature [135]. When autoignition temperature is exceeded, the liquid ignites itself in the combustion chamber. What is important is that autoignition temperature rises

with increasing ambient pressure and decreasing oxygen concentration in the air, so under typical conditions, exceeding the autoignition temperature is tantamount to explosive hazard. Below autoignition temperature and above flash point lies so called explosive range (lower and upper explosive limit), which determines the safety of work in a gaseous phase with flammable substances.

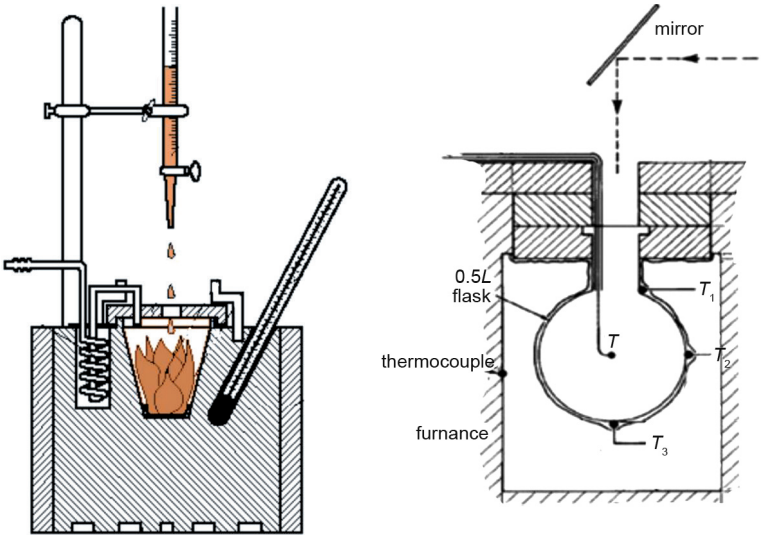


Figure 3.2.5.2. Diagrams of apparatus for autoignition temperature determination. Based on [136, 137]

As [135, 138] states, autoignition temperature for i-component mixtures is an additive value equalling:

$$T_{\text{mix}}, {}^{\circ}\text{C} = \sum_{n=1}^{i} y_i \cdot T_i \tag{3.2.5.6}$$

where:

y_i – molar concentration of component *i*,
T_i – autoignition temperature of component *i*.

3.2.5.3. Cetane number and cetane index

When characterising fuels for high pressure engines, the key parameter is **cetane number** (CN), which determines the fuel's ability to autoignite. This number is determined under standardised conditions by comparing the ignition time of the given fuel with the ignition time of a standard fuel, composed of a mixture of cetane (hexadecane $C_{16}H_{34}$) with a CN = 100 and very short ignition time, and α-methylnaphthalene with zero CN, or heptamethylnonane (CN 15) [139]. For diesel

fuels, the CN value is usually within the 48–55 range, while for biodiesel, according to current regulations, it must be no lower than 55 [4].

Due to the high costs of a cetane number measurement engine, in practice the **cetane index** (CI) value is frequently used, as it is fairly simple to calculate based on fuel density and results of fractional distillation of the fuel (see section 3.2.5.6). Current standards [140] recommend the 4 points method, *i.e.* density at 15°C and temperatures at which 10, 50 and 90% of fuel are distilled away [141, 142]:

$$\mathrm{CI} = 45.2 + 0.0892 \cdot T_{10\mathrm{N}} + (0.131 + 0.901 \cdot \mathrm{B}) \cdot T_{50\mathrm{N}} + \\ + (0.0523 - 0.420 \cdot \mathrm{B}) \cdot T_{90\mathrm{N}} + 0.00049 \cdot (T_{10\mathrm{N}}^2 - T_{90\mathrm{N}}^2) + 107 \cdot \mathrm{B} + 60 \cdot \mathrm{B}^2 \qquad (3.2.5.7)$$

and:

$$T_{10\mathrm{N}},\ ^{\circ}\mathrm{C} = T_{10} - 215 \qquad (3.2.5.8)$$

$$T_{50\mathrm{N}},\ ^{\circ}\mathrm{C} = T_{50} - 260 \qquad (3.2.5.9)$$

$$T_{90\mathrm{N}},\ ^{\circ}\mathrm{C} = T_{90} - 310 \qquad (3.2.5.10)$$

$$\mathrm{B} = e^{-3.5 \cdot \rho\mathrm{N}} - 1 \qquad (3.2.5.11)$$

$$\rho\mathrm{N},\ \mathrm{g/cm^3} = (\rho - 0.85) \qquad (3.2.5.12)$$

where:

T_{10}, T_{50}, T_{90} – the temperatures, °C, at which respectively 10%, 50% and 90% of fuel volume is distilled away at standard pressure 101.3 kPa,

ρ – fuel density at a temperature of 15°C.

3.2.6. Boiling point, distillation and vapour pressure

The boiling point of a pure substance is a tabulated value, characteristic for the given compound. Its measurement not only enables the substance to be identified, but also for its purity to be determined. Relevant regulations [143] recommend measuring boiling points using an ebulliometer, by the distillation method, dynamic method at varying pressure, Sivolobov method recommended for small sample volumes, or by calorimetric methods. The condition for performing the determination is no chemical transformation of the sample (decomposition, oxidation, *etc.*) during its heating. It must also be remembered that the result of the determination will be affected by pressure and presence of impurities.

With minor deviations from standard pressure equalling 101.325 kPa (no more than ±5 kPa), the boiling point is normalised to T_n using the Sidney-Young equation:

$$T_n = T + f_T \cdot (101.325 - p) \qquad (3.2.6.1)$$

where:

p – ambient pressure, kPa,
T – measured boiling point, K,
T_n – boiling point corrected to standard pressure, K,
f_T – rate of temperature change with pressure, K/kPa, which for temperatures between 373.15 and 573.15 K is described by the following equation as a good approximation:

$$f_T = 0.0008 \cdot T - 0.009 \tag{3.2.6.2}$$

3.2.6.1. Boiling point by Sivolobov method

For small volumes of pure samples, distillation temperature is determined in a Thiele apparatus (Figure 3.2.6.1), also used for determining melting points. The apparatus is filled with a silicone oil as the heating medium, and heating occurs only on the side arm, effectively forcing a flow of medium within the apparatus. A thermometer is then placed in a bath prepared in this manner, to which a small test tube (external diameter up to 5 mm) is subsequently affixed, containing the

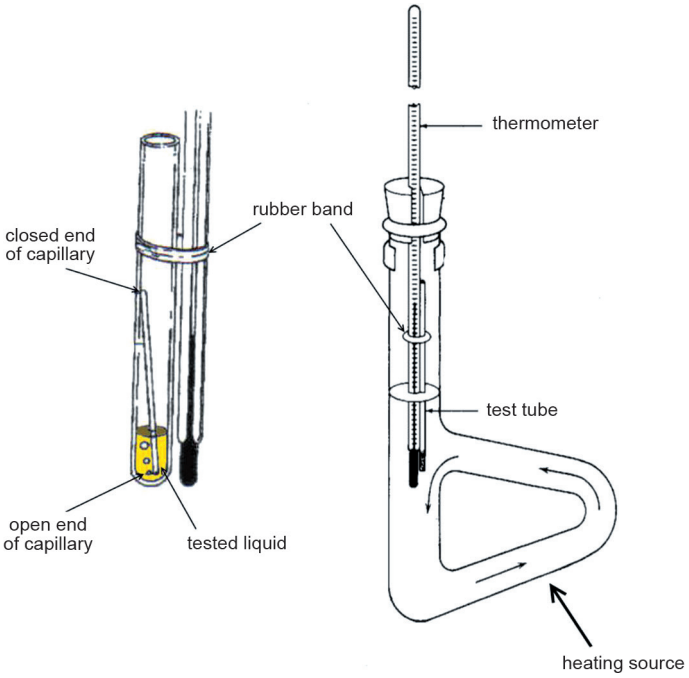


Figure 3.2.6.1. Thiele apparatus with a test tube and a capillary for determining distillation temperatures by the Sivolobov method. Based on [144, 145]

test sample and a small capillary – 1 mm diameter and wall thickness approx. 0.2–0.3 mm – fused at its upper end. Initially, the liquid bath heating is set to a temperature rise at a rate of 3°/min, while at a temperature about 10°K below the expected boiling point, the temperature increase rate is reduced to 1°/min at most. With the boiling point approaching, bubbles being to rise from the capillary. Boiling point is a temperature at which, when momentarily cooled, the bubble chain stops and the liquid level in the capillary begins to rise as a result of equalising pressures.

3.2.6.2. Engler distillation

In the case of single-component liquids, simple distillation, usually carried out under atmospheric pressure, enabled determining the boiling point of the given substance. For two-component A and B mixtures, boiling point under constant pressure is different from boiling points of pure substances and is dependant on the content of both components in the solution. The relation between boiling point under constant pressure and liquid and vapour composition is shown by TPXY curves (Figure 3.2.6.2).

For complex mixtures, such as crude oil products and ester biofuels, initial and final boiling points are determined, *i.e.* the boiling temperature range, while a distilling fraction efficiency test within selected temperature ranges forms the basis for assessing the quality and suitability of crude oil for the given process. Crude oil products are distilled under atmospheric pressures to a temperature of 350°C, above which components of the sample undergo thermal decomposition. The remainder is distilled under reduced pressure, correcting temperatures to atmospheric pressure using formula (3.2.6.1). The curve of distillation efficiency (% m/m) in relation to temperature T_N is referred to as the true boiling point curve, while the Engler curve illustrates the relation between distillate efficiency in % v/v to distillation temperature T. Such a chart, with distillation start and end temperatures marked, as well as temperatures at which selected portions of fuel volume are distilled away (Figure 3.2.6.3), forms a basis for normative determination of fuel fraction composition [146, 147], and is also used to calculate the cetane number (see section 3.2.5.3).

The determination is performed in a standardised apparatus (Figure 3.2.6.4), distilling exactly 100 cm^3 of fuel and reading the temperature of distillation beginning and end (T_{IP}, T_{EP}), as well as **percentage evaporation temperatures** for 5, 10, 20, ... 80, 90 and 95% of fuel distilled (T_5, T_{10}, ... T_{90}, T_{95}, respectively). For petrols, **volumes evaporated** at, successively, 70, 100, 180°C (marked as E_{70}, E_{100}, E_{180}, respectively) and volume evaporated at the end of distillation (E_{EP}) are read. According to standards for diesel oils [147], the test determines the initial point

of distillation (T_{IP}), temperature at which half of the sample volume is evaporated (T_{50}), as well as the volumes evaporated by the time temperatures of 250°C and 350°C are reached, designated E_{250} and E_{350}, respectively. These parameters decide the operating properties of the given fuel.

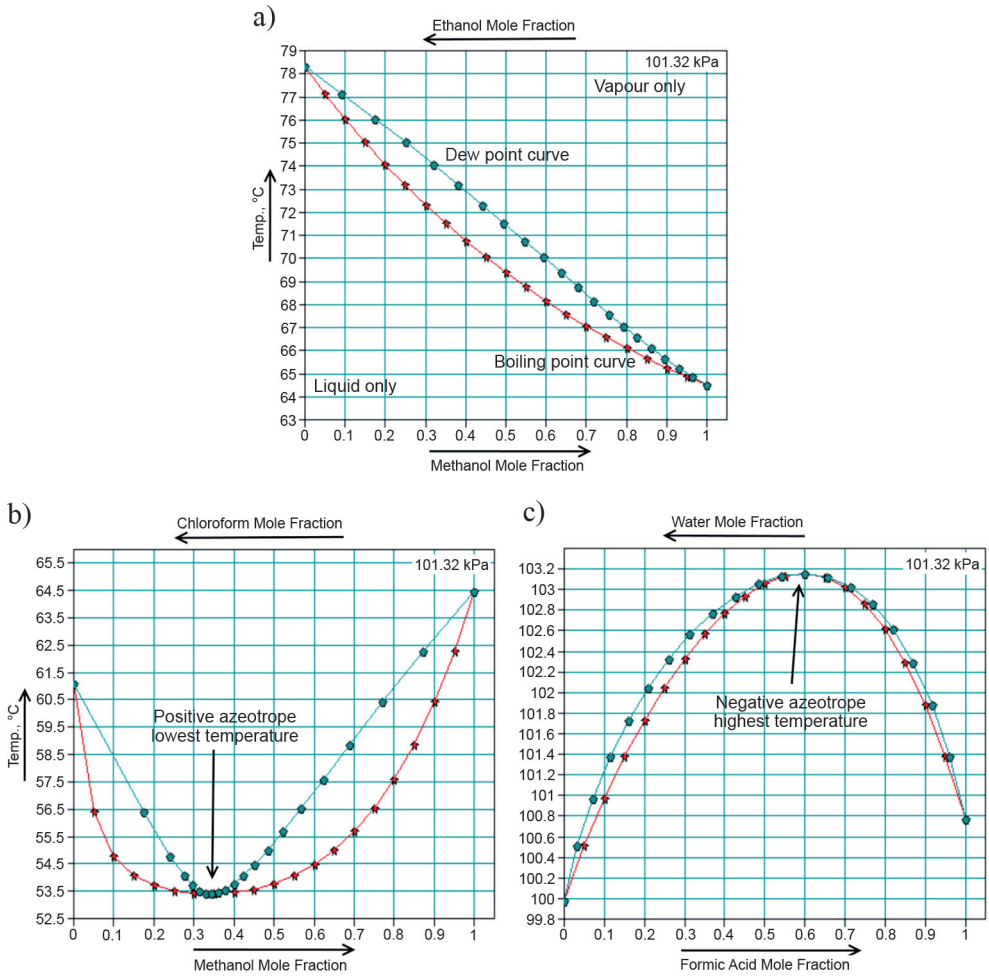


Figure 3.2.6.2. Sample TPXY curves for simple solutions (a) and for solutions forming positive (b) and negative (c) azeotropic mixtures

If the test pressure differs from 1.325 kPa and the temperature correction, mentioned at the beginning of this section, is made (see also 3.2.6.1), then corrections of distillation losses (L_c), total percentage of product volume collected (R_c), and actual percentage of product volume evaporated at the recorded temperature (P_e) must be made:

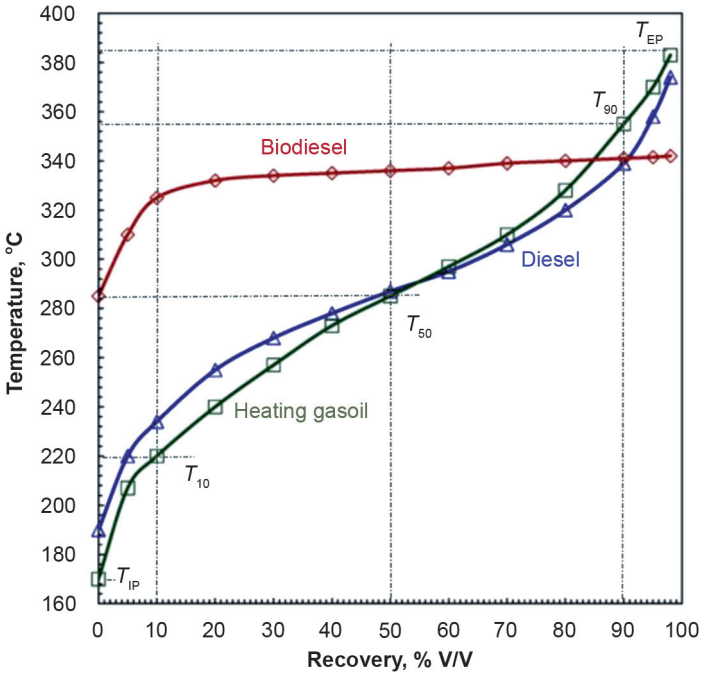


Figure 3.2.6.3. Sample distillation curves for selected fuels, with temperatures of distillation start and end, and temperatures at which selected portions of fuel volume are distilled away. Based on [148]

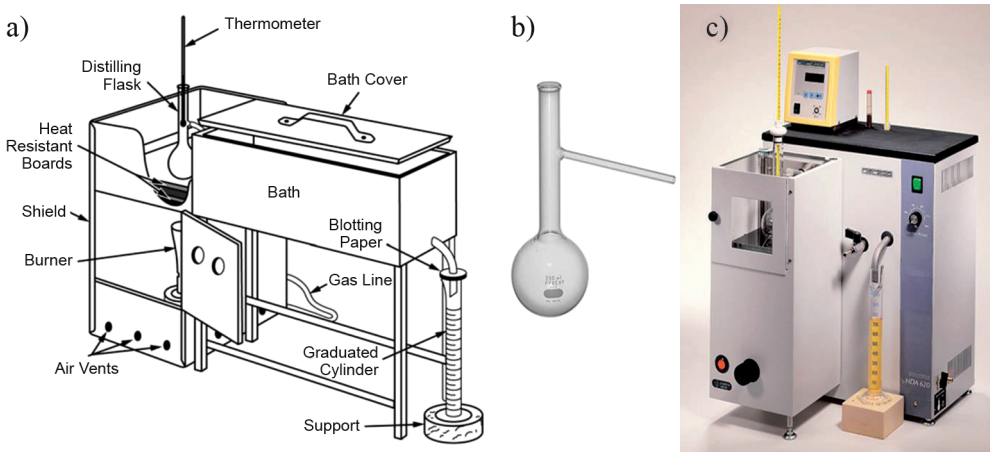


Figure 3.2.6.4. Apparatus for determining the fraction composition using the normal distillation method – general diagram (a), distillation flask (b), sample apparatus (c). Based on [149–151]

$$L_c = 0.5 + \frac{L - 0.5}{1 + \frac{101.325 - P_k}{8}} \tag{3.2.6.3}$$

$$R_c = R + (L - L_c) \tag{3.2.6.4}$$

$$E_e = P_r + L \tag{3.2.6.5}$$

$$L = 100 - V_D - V_R \tag{3.2.6.6}$$

$$E_{ec} = P_{rc} + L_c \tag{3.2.6.7}$$

where:

L, L_c – appropriately determined experimentally and corrected amount of loss, %,

P_k – atmospheric pressure during the test, kPa,

R, R_c – total and corrected total percent of product volume collected, respectively, %,

P_e, P_{ec} – experimental and corrected percent of product volume evaporated, respectively, %,

P_r, P_{rc} – experimental and corrected percent of product volume collected at the given temperature, respectively, %,

V_D, V_R – volume of distillate and volume of residue, cm^3.

It is customary that the distillation curve shows temperatures corresponding to 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 85, 90 and 95% of product evaporated (after corrections for pressure). Next, using appropriate formulae, the actual volume of distillate corresponding to the evaporation percent of 5, 50 and 90% of the volume is calculated, and temperatures are read from the same chart.

3.2.7. Characteristic numbers and related tests

In the case of fat analysis and characterisation, characteristic numbers are used to determine the type and quality of fat. In the former case, saponification value and iodine value are determined, which provide information on the mean molecular mass and degree of unsaturation of the analysed triglyceride. These values are characteristic for the given type of oil and by comparing them with values from literature, they enable the compound to be identified. On the other hand, the acid value and peroxide value provide information on the degree of sample refinement (the more refined the sample, the lower free fatty acid content), and degree of its rancidity due to hydrolytic changes or oxidation. Knowledge of the individual values enables determining the suitability of the given oil for dietary or cosmetic purposes, and enables selecting the correct conditions in soap production, fat hydrogenation, or biodiesel synthesis processes.

The selected analyses are not used for characterising triglycerides only. For example, the saponification number (SN) combined with unsaponificable matter (USM) analysis enables determining the content of ester fuel in mixed biodiesel, acid number (AN) characterises the degree of lubricant oil degradation, while epoxy value (EPN) enables determining the percentage of reacted substrates in the polyol synthesis process. Essentially, the methodology used during analytical assays enables characterising any materials containing ester (saponification number – SN, and ester number – EN), carboxylic (acid number – AN), hydroxylic (hydroxyl number – HN), or epoxy (epoxy number – EPN) groups, as well as unsaturated bonds (iodine number – IN).

3.2.7.1. Acid, saponification and ester numbers

According to the established definition, the **acid number** (AN) is a value specifying the amount of potassium hydroxide, in milligrams, necessary to neutralise free acids (*e.g.* carboxylic) present in 1 g of the test material. For refined fats, it is a measure of rancidity due to hydrolytic changes, although it should be remembered that a certain amount of free fatty acids is always present in freshly expressed natural fat. Appropriate industry standards indicate the maximum acceptable value of the acid number for various fat types intended for nutritional purposes, *e.g.* for refined plant oils, AN should not exceed 0.3 mg KOH/g [152], for animal fats it should be below 1.1 mg KOH/g [153], while for pressed olive oil, values up to 6.6 mg KOH/g are acceptable [154]. For crude oil, the AN value usually does not exceed 0.5–1 mg KOH/g, while for biooil obtained from biomass pyrolysis, the acid number can exceed 100 mg KOH/g. The relevant regulations of the Minister of the Economy concerning requirements for liquid biofuels specify the maximum acceptable value of acid number for B100 biodiesel and diesel fuel containing 20% FAME as 0.5 and 0.2 mg KOH/g, respectively [155].

Acid number (AN, also designated TAN – total acid number) should be distinguished from acidity, which is defined as the number of KOH milligrams necessary to neutralise acids present in 100 cm^3 of a product. **Acidity assays** are usually used for products containing small amounts of free acids, *e.g.* bread (water extract reaction), ash extracts, juices and beverages, petrol, or crude oil--derived solvents.

Acid number is determined by titrating the test material sample in selected organic solvent, suitable for the nature of the analysed material, with a volumetric solution of KOH. Usually it is isopropanol, a mixture of ethanol and toluene (1 : 1 v/v), toluene with isopropanol and water (50 : 49.5 : 0.5 v/v [156, 157]), acetone, etc. To determine the equilibrium point, phenolophthalein is used, while for crude oil products, *p*-naphtholbenzein or alkali blue 6B are recommended, and for

samples with intense dark colours, potentiometric titration is used. Performing the determination at room temperature is crucial. At elevated temperatures, samples can undergo partial saponification, elevating the assay result.

Saponification number (SN) is defined as the number of KOH moles necessary to neutralise fatty acids, both free and formed as a result of hydrolysis of 1 g of fat. The ratio of fat and potassium hydroxide used for saponification can be used to draw conclusions about the length of carbon chains of carboxylic acids (free and fixed) present in the analysed fat. According to the relation expressed in equation (3.2.7.1), the higher the saponification number, the lower the mean molar mass $(\bar{M})$ of the analysed fat, and consequently the higher content of derivatives with short carbon chains. Saponification number is the base for calculating the amount of saponification agents consumed to produce soap from fats:

$$\bar{M} = \frac{56110}{\mathrm{SN}} \tag{3.2.7.1}$$

Determination is performed, adding an excess of alcoholic solution of KOH to a sample of the test material (in a solvent or not) and heating the sample on water bath for 60 minutes. After this time, the unreacted hydroxide is titrated with volumetric hydrochloric acid in the presence of phenolophthalein as indicator [158].

