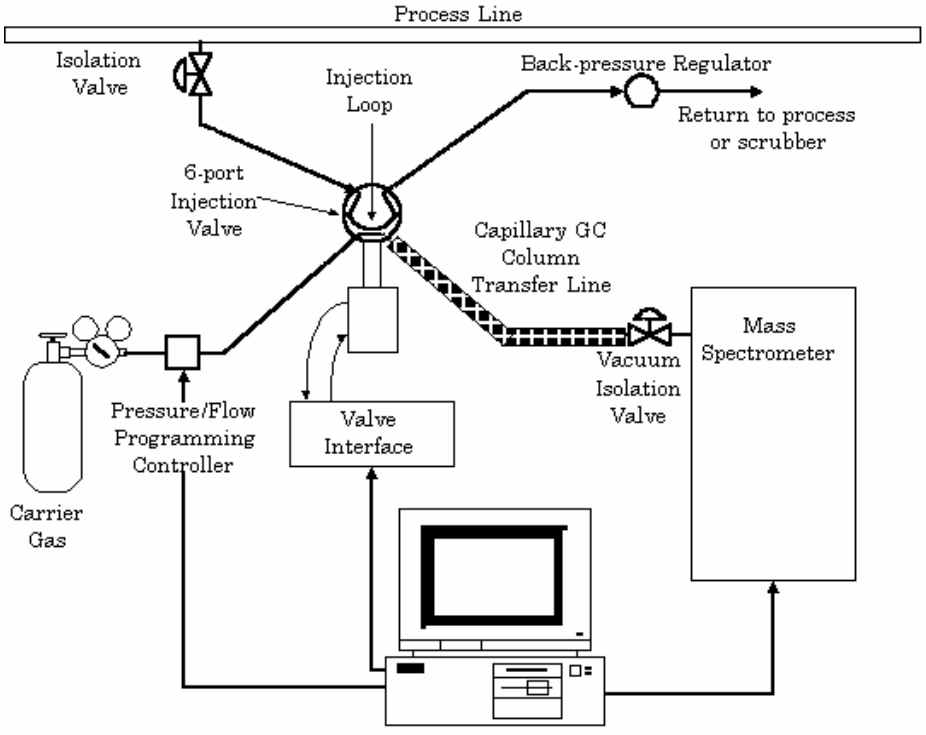
University of Patras, Department of Chemistry

Food Chemistry Laboratory Exercises



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Caution!

Working safely in the Food Chemistry Laboratory is a priority. This text contains the general regulations that we are all obliged to apply from the moment we enter the laboratory area for any exercise.

In addition, in each exercise, the following are highlighted:

- specific points with particular risk
- clear handling instructions for safe execution of the experiments

Registration and training in the course means full acceptance and strict application of the rules.

Failure to follow any rule will result in immediate removal from the laboratory area!

CONTENTS

REGULATIONS

Below are the rules that govern the students' laboratory training.

After a relevant announcement, each student receives from the laboratory teaching staff the notes with the exercises to be carried out during the semester.

The students must come to the laboratory at the exact starting time and not later.

A necessary condition for each exercise is the adequate study of the theoretical part of the exercise. This may be determined by an oral or short written examination by the person in charge of the exercise.

Students must keep the laboratory areas clean.

Students must wear their lab coat in the lab. If they have long hair, it should be tied back. Do not wear open shoes (sandals, flip-flops, etc.). Do not wear contact lenses because they trap toxic substances in the eyes.

Every student must perform all the scheduled exercises, otherwise is excluded from the exams.

The laboratory course is mandatory. Unexcused absence is not allowed. One absence is considered justified when it is certified by a medical certificate or by a judicial authority or by some other government document.

Leaving the laboratory for a few minutes is only allowed with the permission of the laboratory staff.

Before the start of the exercise, the students are obliged to check, whether all the necessary instruments and devices for carrying out the exercise are present in the laboratory. If something is missing, it is reported to the person in charge of the exercise, otherwise it is assumed that the relevant instruments have been received in perfect condition. After the end of the experiments the instruments are cleaned by the trainees and checked by the person in charge.

If a student finishes the experiments earlier than the expected time, may be allowed to leave after the permission of the laboratory staff.

The grade of each exercise (0-10) is the average of the scores of any oral examination, laboratory skills during the performance of the exercise, weekly reports of results, and a possible final written exam for the laboratory exercises. Students are considered to have completed training completely when they perform all the exercises planned by the laboratory staff and have achieved an overall grade of five (5) or above.

Completion of all exercises is a necessary condition to take part in the final course examination. Those who are excused are given the opportunity within only two supplementary laboratory periods to complete the exercises that were not performed. During the above period of time, the trainee is also given the opportunity to repeat an exercise, in which he/she has a grade below five (5). The intern student who has unexcused absences is obliged to complete the remaining laboratory exercises in the following academic years.

HAZARDS IN THE LABORATORY AND RULES

Hazards in the laboratory can be mechanical, fire and explosion hazards, electrical and toxic.

A. Mechanical hazards

They come from the non-proper handling various devices. The person in charge of the exercise will show you how to use them. Beware of broken glassware.

B. Fire and Explosion Hazards

Many chemicals such as ether, alcohol, acetone, acetic acid, etc., are flammable, so their use must be done away from flame.

Other substances become flammable, such as Na_2O_2, Na, K, when they absorb moisture. Therefore, it is forbidden to keep them on paper on the laboratory bench or anywhere else. Mixtures such as acetone vapors and air, acetylene and air, butane and air, are explosive. The replacement of the empty gas containers with new ones (containing butane) will be done by a laboratory staff. Make sure the burner valve is closed when not used. A butane leak creates a risk of explosion and fire from a nearby flame. Gas burners should not be left burning when they are not in use. In the event of a fire, inform the laboratory staff immediately.

Learn how to put out a fire with a fire extinguisher. NO SMOKING in the laboratory.

It is PROHIBITED to perform experiments that are not in the lab notes.

C. Electrical Hazards

Any breakdown of an electrical device must be reported immediately to the person in charge of the exercise. Do not attempt to repair it yourself.

Do not touch electrical appliances and sockets with wet hands. Be careful not to spill liquids or solids on surfaces in contact with electrical cables.

D. Toxic Hazards

Substances that cause poisoning in the human body are called toxic: a) due to absorption of the chemical substance through the skin, b) due to inhalation of gas or vapor and c) due to entry of the chemical substance into the digestive system.

In general, we can say that all chemicals are toxic.

The following rules must be followed:

a) Always use a pipette filler bulb for filling and releasing the liquid.

b) Avoid contact with the skin of any chemical substance (liquid, solid, gas). If this happens, wash with plenty of water and inform the laboratory manager. A visit to the doctor may be necessary. In the case of strong acids and bases, a quick visit to a doctor is imperative.

c) Protect skin wounds or cuts well. Absorption of chemicals is easier through them.

d) When diluting concentrated acids, add the concentrated solution to the water and never vice versa. Special care must be taken in the dilution of H_2SO_4. The concentrated H_2SO_4 must be added to water and never the other way around. The same process occurs when mixing aqueous solutions with conc. H_2SO_4. The conc. H_2SO_4 is added to the aqueous solution. If the process is reversed, drops of conc. H_2SO_4 will be ejected and will cause severe skin burns. When diluting, you must wear safety glasses.

e) Also, when handling conc. NaOH solutions, you must wear protective glasses.

f) Do not inhale vapors produced by a reaction. Experiments with reactions that release toxic gases (SO_2, NO_2, H_2S, HCN, etc.) are carried out in the fume hood. Concentrated solutions of acids and other organic reagents are in fume hoods and should be handled there. When you have a small amount of concentrated acid solution left, pour it into the hood sink with the water tap open. Let the water run abundantly for a few minutes.

g) Again it is repeated that smoking is prohibited in the laboratory. Harmless chemicals, if they come into contact with cigarette smoke, can become very toxic.

h) It is forbidden to take food in the laboratory or keep food in your laboratory position.

i) No reagent should be tested by your tongue.

j) At the end of your experiments, wash your hands thoroughly with soap and water.

INSTRUCTIONS IN CASE OF ACCIDENTS

In the event of an accident, keep calm to deal with it. Notify the laboratory staff immediately.

A. Cutting from glasses

If the wound contains glass fragments, remove it with oxygen peroxide and cover with gauze. If the glass fragments have not been completely removed, a visit to the doctor is necessary.

B. Fire burns

Hold the burned part under running water for several minutes and then apply an ointment. If the burning is extensive see the doctor.

C. Fire

If the fire is small, we can extinguish it with a thick, non-synthetic cloth that we throw over it or with a fire extinguisher. If the fire has spread, the room is evacuated and the fire service is notified (tel. 199). Until it arrives, the use of fire extinguishers, if possible, is deemed necessary to limit the fire.

D. Acids

The affected part is washed with plenty of water. If the injury is serious (face, eyes, skin, etc.) the student must go to the hospital with the Safety Data Sheet (SDS) (available in the lab) of the reagent from which he/she was injured.

E. Caustic alkalis

The affected part is washed with plenty of water. If the injury is serious (face, eyes, skin, etc.) the student must go to the hospital with the Safety Data Sheet (SDS) of the reagent from which he/she was injured.

CLEANING OF LABORATORY, GLASSES, APPARATUSES

It must be understood that the cleanliness of the laboratory, the bench, the glassware, and the devices contributes to a large extent to the success of the analyses.

Regarding the general cleanliness of the laboratory and its maintenance, the students can help to a great extent.

a) Do not pour liquid and solid reagents on the floor, but only in the sinks and let plenty of water run to avoid damage of the drains.

b) Do not throw papers on the floor or in the sinks but only in the waste containers. Clogged sinks help destroy drains faster.

c) Do not spill liquids on the floor (risk of slipping).

d) Solid or liquid reagents spilled into drains or laboratory areas must be removed immediately. Regarding the cleanliness of instruments, e.g. of the weighing scale, students should not leave spilled liquids or solid reagents on them to avoid their destruction by corrosion. After weighing, the scale should be reset to its original state with the "tare" key. Finally, the cleanliness of the dishes is considered very necessary. Glassware is cleaned with a detergent solution and a brush. This is followed by a good rinse with tap water and deionized water.

Exercise 1. Analysis of flour

Introduction

"Wheat flour" or simply "flour" is the product of wheat milling, free of any inorganic or organic substance.

Depending on the percentage of protein content, flours are divided into hard and soft (strong and weak). Hard flour has a high protein content, part of which is gluten, which is responsible for the **baking capacity** of flours (dough gas retention capacity, dough rising, cohesiveness and elasticity, resistance to machine mixing, porosity).

Soft flour contains a lower amount of proteins and in particular gluten, so it is mainly used in recipes where the dough does not need to be elastic, such as in cookies and in confectionery products in general.

As **degree of milling**, we define the percentage of flour obtained from a given unit of whole wheat kernels (flour yield or flour extraction rate). Depending on the degree of milling, we also define the **type** of flour. The lower the degree of milling, the less bran the flour contains:

55% type flour is used for high quality bread rolls, toast bread, rusks, and other products with high protein content.

70% type flour, which has a small amount of bran, is used for common white bread.

85 and 90% type flour is rich in bran and nutrients making it ideal for wholemeal bread.

Amounts of gluten in flour of various types

55% type flour: high protein content, bran-free

70% type flour: minimum 26% gluten

85% type flour: at least 25% gluten

90% type flour: at least 25% gluten

100% type flour: at least 24% gluten

a. Determination of ash in flour

GUIDELINES FOR THE SAFE CONDUCT OF THE EXERCISE -Determination of ash -Keep flammable materials and solvents away from the lit gas. -Handle the hot crucible with wooden tongs. -Do not inhale the smoke from burning flour.

Method

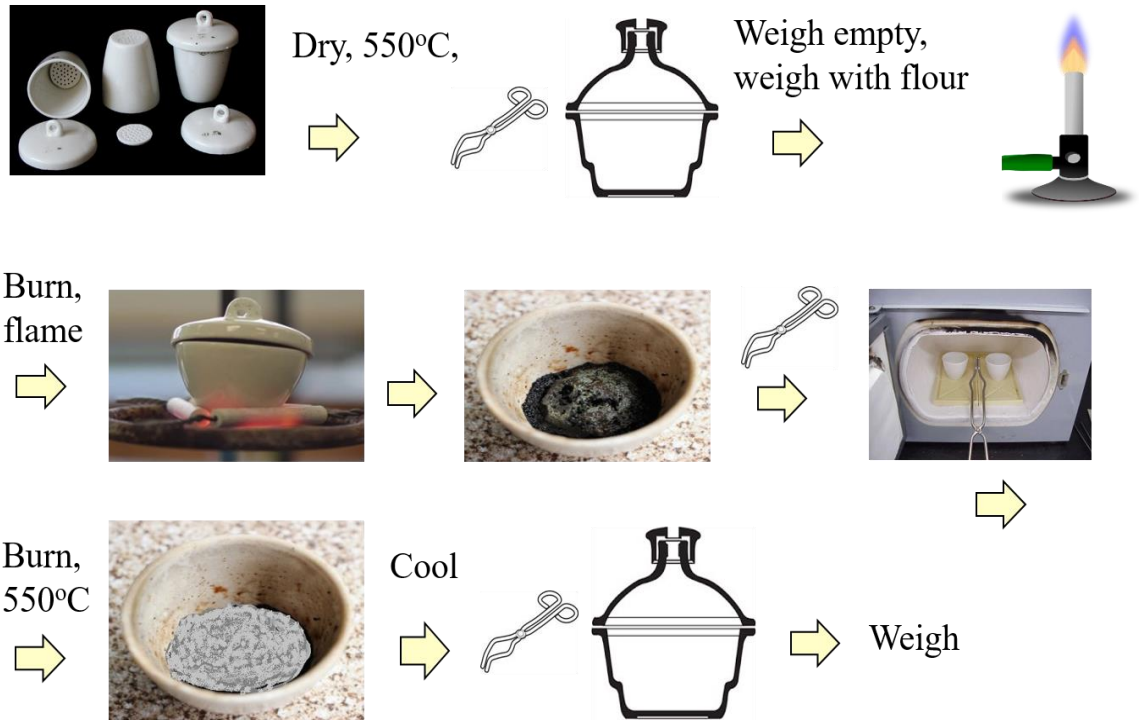
1-2 g of flour sample is weighed into a pre-weighed crucible, which has previously been heated at a temperature of 550 ^{o}C (dark red) and cooled in a desiccator.

This is followed by burning the flour in a burner flame with careful handling of the crucible, with the help of the supervisor (charring).

This is followed by heating in an oven (temperature no higher than 550^{o}C) until carbon-free ash remains.

After cooling in a desiccator, the crucible is weighed. The amount of ash is found from the difference in weight and in **% wt** (g/100 g of flour).

The ash range of different types of flour is **0.0-2.0%** (commonly 0.4-0.5%).



b. Determination of wet and dry gluten

Method

33.33 g of flour are weighed and mixed with 17 mL of cold distilled water in a porcelain evaporating dish, 10-11 cm in diameter, and with the help of a spatula, a soft mass that does not stick to the fingers is formed.

The mass is pressed slightly under the continuous flow of water 15-16^{o}C that is is continued until no starch is detected in the wash water. Detection is done using an iodine solution.

Finally, the gluten is pressed with the fingers to remove the excess water and weighed. The amount weighed if multiplied by 3 provides the % quantity of the wet gluten in flour.

It is then subjected to drying in an oven (100-105^{o}C) until constant weight to find the % amount of dry gluten.
From the difference between the weight of wet and dry gluten, the hydration (water holding capacity) of gluten (as % wt) can be calculated.

Gluten formation in dough (done spontaneously after mixing flour with water and enhanced by oxidative additives):

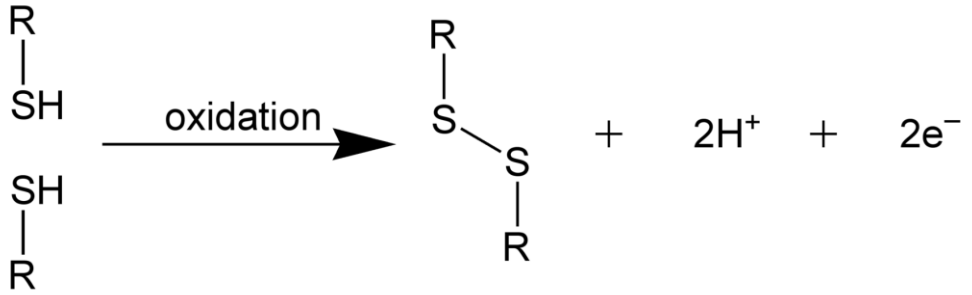
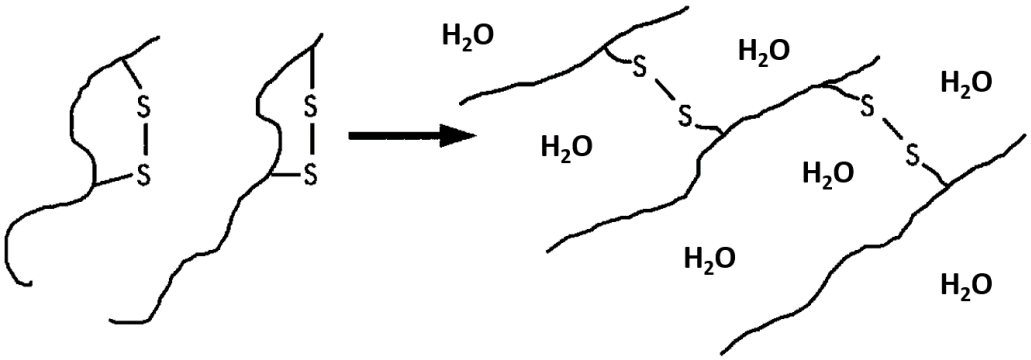


Figure Development of gluten network



c. Detection of oxidant additives

Method

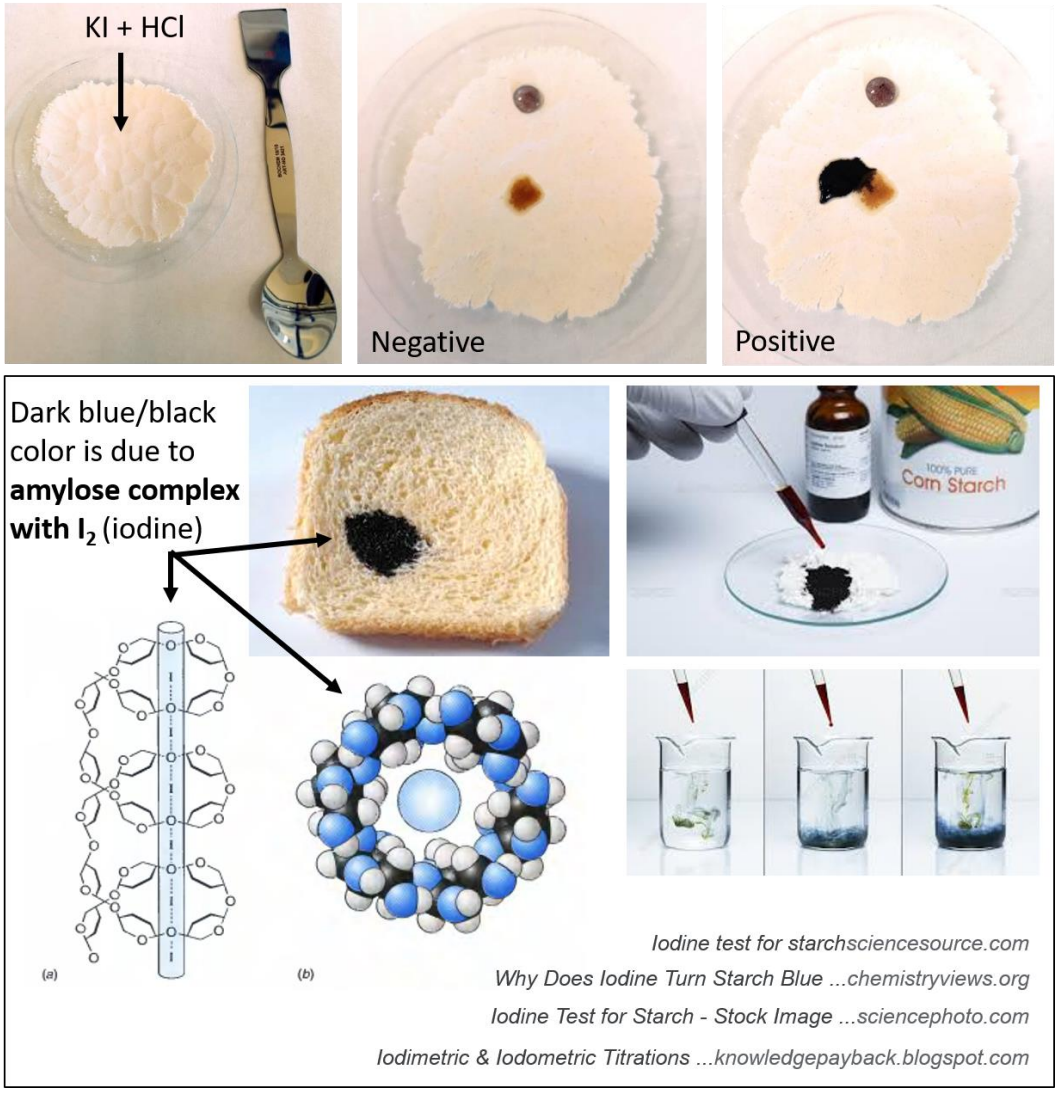
A quantity of flour is pressed with a spatula onto a flat surface (e.g. a Lab Watch Glass) and a few drops of a 10% potassium iodide (KI) and 20% hydrochloric acid (HCl) solution are added on each drop of KI. The appearance of dark black spots proves the presence of oxidizing agents in flour, which may be illegal depending on local legislation.

Reactions:

$BrO_3^- + 6\ I^- + 6\ H^+ \rightarrow 3\ I_2 + Br^- + 3\ H_2O$

(or $KBrO_3 + 6\ KI + 6\ HCl \rightarrow 3\ I_2 + KBr + 6\ KCl + 3\ H_2O$)

I_2 + starch → blue/black spots



Exercise 2. Measuring acidity in food

Definitions of degree of acidity

1. Reichert-Meissl: mL of 0.1 N alkali to neutralize water soluble fatty acids distilled under special conditions from 5 g of oil (**mainly C4-6**).

2. Polenske: mL of N/10 alkali to neutralize the water insoluble fatty acids distilled under special conditions from 5 g of oil (**mainly C8-14**).

3. Kirshner: mL of N/10 alkali to neutralize the water soluble volatile fatty acids which form soluble salts with Ag and distilled under special conditions from 5 g of oil (**butyric**).

4. Köttstorfer: mL N/1 alkali to neutralize **free fatty acids** contained in **100 g** of oil.

5. Burstyn: mL of N/10 alkali to neutralize **free fatty acids** contained in **100 mL** of fat.

a. Determination of degree of acidity in olive oil

INSTRUCTIONS FOR THE SAFE CONDUCT OF THE EXERCISE -Ether and alcohol away from flame. -Wear protective glasses throughout the experimental procedure. -Fill the burette with N/10 NaOH or KOH with the help of a small funnel and after you have lowered it so that the top of the burette is below the level of your eyes.

In food technology:

- fats = solid lipids
- oils = liquid lipids

They all dissolve in organic solvents such as ether, petroleum ether, chloroform, benzene

are insoluble in water.

The acidity of an oil depends on the way of receiving & preserving the oil.

The degree of acidity helps estimate both **quality** and detect **adulteration**.

Acidity determination in oil measures Free Fatty Acids (FFA), which:

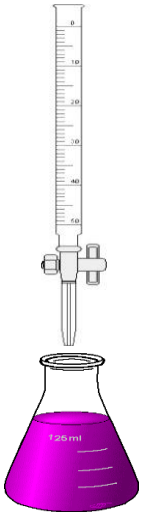
- Are naturally present in all fatty substances
- Their percentage is a quality criterion

Fatty substances with low molecular weight acids (e.g. palm kernel oil, coconut oil, etc.) create problems, e.g. in frying, due to easy hydrolysis to FFA with an unpleasant taste

Usually the result of determining the acidity in vegetable oils is expressed in **oleic acid by %**(the acidity in oleic acid is calculated by multiplying the degree of acidity according to Köttstorfer by the factor 0.2825).

Method

For the determination of degree of acidity of olive oil according to Köttstorfer the following reagent and procedure are used:



1. **Mixture of ether & alcohol:** organic solvent suitable for dissolving both the fat and the aqueous NaOH solution.
2. **NaOH N/10:** standard titration solution.
3. **Phenolphthalein:** indicator (1% alcoholic solution).

Dissolve 8-10 g of fat (accurately weighed) in a mixture (40 ml) of equal parts of ether and alcohol that has been previously neutralized.

Add 3-4 drops of phenolphthalein (1% alcoholic solution) and titrate with N/10 NaOH or KOH solution.

b. Determination of acidity in orange juice

Foods usually contain a mixture of organic acids, with one of them being predominant, which is why acidity is expressed in terms of the predominant acid (tartaric acid in wine, lactic acid in milk, acetic acid in vinegar, citric acid in tomato juice and citrus juices).

Acidity in juices is usually expressed in % w/v (g/100 mL)

In **orange juice,** from the contained acids, 10% is **malic acid**, while the rest (about 90%) is mainly **citric acid** (0.5-1.3% citric acid content). An increase in acidity is observed when fermentation of sugars occurs or when the juice comes from unripe oranges.

The determination of the acidity of orange juice is done by titration with 0.1 M NaOH and phenolphthalein indicator.

Method

Pipette 10 mL of orange juice, add 40-50 mL of water and 3-4 drops of phenolphthalein indicator into a 250 mL conical flask. The solution is titrated with N/10 NaOH until a pink color appears.

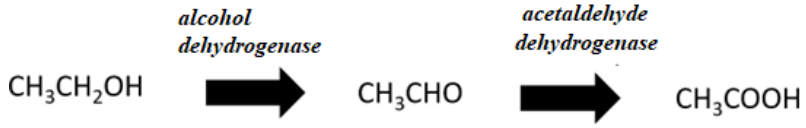
Acidity (%) =

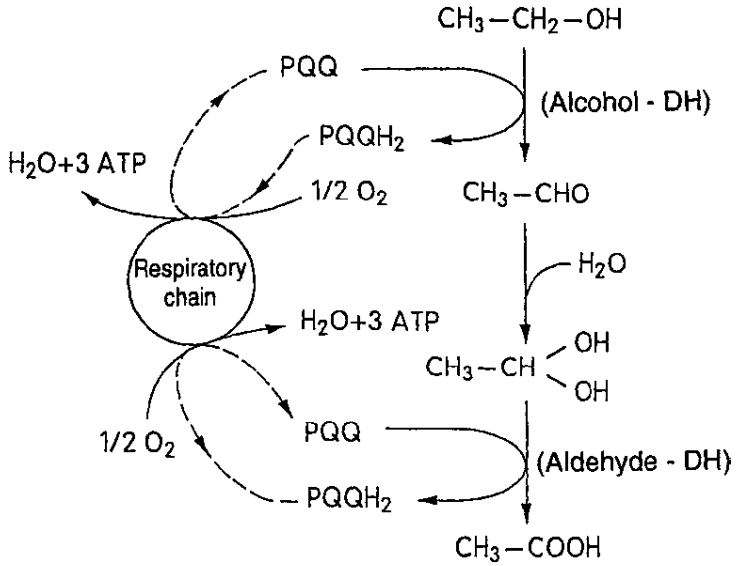
[mL of 0.1 M NaOH consumed] × [NaOH concentration] × 0.064 ×

100/ [sample volume (mL of orange juice)]

c. Determination of acidity of vinegar

Acetic acid bacteria oxidize the alcohol in wine into **acetic acid**, and its further oxidation turns the wine into vinegar. Ethanol is oxidized by two enzymes called alcohol oxidases. One is an *alcohol dehydrogenase* and the other an *aldehyde dehydrogenase* that act in conjunction with NAD^+. Initially ethanol is oxidized to acetaldehyde (CH_3CHO) and then the acetaldehyde is oxidized to acetic acid (CH_3COOH).





Oxidation of ethanol to acetic acid by *Acetobacter* species
(Belitz et al., 2008, p. 984).

The growth of acetic acid bacteria is significantly affected by the presence of oxygen, pH, alcohol content, unfermented sugar, etc.

The acidity of the vinegar is determined by titration with 0.1 M NaOH and phenolphthalein as indicator.

Method

Pipette 1 mL of vinegar, and add 40-50 mL of water and 3-4 drops of phenolphthalein indicator into a 250 mL conical flask. The solution is titrated with N/10 NaOH until a pink color appears.

Acidity (%) =

[mL of 0.1 M NaOH consumed] × [NaOH concentration] × 0.060 ×

100 / [sample volume (mL vinegar)]

d. Determination of acidity in milk

By the general term milk we mean cow's milk, while for milk from another animal origin the corresponding designation is added, e.g. sheep's milk, goat's milk, goat's milk, etc.

Milk, immediately after milking, has low acidity (< 0,002% in lactic acid). Acidity is due to proteins (casein, etc.), phosphates and citrates and carbon dioxide.

Increase in acidity after milking is due to hydrolysis of lactose by lactic acid bacteria and further fermentation to lactic acid. Therefore, the normal acidity of cow's milk is **0.14-0.16% in lactic acid**. A further increase in acidity (0.4%) leads to souring of milk, while a further increase (0.6%) leads to coagulation of milk at normal temperature.

The acidity of goat's milk varies between **0.14-0.23 %** and of sheep **0.22-0.25 %** in lactic acid. The pH of cow's milk is **6.60-6.75** at 25^{o}C.

Method

Pipette 10 mL of milk, and add 40-50 mL of water and 3-4 drops of phenolphthalein indicator into a 250 mL conical flask. The solution is titrated with 0.1 M NaOH until a pink color appears.

$$\textbf{Acidity (\%)} = \frac{\text{consumed mL of 0.1 M NaOH} \times \text{NaOH concentration} \times 0.090 \times 100}{\text{volume of sample (mL milk)}}$$

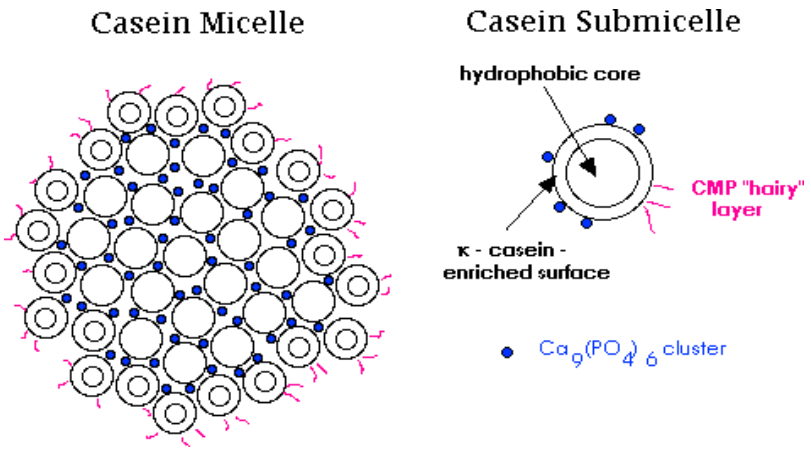
Exercise 3. Precipitation of milk casein

INSTRUCTIONS FOR THE SAFE CONDUCT OF THE EXERCISE
- Wear protective glasses throughout the experimental procedure.
- Fill the burettes with HCl and NaOH with the help of a small funnel after first lowering them so that the top of each burette is below eye level

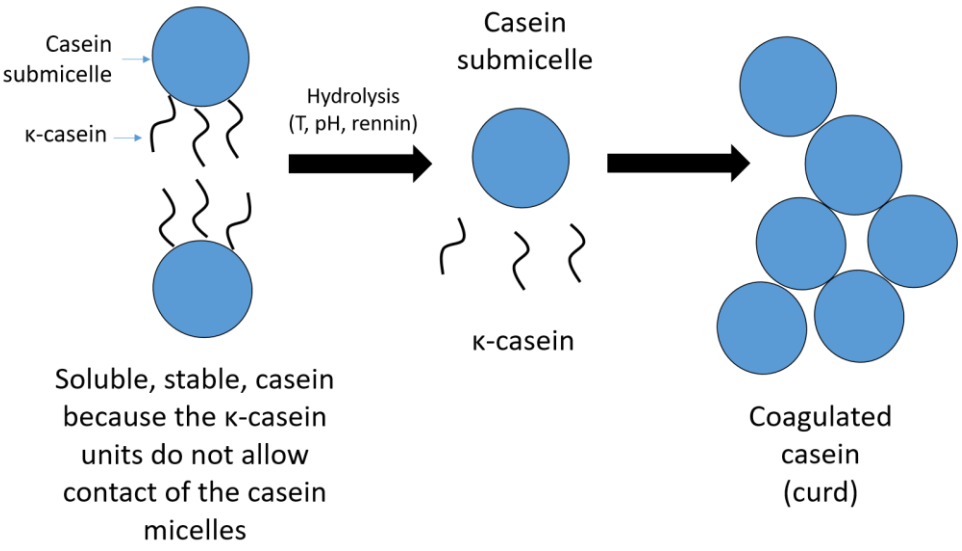
Introduction

Milk proteins are divided into two categories: **casein** and **whey proteins** (lactoglobulin and lactalbumins). The protein content of cow's milk is 3-4%.

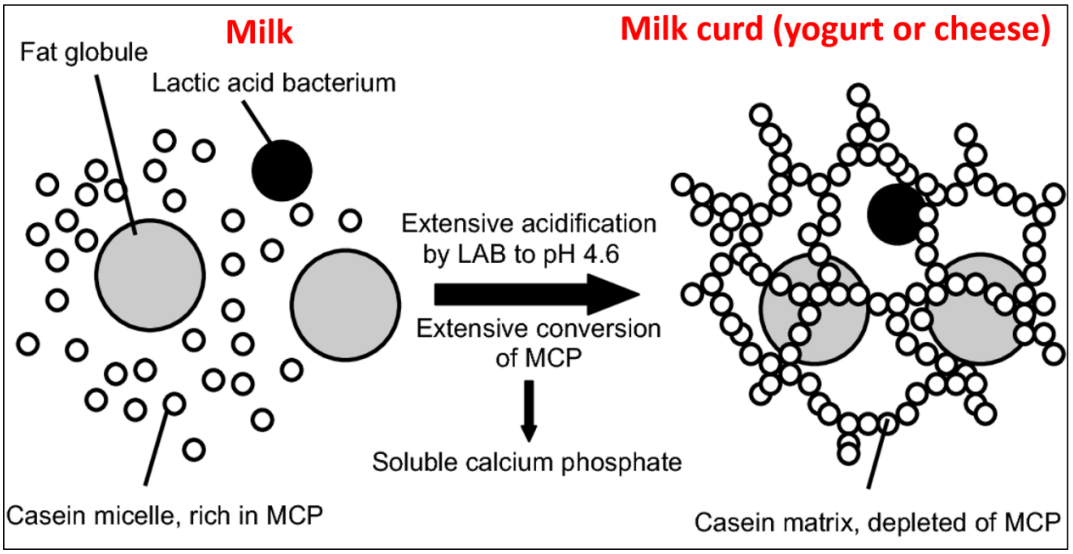
Precipitation of casein (**cheese making**) can be achieved by adding acid, in which case a demineralized casein gel is obtained, or by adding enzyme rennin (rennet), in which case calcium paracasein is obtained (denaturation and coagulation).