Do not confuse saponification number with **base number** (BN), determined for motor lubricant oils. This number is a measure of the amount of enriching alkaline additives present in the oil, such as additives neutralising acidic products of combustion, corrosion inhibitors, and dispersing and washing compounds. Despite having the same unit as saponification number, it is defined as the number of milligrams of KOH determined as equivalent (in terms of acid neutralisation capacity) to alkaline additives present in 1 g of the sample. The BN value depends on sulfur content in the lubricant oil and equals from 12 to 40 mg KOH/g. Titration is performed at room temperature, using volumetric hydrochloric or perchloric acid [159, 160].

Ester number (EN) denotes the difference between saponification and acid numbers, and expresses the number of milligrams of potassium hydroxide necessary to saponify neutral glycerides present in 1 g of fat.

Performing the assay:

Determinations of acid, saponification, and ester numbers can easily be combined and done for a single weighed amount of a sample. To this end, weigh approx. 2–2.5 g oil on an analytical scale directly to a conical flask. Dissolve the sample in 20 cm^3 isopropanol or uniform toluene-ethanol-hexane mixture (Gibbs diagram shown in Figure 3.2.7.1). While stirring, titrate with volumetric alcoholic solution of KOH ($C_{KOH(I)}$ about 0.1 N) until end point in the presence of 5–6 drops

of phenolophthalein. Consider the end point to be the moment when adding one drop of hydroxide causes a weak but visible change of colour that remains for at least 15 seconds.

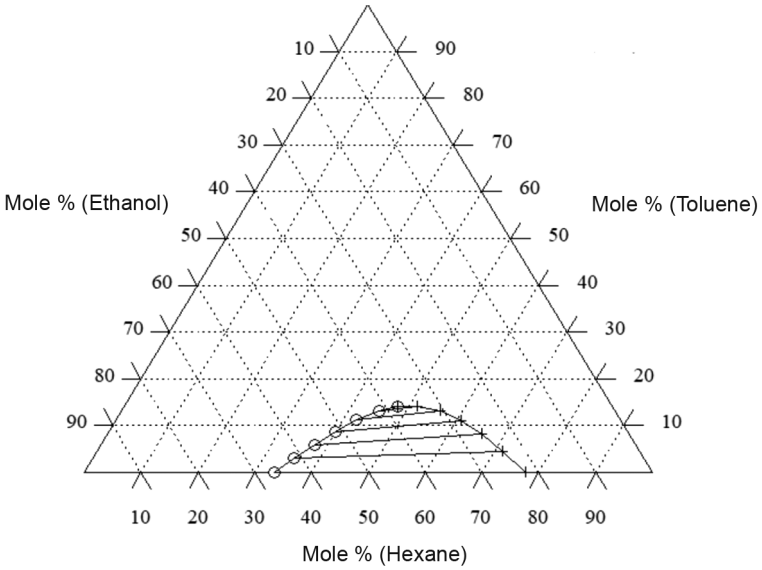


Figure 3.2.7.1. Gibbs diagram for toluene, hexane, and ethanol mixture at 25°C

Record the volume of KOH solution (V_1) and add its excess to a volume of 5 cm^3 for fresh oils or 10 cm^3 for strongly rancid oils. Next, introduce additional 15 cm^3 of 1 N alcoholic solution of KOH, add a few boiling chips and boil on water bath under a reflux condenser for 60 minutes. Afterwards, titrate the mixture while it is still hot with approx. 0.1 N aqueous solution of HCl (C_{HCl}, V_2).

Perform the assay twice, and at the same time perform twice titration of a blank sample (an identical assay without oil, heating time can be reduced to 20 minutes).

Calculate the values of characteristic numbers using the following formulae:

$$\text{AN, mg KOH/g} = \frac{56.1(V_1 - V_{01}) \cdot C_{KOH(1)}}{m} \tag{3.2.7.2}$$

$$\text{SN, mg KOH/g} = \frac{56.1(V_{02} - V_2) \cdot C_{HCl}}{m} \tag{3.2.7.3}$$

$$\text{EN, mg KOH/g} = \text{SN} - \text{AN} \tag{3.2.7.4}$$

where:

m – mass of weighed amount of fat, g,

V_1 – volume of KOH solution with concentration $C_{KOH(I)}$ used to titrate the fat sample, cm^3,
V_{01} – volume of KOH solution used to titrate the blank sample, cm^3,
$C_{KOH(I)}$ – concentration of the first KOH solution used, mol/dm^3,
V_2 – volume of HCl solution with concentration C_{HCl} used to titrate excess alkalis remaining in the flaks with test sample of fat, cm^3,
V_{02} – volume of HCl solution used to titrate blank sample, cm^3,
C_{HCl} – concentration of aqueous HCl solution, mol/dm^3,

ATTENTION! If the pink colour disappears from the solution during heating, add another portion of concentrated alcoholic solution of KOH and continue heating, expecting its duration appropriately. Use an identical volume to determine a standard blank sample.

Due to the possibility of potassium carbonate precipitation and alcohol evaporation in KOH solutions, check the titre of the hydroxide solution **each time** before performing determinations of characteristic numbers, by titrating with a volumetric HCl solution in the presence of phenolophthalein to indicate the end of titration.

In the case of saponification number, literature [161] also states that it can be calculated based on mixture composition (known *e.g.* through gas chromatography):

$$\text{SN, mg KOH/g} = \sum \frac{A_f \cdot 56.106}{MW_f} \tag{3.2.7.5}$$

where:
A_f – content of component *f* in the mixture,
MW_f – molar mass of component *f*.

3.2.7.2. Unsaponifiable matter

An unsaponifiable substance is defined as an organic fraction non-volatile at 100–105°C, produced by extraction from a solution remaining after a saponification number assay. Components that can be present in such a fraction are, among others: fatty alcohols, steroles, phytosterols, aliphatic hydrocarbons (*e.g.* squalane and squalene), fat-soluble vitamins, ceramides, *etc*. [162]. Their content depends on the character of the natural fat and reaches up to 2% in the case of borage, 1–17% in shea soap, 1.2% in ricin oil, and as much as 48% in jojoba oil [163–165].

Performing the assay:

Weight 5–10 g of the test substance (m_{sample}) to a conical flask with ground glass joint and add 50 cm^3 2 M alcoholic solution of KOH. Heat on water bath under a reflux condenser for 60 minutes, frequently stirring the flask contents. After cooling to room temperature, transfer quantitatively to a separator, washing the flask with 100 cm^3 distilled water. Extract three times with 100 cm^3 diethyl ether. Combine the ether fractions and wash with distilled water until neutral reaction disappears. Evaporate solvent off on a rotary evaporator, transfer residue to a Petri dish, washing the flask with 6 cm^3 acetone. Dry to constant mass at 105°C and weigh ($m_{residue}$).

Calculate the content of unsaponifiable substances using the following formula:

$$X_{unsap.},\ \%\ mas. = 100\frac{m_{residue}}{m_{sample}} \tag{3.2.7.6}$$

3.2.7.3. Iodine value (titration and analytic method)

Iodine number is the number of grams of iodine, which under specific conditions attaches to double bonds in the acid radicals present in 100 g of the analysed fat. This number measures the degree of fat unsaturation, so it can be used to identify it. According to standard [166], the source of iodine is Wijs reagent, a solution of iodine chloride (I/Cl ratio of 1.1 ± 0.1) in glacial acetic acid. This solution is obtained by saturating an iodine solution in glacial acetic acid (13 g I_2 in 1 dm^3 CH_3COOH) with dry chloride. An alcoholic solution of iodine (Margosh method), bromide--iodide solution in glacial acetic acid (Hanus reagent), or an alcoholic solution of I_2 in the presence of alcoholic solution of $HgCl_2$ sublimate as catalyst of attachment to unsaturated bond (Hübl reaction) can also be used as iodine sources. It must be stressed, however, that the above methods often provide inconsistent results and iodine number values obtained by different methods cannot be compared directly. As stated in standard [4], for biodiesel, a mathematical equation (calculation method), based on known composition of fatty esters in the test sample, can be used instead of the titration method with Wijs reagent.

Performing the assay – Wijs method [166]:

Dissolve a weighed amount of fat in 20 cm^3 cyclohexane-acetic acid mixture (1 : 1 v/v) in a dry conical flask, then introduce exactly 25 cm^3 Wijs solution to the flask and close it tightly with a plug moistened with a drop of a KI solution. Thoroughly mix the flask contents and leave in a shaded place for 60 minutes. Afterwards, add 20 cm^3 10% KI solution and 150 cm^3 distilled water, washing the plug and flask walls with it. Titrate the mixture with a volumetric solution of sodium thiosulfate with a concentration of $C_{Na2S2O3}$ 0.1 mol/dm^3 to straw yellow colour,

then in the presence of starch until blue colour disappears. At the same time, perform a control test (without fat). Iodine number is calculated using the following formula:

$$\text{IV, g}\ \frac{\text{I}_2}{100\ \text{g}} = 12.69 \cdot \frac{V_0 - V_1}{m} \cdot C_{\text{Na}_2\text{S}_2\text{O}_3} \qquad (3.2.7.7)$$

where:

V_0 – volume of 0.1 N sodium thiosulfate used to titrate the blank sample, cm^3,
V_1 – volume of sodium thiosulfate used to titrate the sample proper.

Performing the assay – Hanus method [167]:

The assay is performed as above, although the fat sample (0.5–1 g) is dissolved in 10 cm^3 chloroform, then 15 cm^3 Hanus reagent is added. Leave the plugged flask in a dark place for 30 minutes, shaking from time to time. Finally, add 15 cm^3 10% potassium iodide solution and 50 cm^3 distilled water. Titrate immediately with 0.1 M solution of $Na_2S_2O_3$. At the same time, perform the blank test. Calculate the iodine number using formula (3.2.7.7).

Performing the assay – Morgosches method [168]:

The iodination is carried out in a water and alcohol environment, where a hydroxylic group is also attached to the unsaturated bond, with a HI molecule released. Perform the analysis within 4–5 minutes, as after this time, the results become inflated.

Weight 1–2 g oil to a flask with ground glass joint and add 15 cm^3 ethanol (heat the solution gently if required). Then introduce 10 cm^3 alcoholic solution of iodine with a concentration of 0.2 M, mix thoroughly and immediately add 100 cm^3 distilled water. Plug the flask and leave for up to 5 minutes in a dark place. After this time, titrate excess iodine with a volumetric solution of $Na_2S_2O_3$ in the presence of starch. The end of titration is signalled by a milky colour of the solution. Perform assay IV two times, concurrently with blank sample tests. Calculate the result in the same manner as in the other methods.

Performing the assay – Hübl method [169]:

Dissolve a weighed amount of fat (about 0.5 g) in 5 cm^3 chloroform and titrate with an alcoholic Hübl solution (26 g iodine and 30 g mercury chloride dissolved in 1 dm^3 ethanol) of known concentration of iodine (determine the titre of the solution by titrating with a volumetric solution of sodium thiosulfate). Titration is considered over when the solution is coloured by free iodine, optionally a few drops of starch can be added. Calculate iodine number using the following formula:

$$\mathrm{IV}_H, \mathrm{g}\,\frac{\mathrm{I_2}}{100\ \mathrm{g}} = 25.681\frac{V_H \cdot C_H}{m} \tag{3.2.7.8}$$

where:

V_H – volume of Hübl solution used for titration, cm^3,
C_H – molar concentration of Hübl solution, mol I_2/dm^3,
m – mass of weighed amount.

Alternatively, excess Hübl solution can be added to the sample in chloroform, and after closing with a glass plug, left for 30 minutes in a dark place. Afterwards, titrate the excess iodine with a volumetric solution of sodium thiosulfate (reaction 3.2.7.9). At the same time, perform the blank test. In this case, calculate iodine number using formula (3.2.7.1).

$$\mathrm{I_2} + 2\mathrm{S_2O_3^{2-}} \rightarrow 2\mathrm{I^-} + \mathrm{S_4O_6^{2-}} \tag{3.2.7.9}$$

Performing the assay – calculation method [170, 171]:

According to literature [170, 171], iodine number can be calculated using formula (3.2.7.10). Moreover, the value of iodine number is additive, and consequently for mixtures such as natural oil, it can be easily calculated using formula (3.2.7.11) based on known mass percentage content of the given component (A_f), its molar mass (MW_f) and number of unsaturated bonds in its structure (db):

$$\mathrm{IV}_{\mathrm{pure}}, \mathrm{g}\,\frac{\mathrm{I_2}}{100\ \mathrm{g}} = 100\frac{253.81 \cdot db}{MW_f} \tag{3.2.7.10}$$

$$\mathrm{IV}_{\mathrm{mixture}}, \mathrm{g}\,\frac{\mathrm{I_2}}{100\ \mathrm{g}} = \sum 100 \cdot A_f\,\frac{253.81 \cdot db}{MW_f} \tag{3.2.7.11}$$

This method provides almost identical values as correction factor values specified in the biodiesel standard, recommended for calculating the iodine number values based on equation (3.2.7.12) and chromatographic analysis of mixtures of methyl esters of fatty acids:

$$\mathrm{IV} = \sum x_i \cdot f_i \tag{3.2.7.12}$$

where:

x_i – mass fraction of selected unsaturated derivative, determined chromatographically,
f_i – calculation factor for selected methyl esters, as per Table 3.2.7.1.

When one compares the equation for calculated iodine number IV (3.2.7.11) with the equation for calculated saponification number SN (3.2.7.5), it can be noticed that the average number of unsaturated bonds in a pure compound or a mixture can be calculated by substituting both values to the following equation:

$$db_{\text{mixture}} = \sum A_f \cdot 2.21 \cdot \frac{\text{IV}}{\text{SN}} \qquad (3.2.7.13)$$

Table 3.2.7.1

Calculation factors indicated in the standard EN 14214 and calculated using equation 3.2.7.11

Fatty acid ester	Cn : *db*	EN 14214 f_i	$\frac{253.81 \cdot db}{MW_f}$
Saturated esters	Cn : 0	0	0
Methyl hexadecenoate (methyl palmitoleate)	C16 : 1	0.950	0.946
Methyl octadecenoate (methyl oleate)	C18 : 1	0.860	0.817
Methyl octadecadienoate (methyl linoleate)	C18 : 2	1.732	1.724
Methyl octadecatrienoate (methyl linolenate)	C18 : 3	2.616	2.604
Methyl eicosenoate	C20 : 1	0.785	0.782
Methyl docosenoate (methyl erucate)	C22 : 1	0.723	0.720

3.2.7.4. Peroxide value

Peroxide number (so called Lea number) is the number of milliequivalents of active oxygen per kilogram of oil, equal to the number of millilitres of volumetric solution of sodium thiosulfate necessary to titrate iodine released from potassium iodide by peroxides present in a fat sample [172]. It is therefore a measure of peroxide content in the material, and is treated as an indicator of its degree of oxidation (rancidity). For refined plant oils, the maximum acceptable value of the peroxide number is 5.0 mEq O_2/kg [152], while for olive oil it can be as much as 20 mEq O_2/kg [154].

Performing the assay:

Weigh about 0.5–1 g oil with accuracy to 0.001 g to a 200 cm^3 conical flask and dissolve in 2 cm^3 chloroform. Add 3 cm^3 acetic acid and 0.2 cm^3 saturated solution of potassium iodide. Close the flask with a glass plug, mix thoroughly and leave in a dark place. After 5 minutes, add 15 cm^3 distilled water and mix thoroughly. Titrate the released iodine with approx. 0.001M solution $Na_2S_2O_3$ in the presence of starch until complete loss of colour remains for at least 30 seconds. Concurrently, perform a blank test without oil added.

Calculate peroxide number (LOO), expressed in milliequivalents of active oxygen per kilogram of sample, using the following formula:

$$\text{LOO, mEq } O_2/\text{kg} = 1000 \cdot \frac{V - V_0}{m} \cdot C_{Na_2S_2O_3} \qquad (3.2.7.14)$$

where:

m – mass of weighed amount of fat, g,
V – volume of $Na_2S_2O_3$ solution used to titrate fat sample, cm^3,
V_0 – volume of $Na_2S_2O_3$ used to titrate blank sample, cm^3,
$C_{Na_2S_2O_3}$ – concentration of volumetric solution of $Na_2S_2O_3$ (approx. 0.01 mol/dm^3).

3.2.8. Oxidation stability

Adverse changes in oils can be initiated even in the seeds of oil plants, and during oil production they are intensified substantially by pressing or extraction. Preconditioning of seeds before pressing, crushing seeds on a piston shaft and its necessary pre-heating result in the temperature reaching in excess of 90°C during pressing. On the other hand, depending on the selected solvent, the temperature during extraction can equal 60–80°C. Degumming, deacidification and decolourisation employ processing at about 90°C, while the highest temperature, of the order of 185–240°C, is applied during deodorisation. Because oxygen solubility, and consequently oil oxidation rate, rises with increasing temperature, fats subjected to all these processes are the most susceptible to autoxidation. Moreover, refining removes natural anti-oxidants, *e.g.* tocopherols, phospholipids, carotenoids and polyphenols, which confer greater oxidation stability to cold-pressed oils.

Numerous changes of various nature occur during autoxidation of fats, although they depend on the conditions of fat storage (temperature, access of light, air, humidity, *etc.*). It also bears stressing that susceptibility to oxidation increases geometrically, proportionally to the number of unsaturated bonds present in individual fatty acids [173], so chemical composition of fat and presence of anti--oxidants and pro-oxidants (*e.g.* moisture and metals) will be important factors determining oxidation stability.

During the first phase of autoxidation, peroxides, epoxides, dienes and free fatty acids are the primary products (Figure 3.2.8.1), and they can be measured by analysis of the epoxy (EP), peroxide (LOO), iodine (IV), and acid numbers (AN). As secondary oxidation products, aldehydes, ketones, oxoacids and trienes appear, which are identified by measuring the anisidine value and measuring the amount of polyunsaturated oxidated derivatives (COP). During the last phase, the content of volatile substance increases, dicarboxylic acids, malonic acid, and epihydrinic aldehyde form. Derivatives can also condensate to macromolecular compounds [174, 175]. All these changes greatly affect the chemical composition of mixtures and their physical properties (*e.g.* density, viscosity, solidification, cloud and smoke points, *etc.*). The content of fat-soluble vitamins and absorbability of protein contained in a food portion decrease substantially. Oxysteroles are particularly

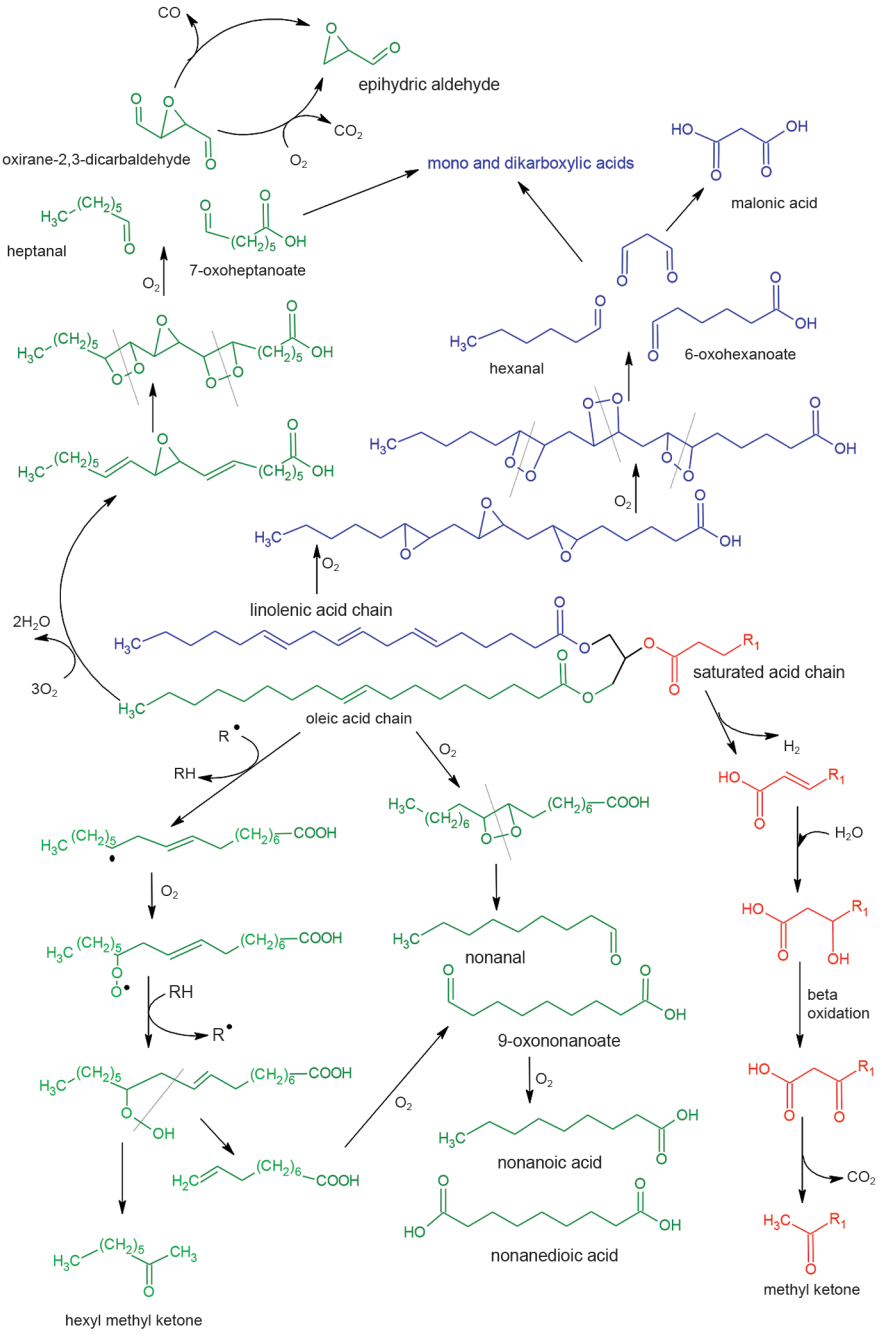


Figure 3.2.8.1. Diagram of reactions typical for aldehydic rancidification, odouriferous rancidification and ketonic rancidifications, tallowing, and forming of epihydrinic aldehyde. Based on [175]

dangerous, as they exhibit mutagenic and carcinogenic effects, and intensify the development of atherosclerosis [176].