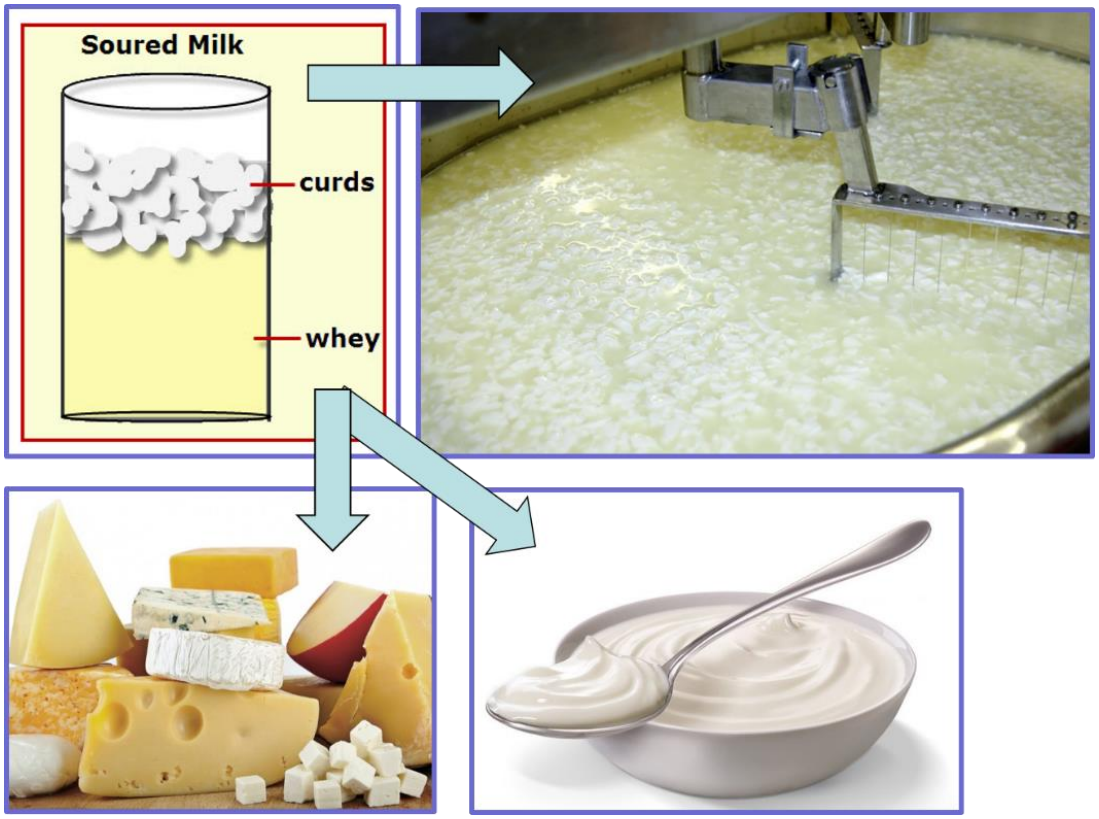
Casein micelle structure
(http://www.foodsci.uoguelph.ca/deicon/casein.gif)



Casein coagulation by enzyme (in cheese making)



Casein coagulation by acid (in yogurt and in cheese making).



Method

In a 400 mL beaker, add 100 mL of milk and 100 mL of water and mix with a glass rod. Set the temperature to 20°C and measure the pH with a pH meter. Add 0.1 M HCl dropwise using a burette to a final pH of 4.6 while stirring with a glass rod. Leave solution at rest. Observe the precipitation of casein.

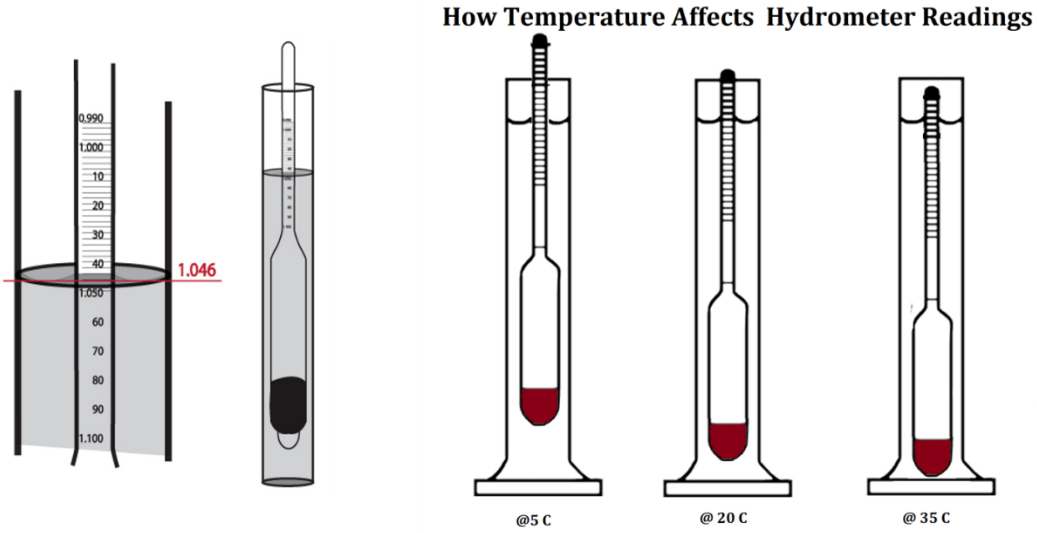
Carefully decant 50 mL of coagulated casein and liquid into a 250 mL beaker and add 0.1 M NaOH dropwise from a burette, stirring with a glass rod while measuring the pH. Observe the behavior of the coagulated casein. Separate the curd from the whey (serum) with a cloth and leave it hanging to drain.

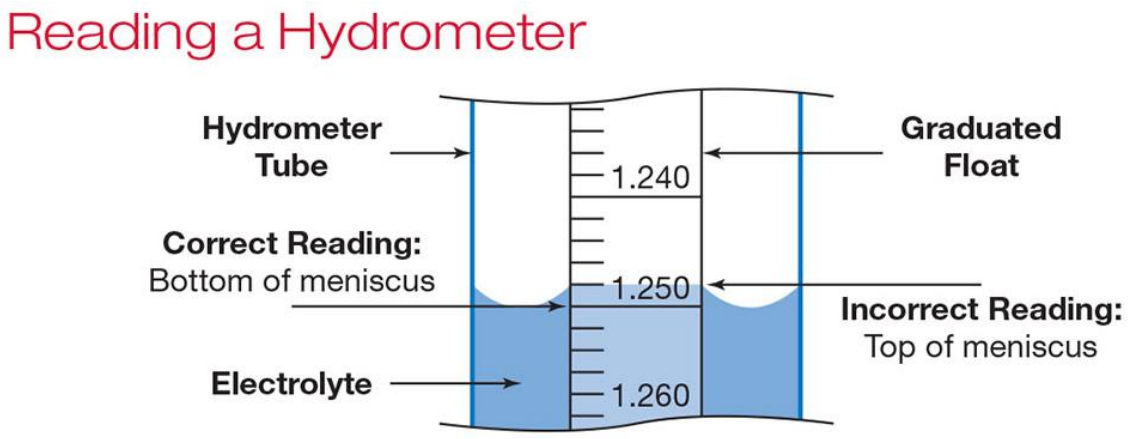
Exercise 4. Determination of sugars in grape must, wort or molasses, and alcohol in wine and spirits

INSTRUCTIONS FOR THE SAFE CONDUCT OF THE EXERCISE
- Wear protective glasses throughout the experimental procedure.
- Make sure water is flowing into the cooler from the beginning of the experiment, and throughout it.

a. Determination of sugars in wort or molasses with a Baumė hydrometer

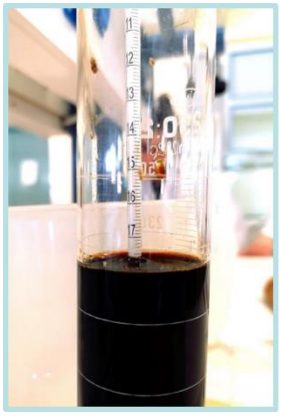
Baumé hydrometers are used to measure the density of liquids that are lighter or heavier than water. For liquids heavier than water, hydrometers are set to read zero (0) in pure water and at 15 ºC, and sixty-six (66) in concentrated sulfuric acid (94-96% with a specific gravity of 1.84 g/cm^3). The interval between 0 and 66 degrees Baumé (ºBe) is divided into 66 equal parts. The degree ºBe is therefore the 1/66 of this interval.

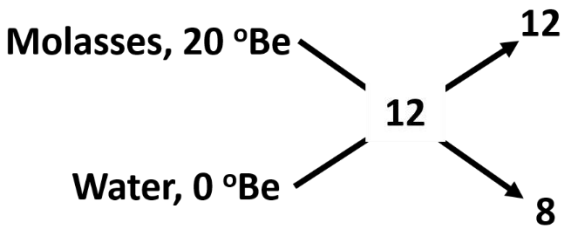




Method

Using concentrated molasses, you can make calculations to obtain various volumes of diluted molasses with desired Baumé densities. An example of fast dilution calculation is shown below:

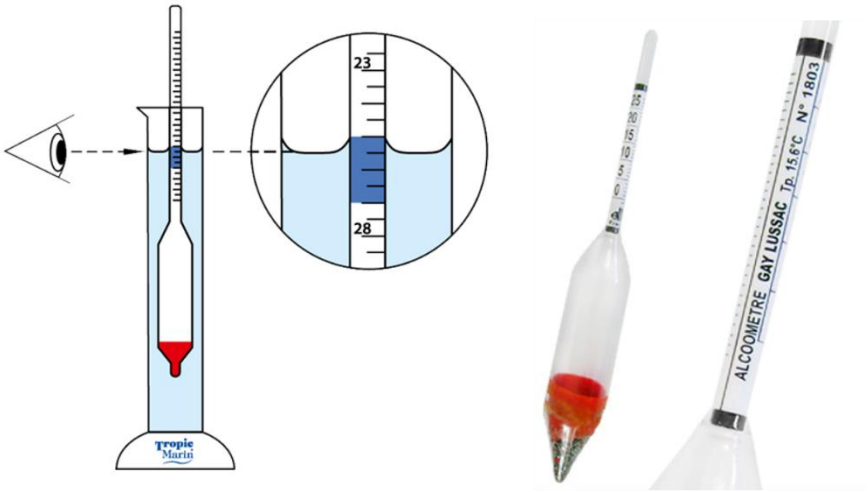




To dilute concentrated molasses of original density 20 °Be, with water (0 °Be), to a final density of 12 °Be, we need to mix 8 parts of water and 12 parts of molasses. Therefore, if you need to produce 200 L of dilute molasses (12 °Be), you need to mix 120 L of concentrated molasses (20 °Be) with 80 L of water. The diluted molasses is transferred to a volumetric cylinder, the Baumé hydrometer is carefully inserted, and the reading at the bottom of the meniscus is noted.

b. Determination of alcohol in wine by distillation

Determination of alcohol by distillation gives safe results and should be performed for accurate determinations. It is based on distilling the entire amount of alcohol contained in a certain volume of fermented must or wine and then making up the distillate with distilled water to the original volume of the sample that was distilled Special hydrometers (**Gay Lussac alcoholmeters**) are usually used for this purpose, which directly show the alcohol content by volume %.

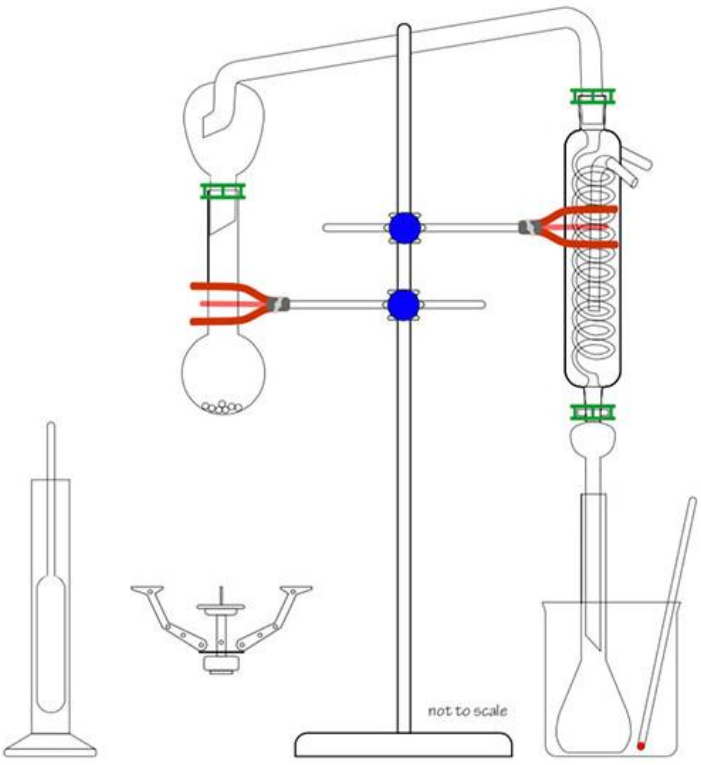


The volatile acids, which are contained in the wine or the fermented must (mainly acetic acid), when co-distilled, cause an increase in the specific gravity, and therefore show a lower amount of alcohol. If the amount of these is small, the error is negligible, but if it is increased (by more than 1.2 g/L in acetic acid), the liquid

must be neutralized with alkali before distillation, the amount of which is calculated on the basis of the acidity of the wine. However, if the distillation residue will be used for the determination of the solid residue of the wine, then the acids are not neutralized. In this case we measure the acidity of the distillate and make the necessary correction on the measured specific gravity of the distillate to properly determine the alcohol.

Method

To determine the alcoholic strength, 200 mL of must or wine, measured in a volumetric flask at the proper temperature (indicated on the flask), and are transferred to a spherical distillation flask. The volumetric flask is washed three times with a few mL of water each time, and the washings are also transferred to the distillation flask. Some wines may foam a lot during distillation causing overflow of liquid to the distillate receiver. Therefore, it is good to add a small amount of tannin to the distillation flask, which binds the proteins that are responsible for the foaming, or a little pumice stone. However, if the distillation residue is used to determine the solid residue of the wine by the indirect method, then antifoaming agents should not be added. Foaming can be avoided if mild heating is applied.



Alcohol distillation apparatus.

The condenser used must be well cooled and preferably vertical. The same volumetric flask with which the liquid to be distilled was measured is used as the receiver. Distillation continues until all the alcohol is transferred to the distillate, which is considered to happen when at least 2/3 of the original liquid is distilled. Then the distillation stops, the receiver volumetric flask is filled up to the mark with distilled water, shaken well, and the alcoholic strength is determined with the alcoholmeter. The temperature is also measured and the correct alcohol content is obtained with the help of a proper alcohol correction table:

Table 1. Determination of the correct alcoholic title, based on the measurement by a Gay-Lussac alcoholmeter calibrated at 15°C.