As certain parameters may initially increase during rancidification, then drop during the subsequent oxidation phase, *e.g.* peroxide number, which reflects only the original products of fat oxidation – *cf.* Figure 3.2.8.2, literature recommends using multiple designations or calculating the TOTOX indicator for determining a credible degree of fat rancidification [177, 178].

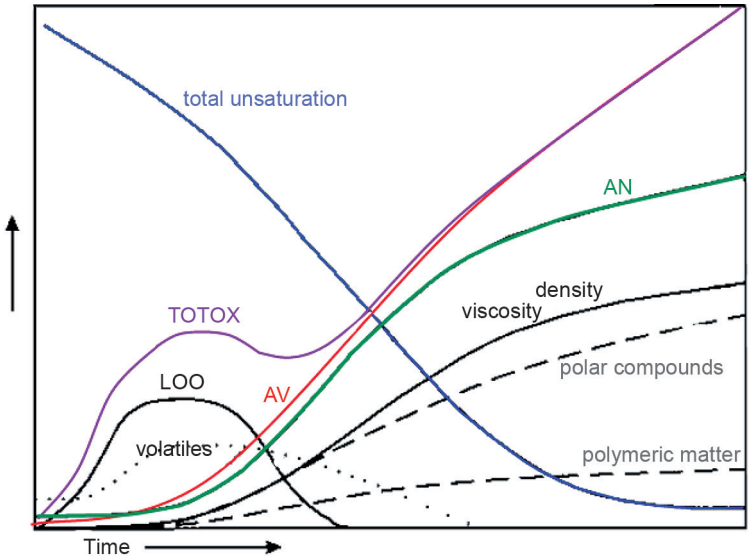


Figure 3.2.8.2. Temporal changes in values of characteristic numbers and basic parameters of oil during rancidification. Key: AV – anisidine value, AN – acid number, LOO – peroxide number, TOTOX – TOTOX indicator. Based on [179, 180]

3.2.8.1. Anisidine value

Measurement of the *p*-anisidine value (AV) enables assessing the amount of secondary products of lipid oxidation, including macromolecular non-volatile carbonyl compounds formed through decomposition of peroxides and hydroperoxides. The assay is based on determining the reactivity between the carbonyl bond of an aldehyde and the amine group of *p*-anisidine. The anisidine value reflects a 100-fold increased absorbance value of the solution formed as a result of reaction between 1 g weighed amount of fat dissolved in 100 cm^3 of mixture of solvent (isooctane) and reaction agent (*p*-anisidine). Absorbance is measured in 1 cm wide quartz cells at a wavelength $\lambda = 350$ nm [2, 181, 182].

Performing the assay:

Weigh the analytical sample to a 25 cm^3 volumetric flask and dissolve in isooctane. Measure solution absorbance with pure isooctane as the standard. Introduce 5 cm^3 fat solution and 1 cm^3 anisidine agent (0.25 g *p*-anisidine dissolved in 100 cm^3 acetic acid) to one test tube, and 5 cm^3 isooctane and 1 cm^3 anisidine agent to another. Plug the test tubes, mix thoroughly and leave in a shaded place at room temperature for exactly 10 minutes. After this time, measure the absorbance of the fat sample against the standard solution, *i.e*. the solution from test tube two.

Perform the determination twice; the difference between consecutive measurements should not exceed 0.5 for solutions with AV between 1 and 20 [183]. The unit of anisidine number is 100-multiple of absorbance and is calculated using the following formula:

$$\mathrm{AV} = 25 \cdot \frac{1.2 \cdot E_b - E_a}{m} \tag{3.2.8.1}$$

where:

m – mass of weighed amount of fat, g,
E_a – absorbance of fat solution with pure isooctane as a standard,
E_b – absorbance of fat-anisidine agent solution with isooctane-anisidine agent as a standard.

3.2.8.2. TOTOX index

The TOTOX indicator is a parameter calculated in the basis of peroxide number and anisidine value analysis. It is defined as the following equation [177]:

$$\mathrm{TOTOX} = \mathrm{AV} + 2\,\mathrm{LOO} \tag{3.2.8.2}$$

3.2.8.3. Conjugable oxidation products

The content of conjugated autoxidation products (*COP*) is determined on the basis of spectrophotometric measurement of alcohol-isooctane solutions of fats subjected to hydrogenation and dehydration. This method was developed almost half a century ago by the employees of ARC Food Research Institute and enables hydroperoxides of fatty acids and products of their further degradation (hydroxyl and carbonyl derivatives) to be determined concurrently. Together they are referred to as "total oxidation products" or "conjugated oxidation products", designated as V_{COP}, while the value V_{oxodiene} identifies the independently conjugated carbonyl compounds. Another property of this test is that it distinguishes between oxidation products originating from diene fatty acids and highly unsaturated fatty acids, which

are defined with the dimensionless ratio R_{COP} [184]. All measurements are made in quartz cells with optical path length of 1 cm.

Performing the assay:

To perform the determination, dissolve fat (analytical weighed amount of approx. 0.25 g) in 5 cm^3 of mixture of equal volumes of isooctane and ethanol. Introduce 1 cm^3 solution to each of three 25 cm^3 volumetric flasks marked with letters "O", "R", and "D", then add 1 cm^3 isopropanol to flask "O" (original) and make up to the mark with ethanol. Introduce 1 cm^3 sodium borohydride solution in isopropanol (1 g/100 cm^3) to flask "R" (reduced), heat for 30 minutes at 60°C, and make up to the mark with ethanol after cooling. Introduce borohydride solution (1 cm^3) to the last flask "D" (dehydrated) and heat as with flask "R". After cooling, add 5 cm^3 20% (w/v) sulfuric acid solution in ethanol and heat again for 30 minutes at 60°C. Finally, after cooling, make up to 25 cm^3 with ethanol. Measure absorbance with ethanol as a standard for all three solutions at wavelengths 268, 275 and 301 nm, additionally diluting the solutions with ethanol if required.

Individual parameters for conjugated oxidation products are calculated using the following formulae:

$$V_{\text{oxodiene}} = \frac{(A^O_{275} - A^R_{275})}{0.8\,m} \tag{3.2.8.3}$$

$$V_{COP} = \frac{(A^D_{268} - A^R_{268}) + (A^D_{301} - A^R_{301})}{0.8\,w} \tag{3.2.8.4}$$

$$R_{COP} = \frac{(A^D_{301} - A^R_{301})}{(A^D_{268} - A^R_{268})} \tag{3.2.8.5}$$

where:

A^D_{268}, A^D_{301} – absorbance of solution *D* measured at wavelengths 268 and 301 nm,
A^R_{268}, A^R_{275}, A^R_{301} – absorbance of solution *R* measured at 268, 275 and 301 nm,
A^O_{275} – absorbance of solution *O* measured at 275 nm,
m – mass of fat sample take to prepare the initial solution.

3.2.8.4. Rancimat test

As most autoxidation processes occur relatively slowly at room temperature, so called accelerated oxidation test has been introduced, where a fat sample is subjected to the effects of air (in the same manner as barbotage) and elevated temperature, usually within the 100–150°C range. Air leaving a measurement cell with fat

subsequently barbotages though a vessel filled with deionised water, whose changing conductivity is constantly measured. After the induction stage, where fat degradation processes are initiated, a rapid leap in conductometer readings is observed, which reflects the free radical nature of the oxidation reaction. A conceptual diagram of the test with a sample conductometer and a curve of relation between conductivity and time is shown in Figure 3.2.8.2.

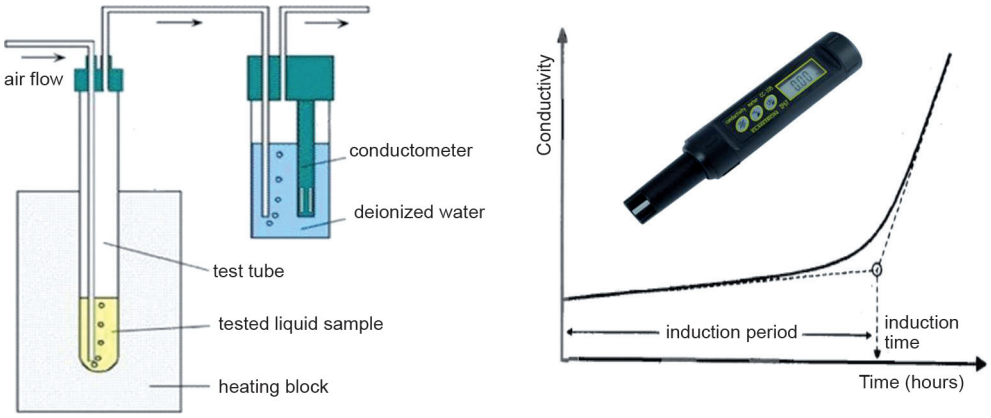


Figure 3.2.8.3. Conceptual diagram of accelerated oxidation test with sample conductometer and curve of relation between conductivity and test duration

As stated in literature [185], a change in oxidation test parameters significantly affects the measured duration of the induction period. The effects of temperature are crucial here, as its increase by 10° results in almost 50% reduction in induction duration. Air flow and fat sample size do not markedly affect the assay's results, provided that sufficient excess of oxygen is provided. The optimum ratio of oil volume to air flow intensity should be 6–10 g per 10–15 dm^3/h at 130°C, although the standard [186] recommends using a 3 g fat sample, 60 cm^3 deionised water and air flow of 20 dm^3/h. Regardless of the proportion selected, tests should be performed under comparable conditions. In a laboratory where a heating block, controlled air flow and Nessler cylinders shown in Figure 3.2.8.3, it is recommended, due to equipment size, to use 20 cm^3 fat sample and 20 cm^3 distilled water, air flow of about 15 dm^3/h (flowmeter reading 12–15), and heating block temperature set to program 1 or 2, *i.e.* 130° or 101.5°C, respectively. Based on graphical representation of conductivity changes (μS/cm) with time, determine induction time for the analysed fat.

ATTENTION! After the determination, dispose of the fat to a waste container – the analysis has a destructive effect on the sample, similar to the analysis of high--temperature properties.

3.2.8.5. Epoxide number

During oxidation, epoxy compounds can form in highly oxidised fat (Figure 3.2.8.1). They are characterised by limited stability and undergo further oxidation, ring opening or decomposition processes.

Performing the assay [187]:

To perform the assay, add 13 cm^3 hydrochloric acid in dioxane (mix 4 cm^3 HCl with 250 cm^3 dioxane in a separatory funnel; remove bottom phase after settling) and a few drops of cresol red solution to about 1 g sample of rancid fat. After 30 minutes, titrate the solution with 0.2 M alcoholic solution of NaOH. Solution colour changes from red to yellow to violet during titration. Perform blank test in the same manner.

Calculate epoxy number [mol/100 g] using the following equation:

$$\mathrm{EP},\ \mathrm{mol/100\ g} = 0.1 \cdot \frac{V_0 - V_1}{m} \cdot C_{\mathrm{NaOH}} \tag{3.2.8.6}$$

where:

V_0 – volume of alcoholic solution of NaOH used to titrate blank sample, cm^3,
V_1 – volume of NaOH solution used to titrate test sample, cm^3,
C_{NaOH} – concentration of alcoholic solution of NaOH, mol/dm^3,
m – mass of sample, g.

3.2.8.6. Identification of oxidation products in colour test reactions

Literature identifies the following among oil and fat autoxidation processes: (i) rancidification to aldehydes; (ii) odouriferous rancidification leading to formation of methylketones, and (iii) fatty acid tallowing processes, where oxyacids, polymers and products of condensation form [175, 188]. Sample reactions leading to the individual products are shown in Figure 3.2.8.1.

Almost any of the mentioned groups has several colourful test reactions proposed, enabling qualitative analysis of selected degradation products [174, 175, 189]. With appropriate modifications, it is also possible to determine selected compounds quantitatively [190]. An example can be the Kreis test, where epihydrinic aldehyde, *i.e.* 2,3-epoxypropenal, forming as a result of autoxidation of unsaturated acids, condenses with resorcinol in the presence of a concentrated mineral acid, forming macromolecular products of intense violet colour – a schematic reaction is shown below:

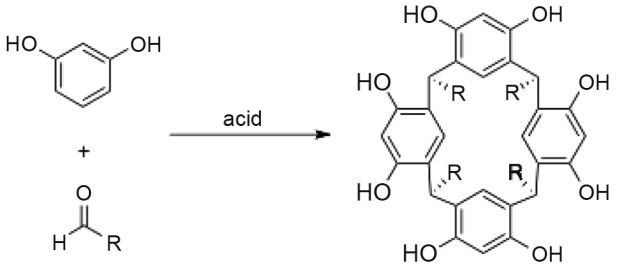


Figure 3.2.8.4. Schematic reaction of aldehyde condensation with resorcinol [191]

If fluoroglucine (1,3,5-benzenetriol) is used, compounds of intensely red colour are formed, similar to the sugar test (*cf.* section 3.1.6.2).

Performing the Kreis test for epihydrinic aldehyde presence [45, 192, 193]:

Mix 1 cm^3 filtered fat and 1 cm^3 concentrated HCl in a test tube with ground plug, and vigorously shake for one minute. Add 1 cm^3 0.1% fluoroglucine solution in ether or 1 cm^3 0.15% resoricinol solution in benzene, mix vigorously again and leave until both phases separate. Red or violet colour of the acidic phase (for fluoroglucine or resoricinol, respectively) indicates aldehyde presence and fat rancidification. The test gives a positive result for epoxyaldehydes and their acetals, as well as malonic aldehyde [191].

Another recognised test enabling malonic aldehyde presence to be identified is the 2-thiobarbituric acid test, occurring according to the reaction shown in Figure 3.2.8.5. While intensity of the generated pink colour is proportional to initial aldehyde concentration, the reaction is not specific. As stated in literature, this test also gives a positive reaction for sulfaguanidine and an entire range of amino acids.

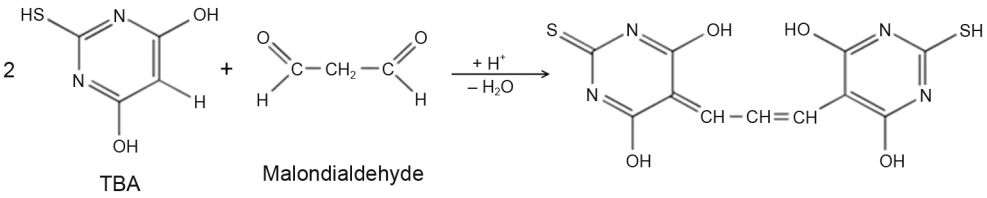


Figure 3.2.8.5. Thiobarbituric acid reaction with malonic aldehyde

Performing the thiobarbituric acid test for malonic aldehyde presence [191, 193]:

Place 5 g oil or fat in a distillation flask, add 100 cm^3 water and 5 cm^3 3M HCl. Evaporate about 45 cm^3 distillate. Add 2 cm^3 2-thiobarbituric acid solution and 2 cm^3 concentrated ortophosphoric (V) acid to 40 cm^3 solution. Place in boiling water

bath for 35 minutes. At the same time, perform a blank test using 40 cm^3 distilled water, processed as above. After cooling, measure absorbance of resulting colour at a wavelength of 530 nm. Record results in absorbance units, testing the samples always at an identical solution layer thickness.

Preparation of the 2-thiobarbituric acid solution: Dissolve 0.5 g 2-thiobarbituric acid in 25 cm^3 distilled water at a temperature of 40°C with 1 cm^3 3M NaOH added. After cooling, add 0.2 cm^3 3M HCl and make up with distilled water to 50 cm^3.

The last test, so called Taufel and Thaler test, enables identifying the products of ketonic rancidification, typical for fats with a low degree of unsaturation [45], although it must be stressed that methylketones can also be produced as a result of oxidative degradation of unsaturated derivatives. The pathway of reactions leading to such products are shown in Figure 3.2.8.1 in red.

Performing the Taufel and Thaler assay:

Add 4 ml warm distilled water and 0.4 ml salicylic aldehyde to 10 ml molten fat placed in a test tube, shake vigorously, then pour 2 ml concentrated sulfuric acid in the centre of the mixture, making sure not to moisten test tube walls with the acid. Place the test tube in boiling water bath without mixing the contents. After 10–15 minutes, the phases separate and in the presence of methylketones, the colour of the top phase (pink to carmine) becomes more intense. At the same time, perform a control test.

When assaying fats, make sure that the test sample does not contain diacetyl, which produces a similar colour with salicylic aldehyde. To remove it, heat the test sample.

3.2.9. General analyses of fat composition

As was mentioned in section 3.1.3, the fat fraction obtained from natural materials comprises primarily free fatty acids and triglycerides (*i.e.* proper fats), accompanied by phospholipids, steroles, fat-soluble vitamins, waxes, fatty alcohols, dyes, and many others. Aside from components desirable from the standpoint of nutrition, including glucosinolates, present in large amounts in fats obtained from crucifer plant seeds and characterised by chemopreventive effects [194], raw fats also contain harmful, and even toxic components of natural or synthetic origin. The former group includes *e.g.* mycotoxins (fungus metabolites), derivatives of erucic acid, cyanides and amines formed as a result of decomposition of certain proteins, as well as poisonous alkaloids, *e.g.* ricin present in castorbean oil. Most of these components are removed from raw fat in refining processes, involving degumming, deacidification, bleaching and deodorisation (for ricin, steam distillation is necessary), which also remove some components from the latter

group, *i.e.* pesticides, heavy metals indicating environmental pollution or imperfections in the processing technology. It must be stressed, however, that some components harmful to health, *e.g.* derivatives of trans fatty acids, do not occur in raw fat naturally. They are characteristic for hardened fats (*e.g.* margarines), *i.e.* ones subjected to deeper chemical and thermal processing. If fats are used for technical purposes (*e.g.* biodiesel production), deep refining is not required, although it affects the efficiency of the end product or requires special modifications to the processing technology. For example: the presence of free fatty acids necessitates carrying out methanolysis employing highly corrosive acidic catalysts or severe process conditions combined with solid catalysts or without a catalyst, while pre-degumming of a material makes phase separation and purification of the end biodiesel significantly easier. However, regardless of the purpose of the fat, one of the most important analyses is determination of the fatty acid profile, which enable not only identifying the origin of the fat and its degree of processing, but also assessing its suitability for nutritional and technical purposes (*e.g.* as raw material in technical processes).

3.2.9.1. Fatty acid profile

Despite a certain variation in fatty acid profiles between individual varieties, the content of the most important fatty acids is usually characteristic and similar within a single species (material). For example: linseed oil is rich in linolelic acid (C18 : 3) derivatives, olive oil is dominated by oleic acid (C18 : 1) esters, derivatives of 12-hydroxy-cis-9-octadecenoic acid only occur in ricin oil, fish fats predominantly contain polyunsaturated derivatives, while animal tallow – saturated derivatives (Table 3.2.9.1).

Table 3.2.9.1

Distribution of basic fatty acids of selected oils and natural fats. Based on [195–200], rounded values

Fat	Fatty acid composition,%							
	$C_{\leq 14}$	C16 : 0	C16 : *n*	C18 : 0	C18 : 1	C18 : 2	C18 : 3	$C_{\geq 20}$
Microalga	14–20	12–38	32–57	1–3,7	7–60	3–20	7–30	
Yeast		11–37	1–6	1–10	28–66	3–24	1–3	
Fungi		7–23	1–6	2–6	19–81	8–40	4–42	
Bacterium		8–10	10–11	11–12	25–28	14–17		
Coconut	16–67	9–11		2–3	6–9	< 1		< 1
Palm oil	32–52	4–6		36–53	6–12			
Olive oil	< 1	12–17		2	66–78	7–16	1–2	< 1

Table 3.2.9.1 continued

Linseed		6	< 1	3–4	20–22	15–21	48–55	< 1
Hemp	< 1	6	< 1	3	11	59	3–4	27
Sunflower	< 1	5–6	< 1	3	28–53	38–62	< 1	
Sesame		10	< 1	5–7	41	41	0–1	< 1
Rapeseed	< 1	5	< 1	1–7	60-63	10–23	1–13	5–15
Soy bean	< 1	9–13	< 1	2–6	17–30	49–57	8–11	
Jatropha	< 1	13–14		5	32	45	< 1	3
Black mustard	< 1	3		1	8	11	3	57
Lard grease	1–4	16–25	1–3	13–18	37–49	10–17	< 1	
Polutry		21–24	8	5–8	40–42	17–27	1–2	
Beef grease	3–6	24–32		12–13	47	3		

Importantly, almost all fats of natural origin contain only derivatives of fatty acids with straight, non-branched chains, even numbers of carbon atoms and cis configuration. Derivatives containing additional functional groups (*e.g.* -OH) occur extremely rarely – *e.g.* ricin oil. An exception are also fats obtained from microorganisms (bacteria and fungi), characterised by the presence of derivatives of fatty acids with odd numbers of carbon atoms, frequently polyunsaturated, containing additional functional groups, methyl substituents and hydroxyl groups [196, 201–206].

Methods recommended for analysing the acid profile of fat are chromatographic methods, where methyl esters of fatty acids are analysed [4, 54, 207]. Material derivatisation is performed in one or multiple stages (transesterification or hydrolysis/saponification and esterification, respectively, and the resulting mixture of esters in a non-polar solvent is injected directly to an appropriate, medium-polar capillary column. Identification of fatty acid derivatives involves comparing retention times with standards (FID) or coupling the chromatograph with a mass detector (GC-MS). On the other hand, quantitative analysis requires calibrating the detector signal beforehand.

Preparation of sample for analysis – BCl_3 method:

Weigh an amount of about 0.5 g fat directly to a small flask with ground glass joint, add 2 cm^3 10% methanol solution of BCl_3 (or BF_3) using a pipette and heat on water bath under a reflux condenser for 20–30 minutes. After cooling, add 1–1.5 cm^3 hexane and 2 cm^3 water and shake vigorously for one minute. Separate and dry the top phase over anhydrous magnesium sulfate, calcium chloride or roasted silica. Additionally dilute solution with hexane if required. When using a method with an internal standard, add a precisely defined amount of standard (100 microlitres of

margaric acid C17 : 0 of known concentration) to a weighed amount of fat before adding BCl_3 solution. For external calibration, adding the internal standard is not necessary.

Follow identical steps for any other fat material, including fragmented oil plant seeds, animal fat samples and materials rich in free fatty acids.

For materials with high water content, extract the fat with an anhydrous solvent (hexane or heptane, light petroleum ether, *etc.*) before adding the methanol solution of BF_3 and standard. On the other hand, for materials that are anhydrous or poor in fatty acids, transformation into methyl esters of fatty acids can be carried out by heating the sample – fat with methanol solution of potassium or sodium hydroxide (about 5–10 cm^3, concentration 0.5% m/m) – with a standard at a temperature of 50–60°C for 30 minutes under a reflux condenser. Subsequently dissolve the resulting esters in a selected organic solvent, thoroughly wash with water and dry before injecting into a chromatographic column.

WORNING! Water, alkalis, and strong acids injected into a FAME-dedicated capillary column significantly reduce its lifetime.

Chromatographic analysis:

The test is performed on a Shimadzu 5A gas chromatograph fitted with a Zebron ZB-WAX capillary column (30 m, diameter 0.25 mm, film thickness 0.25 μm), a split/splitless injector and a FID. Inject an ester sample of 0.2 μl into the column using the following temperature increase program: carrier gas (99.999% helium) flow 1 cm^3/min, split 1 : 10, injector temperature 250°C, detector temperature 350°C, programmed temperature increase at the column: 80°C maintained for 0.5 minuted, then increase by 45°C/min to, in turn: 160°C (2 minutes), 230°C (7 minutes) and 255°C. Maximum temperature was maintained for 8.5 minutes.

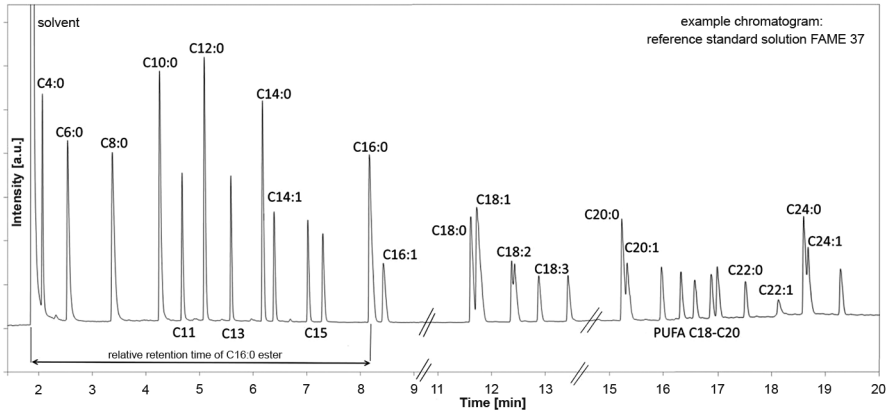


Figure 3.2.9.1. Sample chromatogram of the standard mixture FAME 37

Qualitative analysis of fatty acid composition is performed by comparing retention times of sample components with standard components (Figure 3.2.9.1), although a change of column or in analysis conditions markedly affects results (peaks can appear in a different order! – see [208]).

Quantitative analysis for unidentified components is performed based on detector signal calibration. When analysing methyl esters of fatty acids, a FID is commonly used, although it is characterised by different sensitivity to individual components of the analysed mixture. Therefore, the most convenient and recommended method of qualitative analysis is the external calibration method. In this method, inject a standard solution of several different concentrations (at least 5 injections) into the column, and based on the surface area/number ratio of the analysed compound, prepare a separate calibration curve for each of the analysed components (*e.g.* components 1, 2, 3 – as shown in Figure 3.2.9.2). The curve should generally cross the (0,0) point and be a straight line. However, take into account that with high or very low sample concentrations, linearity of the detector's response may be disrupted. When peaks are symmetrical (Gaussian) and very well separated, peak height or width at half height can be used instead of its surface area.

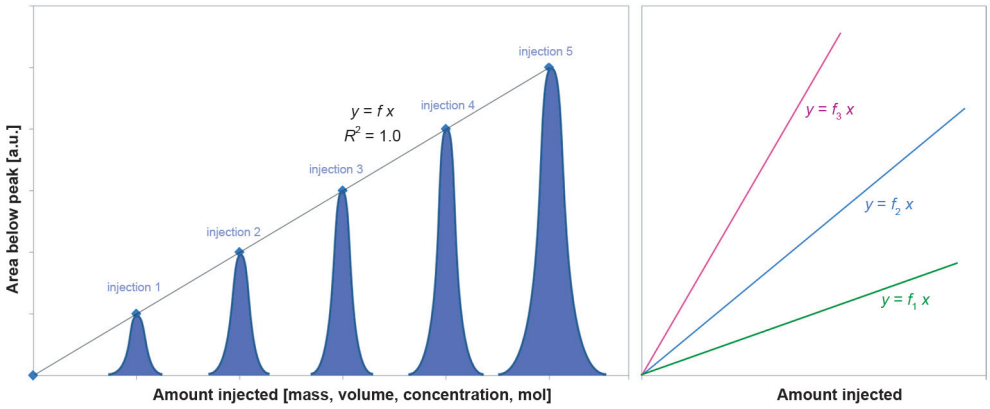


Figure 3.2.9.2. Sample calibration using the external standard method

The unit in which compound amounts are given is mole – a universal unit, volume – fully reliable for gaseous samples or liquid components that do not undergo volume contraction during mixing, as well as concentration and mass, used for liquid (dissolved) samples. The content of each calibrated component in the test mixture (x_i) is determined using the following relation:

$$x_i = \frac{A_i}{f_i} \tag{3.2.9.1}$$

where:

A_i – surface area under the peak with a retention time equal to the analysed compound, a.u.,

f_i – calibration factor for compound *i*, determined as in chart 3.2.9.2.

Percentage content of individual components of the test sample (X_i) is therefore defined as:

$$X_i,\ \% = 100\frac{x_i}{\sum x} \tag{3.2.9.2}$$

If some components are unavailable for calibration, but their character is known, the internal standard method can be applied. In this method, a known amount of internal standard (IS) with properties as close as possible to the analysed substance, but absent in the test mixture, is added to the mixture. When analysing the composition of natural fats, it is usually an ester or margaric acid C17 : 0 [209] or ester of a C21 : 0 acid [210]. This method is recommended e.g. for determining sterol content in fat samples [211], total methyl ester content (FAME, %) in a mixture (*e.g.* biodiesel tests), and determining the concentration of individual mixture components X_x, whose percentage content is limited by the requirements of a fuel standard (*e.g.* linoleic acid esters [209]):

$$\text{FAME},\ \% = 100\frac{(\sum A) - A_{IS}}{A_{IS}} \cdot \frac{C_{IS} \cdot V_{IS}}{m} \tag{3.2.9.3}$$

$$X_x,\ \% = 100\frac{A_x}{(\sum A) - A_{IS}} \tag{3.2.9.4}$$

where:

A, A_x, A_{IS} – surface areas under the peaks of all esters from C14 to C24; surface area under the peak of selected ester *x*, and surface area under the internal standard peak, respectively,

C_{IS}, V_{IS} – concentration and volume of standard solution added to the sample,

m – mass of sample.

The least reliable but simple method of determining the percentage composition of the analysed mixture is the simple normalisation method, where the content of individual components is determined on the basis of surface area shares under individual peaks:

$$x_i = 100\frac{A_i}{\sum A} \tag{3.2.9.5}$$

where:

A_i – surface area under the peak with a retention time equal to the analysed compound, a.u.

ΣA – sum of surface areas under all peaks.

This method can be applied only if the chromatogram shows all components of the mixture, all are well separated and the detector response factor is similar for all components. If the differences between detector responses for individual components of the mixture are known, formula 3.2.9.5 can be supplemented with appropriate correction factors.

3.2.9.2. Free glycerol and glycerides

Determining the content of free and fixed glycerine is of great importance in biodiesel production, as according to current standards [4, 212], the maximum allowed amount of glycerine in biofuel is 200 mg/kg. There are several alternative determination methods: classic titration method using aqueous extracts with periodic acid [213], chromatographic method using a polar capillary column, enabling free and fixed glycerol to be analysed [214], and spectrophotometric method with acetylacetone [215].

Classic titration method using periodic acid [216]:

To determine free glycerine in biofuel, it must first be extracted to an aqueous phase. To this end, heat a biodiesel sample of about 50-100 g for 15 minutes in an Erlenmeyer flask on water bath (60–65°C) with 20 cm^3 5% diluted hydrochloric acid. After separating the aqueous phase, repeat the glycerine washing with, in turn, 20 cm^3 2.5% HCl and water. Wash the separating funnel with an additional portion of water and add to the extract. An identical method can be used to prepare a biofuel sample for determination of total glycerine content (*i.e.* fixed as glycerids), although the biofuel must first be subjected to saponification with an alcoholic solution of NaOH, and the alcohol phase must subsequently be neutralised with 5% HCl solution. After separating the water phase, extract released glycerine using, in turn, 2.5% HCl and water.

Add a few drops of phenol red to combined aqueous extracts and add 2M NaOH until the colour changes to red. Next, titrate with 5% HCl, and when colour changes to yellow, introduce another 0.5 cm^3 5% HCl solution. Heat the combined solution to gentle boil for 3 minutes, cool and add 0.1N NaOH until the colour changes again. Introduce 15 cm^3 of freshly prepared $NaIO_4$ (6 g/100 cm^3) and leave for 30 minutes in a dark place. During this time, glycerine is oxidised and formic acid is released, in accordance with the following reaction:

$$C_3H_8O_3 + NaIO_4 \rightarrow 2HCHO + HCOOH + 2NaIO_3 + H_2O \qquad (3.2.9.6)$$

After this time, add 2 cm^3 ethylene glycol to decompose excess sodium metaperiodate (reaction 3.2.9.7), wash flask walls with distilled water and leave again for 20 minutes in a dark place. After this time, titrate with 0.1N NaOH until colour changes.

$$C_2H_4(OH)_2 + NaIO_4 \rightarrow 2HCHO + NaIO_3 + H_2O \qquad (3.2.9.7)$$

Calculate percentage content of glycerine (x_{GLY}) in the test sample using the following formula:

$$x_{GLY},\ \% = 100 \cdot \frac{V_{NaOH} \cdot 0.0921 \cdot C_{NaOH}}{m} \qquad (3.2.9.8)$$

where:

V_{NaOH}, C_{NaOH} – volume in cm^3 molar concentration of NaOH solution, respectively,

m – mass of biofuel sample in grams.

ATTENTION! If the volume of 0.1 N NaOH exceeds 20 cm^3, repeat the assay with a smaller portion of the sample or increase the amount of $NaIO_4$.

Chromatographic analysis of free and fixed glycerol:

The chromatographic method enables assessing the content of free and fixed glycerine in biofuel samples, although it requires the use of a dedicated column operating at very high temperatures, reaching 400°C (*e.g.* Zebron ZB-5HT Inferno with 15 m × 0.32 mm × 0.10 μm dimensions and an additional 2 m × 0.53 mm pre-column), and a pre-derivatisation of the sample using two internal standards: 1,2,4-butanotriol (IS1) and 1,2,3-tricaprylglycerol (IS2) in pyridine, and a derivatisation agent: N-Methyl-N-(trimethylsilyl)trifluoroacetamid (*i.e.* MSTFA). A sample chromatogram is shown in Figure 3.2.9.3.

To prepare the sample, weigh about 0.1 mg biodiesel to 20 cm^3 closable vials, add 100μl of IS1 and IS2 standards and 100μl MSTFA to each. Close all vials hermetically, stir vigorously and leave for 15-20 minutes at room temperature. After this time, add 8 cm^3 heptane and inject 1 μl of the sample diluted this way to the chromatographic column using the "cold on-column" method. The recommended program of furnace temperature increase is: 50°C 1 min, increase in subsequent steps to 180C (15°/min), 230°C (7°/min) and 370°C (10°/min). Maximum furnace temperature maintained for 5 minutes.

The content of individual components is determined based on the calibration of standard solutions of glycerine, mono-, di- and triglycerides, although the range of applicability is limited to 0.005–0.05% m/m of glycerine (G), 0.25–1.25%

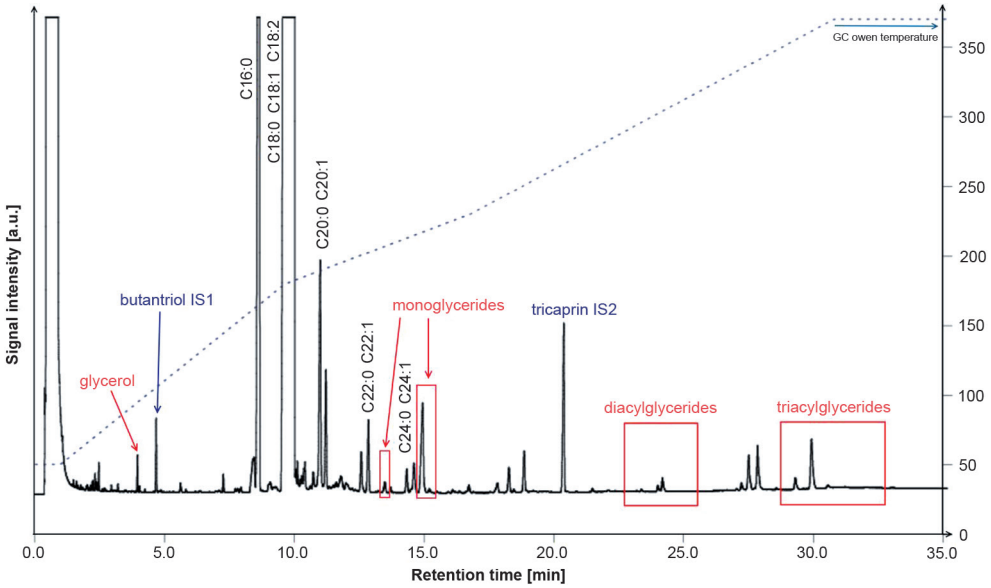


Figure 3.2.9.3. Sample chromatogram of free and fixed glycerine analysis using a method compliant with the standards EN-14105 [217] and ASTM D 6584 [218]. Based on [219, 220]

monoglycerides (M), 0.05–0.5% diglycerides (D) and 0.05–0.4% m/m of triglycerides (T). Total glycerol (GT) in the sample is calculated using the following formula:

$$GT = G + 0.255\,M + 0.146\,D + 0.103\,T \tag{3.2.9.9}$$

Spectrophotometric analysis [215]:

Add 4 cm^3 hexane and 4 cm^3 extraction solvent (mixture of equal volumes of water and 95% ethanol) to 1 g sample of biofuel. Mix the solution vigorously for 5 minutes (vortex), then centrifuge for 15 minutes at 2000 RPM. Separate the top phase and transfer exactly 0.5 cm^3 from the bottom phase to a closable test tube or a 10 cm^3 cylinder. Add 1.2 cm^3 freshly prepared 10 mM solution of sodium metaperiodate and mix for 30 second. Then add 1.2 cm^3 0.2M solution of acetylacetone and thermostat the solution for 1 minute at 70°C (manual stirring). After this time, cool to 20–25°C and analyse spectrophotometrically at a wavelength of 410 nm. Use a 4 cm^3 sample of diluted ethanol prepared in the same manner as the standard solution. Prepare standard ethanol solutions of glycerine in the same way and determine the calibration curve with their use.

Calculate the content of glycerine (x_{GLY}) in biofuel using the following formula:

$$x_{GLY},\ \mathrm{mg/kg} = \frac{GLY}{1000 \cdot m} \tag{3.2.9.10}$$

where:

GLY – content of glycerine read directly from the calibration curve, mg,

m – mass of biofuel sample take for the test, g.

Except for acetic acid, ammonium acetate and diluted alcohol solutions, all reagents must be prepared directly before the assay:

- 10 mM solution of sodium metaperiodate in a pH 5.5 buffer: Dissolve 21 mg sodium metaperiodate in 5 cm^3 1.6 M acetic acid solution (9.6 g/100 cm^3). After dissolving, add 5 cm^3 4 M ammonium acetate solution (30.8 g/100 cm^3);
- 0.2 M acetylacetone solution: Dissolve 200 µl (195 mg) acetylacetone in 5 cm^3 acetic acid solution, then add 5 cm^3 ammonium acetate solution.

3.2.9.3. Phospholipids

Phospholipids, together with lycolipids, belong to complex fats and aside from fatty acids, contain a phosphoric acid radical in their structure. They are a construction material for cell walls, they are partially soluble in fats and organic solvents. Present in fats used at high temperatures, they cause their darkening and are responsible for the release of unpleasant odour. They are removed from raw pressed and extracted oils through fat refining processes, usually in two steps: during pre-degumming by hydratation (mixing and heating with water at 80°C for 20 minutes at a water : oil volumetric ratio of about 1 : 10, followed by centrifuging after cooling), and during acidic refinement, frequently combined with adsorption on bleaching clay. Acidic refinement is performed by heating fat with a small portion of 40% H_3PO_4 or citric acid (volume ratio 50 : 1), and after phase separation, the fat is washed with water or bleaching clay is directly added [221]. Phospholipid content in oil is analysed based on phosphorus content analysis.

Performing the assay using the vanadium and molybdenum method:

Roast quartz crucibles in a muffle furnace at a temperature of 900°C for 60 minutes, then weigh 0.1 g magnesium oxide and 0.5–1 g fat sample to each crucible. Subsequently heat the crucibles in a dryer to 105°C (10 minutes) to oversaturate the magnesium oxide with the sample, incinerate the contents on an electrostatic plate and burn for 60 minutes at 900°C. After cooling, add 1.25 cm^3 nitric acid diluted with water at a 1 : 2 ratio (alternatively, use 10 cm^3 1 N H_2SO_4) and 5 cm^3 of post-reaction mixture prepared using equal amounts of 5% aqueous ammonium molybdate and acidic solution of ammonium methavanadate (dilute 2.5 g NH_4VO_3 in 250 cm^3 hot water, add 10 cm^3 concentrated HNO_3 after

cooling and make up to 500 cm^3 with water). Mix the crucible contents. After 20–30 minutes, transfer the solution to a glass cell and measure absorbance at a wavelength λ = 460 nm with a reagent sample as a standard, which is prepared by weighing 0.1 g magnesium oxide to a clean beaker and adding the remaining solutions, as with the proper samples. Using the calibration curve, calculate phosphorus (P) content in oil with the use of formula (3.2.9.11), then calculate it to percentage content of phospholipids (*FL*) [222]:

$$\mathrm{P},\ \mathrm{mg/kg} = 1000\frac{a_1}{m} \tag{3.2.9.11}$$

$$FL,\ \% = 26 \cdot \mathrm{P} \tag{3.2.9.12}$$

where:

P – phosphorus content, mg/kg,
a_1 – solution concentration read from calibration curve, mg,
m – weighed amount of test sample, g.

Phosphorus assay using the molybdenum method [223]:

Dissolve a sample of material after mineralisation with sulfuric acid and hydrogen peroxide solution, a sample prepared in an identical manner as in the previous assay, or ash residue after dry mineralisation (see section 3.2.9.6) in nitric acid diluted with water, and make up with water to 50 cm^3. Add 1 cm^3 ammonium molybdate solution (2.5% aqueous solution) to 5 cm^3 of the above solution, mix thoroughly for 5 minutes (vortex), then add 1 cm^3 ascorbic acid solution (10% aqueous solution) and mix again for 5 minutes. Close the vessel tightly (or protect against evaporation) and heat at 100°C for 7 minutes. After cooling to room temperature, read absorbance at 820 nm with blank reagent sample. Read phosphorus content from the relevant calibration curve.

3.2.9.4. Sterols

Sterols of animal and plant origin, containing double bonds in their molecules, form products of characteristic colours with concentrated acids. In the case of cholesterol, under the effect of concentrated sulfuric acid (Salkowski reaction, Figure 3.2.9.4) water is eliminated and the red-coloured disulfonic bicholestadien acid is formed. To perform the assay, introduce 5 drops of oil and 1cm^3 chloroform to a dry test tube, and without mixing, add 3 drops of concentrated sulfuric acid. In the presence of cholesterol, the chloroform phase takes on a brown and red colour.

In the Liebermann-Burchard reaction, monosulfonic bicholestadien acid forms in the presence of concentrated sulfuric acid and acetic anhydride. The acid has

a green colour and its radiation absorption maximum is at 625 nm (Figure 3.2.9.5). On the other hand, using a 0.05% $FeCl_3 \cdot 6H_2O$ solution in glacial acetic acid, and concentrated sulfuric acid, a violet-coloured solution with its absorbance maximum at 560 nm is obtained [224]. Regardless of the selected method, to perform the determination of percentage content of cholesterol in the test sample, prepare a calibration curve, which is a function of extinction against concentration of standard cholesterol solution.

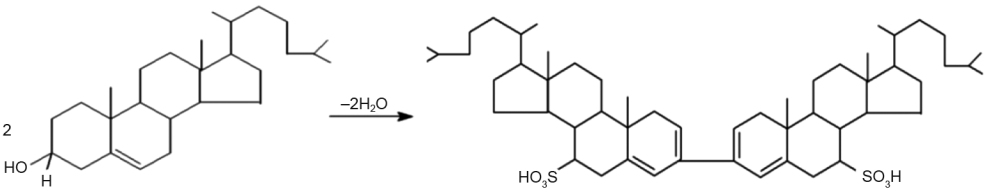


Figure 3.2.9.4. Salkowski reaction

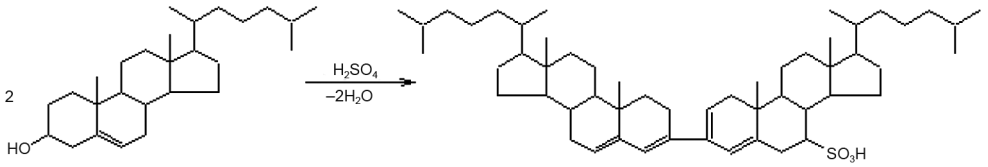


Figure 3.2.9.5. Liebermann-Burchard reaction

Aside from spectrophotometric methods, chromatographic (HPLC) and precipitation methods are commonplace, including the digitonin ($C_{56}H_{92}O_{29}$) method employed, for example, by the company Croda Chemicals.

Cholesterol content assay using the precipitation method:

Dissolve about 1 g sample in 25 cm^3 hot 90% ethanol and hot filtrate the solution through a sintered glass filter. After washing the filter with further 50 cm^3 hot ethanol, combine the resulting filtrates and make up to 100 cm^3 with alcohol. Next, add 40 cm^3 0.5% ethanol solution of digitonin to 10 cm^3 test solution, heat and leave for 18 hours at ambient temperature. Filter the resulting cholesterol digitonide (Figure 3.2.9.6) precipitate through a dried and weighed glass filter, washing, in turn, with 15 cm^3 cold 90% ethanol, and identical portions of cold acetone and hot carbon tetrachloride. Dry the washed precipitate to constant mass at 105°C.