		Alcoholmeter																								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
Temperature	**0**	1,3	2,4	3,4	4,4	5,4	6,5	7,5	8,6	9,7	10,9	12,2	13,4	14,7	16,1	17,5	19,0	20,4	21,7	23,0	24,3	25,7	27,1	28,5	29,9	31,1
	1	1,3	2,4	3,4	4,4	5,4	6,5	7,5	8,6	9,7	10,9	12,2	13,4	14,7	16,0	17,3	18,7	20,1	21,4	22,7	24,0	25,4	26,8	28,1	29,4	30,6
	2	1,3	2,4	3,4	4,4	5,4	6,5	7,5	8,6	9,7	10,9	12,2	13,4	14,7	16,0	17,2	18,6	19,9	21,2	22,4	23,7	25,0	26,4	27,6	28,9	30,2
	3	1,3	2,4	3,4	4,4	5,4	6,5	7,5	8,6	9,7	10,9	12,2	13,3	14,6	15,9	17,0	18,3	19,7	20,9	22,1	23,4	24,7	26,0	27,3	28,6	29,8
	4	1,3	2,4	3,4	4,4	5,4	6,6	7,5	8,6	9,7	10,9	12,2	13,3	14,5	15,8	16,9	18,1	19,4	20,7	21,9	23,1	24,4	25,7	26,9	28,1	29,3
	5	1,4	2,5	3,5	4,5	5,5	6,6	7,7	8,7	9,8	10,9	12,1	13,2	14,4	15,7	16,8	18,0	19,2	20,5	21,6	22,8	24,1	25,3	26,5	27,7	28,9
	6	1,4	2,5	3,5	4,5	5,5	6,6	7,7	8,7	9,8	10,9	12,1	13,1	14,3	15,6	16,7	17,8	19,0	20,3	21,4	22,5	23,7	25,0	26,1	27,3	28,5
	7	1,4	2,5	3,5	4,5	5,5	6,6	7,7	8,7	9,8	10,9	12,1	13,0	14,2	15,4	16,6	17,7	18,8	20,0	21,0	22,1	23,4	24,7	25,8	27,0	28,1
	8	1,4	2,5	3,5	4,5	5,5	6,6	7,7	8,7	9,8	10,9	12,1	13,0	14,1	15,3	16,4	17,5	18,6	19,7	20,7	21,8	23,0	24,2	25,4	26,6	27,7
	9	1,4	2,5	3,5	4,5	5,5	6,6	7,7	8,7	9,8	10,9	12,1	12,9	14,0	15,1	16,2	17,3	18,4	19,5	20,5	21,6	22,7	23,9	25,0	26,2	27,3
	10	1,4	2,4	3,4	4,5	5,5	6,5	7,5	8,5	9,5	10,6	11,7	12,7	13,8	14,9	16,0	17,0	18,1	19,2	20,2	21,3	22,4	23,5	24,6	25,8	26,9
	11	1,3	2,4	3,4	4,4	5,4	6,4	7,4	8,4	9,4	10,5	11,6	12,6	13,6	14,7	15,8	16,8	17,9	19,0	20,0	21,0	22,1	23,2	24,3	25,4	26,5
	12	1,2	2,3	3,3	4,3	5,3	6,3	7,3	8,3	9,3	10,4	11,5	12,5	13,5	14,6	15,6	16,6	17,6	18,7	19,7	20,7	21,8	22,9	24,0	25,1	26,1
	13	1,2	2,2	3,2	4,2	5,2	6,2	7,2	8,2	9,2	10,3	11,4	12,4	13,4	14,4	15,4	16,4	17,4	18,5	19,5	20,5	21,5	22,6	23,7	24,7	25,7
	14	1,1	2,1	3,1	4,1	5,1	6,1	7,1	8,1	9,1	10,2	11,2	12,3	13,2	14,2	15,2	16,2	17,2	18,2	19,2	20,2	21,2	22,3	23,3	24,3	25,3
	15	1,0	2,0	3,0	4,0	5,0	6,0	7,0	8,0	9,0	10,0	11,0	12,0	13,0	14,0	15,0	16,0	17,0	18,0	9,0	20,0	21,0	22,0	23,0	24,0	25,0
	16	0,9	1,9	2,9	3,9	4,9	5,9	6,9	7,9	8,9	9,9	10,9	11,9	12,9	13,9	14,9	15,9	16,9	17,8	18,7	19,7	20,7	21,7	22,7	23,7	24,7
	17	0,8	1,8	2,8	3,8	4,8	5,8	6,8	7,8	8,8	9,8	10,8	11,7	12,7	13,7	14,7	15,6	16,6	17,5	18,4	19,4	20,4	21,4	22,4	23,4	24,4
	18	0,7	1,7	2,7	3,7	4,7	5,7	6,7	7,7	8,7	9,7	10,7	11,6	12,5	13,5	14,5	15,4	16,3	17,3	18,2	19,1	20,1	21,1	22,0	23,0	24,0
	19	0,6	1,6	2,6	3,6	4,5	5,5	6,5	7,5	8,5	9,5	10,5	11,4	12,4	13,3	14,3	15,2	16,1	17,0	17,9	18,8	19,8	20,8	21,7	22,7	23,6
	20	0,5	1,5	2,4	3,4	4,5	5,4	6,4	7,3	8,3	9,3	10,3	11,2	12,2	13,1	14,0	14,9	15,8	16,7	17,6	18,5	19,5	20,5	21,4	22,4	23,3
	21	0,4	1,4	2,3	3,3	4,3	5,3	6,2	7,1	8,1	9,1	10,1	11,0	11,9	12,8	13,7	14,6	15,5	16,4	17,3	18,2	19,1	20,1	21,1	22,1	22,9
	22	0,3	1,3	2,2	3,2	4,1	5,1	6,1	7,0	7,9	8,8	9,9	10,8	11,7	12,6	13,5	14,4	15,3	16,2	17,0	17,9	18,8	19,8	20,7	21,6	22,3
	23	0,1	1,1	2,1	3,1	4,0	4,9	5,9	6,8	7,8	8,7	9,7	10,6	11,5	12,4	13,3	14,1	15,0	15,9	16,7	17,8	18,5	19,4	20,3	21,3	22,2
	24	0,0	1,0	1,9	2,9	3,8	4,8	5,8	6,7	7,6	8,5	9,5	10,4	11,3	12,2	13,1	13,9	14,8	15,7	16,5	17,4	18,2	19,1	20,0	21,0	21,8

Exercise 5. Alcoholic fermentation of molasses

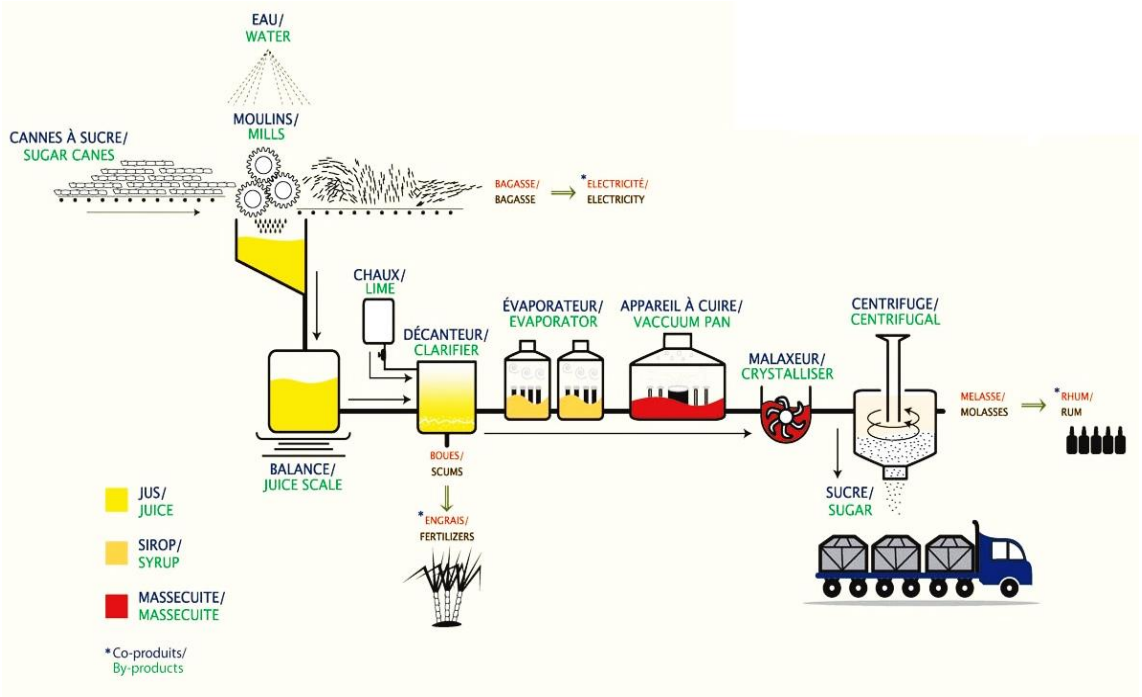
INSTRUCTIONS FOR THE SAFE CONDUCT OF THE EXERCISE
- Wear protective glasses throughout the experimental procedure.
- Attention, the use of concentrated sulfuric acid is only done inside the drain.

Glycolysis and alcoholic fermentation

Molasses is the thick dark-colored syrup remaining as a by-product after a series of crystallization cycles in industrial sugar production. Its composition depends on the raw material (sugar beet or sugarcane), the quality of the raw material as well as the production process followed. In general, however, molasses contains:

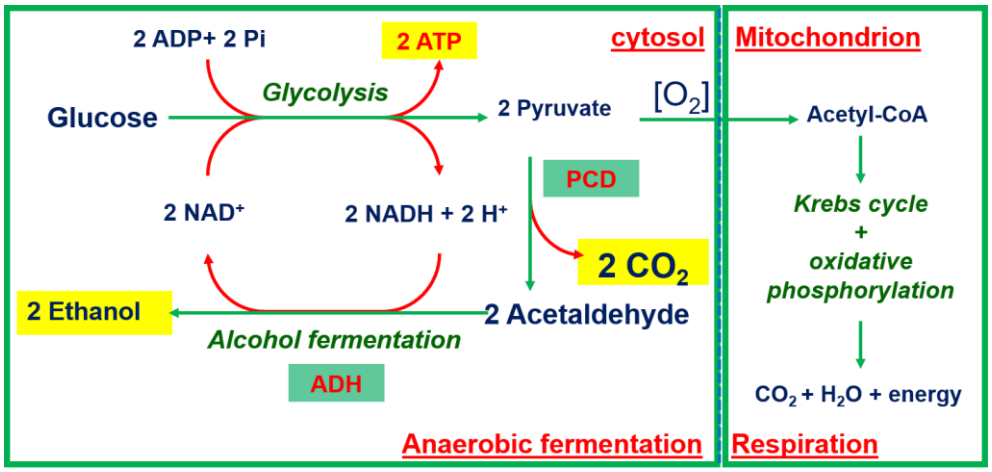
- 80% dry ingredients (**50% sugar**, 30% other dry ingredients)
- 20% water

Specifically, it contains carbohydrates (mainly **sucrose**, glucose, fructose and raffinose), amino acids, betaine, organic acids (citric, malic, acetic, lactic, etc.), inorganic cations (potassium, sodium) and anions (chlorides, nitrates, sulfates, phosphates).



During **alcoholic fermentation**, the sugars (hexoses) $C_6H_{12}O_6$, are converted into ethyl alcohol and carbon dioxide. This conversion takes place with a complex mechanism whose stages are catalyzed by a series of enzymes that are collectively called *zymases*. Hexoses through glycolysis are converted to pyruvate and the two stages of alcoholic fermentation follow, which involve the conversion of pyruvate to acetaldehyde by ***pyruvate decarboxylase (PDC)***, and carbon dioxide and then to ethyl alcohol by ***alcohol dehydrogenase (ADH)***. Overall reaction:

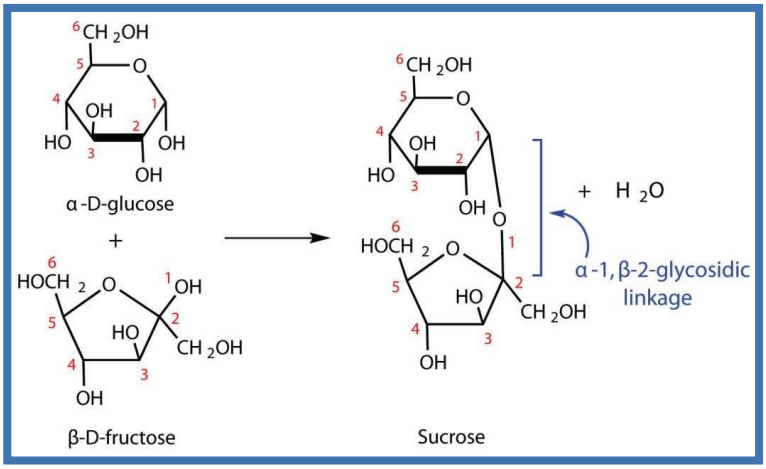
$$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2 + 2\ ATP$$



Synopsis of the alcoholic fermentation in yeast

During alcoholic fermentation, several volatile **by-products** (**congeners**) are produced (higher alcohols, glycerol, acetaldehyde, esters, organic acids, etc.).

In the sugary solutions to be fermented, the pH is adjusted by adding and stirring concentrated H_2SO_4, to **3.2** or **4.7** in the case of hexoses (e.g. raisin extracts which contain glucose and fructose) or sucrose (e.g. molasses), respectively. pH 4.7 is the optimum pH for the enzyme ***invertase,*** which hydrolyzes sucrose into hexoses (equimolar mixture of glucose and fructose or ***invertsugar***).



Method

200 mL of molasses is diluted with water so that the final density is 10 °Be. 500 mL of the diluted molasses are then transferred to 2 conical flasks. Adjust the pH to 4.7 by adding concentrated sulfuric acid. An amount of 0.3 g/L $NH_4H_2PO_4$ is added and *Saccharomyces cerevisiae* yeast is added (20 g/L). The yeast is previously dispersed in 3-5 mL of diluted molasses by vortexing. A few drops of oleic acid are added as an antifoam. The flasks are placed in an incubator set at 25 °C. Fermentation is monitored by recording the density (°Be), and the temperature should not exceed 28 °C. At the end, the fermented liquid will have a residual density of 2.5 °Be due to raffinose, a trisaccharide that cannot be fermented.

Perform the experiments with both sterilized and non-sterilized molasses. Compare their kinetics. Determine the alcohol content by distillation.

Exercise 6. Milk pasteurization control

INSTRUCTIONS FOR THE SAFE CONDUCT OF THE EXERCISE
1) Wear protective glasses throughout the experimental procedure.

Microbiology and pasteurization of milk

Enzymes in milk are either endogenously derived from secretions of milk microorganisms, or enter the milk before or after milking and are of bacterial origin. They are found either free as proteins or bound in complexes, as in lipoproteins. They are divided into hydrolytic (phosphatases, lipases, proteases, etc.) and redox (reductases, catalases, peroxidases, etc.). They participate in the production processes of dairy products, such as lipase which causes the hydrolysis of milk fat to glycerol, free acids, mono- and diglycerides as well as protease which causes the hydrolysis of proteins.

The purpose of **pasteurization** is to free the milk from microorganisms likely to cause a problem for humans, and to significantly reduce the total microbial load in order to preserve the quality of the milk. Pathogens such as the *Mycobacterium tuberculosis* are destroyed by pasteurization.

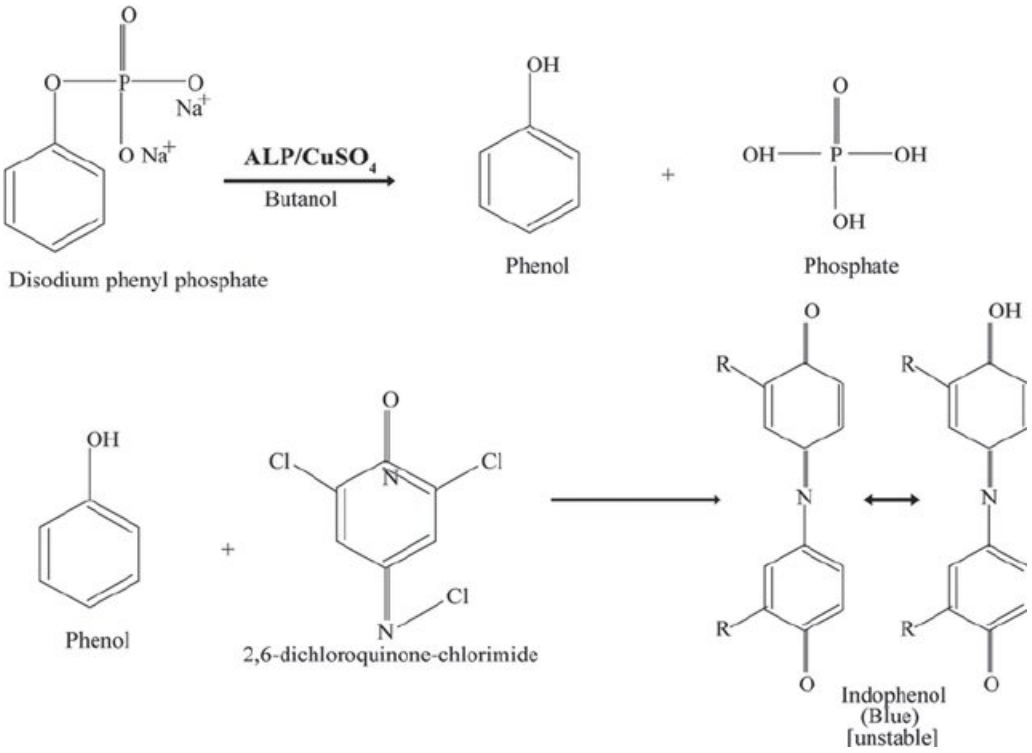
The two currently accepted sterilization methods are: a) batch pasteurization by heating the milk to 63^{o}C for 30 min (holding time), and the continuous process, high-temperature short-time (HTST), which consists in heating the milk to 72 ^{o}C for 15 sec (holding time). The second pasteurization method is the most common at the industrial level. Pasteurized milk is not sterile and must be immediately cooled after sterilization to prevent the proliferation of surviving bacteria. Pasteurization does not have a significant effect on the nutritional value of milk, nor on its organoleptic character, although a slight reduction in vitamins may occur.

Alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) catalyzes the hydrolysis of certain phosphate compounds. By pasteurizing milk, it is 99.9% inactivated, which is why it is an indicator of the good pasteurization of the milk. More specifically, the enzyme catalyzes the hydrolysis of disodium phenyl phosphate producing phenol which is detected with 2,6-dibromoquinone-4-chloroimide or 2,6-dichloroquinone-4-chloroimide (Rankin et al., 2010, Clawin-Rädecker et al., 2021).

Appearance of **blue color** (indophenol) means presence of phosphatase, that means no good pasteurization.

Appearance of **brown or reddish brown** means absence (deactivation) of phosphatase, that means good pasteurization.



a. Milk pasteurization control

Method

Reagents:

Lactognost N °I: alkaline buffer

Lactognost N °II: disodium phenylphosphate

Lactognost N °III: 2,6-dibromoquinone-4-chloroimide

1 mL of pasteurized milk and 1 mL of unpasteurized milk are placed in two clean test tubes. In both tubes, 10 mL of distilled water are added, one Lactognost N °I tablet and one Lactognost N °II tablet.

This is followed by good agitation to dissolve the tablets, and the tubes are placed in a water bath at 37 °C for 15 min.

Then 0.1 g of Lactognost N° III is added to each tube followed by stirring.