Calculate cholesterol content in test sample using the following formula:

$$x_c, \% = \frac{a \cdot 0.239 \cdot 1000}{m} \qquad (3.2.9.13)$$

where:

a – mass of precipitate, g,

m – weighed amount of sample, g.

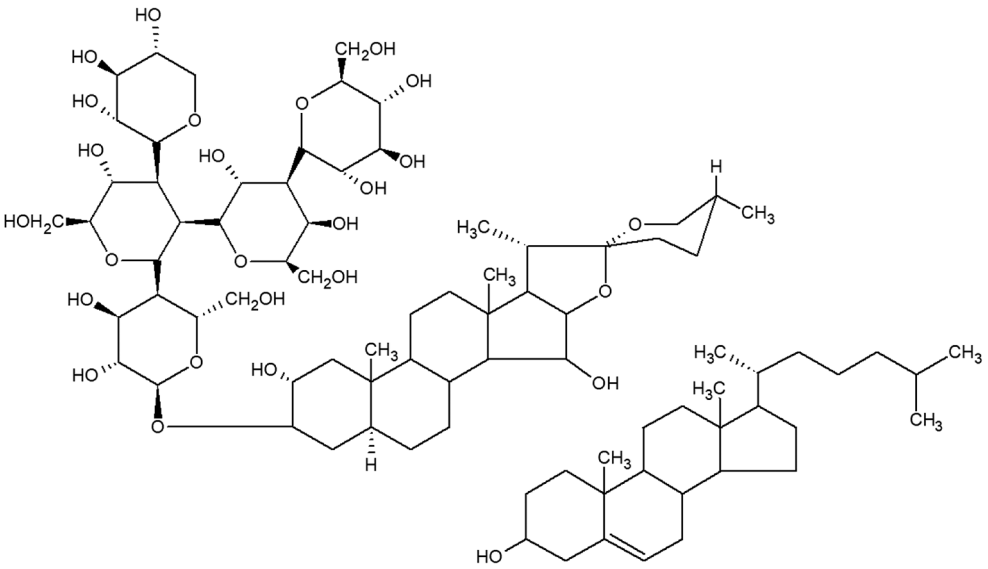


Figure 3.2.9.6. Cholesterol Digitonide, $C_{83}H_{138}O_{26}$, M = 1551.99 g/mol

Cholesterol content assay using the spectrophotometric method:

Weigh about 0.1–0.5 g test substance in a volumetric flask and dissolve in glacial acetic acid (20–25 cm^3). Take 0.2 cm^3 to a test tube and add 1 cm^3 colour fixing solution (12 g *p*-toluenesulfonic acid dissolved in 100 cm^3 glacial acetic acid) and 3 cm^3 acetic anhydride. Due to the exothermic nature of the reaction, leave the test tube contents to cool without mixing. Next, add 0.5 cm^3 concentrated sulfuric acid, mix thoroughly and leave for 20 minutes. After this time, read absorbance (A_1) at a wavelength of 550 nm with a reagent sample (0.2 cm^3 acetic acid subjected to all steps of the procedure) as a standard. To rule out interfering, non-sterol compounds, perform another blank test, with the sample containing 0.2 cm^3 test sample solution and 4.5 cm^3 colour fixing solution.

Read absorbance A_2 after 20 minutes, with *p*-toluenesulfonic acid solution as a standard. Calculate cholesterol content in the sample using the formula:

$$x_c,\ \% = \frac{V \cdot C}{m} \qquad (3.2.9.14)$$

where:

V – volume of acetic acid used to dissolve the weighed amount of sample m (20–25 cm^3),

C – cholesterol concentration read from a calibration curve prepared for cholesterol solutions of known concentration, or calculated using formula 3.2.9.15 after taking into account the interference by non-steroid components in the sample:

$$C,\ g/cm^3 = \frac{A_1 - A_2 - 0.0542}{85.587} \qquad (3.2.9.15)$$

3.2.9.5. Mineral content (ash estimation in liquid samples)

For liquid samples, ash content can be determined by pre-coking and roasting a weighed amount of sand with the test sample added, or using the filter paper method described in the standard [225]. To this end, place an ashless filter paper in a roasted and weighed crucible or evaporator dish in such a way so it tightly adheres to the walls and bottom of the vessel. Next, introduce a weighed amount of the analysed liquid product (2–5 g, depending on predicted ash content) to the vessel and place another filter paper, folded in two and formed in a conical shape, in the vessel – it will serve as a wick (Figure 3.2.9.7). Leave the contents for 30–40 minutes so the filter papers soak with the sample. If the test sample is poorly absorbed by the filter paper, is semi-liquid, contains moisture, or visibly foams during heating – gradually heat the contents of the crucible to a temperature of 100–105°C. This way, the sample should liquefy, water should evaporate, and the filter papers will be sufficiently soaked with the test product. Finally, carefully ignite the top of the wick-filter paper, and carefully heating the vessel on an electric machine, burn the sample completely. When the combustion process ends, place the vessel and

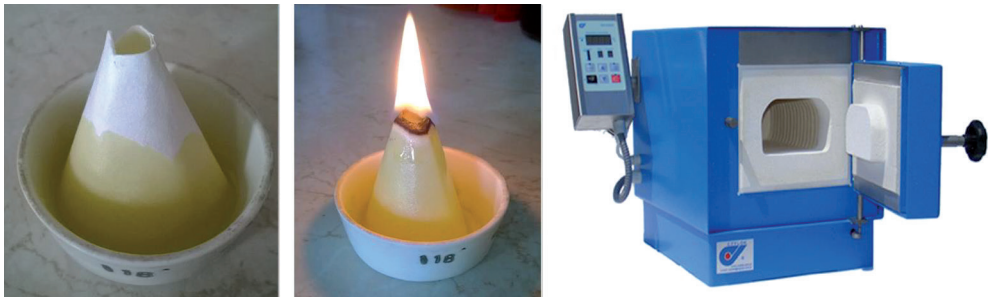


Figure 3.2.9.7. Crucible with liquid sample for ash determination, and muffle furnace for calcination

its contents in a muffle furnace (**CAREFULLY!** The crucible becomes hot during incineration!) and roast to constant mass at a temperature of 600–650°C (at least 3–5 hours).

If the residue incinerates too slowly, which can be recognised by the unchanging amount of black particles of coal, remove it from the furnace, cool, and moisten with a few drops of ammonium nitrate solution. Carefully evaporate the contents and roast again at 600–650°C.

Calculate the ash content using the following formula:

$$\text{Ash, } \% = 100\frac{m_1 - m_2 - m_3}{m} \tag{3.2.9.16}$$

where:

m_1, m_2, m_3 – indicate mass of vessel with ash after calcination, mass of empty vessel, mass of ashless filter papers used (value read from the label and multiplied by the number of papers), respectively,

m – mass of weighed amount of liquid sample.

3.2.10. Analyses of ethanol and light alcohols

Depending on the method of ethyl alcohol production and the raw materials used, a product polluted with a varied range of organic compounds is produced. Classic methods, involving glucose fermentation to ethanol and CO_2, are based on raw materials rich in starch, which – after pre-liquefaction and saccharification – are subjected to fermentation with yeasts. Following the fermentation and separation of sediments, ethyl alcohol is subjected to refinement, which – aside from the main product (bioethanol) – generates byproduct such as esters with small molecules, aldehydes (*e.g.* acetic aldehyde, acrolein), methanol, higher alcohols – so called fusels – which include pentyl, propyl, and isobutyl alcohols, as well as organic acids, *e.g.* lactic, acetic, succinic, malonic, and butyric acids, and small amounts of glycerine [226–228]. To date, over 100 chemical compounds have been identified that transfer to the distillate during alcohol desorption from fermented mash, although their content usually does not exceed 1% [229]. Most of these compounds are removed during refinement of raw spirit, and their acceptable content in alcohol products is limited by the relevant standards [230].

From the perspective of biofuel production, the presence of most organic components is less important than the presence of water, which adversely affects low-temperature properties of fuel with a bioethanol addition, and also contributes to phase separation of fuel mixtures. As water forms an azeotropic mixture with alcohol (boiling point 78.15°C for 95.6% ethanol), complete dehydration of alcohol is usually done in several steps, by variable pressure distillation, distillation with an

additional azeotroping agent (cyclohexane, benzene, toluene), and final drying on molecular sieves.

Table 3.2.10.1

Properties of refined spirit according to standard [230]

Property\spirit	Raw	Technical	Neutral	Superior	Luxurious
Alcohol, % vol. (20°C)	91–94	95.0	96.0	96.5	96.5
Aldehydes, g/L (heads)	0.03–0.1	n.a.	0.005	0.002	0.001
Fusel, g/L (tails)	1.9–6.5	0.1	0.005	0.002	0.001
Methanol, g/L (heads)	0.04–0.24	1.0	1.0	0.5	0.3
Esters, g/L	0.2–0.6	0.07	0.05	0.03	0.03
Dry extract, g/L	< 0.1	0.01	0.01	0.01	0.01
Furfural (tails)	n.a.	n.a.	0 (unacceptable)		

The alcohol content in a sample is usually determined using the chromatographic method, enabling accurate determination of ethanol and impurity concentrations, or by the pycnometer method – literature provides detailed tables of the relation between the density of aqueous alcohol solution and its concentration. Additionally, it is possible to determine alcohol content using the refractometric method, which – despite its low precision – enables concentration to be determined very quickly.

3.2.10.1. Chromatographic method for light alcohols analyses

Analysis of ethanol and other alcohol content can be performed on a Shimadzu--17A gas chromatograph, fitted with a FID and a universal capillary column DB-5 or one dedicated for methyl ester analysis (ZB-WAX) with a length of 30 m, or on a Chromatron gas chromatograph, fitted with a FID and a 2 m packed column filled with Porapak Q, or a packed column BS-1 with a length of 3 m. In the first case, the volume injected into the column should not exceed 0.2 µl, while in the second case, the recommended volume is 1µl. A sample analysis of liqueur on a capillary column is shown in Figure 3.2.10.1, while Figure 3.2.10.2 shows a chromatogram and detector signal calibration for ethanol on a packed column.

Essentially, it is recommended that highly polluted samples or ones containing a lot of water should be analysed on a packed column, characterised by much higher resistance to damage and signal interference than capillary columns.

Knowing the composition of a sample (identification based on retention times) and having calibrations for individual mixture components, it is possible to calculate the precise composition of the analysed mixture, even if one component remains unknown. For example – the chromatogram of a 1 microlitre

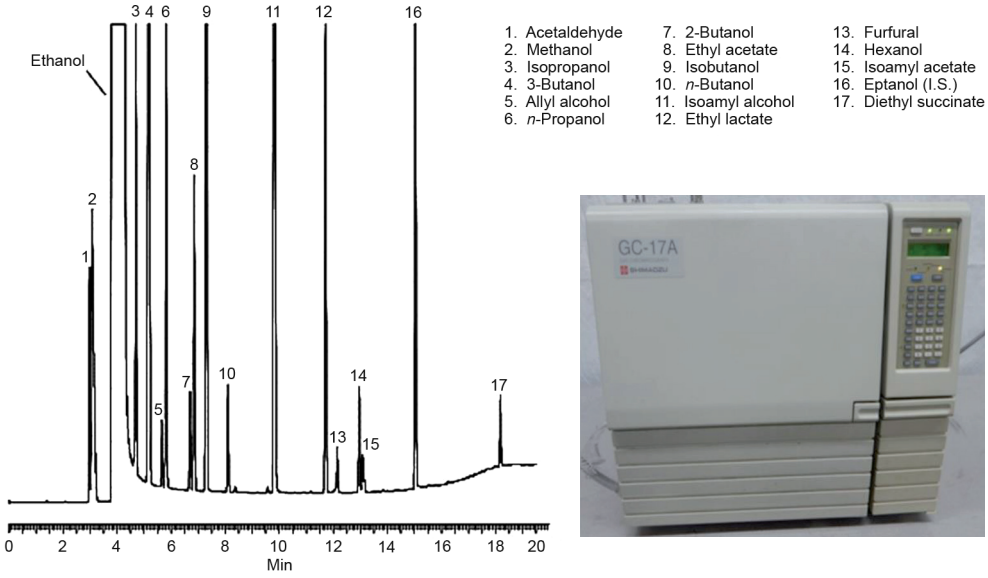


Figure 3.2.10.1. Chromatogram illustrating liqueur components – based on [231]

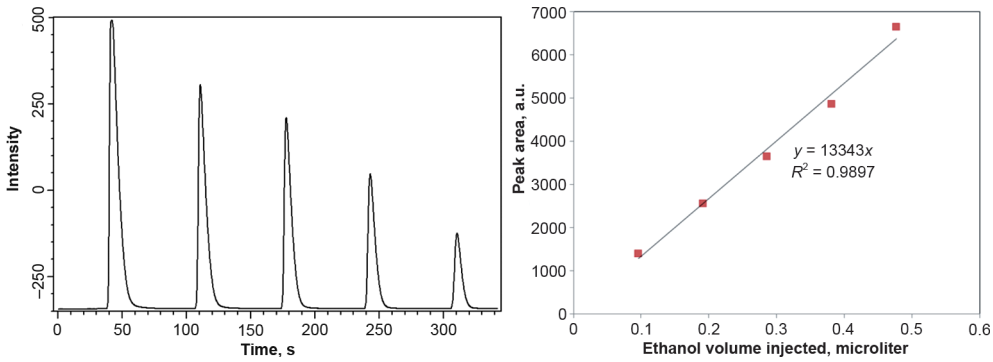


Figure 3.2.10.2. Signal calibration for ethanol and a calibration curve. Analysis performed using chromatograph equipped with FID detector and 2 m Porapak Q packed column

of an anhydrous mixture of isopropanol and acetone has revealed the presence of a third component *X* (Figure 3.2.10.3). Having done the calibrations for known components and using formula (3.2.10.1), it is possible to calculate the volume composition of the sample and the content of the third component.

$$X_A,\ \%\ \text{vol.} = \frac{V_A}{V_{\text{inj}}} \tag{3.2.10.1}$$

$$X_{A_i},\ \text{vol.} = \frac{A_i}{f_{A_i}} \tag{3.2.10.2}$$

$$X_{A_2},\ \text{vol.} = V_{\text{inj}} - (V_{A_1} + V_{A_3}) \tag{3.2.10.3}$$

where:

- X_A – percentage volume of component A in the mixture (any of the analysed),
- V_{Ai} – volume of component i for which detector signal calibration has been performed,
- A_i – surface area of peak i, for which detector signal calibration has been performed,
- f_{Ai} – calculation factor for component i (read from the chart of injected volume against peak surface area, similar to than shown in Figure 3.2.10.2),
- V_{inj} – total volume injected to the chromatograph column.

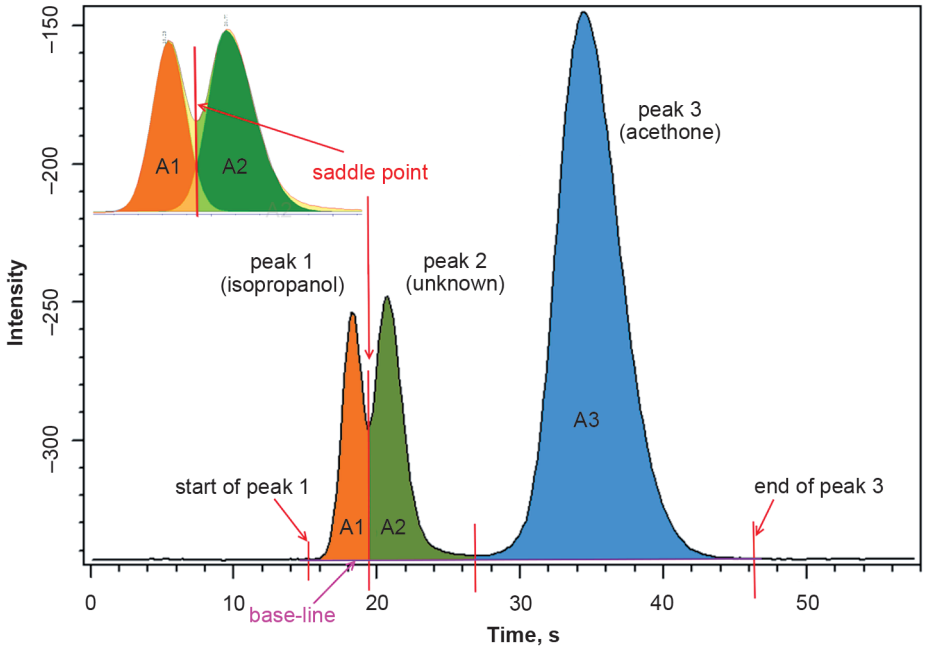


Figure 3.2.10.3. Deconvolution of detector signal for poorly separated peaks

When processing chromatograms, attention must be paid to correct placing of base line under peaks and to their correct separation – for poorly separated peaks, select one saddle point as the end of one and beginning of the other peak (very good results for low-laying saddle points), or select the option of automatic matching of

Gaussian curves for optimum matching of surface areas. If calibration coefficients are known for all components of the mixture, the sum of volumes should equal the total volume injected into the chromatograph column (V_{inj}). A difference between these may indicate the presence of components not observed on the chromatogram, which may evidence that water, invisible to FIDs, or components not eluted from the column (too low pressure of the compound – below lower detectability threshold, too long retention times caused by strong sorption on the column, low sensitivity of detector response to the given compound, or its decomposition under assay conditions) are present.

3.2.10.2. Ethanol concentration by density measurement

The densimetric method is one of the simpler methods of determining alcohol concentration. It involves measuring the density of a liquid at a temperature of 20°C (see section 3.2.2), which can subsequently be calculated to mass or volume content of ethanol using appropriate tables [232]. Figure 3.2.10.4 shows charts illustrating the relation of actual mass concentration and volume concentration of ethanol to solution density in kg/m^3 (for 20°C), including relevant conversion equations.

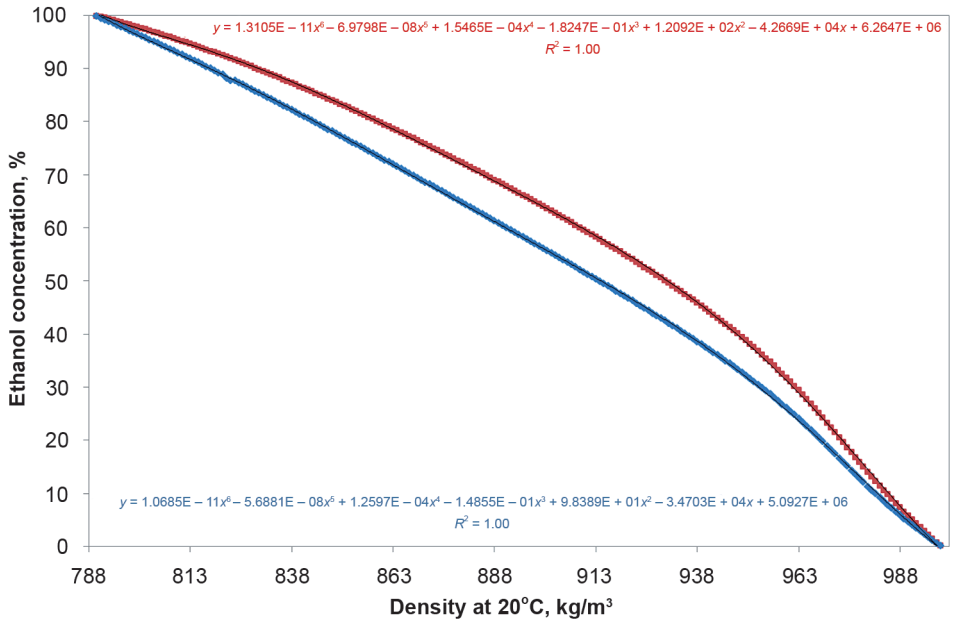


Figure 3.2.10.4. Relation between solution density at 20°C and mass and volume concentration of ethanol (red and blue curve respectively). Based on [232]

In a similar, simplified manner, based on tabular data, the relation between volume concentration (x) and mass concentration (y) of an ethanol solution at 20°C can be derived:

$$y = 2.3827\text{E} - 05x^3 - 6.4251\text{E} - 04x^2 + 8.1907\text{E} - 01x \qquad (3.2.10.4)$$

3.2.10.3. Ethanol concentration by refraction index measurement

Measurement of refractive index of light is another simple method of determining ethanol content in aqueous solutions. The principle of this assay is explained in section 3.1.6.1, while tabular values for the temperature of 20°C [233] are shown in graphical form in Figure 3.2.10.5, together with the best fitting function.

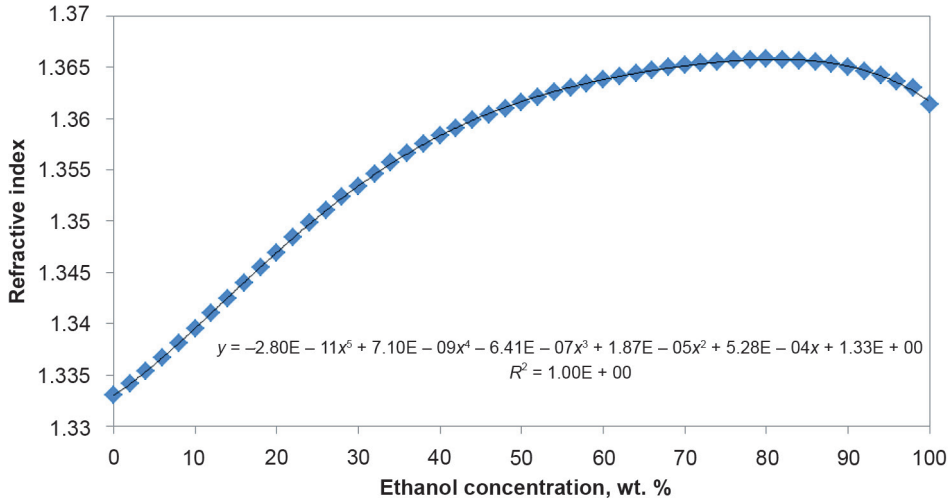


Figure 3.2.10.5. Relation between refractive index of light at 20°C and mass concentration of ethanol. Based on [233]

3.2.11. Analyses of water content

The presence of water in bioresources and biofuels is commonplace and is a direct result of the presence of water in all living organisms, both plants and animals. For natural oil production, the presence of 5–7% of moisture in raw materials (seeds) is even beneficial, as it facilitates the pressing process. Use of dried seeds increases pressing resistances and raises the temperature at the worm shaft, at the same time reducing oil yield, as does too high moisture content, which in extreme cases causes an oil emulsion to be pressed. Thanks to fat rafination, commercial--grade raw materials are usually completely devoid of moisture to increase its

hydrolytic and oxidating stability. If moisture is present, foaming during heating and increased cloud temperatures have also been observed. For similar reasons, the presence of water in ester biofuels is limited to the level of 500 mg/kg [4]. Although raw materials directed for biodiesel synthesis are usually anhydrous, so as to avoid undesirable losses due to saponification and emulsification, large amounts of water can, however, penetrate into the product during the biofuel purification stage (washing with water). In the case of bioethanol, the presence of water in the product is a natural consequence of the aqueous environment of the fermentation reaction, and of the forming of the water-ethanol azeotrope (see section 3.2.10). For fuel purposes, water content in bioethanol is limited to 0.5% [234].

Some methods of water content determination in solid products (section 3.1.1) can also successfully be applied to liquid products, especially those with high thermal stability and low volatility. In the case of hydrophobic liquids, azeotropic distillation with toluene, benzene or cyclohexane is particularly practical, while for hydrophilic single-component liquids (often highly hygroscopic, such as ethanol and glycerine), it is possible to determine water content by measuring density or refractive index of light (*cf*. section 3.2.10). Neither method, however, is suitable for multi-component mixtures, for which it is the most convenient to determine water content using the chromatographic method or Karl Fischer method.

3.2.11.1. Chromatographic method

Determination of water content in liquid samples is possible using a chromatograph with a thermal conductivity detector (TCD). It is recommended to use a short packed universal column (*e.g*. Porapak Q) with a zero degree of water retention. However, carbon and aluminosilicate packings should be avoided, as they absorb water relatively strongly, changing the parameters of the analysis. To ensure sufficient elution of all components of the test solution, it is preferable to maintain the column operation temperature above water boiling point, and to periodically heat the column with carrier gas flowing.

In order to perform the analysis, pre-heat the chromatographic column to 140°C with helium flowing (pressure at the column 1 atm), and activate the TCD sufficiently in advance. After stabilising the base line, inject the test samples in volumes of 0.5–1 μl. Calibrate the detector signal for water in an identical manner as it was described for ethanol in section 3.2.10.1. A sample chromatogram from an assay of watered methanol, and water signal calibration are shown in Figure 3.2.11.1, together with the relevant calibration curves for both components of the mixture. Calculate the content of individual components using equations (3.2.10.1)–(3.2.10.3).

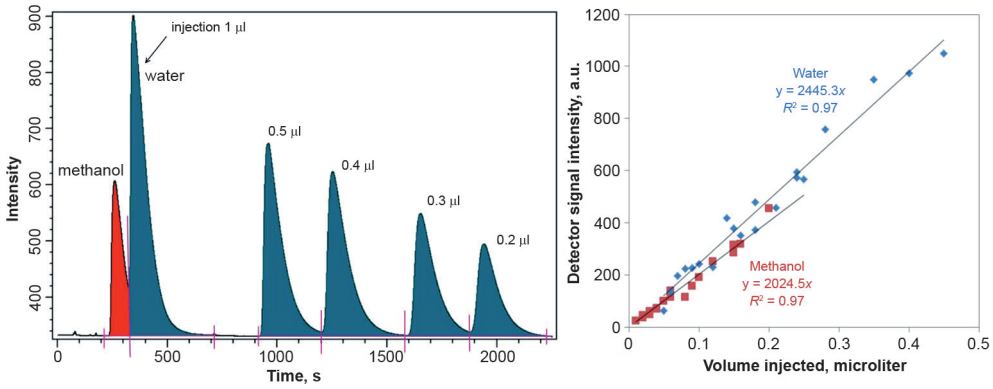


Figure 3.2.11.1. Chromatogram from analysis of a methanol-water mixture on a 2 m packed column Porapak Q (TCD detector), including water signal calibration and relevant calibration curves

3.2.11.2. Karl-Fisher method

The Fischer method is applied to products containing from 0.0005% to 0.5% water [235]. It involves volumetric or coulometric titration of water with the Fisher reagent in a special apparatus (Figure 3.2.11.2), with electrometric determination of the equilibrium point. At very low water concentrations, extraction with ethylene glycol is applied before titration, while for contents exceeding 0.005%, titration is done directly. The determination is based on the following reaction:

$$SO_2 + I_2 + 2H_2O \rightarrow H_2SO_4 + 2HI \qquad (3.2.11.1)$$

In the volumetric method, use a pipette to introduce the test product in an amount of 50 cm^3 for predicted water content 0.005–0.1%, to 10 cm^3 for higher water contents, to 50 cm^3 of an anhydrous methanol-chloroform solution titrated with the Fischer reagent until the end point. Titrate with Fischer reagent until the end point, *i.e.* until the microamperometer pointer remains deflected for more than 30 seconds. Calculate water content in the sample using the following formula:

$$X_w,\ \%\ \text{wt.} = \frac{T \cdot V_F}{10 \cdot V_s \cdot \rho} \qquad (3.2.11.2)$$

where:

T – titre of Fischer solution, mg H_2O/cm^3,
V_F – volume of Fischer solution used to titrate the analysed product, cm^3,
V_s – volume of test sample, cm^3,
ρ – density of test sample, determined using an areometer or pycnometer, g/cm^3.

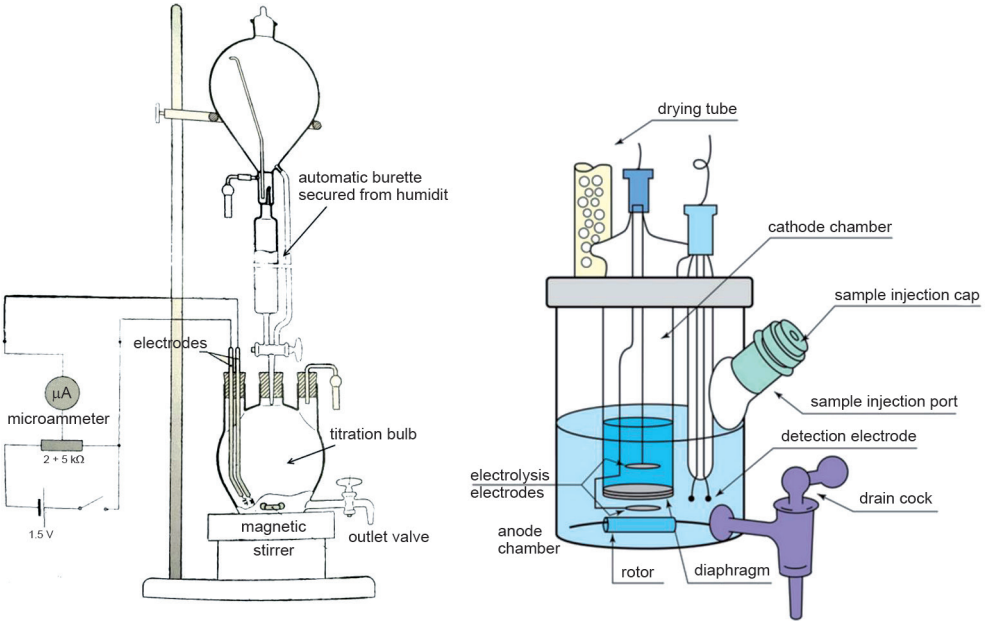


Figure 3.2.11.2. Apparatus for water determination using the Fischer method. From the left – classic apparatus used on the volumetric method (based on the standard [235]), and apparatus for the coulometric method [236]

For very low water content values in the analysed product, add 50 cm^3 anhydrous ethylene glycol to 1 dm^3 of test sample, plug tightly and shake for 20 minutes. After the solution settles, take 25 cm^3 of the glycol phase (bottom) and introduce into a measurement cell containing 25 cm^3 of anhydrous ethylene glycol, previously titrated with Fischer reagent. Titrate until the end point. Perform a blank sample test in the same manner, *i.e.* titration of 25 cm^3 glycol used for extraction. Calculate water content using the following formula:

$$X_w,\ \%\ \text{wt.} = \frac{(V_F - V_0)T \cdot V_{EG1}}{10 \cdot V_{EG2} \cdot m} \tag{3.2.11.3}$$

where:

T – titre of Fischer solution, mg H_2O/cm^3,
V_F – volume of Fischer solution used to titrate the analysed product, cm^3,
V_0 – volume of Fischer solution used to titrate the blank sample, cm^3,
V_{EG1} – volume of glycol used for extraction, cm^3,
V_{EG2} – volume of extract used for titration, cm^3,
m – mass of test sample taken for extraction, g.

Preparing the Fischer reagent:

Dissolve 85 g iodine in 270 cm^3 dehydrated pyridine and 670 cm^3 dehydrated methanol. After cooling the mixture to 0°C, slowly saturate with gaseous SO_2, dried in a scrubber with concentrated sulfuric acid. Saturation is carried out until solution volume increases by 50 cm^3. Alternatively, the solution can be saturated (while stirring) with 50 cm^3 condensed sulfur dioxide. 1 cm^3 of reagent prepared this way corresponds to 5–6 mg water, and its titre should be set to a water drop in a chloroform and methanol solvent.

Calculate the titre of Fischer reagent using the following formula:

$$T = m / V \tag{3.2.11.4}$$

where:

T – titre of Fischer reagent in mg H_2O/cm^3,
m – mass of added water,
V – volume of Fischer reagent used to titrate the added water.

3.3. Selected analyses for gaseous samples

Gaseous products can be analysed by classic methods, after their absorption in appropriate solid or liquid sorbents, or by instrumental methods, using the gas chromatography technique. This method is particularly useful for instances of forced gas circulation, enabling the injection loop to the chromatographic column to be filled easily. Otherwise, the gaseous sample should be collected in a sealed gas pipette or a glass jar with a rubber membrane, enabling gas samples to be taken with an appropriate gas syringe. To isolate the gas sample and minimise contact with air, gas can be introduced directly above the surface of the isolation solution, selected to fit the test sample (*e.g.* saturated brine). All elements mentioned above are shown in Figure 3.3.1.

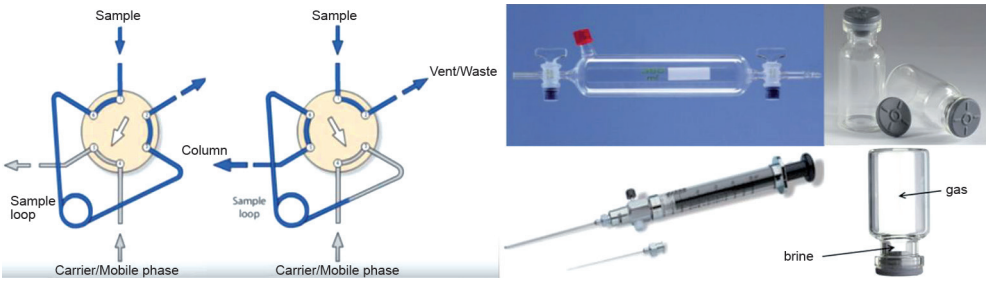


Figure 3.3.1. Diagram of injection loop operation (6-way valve), gas pipette and syringe, and jars with rubber membranes, enabling gas sample storage over a solution isolating from external agents (*e.g.* saturated brine)

3.3.1. Analysis of CO_2

Determination of carbon dioxide content in gases released during anaerobic fermentation, biomass pyrolysis or gasification is the most convenient when done using the chromatographic method, described in the next section. For alcohol fermentation, during which CO_2 is released from a sugar molecule in an amount stoichiometric to alcohol, its quantity can be established by the gravimetric method (loss of mass of the fermentation solution), although this method is relatively inaccurate. More reliable results can be obtained with the titration method.

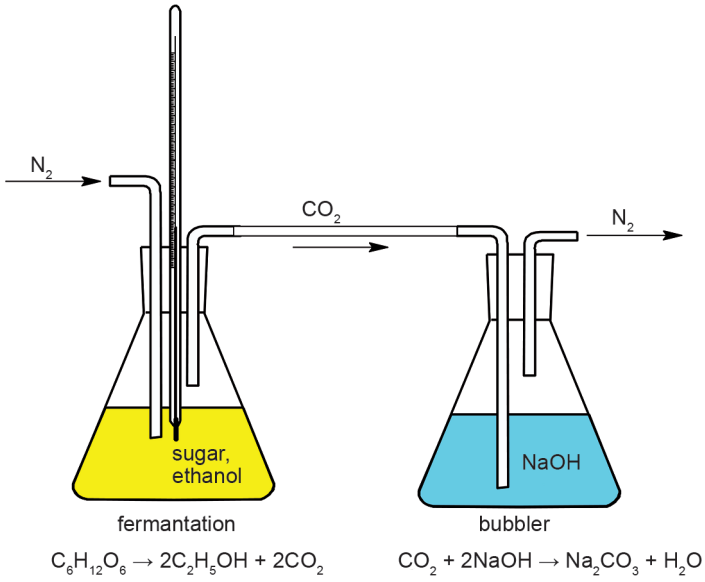


Figure 3.3.2. Conceptual diagram of alcohol fermentation and CO_2 fixing in an alkali solution

To perform the assay, connect a fermentation tank filled with the fermentation solution to a bubbler, filled with an alkali solution of known concentration, using a rubber hose (Figure 3.3.2). Flush the system with an inert gas that does not contain carbon dioxide (*e.g.* nitrogen) and leave to ferment. Maintain inert gas flow at very low level (100 cm^3/h) to avoid suction of alkali solution to fermentation bottle. When the fermentation process is over, push the remaining gas through the bubbler using an inert gas flush, and titrate the solution in the bubbler using an aqueous solution of HCl in the presence of phenolophthalein. Calculate the amount of carbon dioxide using the following formula:

$$CO_2,\ g/g = \frac{\left(\frac{m_o}{39.997} - C \cdot V \cdot 1000\right) \cdot 44.01}{m} \tag{3.3.1}$$

where:

m_o – mass of NaOH introduced into the bubbler, g,
C – concentration of HCl solution used for titration, mol/dm^3,
V – volume of HCl solution used for titration, cm^3,
m – mass of glucose in the fermentation solution, g.

With complete fermentation, the ratio of released CO_2 mass to the mass of glucose should be near 0.5.

3.3.2. Analysis of biogas composition – GC method

Analysis of carbon dioxide and other gases released during anaerobic fermentation (biogas production) can be performed using the chromatographic method, employing a gas chromatograph with a TCD detector, fitted with a 1–2 m packed column filled

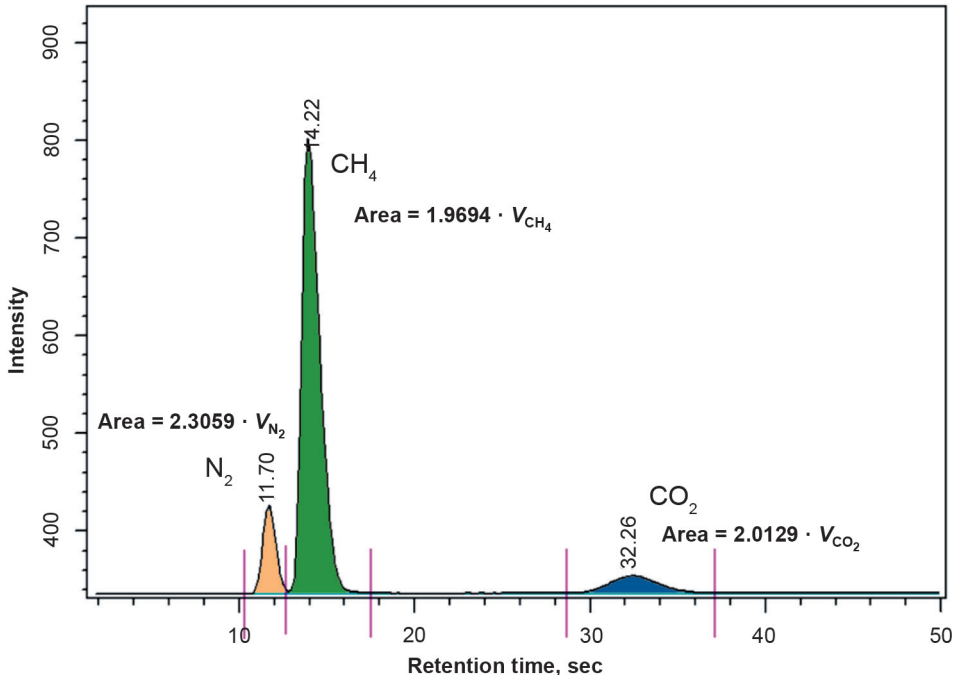


Figure 3.3.3. Chromatogram of biogas sample with component identification and calibration curve equations. Isothermal assay at room temperature on a 1 m column packed with Separon SDA and using 30 cm^3/min He as a carrier gas

with Porapak Q, or a 1 m column filled with Separon SDA. Perform the analysis at room temperature, injecting a 0.5–1 cm^3 gas sample directly into the column. Perform qualitative analysis based on comparison of retention times with standard samples, while quantitative analysis is done after calibrating the detector signal for the analysed gas (methodology identical as in section 3.2.10.1). For gaseous samples it is more convenient, however, to use volumes or moles of individual components, so calibration curves are prepared by dosing specific volumes of pure gases and reading the surface areas of their corresponding peaks. The sum of calculated volumes of individual components of the gas mixture should therefore be equal to the total volume of the sample introduced into the column.

A sample chromatogram for a biogas sample, including identification of individual components and calibration curve equations is shown in Figure 3.3.3.

3.3.3. Analysis of gasification and pyrolysis products composition

For analyses of gaseous mixtures of more complex composition, *e.g.* gases from pyrolysis or biomass gasification, it may be necessary to analyse the composition in two steps, using two different chromatographic systems, one equipped with a FID and another with a TCD. It is related to the low sensitivity of the thermal conductive detector to components of higher molecular masses, and the lack of ability to determine inert components (nitrogen, oxygen, carbon or nitrogen oxides) on a FID. The solution is to inject the same sample separately to a TCD chromatograph column, operating at room temperature (to analyse inert components), and introducing the

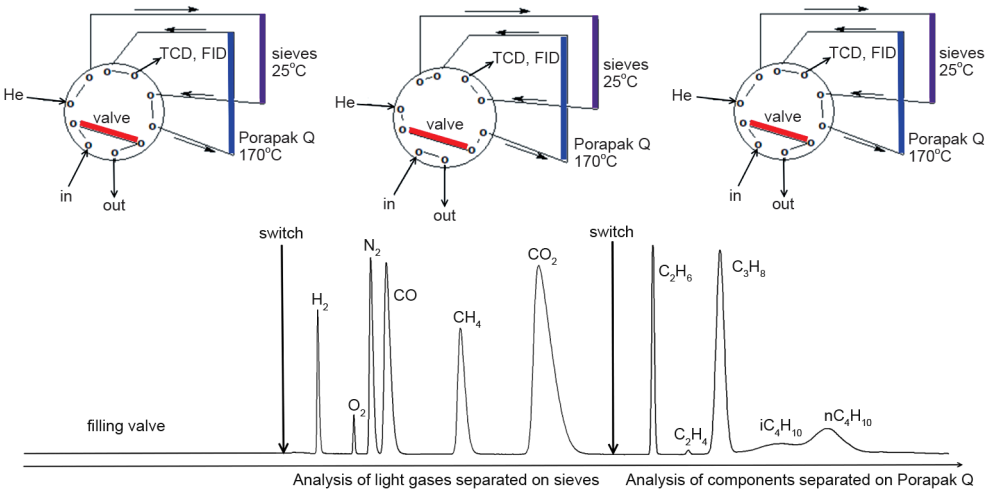


Figure 3.3.4. Operation of 12-way valve and a chromatograph with two columns and two detectors

same gaseous mixture again to a separate device fitted with a FID and a column operating at a higher temperature (*e.g*. 2 m Porapak Q at a temperature of 130°C for analysing light hydrocarbons). Calibration must be done for each system separately, while results are analysed together. Having an SRI chromatograph with a 12-way loop n to detectors connected serially, the entire task can be performed within a single analysis. As is shown in Figure 3.3.4, with the valve in the starting position, the injection loop is filled; after switching, gases are directed successively to Porapak Q and sieves. Light gases, not retained on the first column, are separated on the second column, while heavier compounds with longer retention times are analysed later, bypassing the column with molecular sieves (another switch of the valve).

References

[1] European Pharmacopoeia 5.0 with Supplements 5.1 and 5.2, Council of Europe, July 2004 EDQM – European Directorate for the Quality of Medicines.

[2] Rozporządzenie Ministra Rolnictwa i Rozwoju Wsi z dnia 10 lipca 2007 w sprawie znakowania środków spożywczych, Dziennik Ustaw 2007, nr 137, poz. 966; Rozporządzenie Ministra Zdrowia z dnia 25 lipca 2007, Dz.U. 2007, nr 137, poz. 967.

[3] Sałata B., Centrum Doradztwa Rolniczego w Brwinowie, Oddział w Radomiu, *Wymagania w zakresie znakowania produktów spożywczych*, Radom 2015, http://www.cdr.gov.pl/images/wydawnictwa/2015/2015-WYMAGANIA-W-ZAKRESIE-ZNAKOWANIA-PRODUKTOW-SPOZYWCZYCH.pdf (online: 28.10.2017).

[4] PN-EN 14214, Ciekłe przetwory naftowe – Estry metylowe kwasów tłuszczowych (FAME) do użytku w silnikach samochodowych o zapłonie samoczynnym (Diesla) i zastosowań grzewczych – Wymagania i metody badań.

[5] ASTM D6751, Standard Specification for Biodiesel Fuel Blend Stock (B100) for Middle Distillate Fuels.

[6] PN-EN 16723-1:2016-12, Gaz ziemny i biometan używany w transporcie oraz biometan zatłaczany do sieci gazu ziemnego – Część 1: Wymagania dotyczące biometanu zatłaczanego do sieci gazu ziemnego.

[7] PN-C-04753:2011, Gaz ziemny – Jakość gazu dostarczanego odbiorcom z sieci dystrybucyjnej.

[8] PN-EN ISO 17225-1:2014-07, Biopaliwa stałe – Specyfikacje paliw i klasy – Część 1: Wymagania ogólne.

[9] PN-A-79522:2001, Spirytus rektyfikowany.

[10] EN 15376:2007, Automotive fuels – Ethanol as a blending component for petrol – Requirements and test methods; ZN/MGiPS/NF-214/2003, Etanol paliwowy – Bioetanol.

[11] ASTM D-5798 Standard Specification for Fuel Ethanol (Ed75-Ed85).

[12] PN-A-86908:2000P, Oleje i tłuszcze roślinne oraz zwierzęce – Rafinowane oleje roślinne.