Exercise 7. Determination of milk ash and its alkalinity

INSTRUCTIONS FOR THE SAFE CONDUCT OF THE EXERCISE
- Wear protective glasses throughout the experimental procedure.
- Great care when using the burner and furnace
- Fill the burettes with HCl and NaOH with the help of a small funnel and after lowering them so that the top of each burette is below eye level

Introduction

Ash is the residue (**inorganic matter**) that remains after the charring and further ashing of milk at 500-550 ^{o}C. Milk contains about 0.7% ash. Ash consists of oxides of Na, K, Ca, Mg, Fe, P and S. The inorganic salts are mainly the PO_4^{3-},Cl^- as well as citrate salta of K, Na, Ca, Mg. By ashing, the salts of organic acids are converted into carbonates, which give an alkaline reaction.

Alkalinity of the ash is the sum of all alkaline components, expressed as potassium or sodium hydroxide (KOH, NaOH). The alkalinity number (according to Buttenberg) is the number of mL of normal acid solution required to neutralize 1 g of ash. The determination of alkalinity is important in detecting whether soda or potassium hydroxide or other neutralizing agent has been added to the milk to neutralize its acidity. The alkalinity of normal milk ash barely exceeds 1 mL of normal acid solution per 100 mL of milk.

a. Determination of ash

In a crucible of known weight (which has heated at 550^{o}C and cooled in a desiccator prior to weighing), accurately weigh 10 mL of milk. Add 2-3 drops of 10% acetic acid to coagulate the proteins, stir, and dry in a boiling water bath to evaporate the water (solid residue). This is followed by burning the residue in a burner flame with careful handling and the help of the supervisor.

This is followed by ashing in a furnace (temperature no higher than 550^{o}C) until carbon-free ash remains. After cooling in a desiccator, the crucible is weighed.

The amount of ash is found from the difference in weight and expressed as % wt.

- Milk solid residue: Cow 12.4%, goat 14.3%, sheep 18.5%
- Milk ash: Cow 0.75%, Goat 0.80%, Sheep 1.00%

b. Determination of ash alkalinity

The ash of the 10 mL of milk (a) is transferred with a little hot water to a conical flask and 20-30 mL of 0.1 N H_2SO_4 are added. This is followed by heating in a boiling water bath and careful stirring with a glass rod to dissolve the ash. The whole process is done in the hood wearing protective glasses. The solution is left to cool, and 2-3 drops of 1% methyl orange (helianthine) indicator are added and the excess acid is titrated with 0.1 N NaOH solution.

Exercise 8. Steam distillation of essential oils

INSTRUCTIONS FOR THE SAFE CONDUCT OF THE EXERCISE
-Wear protective glasses throughout the experimental procedure.
- Ensure water flow in the condenser from the beginning of the experiment, during the distillation and afterwards.

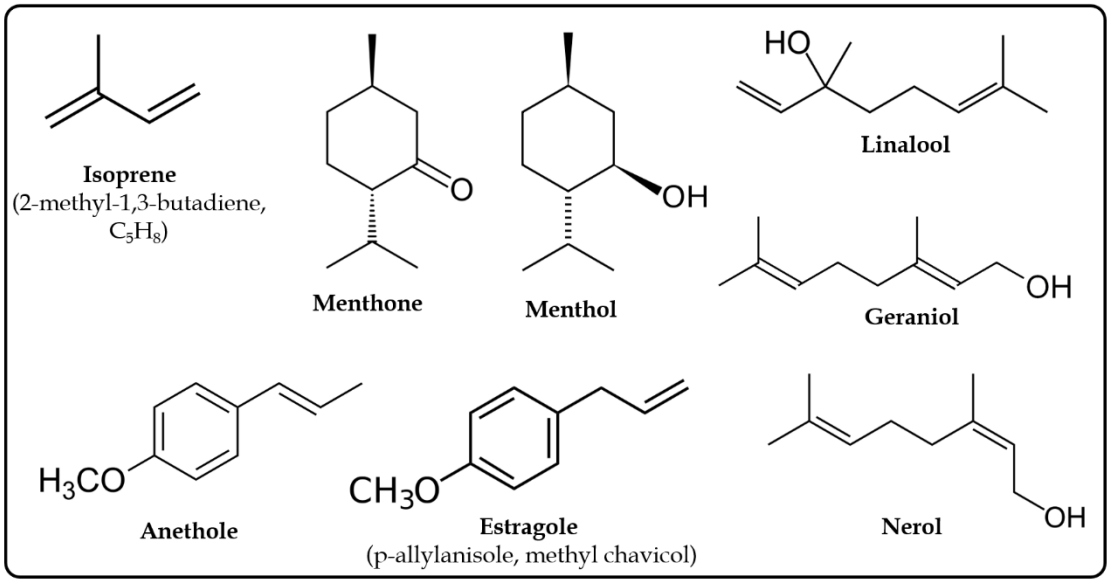
Introduction

Steam distillation applied for the recovery and purification of substances, such as essential oils, at temperatures below their boiling point and even below the boiling point of water (100°C) under atmospheric pressure.

Essential oils are substances responsible for the characteristic aroma of some plants. They are mixtures of hydrocarbons, alcohols and carbonyl compounds, acyclic or cyclic or aromatic, which mostly belong to the category of terpenes or their derivatives.

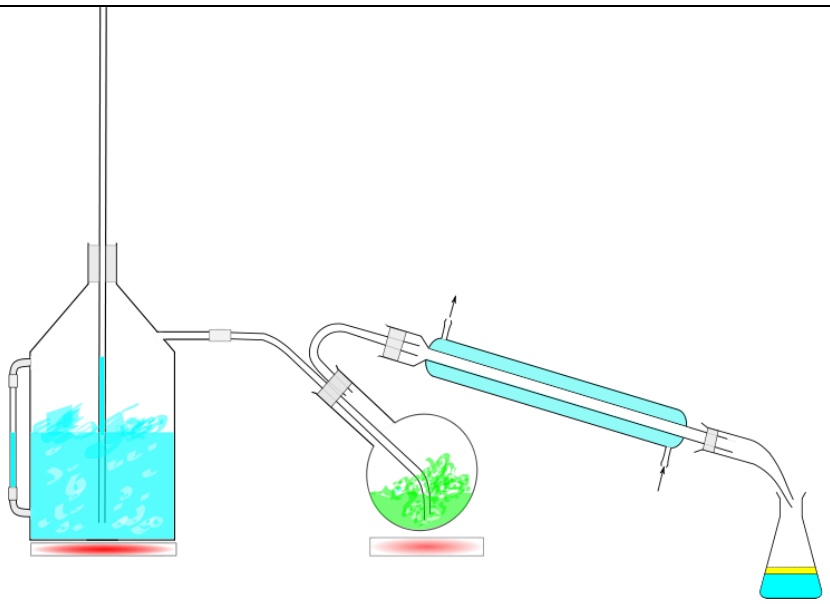
Terpenes are the hydrocarbons consisting of one (semiterpenes) or more **isoprene** units (e,g, monoterpenes, sesquiterpenes, diterpenes, etc.). In addition to carbon and hydrogen, terpenoids often contain oxygen atoms.

For example, the main components of the essential oil of mint are menthol and menthone, of anise is anethole (or anisole) and p-allylanisole, etc.



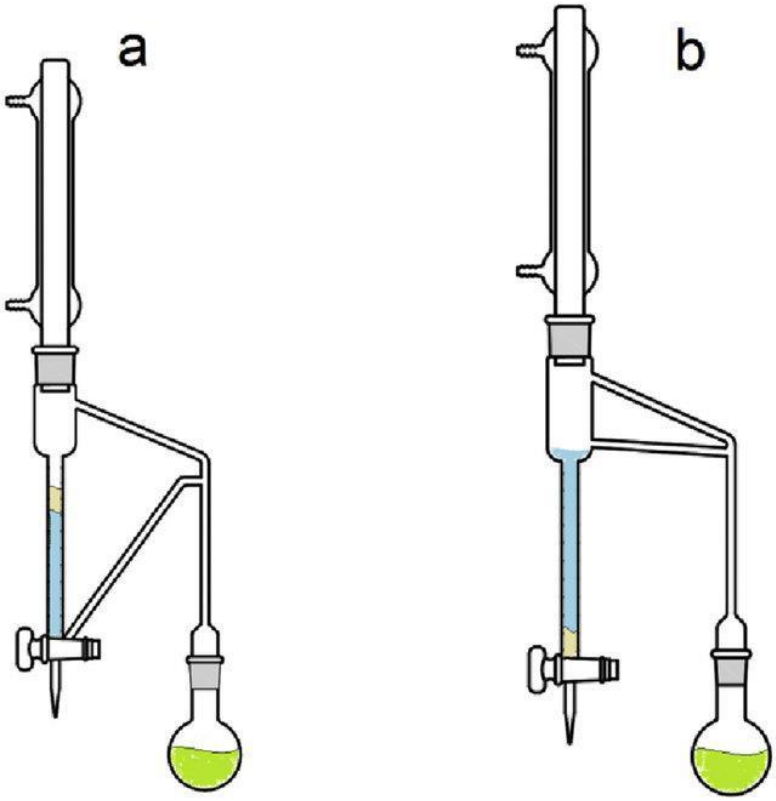
By steam distillation the essential oils from an aromatic plant, such as anise, can be obtained and their % content in the plant can be determined.

The steam distillation is carried out by an apparatus such as in the figure below, with a flask to which finely chopped plant is added and the distillation is done until all the contained essential oil is obtained (about 2-4 h). The water distilled from the flask is replaced by the gradual addition of water.



Simplified steam distillation apparatus for essential oil extraction
(source: Wikipedia)

After the end of the distillation, the essential oils are obtained from the water phase by extraction with a suitable solvent (e.g., chloroform) and their amount is determined gravimetrically after removal of the solvent in a rotary evaporator. If appropriate essential oil traps are available, then receiving and measuring its quantity is done directly.

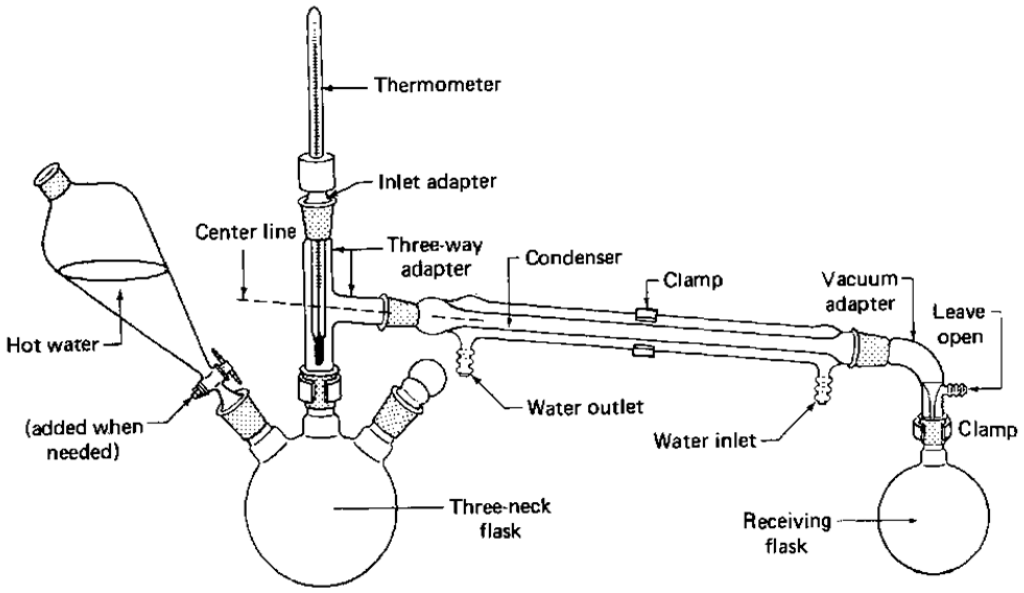


Clevenger Steam distillation apparatus with essential oil traps.
Left: Lighter than water. Right: Heavier than water
(source: Vinatoru et al., 2017).

a. Steam distillation of essential oils

Instruments & reagents

(1) 3-decimal precision balance, (2) 500 mL three-necked round flask, (3) Boiling stones, (4) Condenser, (5) Distillation adapter, (6) 150 mL separatory funnel, (7) Heating mantle, (8) Ground-stoppered conical flask, (9) Rotary evaporator flask and device, (10) Essential oil traps, (11) Aromatic plant (anise or mint), (12) Antifoam, (13) Chloroform, (14) Anhydrous sodium sulfate (Na_2SO_4).



Steam distillation apparatus
(http://what-when-how.com/organic-chemistry-laboratory-survival-manual/distillation-part-3-laboratory-manual/).

Method

1. The aromatic plant is cut into small pieces and about 10 g of them are accurately weighed ($\mathbf{w_a}$).
2. The pieces are placed in a 500 mL round-bottomed flask along with about 200 mL of water and boiling stones and a small amount of antifoam.
3. A side condenser with a distillation adapter is attached to the middle outlet of the three- neck flask, and a 150 mL separatory funnel with water is attached to one of the side necks, while the other side neck is stoppered.
4. The three-necked flask is heated with a heating mantle, and during the distillation the distilled water (carrying the essential oils) is slowly replenished from the separatory funnel.
5. After 1-2 h of distillation, all the drops of the oily essential oil are collected in the aqueous distillate in a receiver flask. In the final distillate, which must be at least 100 mL, 10 mL of chloroform are added and after shaking it is transferred to a 150 mL separatory funnel where the lower chloroform layer is separated and transferred into a conical flask.

6. The lower chloroform layer is again separated and combined with the first chloroform layer.
7. 1-2 g of anhydrous sodium sulfate are added to the combined chloroform layers and after standing for 15-30 min, to absorb the moisture, the solvent is transferred to a pre-weighed rotary evaporator flask, where it is evaporated at 35°C under vacuum.
8. After evaporation, the essential oils remain as an oily layer inside the bottle which is reweighed and the amount of oils contained is determined (w_b).
9. If a device with an essential oil trap is used, then the trap is fitted between the three-necked flask and the condenser, and the essential oils are trapped in it and their quantity (volume) is measured directly on the scale of the trap. With the oil trap method, steps 1) to 4) are performed and steps 5) to 8) are omitted.

Calculations and results

a) By weighing: % essential oil = $(w_a/w_b) \times 100$ (w/w)

b) By trap: % essential oil = mL / 100 g plant (w/w)

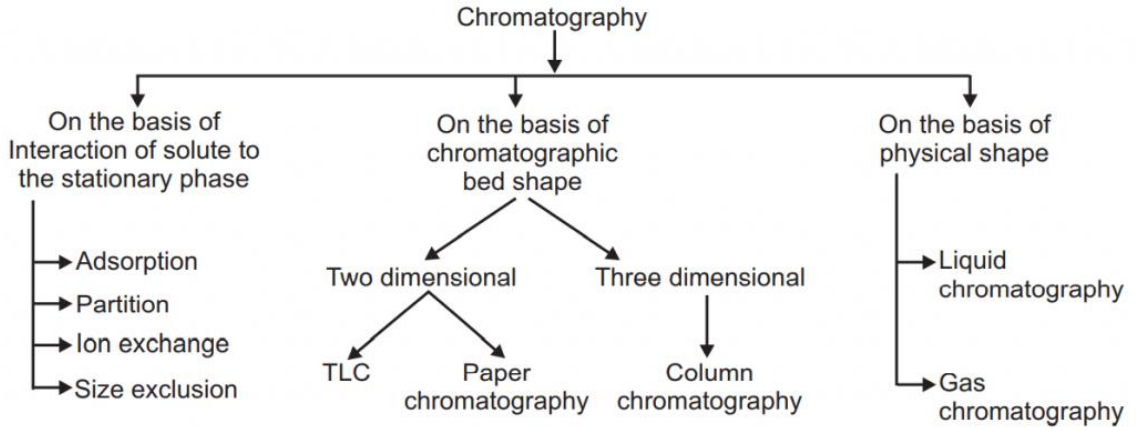
Chromatographic analysis - Introduction

Introduction

The principle of chromatographic analysis lies in the physical separation and determination of a mixture of components, inorganic or organic compounds, which is achieved by the distribution of the components between 2 phases, one mobile and one static, and is based on certain properties of the components of the mixture such as the boiling point, polarity, molecular size, volatility, etc. Thus, the mobile phase, passing through the static phase, causes a different displacement of the components of the mixture, which elute from each other at different times.

The qualitative and quantitative determination of each component is achieved by a detection and counting system located at the exit of the stationary phase.

Chromatographic analysis methods can be classified based on the nature of the mobile phase, the nature and form of the stationary phase, the separation mechanism and the way the sample is introduced into the stationary phase and moved through it.



Types of chromatographic techniques
(https://solutionpharmacy.in/chromatography/)

- ***Liquid Chromatography*** if the mobile phase is liquid. Depending on the nature of the stationary phase it is distinguished into ***Liquid-Solid Chromatography***, if the stationary phase is solid, and ***Liquid- Liquid Chromatography***, if the stationary phase is liquid.

- ***Gas Chromatography*** if the mobile phase is a gas. Depending on the nature of the stationary phase it is distinguished into ***Gas-Solid Chromatography***, if the stationary phase is solid and ***Gas-Liquid Chromatography***, if the stationary phase is liquid.

Depending on the **mechanism** by which the components of the mixture are retained by the stationary phase, the following methods of analysis are distinguished.

- ***Adsorption Chromatography***. The separation is achieved by the distribution of adsorbed particles and of particles in the mobile phase, which may be liquid or gaseous.

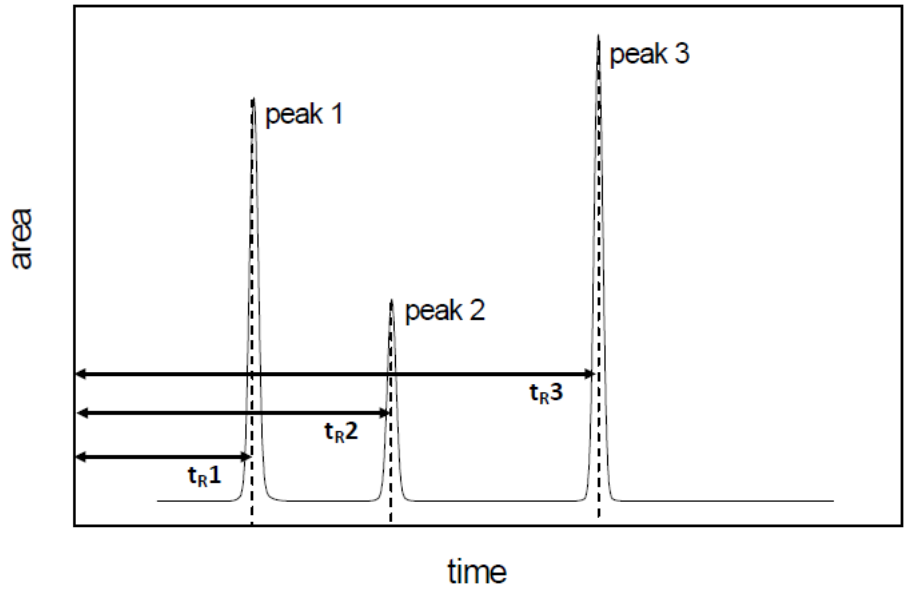
- ***Ion Exchange Chromatography***, where ion-exchange resins or gels are used as a solid stationary phase and a liquid as a mobile phase.

- ***Partition Chromatography***, where the components are distributed between a liquid bed of a stationary phase and a liquid mobile phase.

- ***Molecular Exclusion Chromatography***, where molecules are separated according to their size, with larger molecules exiting first. It is also known as gel permeation chromatography or gel permeation chromatography.

- ***Affinity Chromatography,*** which is a younger and more selective technique based on the specific interaction of a sample molecule with a molecule immobilized in the solid stationary phase.

The **quantification** of the components of a mixture by GC or HPLC is done by the following methods:



Three-component chromatogram. Area= peak area. Time= Elution time.

- ***Standard reference curve method***

A standard peak area-to-concentration reference curve is constructed by GC or HPLC analysis of a number of different standard substances of known concentrations, the same as the analytes in the unknown mixture.

- ***Internal standard method***

A chromatogram of a substance of unknown concentration is obtained (C_x) and the response is recorded (peak area E_x). A known amount of the substance to be analyzed (Cy) is then added to the unknown sample, and the new response is recorded (peak area E_{xy}) corresponding to the total concentration C_x+C_y. The unknown concentration of C_x will be:

$$\frac{Ex}{Ex\gamma} = \frac{Cx}{(Cx + C\gamma)} \Rightarrow Cx = C\gamma \frac{Ex}{(Ex\gamma - Ex)}$$

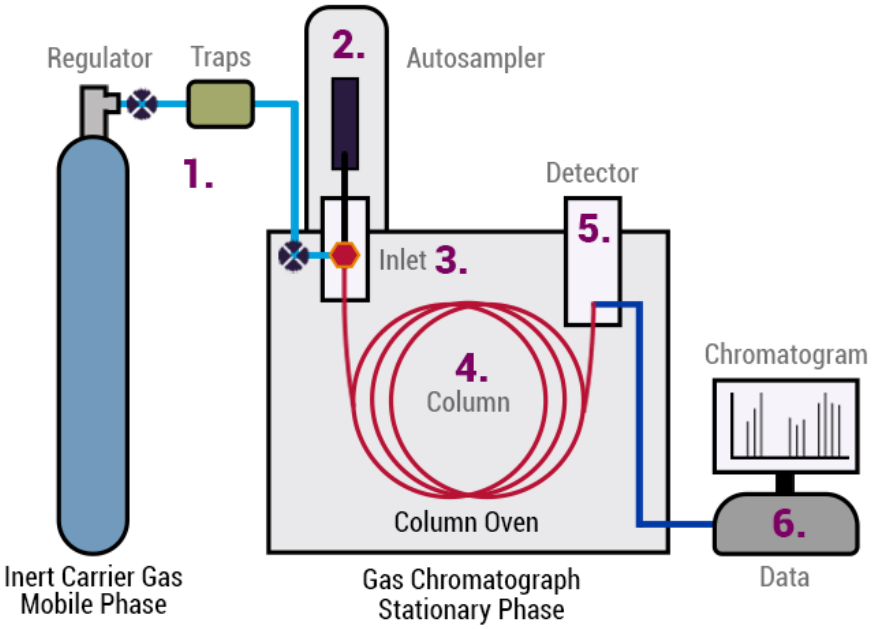
- ***External standard method***

Quantification of several unknown components in a single chromatogram by adding a known amount of a substance that must be of the same nature but not the same as any of the components to be analyzed, and must elute without overlapping its peak with the peaks of the other components. If C_x the unknown concentration of one of the components of the mixture with response E_x and C_y, the concentration of the external standard with response E_y, then the unknown concentration C_x will be:

$$\frac{Ex}{E\gamma} = \frac{Cx}{C\gamma} \Rightarrow Cx = C\gamma \frac{Ex}{E\gamma}$$

Gas-Chromatographic instrumentation

A gas chromatograph consists of (a) a source of carrier gas with one or more pressure reduction valves; (b) an inlet (injection port) that can be heated; (c) a column in a thermostatic air bath; d) a detector suitable for vapour phase samples. The high temperatures are needed to vaporise the solutes of interest and maintain them in the gas phase. The injection port and the detector are generally maintained at a temperature approximately 10% (in ^oC) higher than that of the column to ensure rapid volatilisation of the sample. The temperature of the column is usually set at least 25 ^oC higher than the boiling point of the solute.



Gas Chromatography schematic
(Perkin Elmer, 2023)

Carrier gas

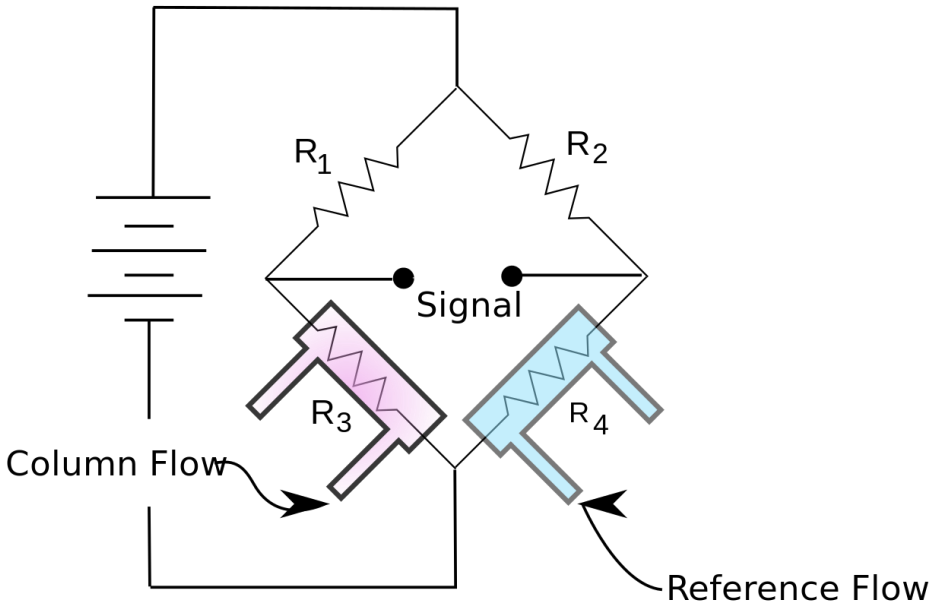
At normal pressures and temperatures, He, N_2, H_2, and Ar_2 are used as carrier gases, because they are considered to be inert. The choice of carrier gas depends on the detector type and the kind of analysis. Thermal conductivity detectors work better with He and H_2, and flame ionisation detectors work better with N_2.

Detectors

The three common detectors in gas chromatography are those based on thermal conductivity, flame ionisation and electron capture. The first measures heat conductivity, which is different for different gases; the second and the third type, responds to changes in electron currents. The electrons are produced in a flame by burning the sample, or by exposing the sample to a radioactive source.

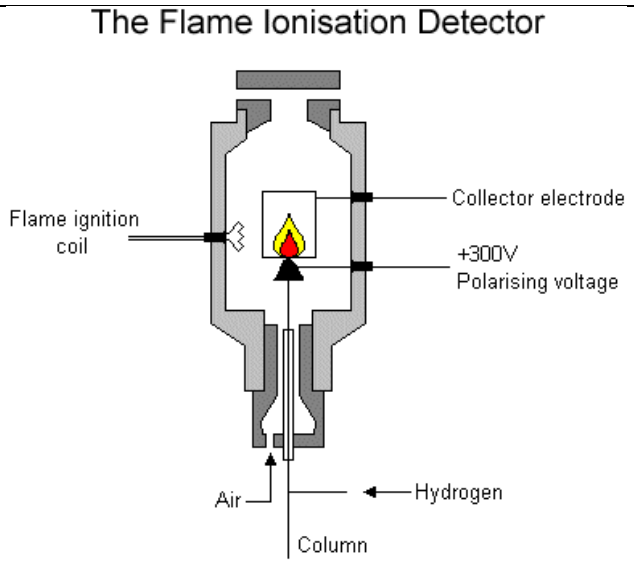
Thermal conductivity detector. The thermal conductivity detector (TCD) is a simple universal detector that produces large signals requiring no amplification. The detector cell has either two or four filaments arranged in a *Wheatstone bridge* circuit. A schematic of a classic thermal conductivity detector design utilizing a Wheatstone bridge circuit is shown in the figure below. The reference flow across resistor R4 of the circuit compensates for drift due to flow or temperature fluctuations. Changes in the thermal conductivity of the column effluent flow across resistor R3 will result in a temperature change of the resistor and therefore a resistance change which can be measured as a signal.

The TCD is reliable, simple, non-destructive and moderately sensitive, it responds to essentially all compounds and is widely used for preparative work. The detector has a concentration detection limit of about 5-10 x 10^{-6} g/mL of eluting gas and a dynamic range of about 10^5.



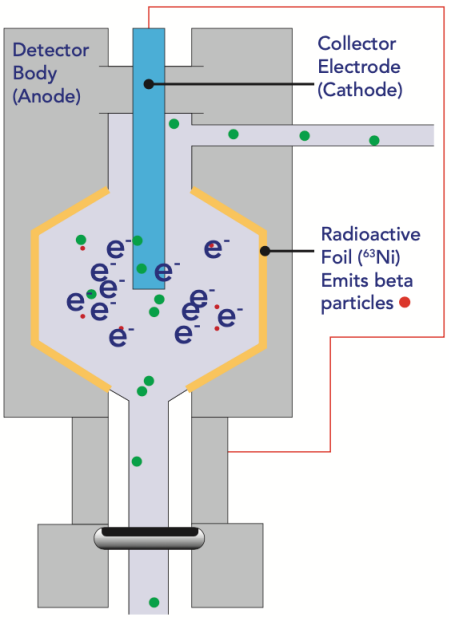
Typical bridge configuration for a TCD
(https://en.wikipedia.org/wiki/Thermal_conductivity_detector)

Flame-Ionisation Detector. The flame-ionisation detector (FID) has a wide linear range, high sensitivity and is quite reliable. It consists of a hydrogen-air flame polarised in an electrostatic field (Fig.). The flame ignites and ionises the combustion sample components as the carrier gas passes into it, after which the ions (primarily carbon compounds) are collected at the electrodes, producing a current. The FID does not respond fully to oxygenated carbons such as carbonyls, carboxylic acids, or their sulphur analogues (e.g. cyclohexane). However, it does not respond at all to water or to permanent gases (N_2, O_2, CO_2, etc.), making it ideally suited for trace analysis in aqueous solutions and atmospheric samples. The FID is a destructive detector with detection limit 1-5×10^{-9} g/mL of eluting gas and with dynamic range of 10^8.



Flame-ionisation detector
(https://teaching.shu.ac.uk/hwb/chemistry/tutorials/chrom/gaschrm.htm)

***Electron Capture Detector*.** The electron-capture detector (ECD) takes advantage of the affinity of certain functional groups for free electrons. The principle is almost identical to flow-through proportional counting of a radioactive source. The carrier gas is passed through a cell containing a beta source (e^- for nuclear decay), which ionises the carrier gas. The source can be Pt foil saturated with $^3H_2$, but a ^{63}Ni foil is used more commonly due to its higher temperature stability. The beta particles ionise the carrier molecules and produce electrons, which migrate to the anode (Fig.) under a potential of 1 to 100 V. An electron capturing species eluting from the column will react with the electrons to form an ion or neutral molecule, which swept from the cell. The result is a reduction in the number of electrons found at steady state or a drop at the standing current. The major advantage of the ECD is its selectivity. The ECD is insensitive to amines, alcohols and hydrocarbons, but very sensitive to halogens, anhydrides, peroxides, ketenes and nitro groups. Its response is not linear, but a linear range of $0.5\text{-}1\times10^3$ can be achieved by pulsing the polarising voltage. The limit of detection is about 1×10^{-12}.

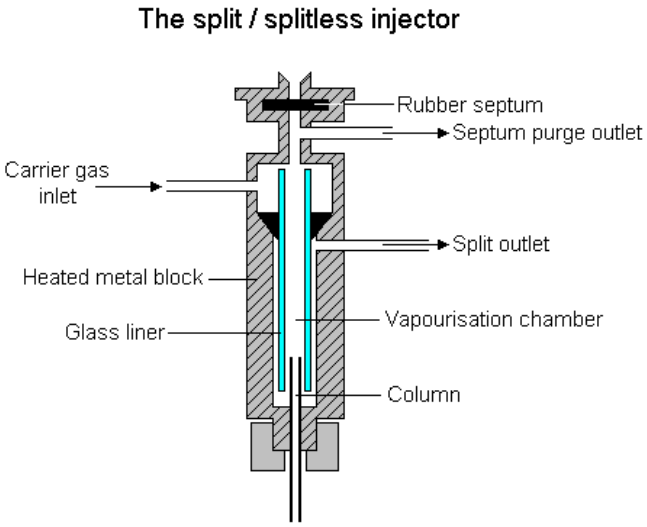


https://www.chromatographyonline.com/

Column inlets (injection ports)

In gas chromatography sample is introduced into the column through a specially designed inlet, generally by injecting it in nanolitre amounts through a rubber septum with a microlitre syringe. The injection port should be hot enough to flash evaporate the sample, and large enough to expand without blowing back through the septum (Fig.). The injector can be used in split or splitless mode. The injector contains a heated chamber containing a glass liner into which the sample is injected through the septum. The carrier gas enters the chamber and can leave by 3 routes (when the injector is in split mode). The sample vapourises to form a mixture of carrier gas, vapourised solvent and vapourised solutes. A

proportion of this mixture passes onto the column, but most exits through the split outlet. The septum purge outlet prevents septum bleed components from entering the column.



An injection port commonly used in gas chromatography
(https://teaching.shu.ac.uk/hwb/chemistry/tutorials/chrom/gaschrm.htm)

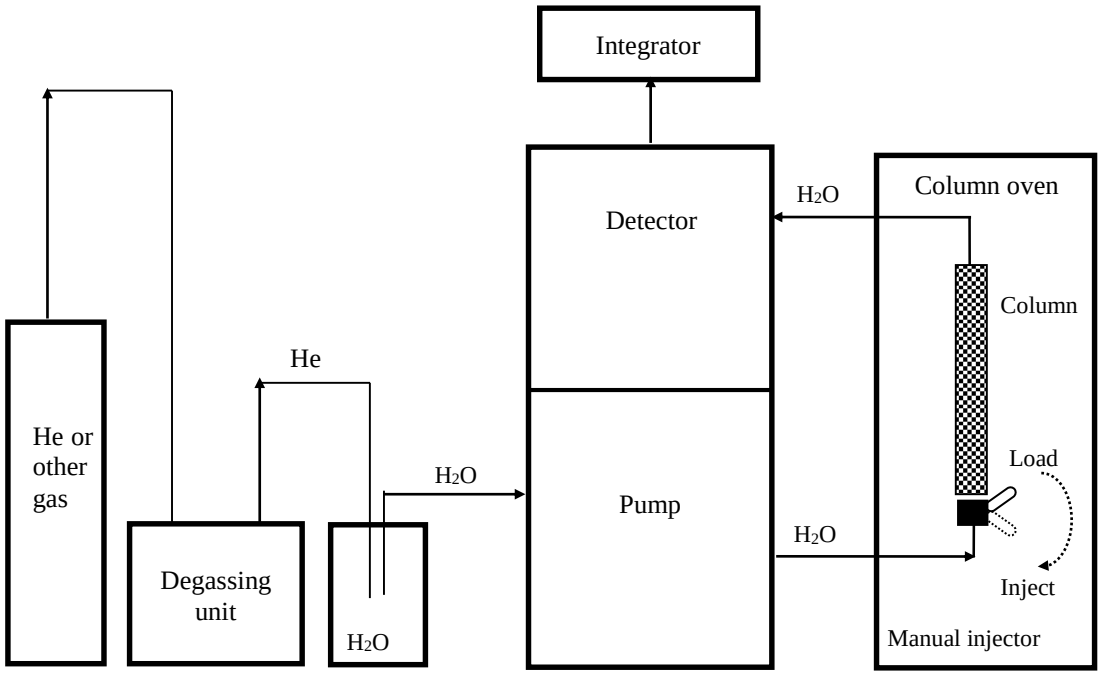
Columns for gas chromatography

There are two kinds of columns usually used: a) packed columns and b) capillary columns. The most commonly used GC columns are the packed ones, consisting of a tube filled with particles of fairly uniform size, coated with the liquid stationary phase. Those columns are of 1-20m length, 3-10mm diameter (1/8-3/8 inches) but there are also preparative columns with a diameter of 10cm or bigger. They are easy to make, cheap, with bigger capacity and long working life. With capillary columns the pressure drop is smaller, so they can have bigger length, (10-50m) and diameter of 0.2-1.2mm. The major advantage of capillary columns is not the plate height, which is generally larger than well-packed columns, but the number of plates achieved, with a relatively small pressure drop. For example if 20,000 theoretical plates is a good upper limit for packed columns, the capillaries can have 75,000 to 150,000 plates. Capillary GC is a technique complementary to the use of packed columns. The latter are to be preferred when available resolution is adequate and the highest quantitative precision is desired.