[13] *Biogaz – produkcja i wykorzystywanie*, Poradnik dostępny na stronie: http://ieo.pl/dokumenty/obszary_badan/Biogaz%20-%20Produkcja%20Wykorzystywanie.pdf (online: 28.10.2017); Opracowanie: *Landfill gas –*

industry code of practise, dostępne na stronie: http://www.esauk.org/reports_press_releases/esa_reports/LandfillgasICoP2012web.pdf (online: 07.08.2018).
[14] Klimiuk E., Pokój T., Pawłowska M., *Biopaliwa. Technologie dla zrównoważonego rozwoju*, Wydawnictwo Naukowe PWN, Warszawa 2012.
[15] Demibras A., *Biofuels*, Springer, Berlin 2010.
[16] Lewandowski W. M., Ryms M., *Biopaliwa. Proekologiczne odnawialne źródła energii*, WNT, Warszawa 2013.
[17] Dobrosz-Teperek K., Dasiewicz B., *Procesy fermentacyjne*, Przemysł chemiczny, 87/9 (2008), 896-901.
[18] Elimer E., Gajos E., *Mikrobiologia techniczna. Higiena żywności*, Wyd. Akademii Rolniczej we Wrocławiu, Wrocław 1994.
[19] Viikari L., Vehmaanperä J., Koivula A., *Lignocellulosic ethanol: From science to industry*, Biomass and Bioenergy, 46 (November 2012), 13-24.
[20] Stelmach S., Wasielewski R., Figa J., *Zgazowanie biomasy – przykłady nowych technologii,* Archiwum Gospodarki Odpadami i Ochrony Środowiska, 7 (2008), 9-20.
[21] Grochal B. J., *Wybrane aspekty zgazowania biomasy*, Prezentacja Gaz Ziemny w Produkcji Rolnej i w Przemyśle Spożywczym, Toruń 12–13.12.2006, http://zssplus.pl/galerie/Ekologia/_private/biogaz_2.pdf (online: 28.10.2017).
[22] Jahirul M. I., Rasul M. G., Chowdhury A. A., Ashwath N., *Biofuels Production through Biomass Pyrolysis*, Technological Review, Energies, 5 (2012), 4952-5001.
[23] Raveendran K., Ganesh A., *Heating value of biomass and biomass pyrolysis products*, Fuel, 75 (15) (November 1996), 1715-1720.
[24] Niewiadomski H., *Technologia tłuszczów jadalnych*, WNT, Warszawa 1993.
[25] http://www.abcmach.com/uploads/allimg/oil-refinery-plant-processing.jpg (online: 28.10.2017).
[26] Głodek E., *Przewodnik Spalanie i współspalanie biomasy*, Opole 2009, www.oze.opole.pl/zalacznik.php?id=364&element=470 (online: 15.07.2017).
[27] Witrowa-Rajchert D., *Ekspertyza – nowe trendy w suszeniu żywności*, 2009, http://www.agengpol.pl/LinkClick.aspx?fileticket=wq81Lx050Uc%3D&tabid=144 (online: 28.10.2017).
[28] Klimek B., *Analiza fitochemiczna roślinnych substancji leczniczych*, Wydawnictwo Uniwersytetu Medycznego w Łodzi, Łódź 2011.
[29] Kumirska J., Gołębiowski M., Paszkiewicz M., Bychowska A., *Skrypt z ochrony środowiska, analiza żywności*, Wydawnictwo Uniwersytetu Gdańskiego, Gdańsk 2010.
[30] Łuczak H., Jeżewska M., Białas M., Kulczak M., *Zawartość popiołu w wybranych herbatach ekspresowych*, Bromatologia i Chemia Toksykologiczna, XLV (3), 2012, 500-506.

[31] PN-G-04512:1980/Az1:2002, Popiół z sadzy, norma kompletna oznaczania popiołu w paliwach stałych.
[32] BN-78/8191-06, Herbata – oznaczanie popiołu oraz alkaliczności popiołu rozpuszczalnego w wodzie.
[33] Pierwsza dyrektywa Komisji z dnia 15 czerwca 1971 r. ustanawiająca wspólnotowe metody analizy do celów urzędowej kontroli pasz, 71/250/EWG, ec.europa.eu/assets/near/neighbourhood-enlargement/ccvista/pl/31971l0250-pl.doc (online: 28.10.2017)
[34] Matusiewicz H., *Rozdział 16. Metody rozkładu próbek na mokro*, http://www.pg.gda.pl/chem/CEEAM/Dokumenty/CEEAM_ksiazka_polska/Rozdzialy/rozdzial_016.pdf (online: 28.10.2017).
[35] PN-R-04014:1991, Analiza chemiczno-rolnicza roślin – Metody mineralizacji materiału roślinnego do oznaczania makro- i mikroelementów.
[36] http://sklep.pkn.pl/ (online: 28.10.2017).
[37] http://www.caslab.com/EPA-Methods-3000-Series/; https://www.epa.gov/measurements/index-epa-test-methods (online: 28.10.2017).
[38] Özcan M. M., Chalchat J. C., *Chemical composition of carrot seeds (Daucus carota L.) cultivated in Turkey: characterization of the seed oil and essential oil*, Grasas y acetites, 58 (4), October–November 2007, 359-365.
[39] PN-ISO 6492:2005, Pasze – Oznaczanie zawartości tłuszczu.
[40] PN-EN ISO 659:2010, Nasiona oleiste – Oznaczanie zawartości oleju (Metoda odwoławcza).
[41] PN-EN ISO 734-1:2008, Śruta nasion oleistych – Oznaczanie zawartości oleju – Część 1: Metoda ekstrakcji heksanem (lub benzyną lekką).
[42] PN-EN ISO 11085:2010, Ziarno zbóż, przetwory wyprodukowane na bazie zbóż i pasze – Oznaczanie zawartości tłuszczu surowego i tłuszczu całkowitego metodą ekstrakcji Randalla.
[43] Bockisch M., *Fats and Oils Handbook*, AOCS Press, Champaign 1998.
[44] PN-EN ISO 1211:2011, Mleko – Oznaczanie zawartości tłuszczu – Metoda grawimetryczna (Metoda odniesienia).
[45] Borawska M. H., Markiewicz-Żukowska R., Naliwajko S. K., Socha K., Skrypt do wybranych ćwiczeń z analizy żywności, Zakład Bromatologii, Wydział Farmaceutyczny, Uniwersytet Medyczny w Białymstoku, Białystok 2017, https://www.umb.edu.pl/skrypt_do_wybranych_cwiczen_z_analizy_zywnosci (online: 28.10.2017).
[46] Leffler T. P., Moser C. R., McManus B. J., Urh J. J., Keeton J. T., Claflin A., *Determination of moisture and fat in meats by microwave and nuclear magnetic resonance analysis: collaborative study*, Journal of AOAC International, 91(4) (2008), 802-810.

[47] Gurczyńska E., Faszcza E., Koczoń P., Kowalski B., *Oznaczanie zawartości tłuszczu i wody w majonezach metodą spektroskopii w podczerwieni z transformacją Fouriera*, Żywność, 2(43) (2005), 95-107.
[48] http://www.foss.pl/rozwi%c4%85zanie-dla-bran%c5%bcy/products/nirs-da1650-oilseed-crush-analyser (online: 28.10.217).
[49] *Analiza techniczna węgla i biomasy*, Materiały dydaktyczne Politechniki Wrocławskiej, http://www.polymer-carbon.ch.pwr.edu.pl/instrukcje/SNE_techniczna.pdf (online: 28.10.2017).
[50] *Surowce energetyczne i ich przetwarzanie. Cz. II – paliwa stałe*, Materiały dydaktyczne Akademii Górniczo-Hutniczej, http://home.agh.edu.pl/~kepw/student/plik/o_c-l_k.pdf (online: 28.10.2017).
[51] AOAC, 1995, *Official methods of analysis of the association of official analytical chemists*, 16th ed. AOAC, Arlington, Method 925.10 (Kjeldahl).
[52] FAO food and nutrition paper 77, *Food energy – methods of analysis and conversion factors, Report of a technical workshop*, Rome, December 3–6, 2002, ftp://ftp.fao.org/docrep/fao/006/y5022e/y5022e00.pdf (online: 28.10.2017).
[53] Klepacka M. (ed.), *Analiza żywności*, Fundacja Rozwój SGGW, Warszawa 2005.
[54] Nielsen S. S. (ed.), *Food analysis*, 4th Edition, Springer, New York 2010.
[55] Kumirska J., Gołębiowski M., Paszkiewicz M., Bychowska A., Skrypt z Ochrony środowiska – analiza żywności, Wydawnictwo Uniwersytetu Gdańskiego, Gdańsk 2010, ttps://inf.ug.edu.pl/kierunkizamawiane/materialy.chemia/Analiza_Zywnosci.pdf (online: 28.10.2017).
[56] Wójciuk K., *Oznaczanie ilościowe białek – porównanie metod*, http://laboratoria.net/pl/artykul/Oznaczanie%20ilo%C5%9Bciowe%20bia%C5%82ek%20-%20por%C3%B3wnanie%20metod;19922.html (online: 28.10.2017).
[57] Gniot-Szulżycka J., Leźnicki A., Wojczuk B., *Białka. Metody ilościowego oznaczania, rozdziału i oczyszczania*, Materiały do ćwiczeń z biochemii, Wydawnictwo Naukowe UMK, Toruń 2005.
[58] Pace C. N., Vajdos F., Fee L., Grimsley G., Gray T., *How to measure and predict the molar absorption coefficient of a protein*, Protein Science, 4 (1995), 2411-2423.
[59] Held P. G., *Nucleic acid purity assessment using A260/A280 ratios*, BioTek, https://www.biotek.com/resources/docs/PowerWave200_Nucleic_Acid_Purity_Assessment.pdf (online: 28.10.2017).
[60] Gornal A. G., Bardawill C. S., Dawid M. M., *Determination of serum proteins by means of the biuret reaction*, Journal of Biological Chemistry, 177 (1949), 751-766.

[61] Hortin G. L., Meilinger B., *Cross-Reactivity of Amino Acids and Other Compounds in the Biuret Reaction: Interference with Urinary Peptide Measurements*, Clinical Chemistry, 51, 8 (2005), 1411-1419.
[62] seilnacht.com (online: 28.10.2017).
[63] Georgiou Ch. D., Grintzalis K., Zervoudakis G., Papapostolou I., *Mechanism of Coomassie brilliant blue G-250 binding to proteins: a hydrophobic assay for nanogram quantities of proteins*, Analytical and Bioanalytical Chemistry, 391 (2008), 391-403.
[64] Preejith P. V., Lim C. S., Kishen A., John M. S., Asundi A., *Total protein measurement using a fiber-optic evanescent wave-based biosensor*, Biotechnology Letters, 25 (2003), 105-110.
[65] http://www.ou.edu/OpenEducation/ou-resources/biochemical-methods/lab-8/ (online: 28.10.2017).
[66] Skale pomiarowe zawartości cukru Brix, Balling, Plato, ČNM, KMW, Oechsle, Baumé, https://www.refraktometr.eu/skale-pomiarowe-zawartosci-cukru (online: 28.10.2017).
[67] https://www.aliexpress.com/item-img/New-Arrival-3-Scale-Hydrometer-Home-brew-Wine-Beer-Cider/32499926469.html?spm=a2g17.10010108.1000017.2.JpJJQA (online: 28.10.2017).
[68] http://beersmith.com/blog/2016/10/09/using-a-refractometer-with-beersmith-brewing-software/ (online: 28.10.2017).
[69] https://www.umb.edu.pl/photo/pliki/WF_jednostki/zaklad.../polarymetria.doc (online: 28.10.2017).
[70] Dubois M., Gilles K. H., Hamilton J. K., Rebers P. H., Smith F., *Colorimetric Method for Determination of Sugars and Related Substances*, Analytical Chemistry, 28 (1956), 350-356.
[71] Agrawal N., Minj D. K., Rani K., *Estimation of Total Carbohydrate Present in Dry Fruits*, IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT), 1 (6) (September 2015), 24-27.
[72] PN-67/A-86430, Mleko i przetwory mleczarskie – Lody – Metody badań chemicznych.
[73] Pierwsza dyrektywa Komisji z dnia 15 czerwca 1971 ustanawiająca wspólnotowe metody analizy do celów urzędowej kontroli pasz, 71/250/EWG, ec.europa.eu/assets/near/neighbourhood-enlargement/ccvista/pl/31971l0250-pl.doc (online: 28.10.2017).
[74] PN-A-82100:1985P, Wyroby garmażeryjne – Metody badań chemicznych.
[75] Oznaczanie zawartości cukrów redukujących oraz cukrów redukujących po inwersji. Część I – oznaczenie zawartości cukrów redukujących oraz cukrów redukujących po inwersji w fermentowanych napojach winiarskich,

z wyłączeniem miodów pitnych (metoda Luff-Schoorla), http://pdfy.polbi.pl/Dz_U_/2013/0624/d130624z5.pdf (online: 28.10.2017).
[76] Dz.U.04.37.334, Rozporządzenie Ministra Rolnictwa i Rozwoju Wsi z dnia 13 lutego 2004 r. w sprawie metod analiz niektórych produktów i półproduktów przemysłu cukrowniczego, http://www.abc.com.pl/du-akt/-/akt/dz-u-2004-37-334 (online: 28.10.2017).
[77] Goni L., Garcia-Alonso A., Saura-Calixto F., *A starch hydrolysis procedure to estimate glycemic index*, Nutrition Research, 17 (1997), 3427-3437.
[78] BN/91-8080-01, Norma branżowa, Przetwory ziemniaczane i skrobiowe, metody kontroli procesu technologicznego krochmalni ziemniaczanej.
[79] Drożdż B., *Analiza jakościowa związków organicznych*, Katedra Chemii Organicznej Uniwersytetu Jagiellońskiego, Kraków 2013, http://www.farmacja.cm.uj.edu.pl/documents/41668/102342567/Analiza+organiczna.pdf/fd074673-5824-4112-a62a-ba1562f26337 (online: 28.10.2017).
[80] http://allmedtests.com/bials-test/ (online: 28.10.2017).
[81] http://www.harpercollege.edu/tm-ps/chm/100/dgodambe/thedisk/carbo/bial/bials.htm (online: 28.10.2017).
[82] http://nlabtech.blogspot.com/2016/01/benedicts-test-principle-composition.html (online: 28.10.2017).
[83] Watson S. A., Whistler R. L, *Estimation of Iodine Color of Starches and Starch Fractions*, Industrial and Engineering Chemistry, Analytical Edition, 18 (1) (1946), 75, 76.
[84] Baldwin R. R., Bear R. S., Rundle R. E., *The relation of starch-iodine absorption spectra to the structure of starch and starch components*, Journal of the American Chemical Society, 66 (1) (1944), 111-115.
[85] BN-86/8130-02, Norma branżowa – wyroby przemysłu spożywczego, Koncentraty spożywcze, Oznaczanie błonnika pokarmowego.
[86] European Commission guidance to methods of analysis for determination of the fibre content declared on a label, December 2012, https://ec.europa.eu/food/sites/food/files/safety/docs/labelling_legislation_guidance_methods_2012_en.pdf (online: 28.10.2017).
[87] Dyrektywa Komisji 92/89/EWG z dnia 3 listopada 1992 – wspólnotowe metody analizy do celów urzędowej kontroli pasz, ec.europa.eu/assets/near/neighbourhood-enlargement/ccvista/pl/3199l0089-pl.doc (online: 28.10.2017).
[88] Sato M. F., Rigoni D. C., Giovanetti Canteri M. H., de Oliveira Petkowicz C. L., Nogueira A., Wosiacki G., *Chemical and instrumental characterization of pectin from dried pomace of eleven apple cultivars*, Acta Scientiarum, Agronomy Maringá, 33 (3) (2011), 383-389.

[89] Zadernowski R., Piłat B., *Zmiany w zawartości błonnika pokarmowego w dojrzewających korzeniach marchwi*, Seria: Zeszyty Naukowe, 61, Wydawnictwo Wyższej Szkoły Agrobiznesu w Łomży, Łomża 2016, 45-57.
[90] http://www.zcha.pwr.wroc.pl/chc4172l/cw8.pdf (online: 28.10.2017).
[91] Shelukhina N. P., Fedichkina L. G., *A rapid method for quantitative determination of pectic substances*, Acta Botanica Neerlandica, 43(2) (June 1994), 205-207.
[92] Ranganna S., McHraw-Hill T., *Handbook of Analysis and Quality control for fruit and vegetable products*, 2nd edition, Publishing Company Limited, New Delhi 1977.
[93] BN-71/6020-02, Norma branżowa – Oznaczanie barwy produktów organicznych.
[94] Li Y., Scales N., Blankenship R. E., Willows R. D., Chen M., *Extinction coefficient for red-shifted chlorophylls: Chlorophyll d and chlorophyll f*, Biochimica et Biophysica Acta, 1817 (2012), 1292-1298.
[95] http://www.citruscollege.edu/lc/archive/biology/Pages/Chapter06-Rabitoy.aspx (online: 28.10.2017).
[96] http://plantphys.info/plant_physiology/lightrxn.shtml (online: 28.10.2017).
[97] PN-A-86934:1995, Oleje i tłuszcze roślinne oraz zwierzęce – Spektrofotometryczne oznaczanie barwy ogólnej.
[98] Sumanta N., Haque Ch. I., Nishika J., Suprakash R., *Spectrophotometric Analysis of Chlorophylls and Carotenoids from Commonly Grown Fern Species by Using Various Extracting Solvents*, Research Journal of Chemical Sciences, 4(9) (September 2014), 63-69.
[99] http://pulpbits.net/wp-content/uploads/2013/12/Spinach-Leaf-Pigments.jpg (online: 28.10. 2017).
[100] http://www.pitt.edu/~ceder/lab5/exp5text.html (online: 28.10.2017).
[101] *Rozdział barwników fotosyntetycznych przy pomocy TLC i RP-HPLC*, Materiały dydaktyczne Katedry Biochemii i Neurobiologii, Wydział Inżynierii Materiałowej i Ceramiki AGH, http://www.neuro.agh.edu.pl/uploads/przemek/Foto.pdf (online: 28.10.2017).
[102] Masłowska J., Janiak J., *Badania nad zawartością syntetycznych barwników spożywczych w barwionych galaretkach żelatynowych*, r. PZH, XLVI, nr 1, 1995, 21-29.
[103] Forgacs E., Cserhati T., *Thin-layer chromatography of natural pigments: new advances*, Journal of Liquid Chromatography and Related Technologies, 25, 10–11 (2002), 1521-1541.
[104] PN-EN ISO 3838:2008, Ropa naftowa i ciekłe lub stałe przetwory naftowe – Oznaczanie gęstości lub gęstości względnej – Metody z użyciem piknometru z korkiem kapilarnym i piknometru dwukapilarnego z podziałką.

[105] https://destylacja.com/pl/p/CUKROMIERZ-WINOMIERZ-4-34-Blg-dokl-2Blg/2283 (online: 28.10.2017).
[106] http://www.gasconcept.com/oferta/termodensymetr/termodensymetr-lpg.html (online: 28.10. 2017).
[107] https://www.helago-cz.cz/eshop-gay-lussacuv-pyknometr-139518.html (online: 28.10.2017).
[108] http://www.e-laboratoria.pl/piknometry-z-termometrem (online: 28.10.2017).
[109] http://www.kfich.am.szczecin.pl/images/images/files/Instrukcje_lab_paliw/01_gestosc_2014.pdf (online: 28.10.2017).
[110] Bigg P. H., *Density of water in SI units over the range 0–40°C*, British Journal of Applied Physics, 18 (1967), 521-525.
[111] Krupowies J., Wiznerowicz C., *Ćwiczenie laboratoryjne – Pomiar gęstości oraz wyznaczanie temperaturowego współczynnika gęstości produktów naftowych*, Materiały dydaktyczne Akademii Morskiej w Szczecinie, Szczecin 2014.
[112] http://www.sigma2c.com/16api_gravity.html (online: 28.10.2017).
[113] *Badanie odporności olejów smarowych na ścinanie. Lepkość kinematyczna*, Materiały dydaktyczne Wydziału Maszyn Roboczych i Transportu Politechniki Poznańskiej, www.fwmt.put.poznan.pl/plik_pobierz.php?id=90 (online: 28.10.2017).
[114] Demirbas A., *Relationships derived from physical properties of vegetable oil and biodiesel fuels*, Fuel, 87 (2008), 1743-1748.
[115] Toscano G., Maldini E., *Analysis of the physical and chemical characteristics of vegetable oils as fuel*, Journal of Agricultural Engineering – Rivista di Ingegneria Agraria, 3 (2007), 39-47.
[116] Lakshmi Narayana Rao G., Ramadhas A. S., Nallusamy N., Sakthivel P., *Relationships among the physical properties of biodiesel and engine fuel system design requirement*, International Journal of Energy and Environment (IJEE), 1(5) (2010), 919-926.
[117] Krisnangkura K., Yimsuwan T., Pairintra R., *An empirical approach in predicting biodiesel viscosity at various temperatures*, Fuel, 85 (2006), 107-113.
[118] http://www.staff.amu.edu.pl/~chemfiz/pliki/Cwiczenie_7.pdf (online: 28.10.2017).
[119] Zarządzenie nr 178 Prezesa Głównego Urzędu Miar z dnia 19 grudnia 1995 w sprawie wprowadzenia przepisów metrologicznych w wiskozymetrach Hopplera, https://www.gum.gov.pl/download/2/2843/pxdu331995.pdf (online: 28.10.2017).