Types of GC columns (Packed and and capillary) (www.ncids.com)

HPLC instrumentation

A HPLC System is basically composed of (1) a solvent reservoir, (2) a high pressure pump, (3) an injection port, (4) a pre-column (optional), (5) a column, (6) a column oven, (7) a detector and (8) a recorder, as shown in the Fig. below.



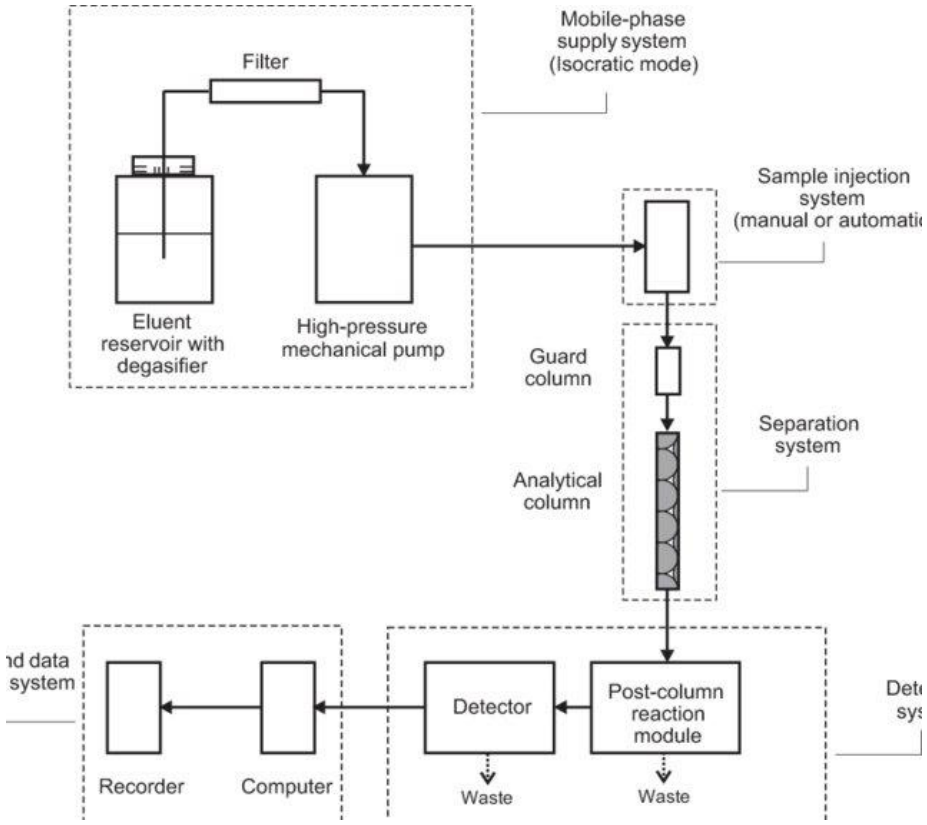
A typical HPLC system

The pump keeps the flow rate of the liquid (mobile phase) constant, and when sample is injected at the injection port, it is transferred into the column by the mobile phase and separated to its components. Each component elutes from the column one by one and is detected. Simultaneously, the chromatogram is plotted and when a component is detected, a peak appears on the chromatogram, as described in the case of GC. Using the value of retention time (t_R), we can determine the eluted compound qualitatively in comparison with that of a standard sample. The peak area or peak height serves to quantify the unknown compound, as it is directly proportional to its concentration in the sample.

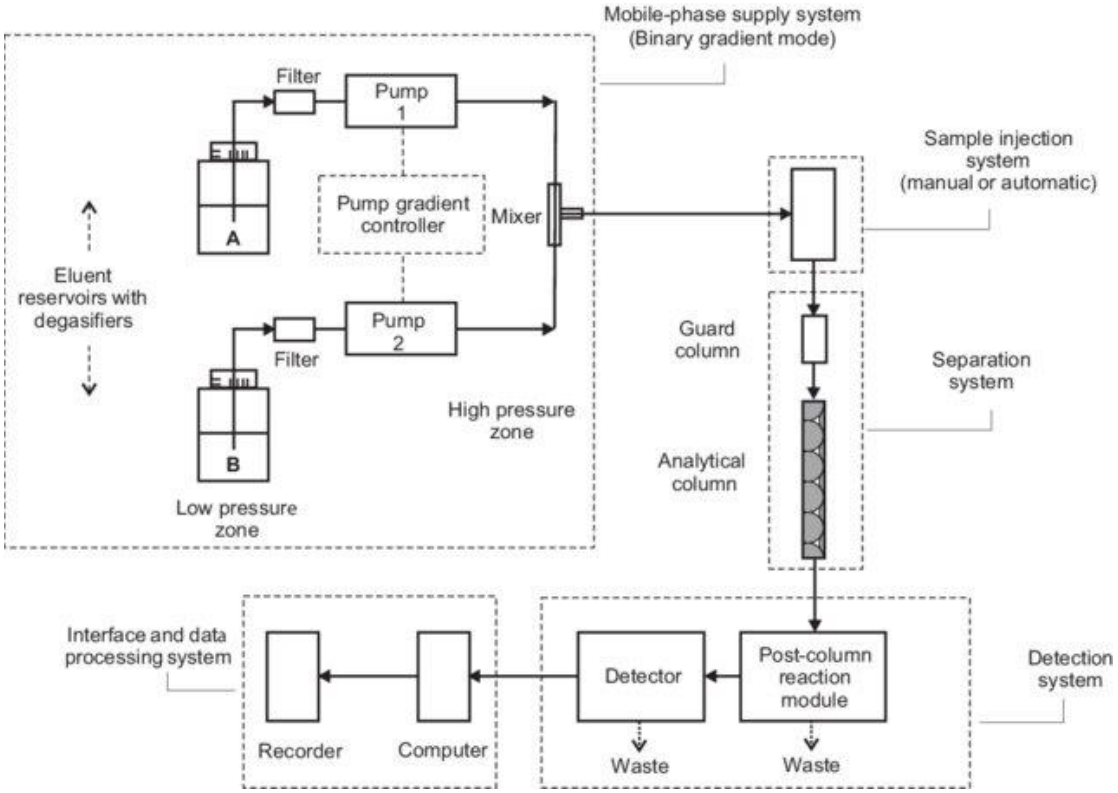
There are two different ways by which the elution of a sample can be done in HPLC The **isocratic elution** and **gradient elution**.

In **isocratic elution**, a mixture of mobile phase or a solvent system used to separate the sample components and it is consistent over the complete testing time. There are no changes in the mobile phase composition that can be made between the entire run of the isocratic system (https://whatishplc.com).

In **gradient elution** the composition of the mobile phase varies throughout the chromatographic run and therefore affects the retention of the analysis. Separation in gradient mode can be either accelerated or decelerated. In the gradient system, the composition of the mobile phase should be changed concerning time so it is a flow gradient if the mobile phase ratio is not changed throughout the process of analytes separation then it is considered as isocratic elution (https://whatishplc.com).



Isocratic elution HPLC system
(https://www.researchgate.net/publication/227934976)



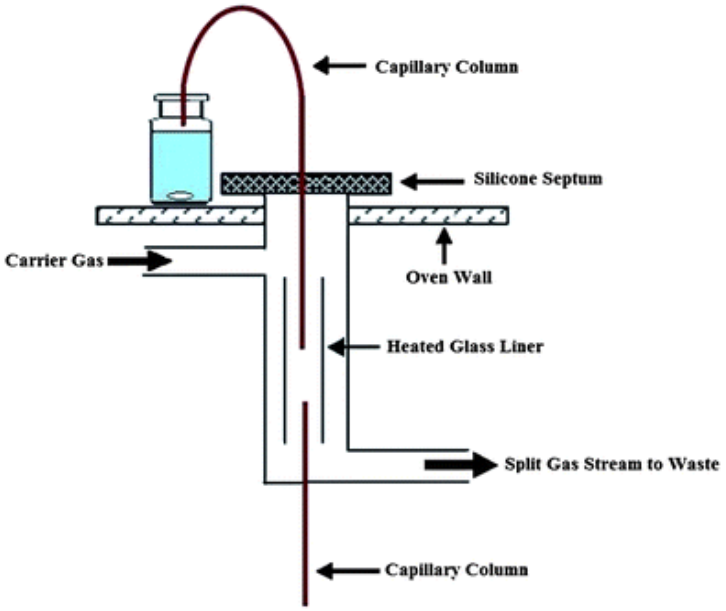
Gradient elution HPLC system
(https://www.researchgate.net/publication/227934976)

Chromatography sample preparation methods

1) Headspace sampling methods

a) Direct headspace

In a closed container, a sample of the headspace air above the sample is taken and analyzed directly in the chromatograph.

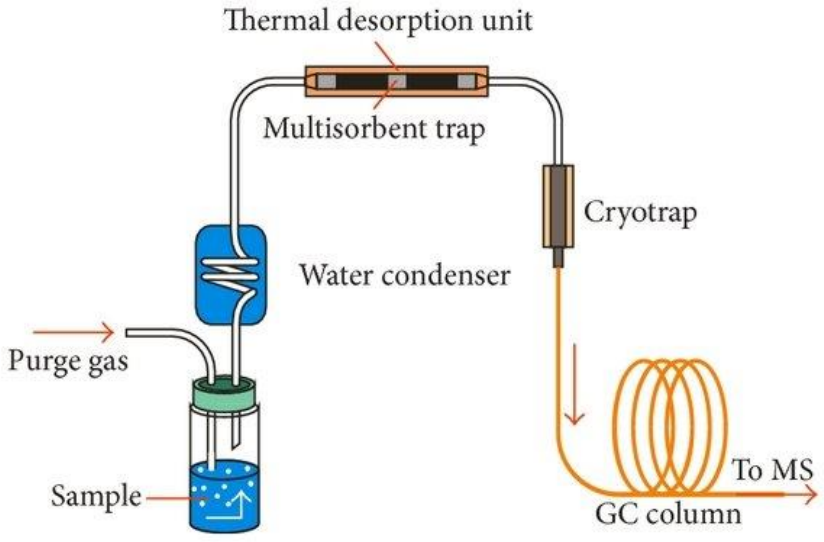


Direct headspace GC (http://pubs.rsc.org)

b) Dynamic headspace

The sample is placed in a closed container and an inert gas, e.g. N_2, is channeled to purge the overlying gas phase in a trap or an adsorbent → **"purge & trap**"

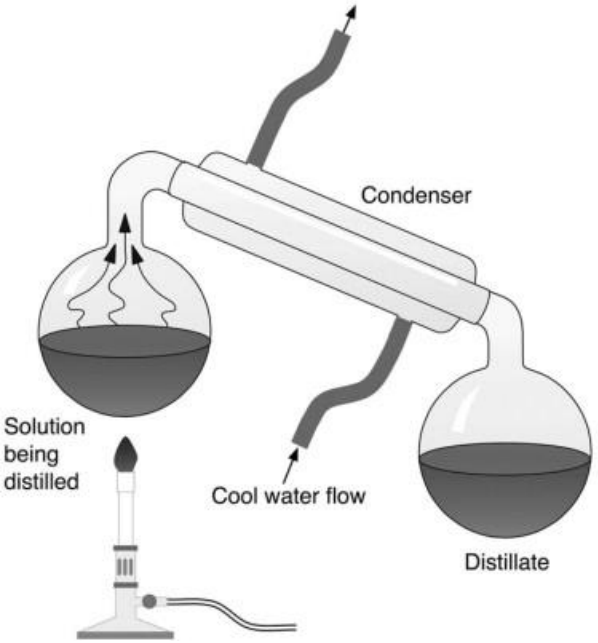
The trap is then extracted with a solvent and the extract analyzed by GC.



Dynamic (purge and trap) headspace GC (Schmidt & Podmore, 2015).

2) Distillation methods

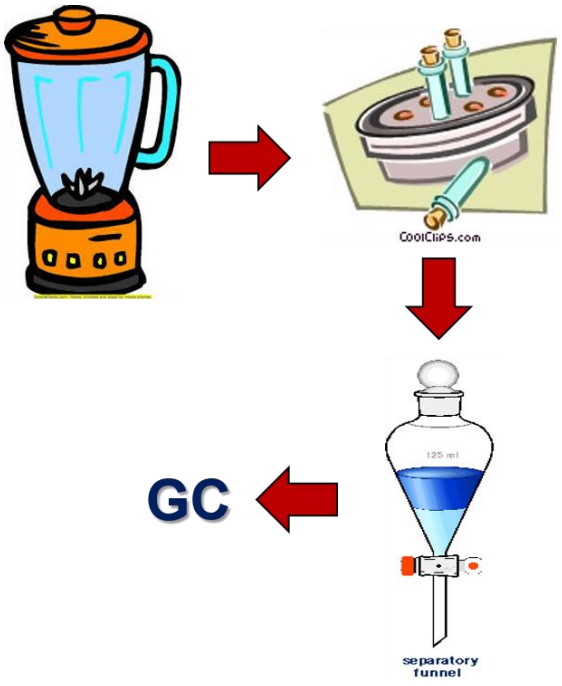
The sample is distilled to separate the volatile components, and the distillate is analyzed by GC.



Distillation of samples before GC
(https://www.sciencedirect.com/topics/engineering/distillation)

3) Solvent Extraction methods

They consist of mixing the sample with and immiscible solvent, followed by centrifugation to separate the solid residues, and solvent separation and concentration prior to GC.



Solvent extraction of samples before GC

4) Solid-Phase Micro-extraction (SPME) methods

Solid Phase Microextraction (SPME) is an extraction process for both volatile and semi volatile compounds in preparation for GC or HPLC analysis. One of its major advantages is that by choosing the proper SPME fiber (with an extracting phase coated on it), specific target analytes can be extracted leaving unwanted compounds behind in the matrix. This makes SPME very popular in applications where the resulting chromatograms are complex, with many different types of compounds. E.g. for polar compounds that are typically related to a food's taste or smell, a polar extraction phase can be chosen to target these compounds, resulting in a simpler chromatogram. Since the extraction uses no solvent, detection levels of even parts per trillion (ppt) are possible and an analyst would not come in contact with potentially dangerous solvents during the analysis.

SPME is performed with a **SPME fiber**. The fiber is coated with a liquid phase polymer and/or a solid sorbent and is mounted within a needle. There are many different phases available and choosing the correct fiber that is optimal for a specific analysis is part of the method development process. The fiber can be extended out of the needle during extraction or desorption, but retracted inside the needle when the needle needs to pass through septa.

(https://estanalytical.com/industries/food-flavor-consumer-products/what-is-spme/).