[120] PN-EN ISO 3104:2004, Przetwory naftowe – Ciecze przezroczyste i nieprzezroczyste – Oznaczanie lepkości kinematycznej i obliczanie lepkości dynamicznej.
[121] PN-EN 590:2013-12, Paliwa do pojazdów samochodowych – Oleje napędowe – Wymagania i metody badań.
[122] PN-ISO 3016:2005, Przetwory naftowe – Oznaczanie temperatury płynięcia.
[123] PN-84/C-04017, Przetwory naftowe – Oznaczanie temperatury mętnienia i początku krystalizacji paliw silnikowych.
[124] García M., Gonzalo A., Sánchez J. L., Arauzo J., Peña J. A., *Prediction of normalized biodiesel properties by simulation of multiple feedstock blends*, Bioresource Technology, 101 (2010), 4431-4439.
[125] Ramos M. J., Fernández C. M., Casas A., Rodríguez L., Pérez A., *Influence of fatty acid composition of raw materials on biodiesel properties*, Bioresource Technology, 100 (2009), 261-268.
[126] https://secure.hastdeer.com.au/hdsosweb/HDLabServices/images/Cloud Point_small.jpg (online:28.10.2017).
[127] http://filtertechno.com/biodiesel-filtration-systems/ (online: 28.10.2017).
[128] http://journeytoforever.org/biodiesel_winter.html (online: 28.10.2017).
[129] PN-EN 116:2015-09, Oleje napędowe i oleje opałowe lekkie – Oznaczanie temperatury zablokowania zimnego filtru – Metoda stopniowego schładzania.
[130] http://www.lin-tech.ch/english/cf254000eng.html (online: 28.10.2017).
[131] Knothe G., Krahl J., Gerpen J., *The Biodiesel Handbook*, AOCS, Urbana 2010.
[132] Materiały Wydziału Inżynierii Transportu Politechniki Poznańskiej, www.fwmt.put.poznan.pl/plik_pobierz.php?id=94 (online: 05.10.2018).
[133] Catoire L., Paulmier S., Naudet V., *Estimation of closed cup flash points of combustible solvent blends*, Journal of Physical and Chemical Reference Data, 35 (2006), 9-14.
[134] https://www.ijee.ieefoundation.org/vol1/issue5/IJEE_16_v1n5.pdf (online: 28.10.2017).
[135] https://www.mech.kuleuven.be/en/tme/research/Thermotech/Contents/2008_Norman.pdf (online: 28.10.2017).
[136] http://anko-lab.com/en/a/mit-apparatusmodel-mit-3 (online: 28.10.2017).
[137] http://www.wha-international.com/autoignition_temperature.php (online: 28.10.2017).
[138] Ryng M., *Bezpieczeństwo techniczne w przemyśle chemicznym – poradnik*, WNT, Warszawa 1985.
[139] Jaworski A., Kuszewski H., Lejda K., Ustrzycki A., Woś P., *Oznaczanie liczby cetanowej wybranych paliw na podstawie spalania w komorze o sta-*

łej objętości w aspekcie ich zastosowania w silniku o zmiennym stopniu sprężania VCR, Czasopismo Techniczne, 3-M/2012, 275-284.
[140] PN-91/C-04031, Przetwory naftowe – Oznaczanie indeksu cetanowego paliw.
[141] ASTM D4737. Standard test method for calculated cetane index by four variable equation.
[142] PN-EN ISO 4264:2010/A1, Przetwory naftowe – Obliczanie indeksu cetanowego paliw ze średnich destylatów metodą równania czterech zmiennych.
[143] Rozporządzenie Komisji (WE) nr 440/2008 z dnia 30 maja 2008, http://eur-lex.europa.eu/legal-content/PL/TXT/?uri=CELEX:32008R0440 (online: 28.10.2017).
[144] https://www.quora.com/Why-is-a-Thiele-tube-preferred-for-melting-point-determination-rather-than-an-open-beaker-of-oil (online: 28.10.2017).
[145] http://www.ebah.com.br/content/ABAAAfPlwAA/identificacao-substancia-desconhecida (online: 28.10.2017).
[146] PN-81/C-04012, Oznaczanie składu frakcyjnego metodą destylacji normalnej; PN-EN ISO 3405:2012 Przetwory naftowe – Oznaczanie składu frakcyjnego metodą destylacji pod ciśnieniem atmosferycznym.
[147] ASTM D86, Standard Test Method for Distillation of Petroleum Products and Liquid Fuels at Atmospheric Pressure.
[148] https://upload.wikimedia.org/wikipedia/commons/7/76/Positive_Azeotrope.png (online: 28.10.2017).
[149] http://chemicalformulas.blogspot.com/2015/09/this-is-apparatus-used-to-test-for-Distillation-of-Petroleum-Product.html (online: 28.10.2017).
[150] http://www.thomassci.com/Laboratory-Supplies/Distilling-Flasks/_/PYREX-DISTILLING-FLASKS (online: 28.10.2017).
[151] http://www.inkom.com.pl/produkt/items/504 (online: 28.10.2017).
[152] PN-A-86908: Oleje i tłuszcze roślinne oraz zwierzęce – Rafinowane oleje roślinne.
[153] PN-90/A-85802: Tłuszcze zwierzęce jadalne topione.
[154] Norma BN-91/8052-01: Oliwy z oliwek.
[155] Rozporządzenie Ministra Gospodarki z dnia 22 stycznia 2009 (Dz. U. z dnia 4 lutego 2009).
[156] ASTM D974 – 14e2 Standard Test Method for Acid and Base Number by Color-Indicator Titration.
[157] PN-85/C-04066, Przetwory naftowe – Oznaczanie kwasowości i liczby kwasowej.
[158] PN-ISO 3657:1994. Oleje i tłuszcze roślinne oraz zwierzęce – Oznaczanie liczby zmydlania.
[159] ASTM D-974 Standard Test Method for Acid and Base Number by Color – Indicator Titration.

[160] *Oznaczanie odczynu chemicznego wyciągu wodnego oraz całkowitej liczby kwasowej lub liczby zasadowej olejów smarowych*, Materiały dydaktyczne Akademii Morskiej w Szczecinie, Wydział Mechaniczny – laboratorium paliw, olejów i smarów, http://www.kfich.am.szczecin.pl/images/images/files/Instrukcje_lab_paliw/05_OWW_BN_2014.pdf (online: 28.10.2017).
[161] Knothe G., *Structure Indices in FA Chemistry. How Relevant is the Iodine Value?*, JAOCS, 79 (9) (2002), 847-854.
[162] Arct J., Pytkowska K., *Importance of Non-Glyceride Fractions of Plant Oil on Cosmetics, Cosmetic and Household Ingredients*, Conference Proceedings, Augsburg 2000, 117-131.
[163] Arct J., Pytkowska K., *Naturalne składniki anti-age z frakcji niezmydlalnych olejów roślinnych* (*Natural anti-aging ingredients from unsaponifiable fraction of plant oils*), SÖFW-Journal, wyd. polskie, 1, 2, 2008, 24-37.
[164] Sandha G. K., Swami V. K., *Jojoba oil as an organic shelf stable standard oil-phase for cosmetic industry*, Rasayan Journal of Chemistry, 2 (2) (2009), 300-306.
[165] Kohlmunzer S., *Farmakognozja – podręcznik dla studentów farmacji*, wyd. 5, Wydawnictwo lekarskie PZWL, Warszawa 1998.
[166] PN-ISO 3961:1998. Oleje i tłuszcze roślinne oraz zwierzęce – Oznaczanie liczby jodowej.
[167] http://www.farmacja.cm.uj.edu.pl/documents/41668/134534151/Protokol+7.pdf/7b502542-2de3-4900-af84-cd0381ee3dae (online: 28.10.2017).
[168] Kurek J., Przybył A. K., Chrzanowska M., *Chemia produktów naturalnych – wybór ćwiczeń*, Materiały dydaktyczne Uniwersytetu im. Adama Mickiewicza, Poznań 2016, http://www.staff.amu.edu.pl/~psorg/c_s1c.pdf (online: 28.10.2017).
[169] Gupta R. Ch., Kanwar G., *Determination of Iodine Numbers of Edible Oils*, Biochemical Education, 22(1) (1994), 47.
[170] http://lipidlibrary.aocs.org/History/content.cfm?ItemNumber=39267 (online: 28.10.2017).
[171] Knothe G., *Structure Indices in Fatty Acid Chemistry. How Relevant Is the Iodine Value?*, Journal of the American Oil Chemists' Society, 79 (2002), 847-854.
[172] PN-ISO 3960:2017-03, Oleje i tłuszcze roślinne oraz zwierzęce – Oznaczanie liczby nadtlenkowej – Jodometryczne (wizualne) oznaczanie punktu końcowego.
[173] Cichosz G., Czeczot H., *Stabilność oksydacyjna tłuszczów jadalnych – konsekwencje zdrowotne*, Bromatologia i Chemia Toksykologiczna, XLIV (1) (2011), 50-60.

[174] Sporzyńska K., *O metodach wykrywania psucia się tłuszczów i olejów jadalnych*, Przemysł Chemiczny, 22 (1938), 3-8, http://delibra.bg.polsl.pl/Content/2844/PChem1938.z1.pdf (online: 28.10.2017).
[175] Kamal-Eldin A. (ed.), *Lipid oxidation pathway*, AOCS Pres, Champaign 2003.
[176] Derewiaka D., Obiedziński M. W., *Modelowe badania nad utlenianiem steroli*, Żywność. Nauka. Technologia. Jakość, 5 (54) (2007), 337-345.
[177] Wai W. T., Saad B., Peng Lim B., *Determination of TOTOX value in palm oleins using a FI-potentiometric analyzer*, Food Chemistry, 113 (2009), 285-290.
[178] Steele R. (ed.), *Understanding and measuring the shelf-life of food*, 10th May 2004, Woodhead Publishing Limited and CRC Press LLC, eBook ISBN: 9781855739024.
[179] https://www.oilsfats.org.nz/documents/Oxidation%20101.pdf (online: 28.10.2017).
[180] Akoh C. C., Min D. B., *Food Lipids. Chemistry*, Nutrition, and Biotechnology, 2nd Edition Revised and Expanded, Marcel Dekker Inc., New York–Basel 2002.
[181] PN-EN ISO 6885:2008, Oleje i tłuszcze roślinne oraz zwierzęce – Oznaczanie liczby anizydynowej.
[182] AOCS Official Method Cd 18-90, Anisidine value.
[183] IAFMM, Fish oil bulletin. No 8, June 1981, Recommended method of analysis for determination of anisidine value of fish oil, http://www.iffo.net/system/files/FOB12.PDF (online: 28.10.2017).
[184] Parr L. J., Swoboda P. A. T., *The assay of conjugable oxidation products applied to lipid deterioration in stored foods*, Journal of Food Technology, 11 (1976), 1-12.
[185] Farhoosh R., *The Effect of Operational Parameters of the Rancimat Method on the Determination of the Oxidative Stability Measures and Shelf-Life Prediction of Soybean Oil*, Journal of the American Oil Chemists' Society, 84 (2007), 205-209.
[186] PN-EN ISO 6886:2009, Oleje i tłuszcze roślinne oraz zwierzęce – Oznaczanie stabilności oksydacyjnej (test przyspieszonego utleniania).
[187] PN 75/C-89085, Żywice epoksydowe – Metody badań – Oznaczanie barwy.
[188] http://delibra.bg.polsl.pl/Content/2844/PChem1938.z1.pdf (online: 28.10.2017).
[189] Gray J. I., *Measurement of Lipid Oxidation: a Review*, Journal of American Oil Chemists' Society, 55 (1977), 539-549.
[190] Holm G. E., Greenbank G. R., *Quantitative Aspects of the Kreis Test*, Industrial and Engineering Chemistry, 15 (10) (1923), 1051-1053.

[191] Högberg A. G. S., *Two stereoisomeric macrocyclic resorcinol-acetaldehyde condensation products*, Journal of Organic Chemistry, 45(22) (1980), 4498--4500.
[192] PN-60/A-86924, Tłuszcze roślinne jadalne – Metody badań – Wykrywanie obecności aldehydu epihydrynowego.
[193] Guillén-Sans R., Guzmán-Chozas M., *The thiobarbituric acid (TBA) reaction in foods: a review*, Critical Reviews in Food Science and Nutrition, 38(4) (May 1998), 315-330.
[194] Szwejda-Grzybowska J., *Antykancerogenne składniki warzyw kapustnych i ich znaczenie w profilaktyce chorób nowotworowych*, Bromatologia i Chemia Toksykologiczna, XLIV (4) (2011), 1039-1046.
[195] Knothe G., Van Grepen J., Krahl J., *The biodiesel handbook*, AOCS Press, Champaign 2005.
[196] Meng X., Yang J., Xu X., Zhang L., Nie Q., Xian M., *Review – Biodiesel production from oleaginous microorganisms*, Renewable Energy, 34 (2009), 1-5.
[197] Skrzyńska E., Matyja M., *Porównanie właściwości fizykochemicznych wybranych tłuszczy naturalnych oraz ich estrów metylowych*, Chemik, 65(10) (2011), 923-929.
[198] Kostik V., Memeti S., Bauer B., *Fatty acid compoisition of edible oils and fats*, Journal of Hygienic Engineering and Design, http://www.jhed.mk/filemanager/JHED%20Vol%204/02.%20FPP/06.%20Full%20paper%20-%20Vesna%20Kostik%202.pdf (online: 30.10.2017).
[199] Rueda A., Seiquer I., Olalla M., Giménez R., Lara L., Cabrera-Vique1 C., *Characterization of Fatty Acid Profile of Argan Oil and Other Edible Vegetable Oils by Gas Chromatography and Discriminant Analysis*, Journal of Chemistry, 2014, Article ID 843908, 8, http://dx.doi.org/10.1155/2014/843908 (online: 05.10.2018).
[200] Myczko A., Golimowska R., *Porównanie właściwości estrów metylowych w zależności od pochodzenia surowca*, Journal of Research and Applications in Agricultural Engineering, 56(2) (2011), 111-117.
[201] Sasser M., *Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids*, Technical Note #101, May 1990, www.midi-inc.com (online: 30.10.2017), http://natasha.eng.usf.edu/gilbert/courses/Biotransport%20Phenomena/pdf/bacteria_gc_1.pdf.
[202] Volkman J. K., Jeffrey S. W., Nichols P. D., Rogers G. I., Garland C. D., *Fatty acid and lipid composition of 10 species of microalgae used in mariculture*, Journal of Experimental Marine Biology and Ecology, 128(3) (June 29, 1989), 219-240.

[203] Tyrrell D., *The fatty acid composition of 17 entomophthore isolates*, Canadian Journal of Microbiology, 13(7) (1967), 755-760.
[204] Graham J. H., Hodge N. C., Morton J. B., *Fatty Acid Methyl Ester Profiles for Characterization of Glomalean Fungi and Their Endomycorrhizae*, Applied and Environmental Microbiology, 3 (January 1995), 58-64.
[205] Olsson P. A., Johansen A., *Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages*, Mycological Research, 104(4) (April 2000), 429-434.
[206] Almyasheva N. R., Sharipova D. A., Barkov V., Karakhanov A., Kulikov B., Maksimov L., Vinokurov A., *Lipids of Basidial Fungi as Feedstock for Biodiesel Fuel Production*, Chemistry and Technology of Fuels and Oils, 51(4), (September 2015), 411-421.
[207] PN-EN ISO 12966-1:2015-01, Oleje i tłuszcze roślinne oraz zwierzęce – Chromatografia gazowa estrów metylowych kwasów tłuszczowych – Część 1: Przewodnik do nowoczesnej chromatografii gazowej estrów metylowych kwasów tłuszczowych.
[208] Eder K., *Review – Gas chromatographic analysis of fatty acid methyl esters*, Journal of Chromatography B, 671 (1995), 113-131.
[209] EN 14103, 2003, Fat and oil derivatives – Fatty Acid Methyl Esters (FAME) – Determination of ester and linolenic acid methyl ester contents.
[210] McCurry J. D., Wang Ch., *Analysis of fatty acid methyl ester (FAME) content and distribution in biodiesel blend using heart-cutting 2D gas chromatography*, Materials of Agilent Technologies Inc., 2008.
[211] ISO 12228:1999, Animal and vegetable fats and oils – determination of individual and total sterols contents – gas chromatographic method.
[212] EN 14213:2003, Oleje opałowe – Estry metylowe kwasów tłuszczowych (FAME) – Wymagania i metody badań.
[213] ISO 2879:1975, Glycerine for industrial use – Determination of glycerol content – Titrimetric method.
[214] EN 14106:2003, Fat and oil derivatives – Fatty Acid Methyl Esters (FAME) – Determination of free glycerol content.
[215] Bondioli P., Della Bella L., *An alternative spectrophotometric method for the determination of free glycerol in biodiesel*, European Journal of Lipid Science and Technology, 107 (2005), 153-157.
[216] Pisarello M. L., Dalla Costa B. O., Veizaga N. S., Querini C. A., *Volumetric Method for Free and Total Glycerin Determination in Biodiesel*, Industrial and Engineering Chemistry Research, 49 (2010), 8935-8941.
[217] EN-14105, Fat and oil derivatives – Fatty Acid Methyl Esters (FAME) – Determination of free and total glycerol and mono-, di-, triglyceride content.
[218] ASTM D 6584, The Analysis of Glycerin and Glycerides.

[219] Oostdijk J., *Analysis of free and total glycerol and triglyceride content in B-100 biodiesel methyl esters using Agilent select biodiesel for glycerides*, https://www.agilent.com/cs/library/applications/5990--8994EN.pdf (online: 30.10.2017).
[220] McCurry J. D., Wang Ch.-X., *Analysis of Glycerin and Glycerides in Biodiesel (B100) Using ASTM D6584 and EN14105*, Application note, https://www.agilent.com/cs/library/applications/5989-7269EN.pdf (online: 30.10.2017).
[221] Rotkiewicz D., Tańska M., Ambrosiewicz-Walczak M., *Przewodnik do zajęć laboratoryjnych – Przetwórstwo nasion oleistych*, Katedra Przetwórstwa i Chemii Surowców Roślinnych, Wydział Nauki o Żywności, Uniwersytet Warmińsko-Mazurski w Olsztynie, http://uwm.edu.pl/kpichsr/ftp/pno2016L.pdf (online: 30.10.2017).
[222] PN-88/A-86930, Tłuszcze roślinne jadalne. Metody badań. Oznaczanie zawartości fosforu.
[223] *Determination of Total Phosphorus*, https://avantilipids.com/tech-support/analytical-procedures/determination-of-total-phosphorus/ (online: 30.10.2017).
[224] Kokot F., *Metody badań laboratoryjnych stosowanych w klinice*, PZWL, Warszawa 1969.
[225] PN-82/C-04077, Przetwory naftowe. Oznaczanie pozostałości po spopieleniu i popiołu siarczanowego.
[226] Stanisz M., Sapińska E., Pielech-Przybylska K., *Charakterystyka zanieczyszczeń występujących w spirytusach surowych*, Zeszyty Naukowe Politechniki Łódzkiej, nr 1058, Chemia Spożywcza i Biotechnologia, 73 (2009), 105-121.
[227] Kotarska K., Żakowicz A., Czupryński B., *Ocena zawartości produktów ubocznych weryfikujących jakość spirytusu surowego*, Inżynieria i Aparatura Chemiczna, 50(3) (2011), 41-42.
[228] Lipińska E., Bonin S., Leoniuk R., Hać-Szymańczuk E., *Wpływ składu podłoża fermentacyjnego i zastosowanych szczepów drożdży na przebieg fermentacji alkoholowej oraz jakość spirytusu surowego*, Bromatologia i Chemia Toksykologiczna, XLIV(3) (2011), 964-969.
[229] Czupryński B., Kotarska K., *Zanieczyszczenia chemiczne spirytusów surowych związkami karbonylowymi*, Inżynieria i Aparatura Chemiczna, 48(2) (2009), 31-32.
[230] PN-74/A-79522, Spirytus rektyfikowany.
[231] GC Analysis of Alcoholic Spirit on Equity®-1 Coupled to SUPELCOWAX 10 (Column Set 2), http://www.sigmaaldrich.com/technical-documents/articles/analytical-applications/gc/gc-analysis-of-alcoholic-spirit-g003995.html (online: 30.10.2017).

[232] Rozporządzenie ministra gospodarki z dnia 25 maja 2006 w sprawie liczbowych danych odniesienia dla mieszanin alkoholu etylowego i wody, Dz.U. 106, poz. 716.
[233] http://www.refractometer.pl/refraction-datasheet-ethanol (online: 30.10.2017).
[234] Wyman Ch., *Handbook on Bioethanol: Production and Utilization*, Applied Energy Technology Series, Taylor & Francis, Washington 1996.
[235] PN-65/C-04086, Przetwory naftowe. Oznaczanie zawartości wody metodą Fischera.
[236] http://mcckf.com/english/kf-basic/what.html (online: 30.10.2017).

S u m m a r y

The work is a compendium of laboratory methods enabling determination of key physical and chemical properties of almost all types of biomass and its components of significant technological importance. Particular emphasis was placed on raw materials, fractions and final products crucial for the biofuel industry and referring to binding strict quality standards.

The syntheses procedures presented in the first part show how to carry out in the laboratory high-temperature biomass conversion (pyrolysis and gasification), biodiesel synthesis from various fatty raw materials, fermentation of starch hydrolysates to bioethanol and how to produce the biogas. The second part is a wide collection of analytical procedures necessary for the qualitative and quantitative characterisation of bioresources and biocomponents. For simplicity, this part was divided into three sections showing successively the methods dedicated for characterisation of solid, liquid and gas fractions.

Biocarburants et biomatériaux

Recueil d'analyses de laboratoire sélectionnées pour les Technologies Chimiques Innovantes

R é s u m é

Ce travail est un recueil de méthodes de laboratoire permettant de déterminer les caractéristiques physiques et chimiques de presque tous les types de biomasse et de ses constituants technologiquement importants. L'accent a été mis sur les matières brutes, les fractions et les produits finaux, essentiels pour l'industrie des biocarburants, à l'égard des rigoureuses normes applicables en matière de la qualité.

Exposées dans la première partie, les méthodes de synthèse montrent comment effectuer en laboratoire, entre autres, la conversion de la biomasse à haute température (pyrolyse et gazéification), la synthèse du biodiesel à partir des matières grasses brutes de différentes qualités, la fermentation des hydrolysats d'amidon en bioéthanol et comment produire du biogaz. La seconde partie présente un vaste éventail de méthodes d'analyse, essentielles pour la caractérisation qualitative et quantitative

des biomatériaux et biocomposants. Pour simplifier, cette partie a été divisée en trois sections qui présentent successivement les méthodes de caractérisation des matières brutes et des fractions solides, liquides et gazeuses.

S t r e s z c z e n i e

Praca stanowi kompendium metod laboratoryjnych umożliwiających określenie kluczowych właściwości fizycznych i chemicznych niemal wszystkich rodzajów biomasy i jej składników o istotnym znaczeniu technologicznym. Szczególny nacisk położono na surowce, frakcje i produkty końcowe, kluczowe dla przemysłu biopaliwowego, odnosząc się do obowiązujących surowych norm jakościowych.

Przytoczone w pierwszej części procedury syntezy ukazują jak w skali laboratoryjnej można przeprowadzić m.in. wysokotemperaturową konwersję biomasy (piroliza oraz zgazowanie), syntezę biodiesla z surowców tłuszczowych o różnej jakości, fermentację hydrolizatów skrobiowych do bioetanolu, oraz otrzymać biogaz. Druga część stanowi szeroki zbiór procedur analitycznych niezbędnych do jakościowej i ilościowej charakterystyki biosurowców oraz biokomponentów. Dla ułatwienia została ona podzielona na trzy części, ukazujące kolejno metody charakterystyki dedykowane dla surowców i frakcji stałych, ciekłych oraz gazowych.