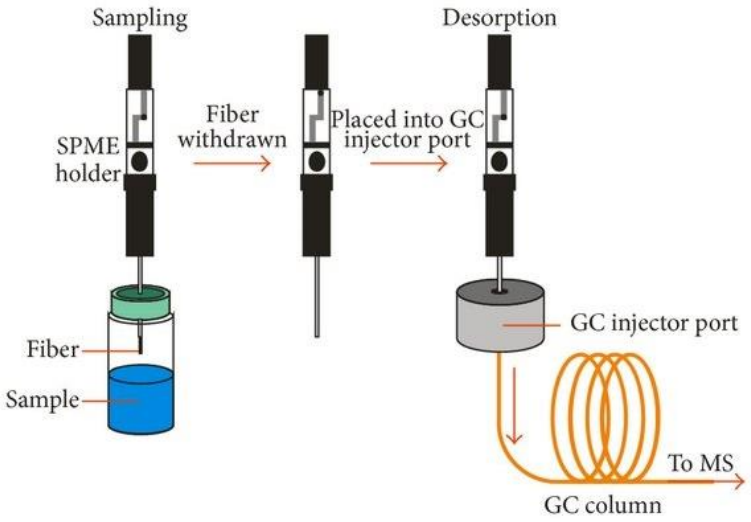
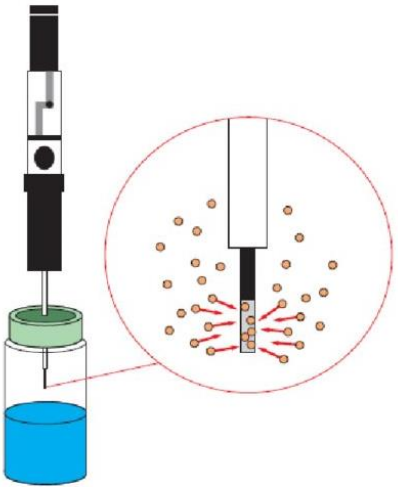
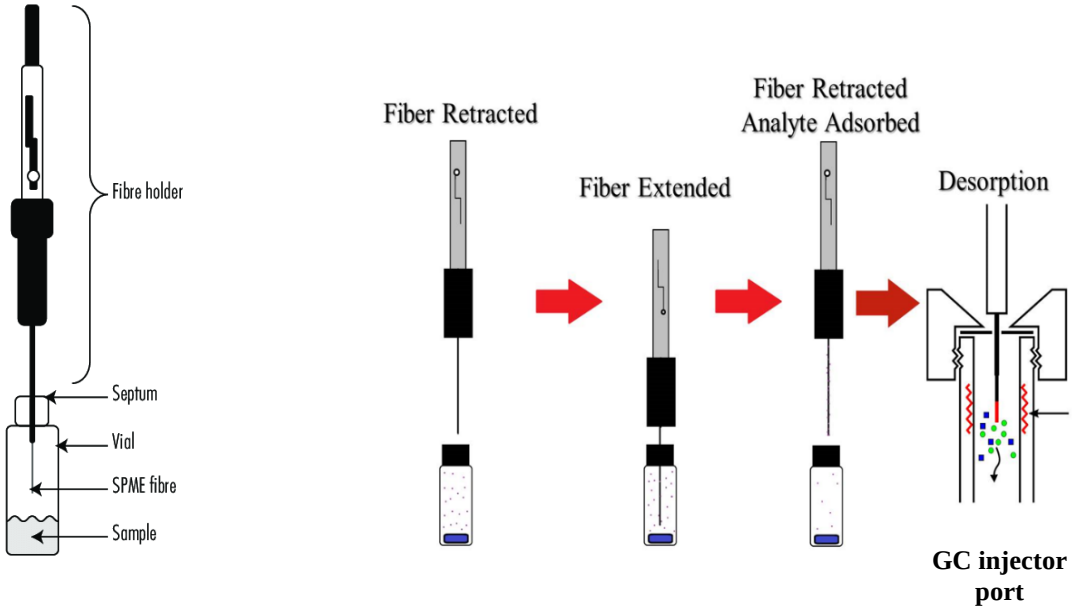


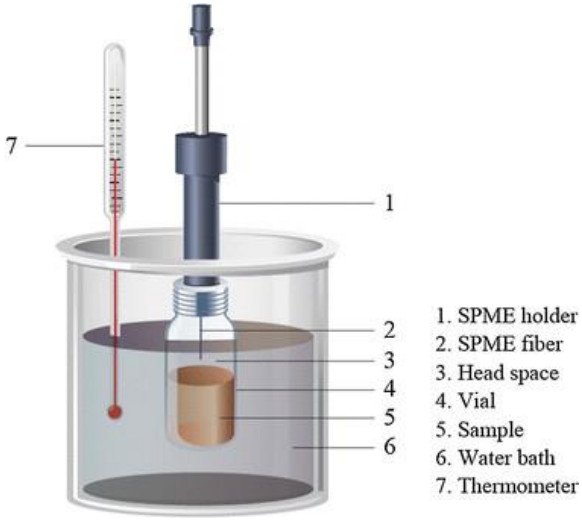
Diagram of analysis with SPME-GC-MS (Schmidt & Podmore, 2015)

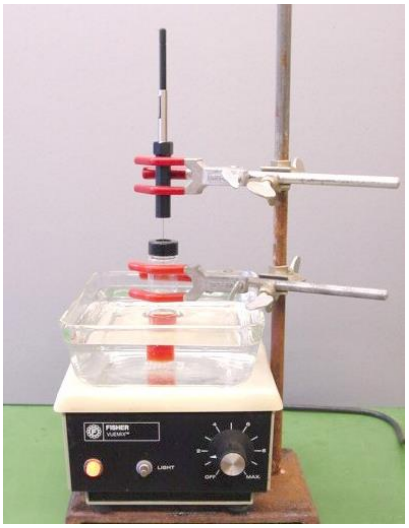


SPME fibre (Schmidt & Podmore, 2015).



SPME fibre insertion and retraction to the sample vial, and exposure to the GC injection port





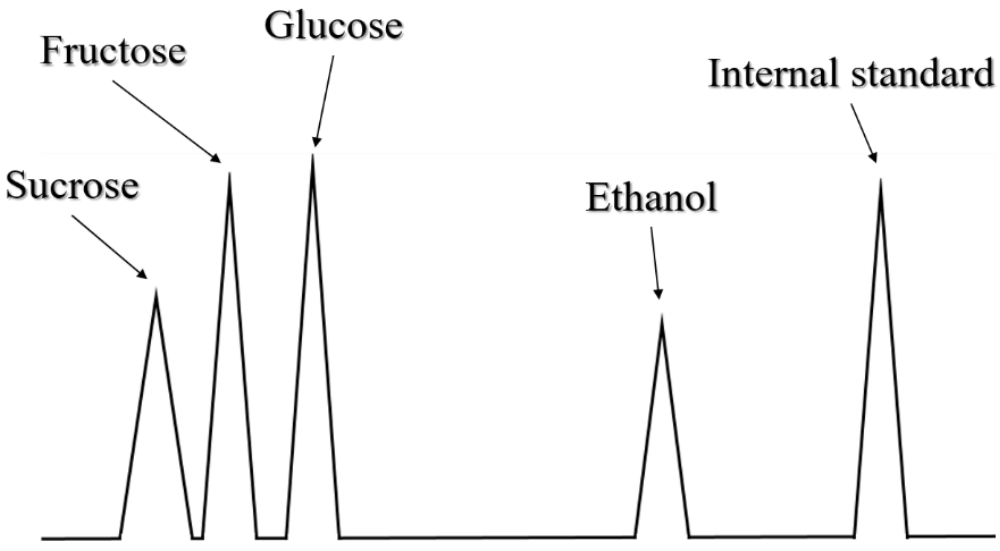
SPME set-ups

Exercise 9. Determination of sugars, ethanol and organic acids by HPLC

INSTRUCTIONS FOR THE SAFE CONDUCT OF THE EXERCISE Determination of degree of acidity - Wear protective glasses throughout the experimental procedure. - Gas chromatography: any adjustment/manipulation regarding exercise gases (N_2, H_2, air) is strictly done by the teaching staff

a. Determination of sugars and ethanol by HPLC with refractive index detector (HPLC-RID)

For the determination of sugars and ethanol by high-performance liquid chromatography (HPLC), a standard curve is first constructed with standard solutions of 1-10 % v/v ethanol and various sugars (glucose, fructose, sucrose, etc., according to the supervisor's instructions) in well degassed and filtered high purity water and concentrations of 1-10 % w/v, with 0.1 % v/v butanol-1 as internal standard. The **isocratic** determination is done on a Shimadzu LC-9A chromatograph with Shim-pack SCR-101 N column, column temperature 60-C, mobile phase, well-degassed and filtered high-purity water, flow rate 0.8 mL/min and refractive index detector (RID) . The samples are introduced into the column after being properly diluted with triple distilled water to a concentration of 1% v/v and filtered with a 0.45 μm microfilter.

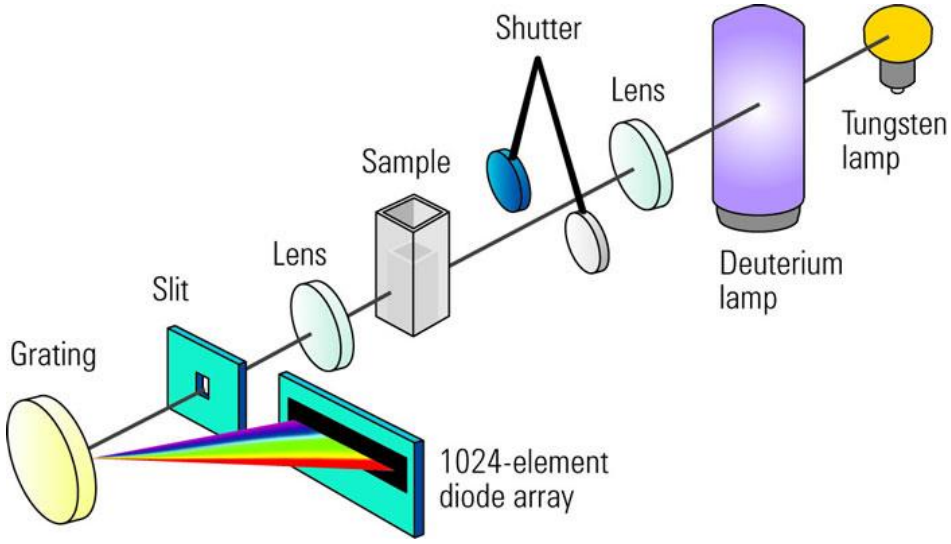


Sugar/alcohol HPLC chromatogram expected in the frame of the exercise

b. Determination of organic acids by HPLC with UV Diode Array Detector (HPLC-DAD)

For the determination of organic acids by HPLC, a standard curve is first constructed with standard solutions of various organic acids (lactic, acetic, citric, succinic, etc., according to the instructions of the supervisor) in well degassed and filtered high purity water. The **isocratic** determination is performed on a Jasco LC-2000 Series HPLC system chromatograph (Jasco Inc., Japan) with a Biorad Aminex HPX-87H column (300 x 7.8 mm id, 9 μm particle size), CO-2060 PLUS oven, PU-2089 pump, AS 2050 PLUS auto sampler, and MD-2018 Photodiode Array detector. The separation is done at 50°C with mobile phase 0.008 N H_2SO_4 at a flow rate of 0.6 mL/min. Detection is done at 210 nm. Sample amounts of 20 μL are analyzed after filtration with 0.22 μm syringe microfilters.

Note: A **Diode array detector** is an apparatus used to detect organic compounds and consists of a two-dimensional pattern of diodes and a prism. During the HPLC analysis, as the sample passes through the detector cell, it is illuminated with light in the region 190-1100 nm and any light transmitted through the sample is dispersed by the prism, so that light of different wavelengths falls on different diodes. The output from the array is used to construct an absorption spectrum that can be compared with standard spectra for identification purposes. Alternatively, a single diode can be used to monitor a specific wavelength at which there is maximum absorption (The Royal Society of Chemistry, 2023).



Photodiode array detection schematic
(https://lab-training.com/benefits-of-photodiode-array-detection-over-conventional-scanning-detection//)

A typical photodiode array with **1024** elements can split the emerging light from the cell into 1024 separate segments, i.e. each diode can measure light in a wavelength range of <1 nm and each individual wavelength in the selected range can be measured individually if required. The UV range is 100-400 nm. DAD detectors range may be 190-950 nm (e.g. a deuterium lamp emits in the UV region 190-400 nm and a tungsten lamp emits in the visible and near-infrared region of 400-950 nm).

Exercise 10. Analysis of alcoholic beverages with gas chromatography with flame ionization detector (GC-FID) and adulteration detection

INSTRUCTIONS FOR THE SAFE CONDUCT OF THE EXERCISE
- Wear protective glasses throughout the experimental procedure.
- Gas chromatography: any regulation/handling concerning exercise gases (N_2, H_2, Air) is strictly done by the teaching staff.

a. Determination of ethanol and methanol by GC-FID

For the determination of ethanol and methanol in carbonated alcoholic beverages by GC, a standard curve is first constructed with standard solutions of 50-200 mg/L methanol and 1-10 % ethanol, and with 0.05 % v/v butanol-1 as an internal standard.

The determination is done on a SHIMADZU GC-8A gas chromatograph with flame ionization detector (FID) connected to a SHIMADZU C-R6A integrator. The combustion gas in the detector is a mixture of pure hydrogen and oxygen gases at pressures of 0.6 and 0.2 kg/cm, respectively. The chromatographic column is a Porapac S type, 2 m long stainless steel, 1/8 inch ID. Column temperature is 130-180^{o}C, programmed to increase at a rate of 3^{o}C/min. The temperature at the sample injection port and detector is 210^{o}C. High purity nitrogen with a flow of 60 mL/min is used as carrier gas. Sample injections (2 μL) are made directly into the chromatograph without dilution.

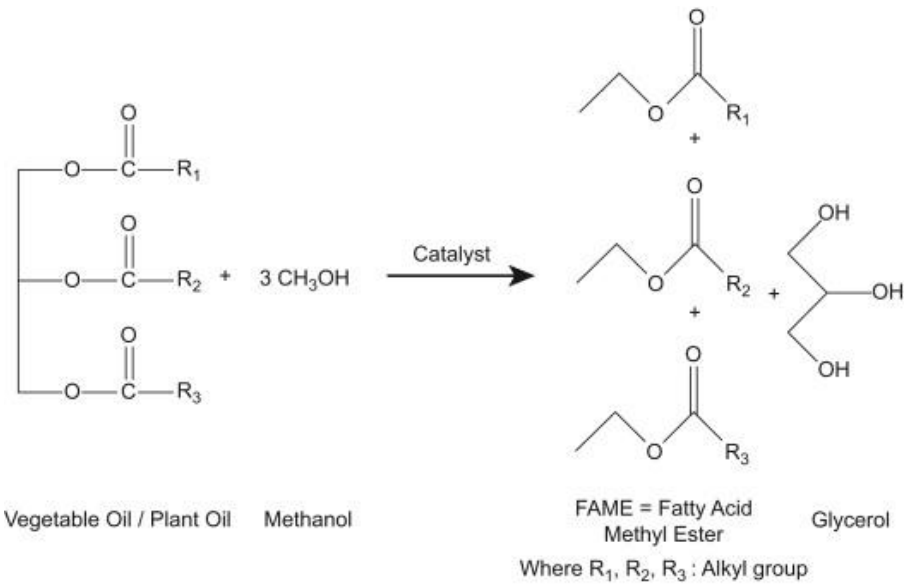
Exercise 11. Transesterification of fatty substances

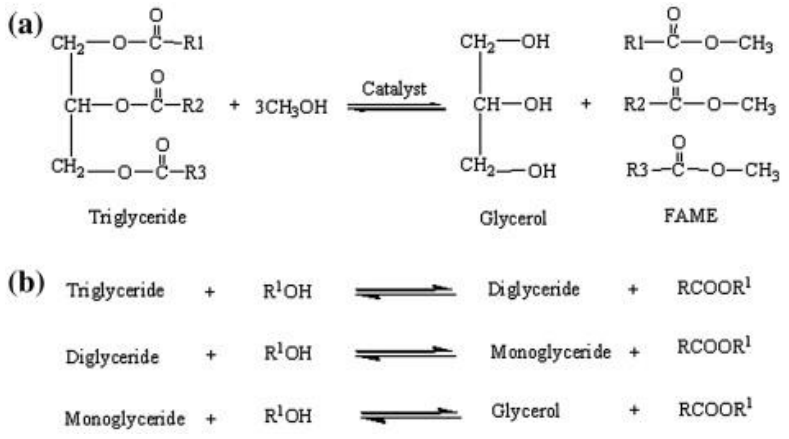
INSTRUCTIONS FOR THE SAFE CONDUCT OF THE EXERCISE
- Be careful to use concentrated acids and solvents according to the general instructions.
- Ether and alcohol away from flame.
- Wear protective glasses throughout the experimental procedure.
- GC: any regulation/manipulation concerning the gases of the exercise (N_2 H_2, Air) is strictly done by the teaching staff

Principle

To analyze fats and oils with GC, the samples must be processed to convert glycerides into saturated and unsaturated fatty acid methyl esters (***derivatisation***).

The treatment is done with methanol according to the reaction (***transesterification***):





Glyceride transesterification
(https://www.sciencedirect.com/topics/chemical-engineering/transesterification)

The above reaction is necessary because the direct injection of crude oils is not recommended due to the low volatility but also the presence of many glyceride isomers, which give separate peaks and confuse the chromatogram. The high content of glyceride isomers is attributed to the many possible combinations that can be achieved by esterification of various higher fatty acids with the three hydroxyl groups of the glycerol molecule. Through this transesterification reaction the formation of methyl esters reduces the number of different compounds in the mixture and hence the peaks in the chromatogram are fewer.

Each oil results in a specific qualitative and quantitative composition of methyl esters, which is characteristic and can be used in analysis to decide whether a sample is normal or not.

a. Transesterification of olive oil and analysis of methyl esters by GC-FID

Reagents

A mixture of a total volume of 230 mL consisting of methanol and benzene 3:1 v/v is prepared. 0.8 g of 4-toluenesulfonic acid (p-TSA) are then carefully diluted into it.

Procedure

In a 200 mL round-bottomed flask, weigh 1 g of olive oil or seed oil and then add 60 mL of the above reagent. Connect the flask to a vertical condenser and heat in a water bath for 2 hours. It is then cooled and transferred to a 250 mL separatory funnel. 100 mL of distilled water is added and then extracted twice with 50 mL of petroleum ether (b.p. 30-60°C). The extracts are washed several times, each time with 20 mL of deionized water, until the acid is completely removed. Then, an amount of sodium sulfate is added to dehydrate the solution, and then the liquid is transferred to a round bottom flask to remove the solvent with a rotary evaporator. The residual viscous liquid consists of fatty acid methyl esters. It is diluted with toluene to prepare a 0.1-1% solution. Toluene contains 0.7 g/100 mL of anthracene as an internal standard added just prior to use. This solution is injected into the chromatograph (injector) and the chromatogram of the unknown sample is obtained.

Similarly, a standard solution of known amounts of **methyl oleate** and **methyl palmitate** is prepared, using toluene as solvent and **anthracene** as internal standard. The latter is diluted to a concentration equal to that used for the unknown sample. The total concentration of methyl esters should be 0.1-1%. Directly inject this solution into the injector port of the instrument and obtain the chromatogram.

Assuming that the concentration of methyl linoleate is equal to that of oleate, the concentration of methyl linoleate is also calculated.

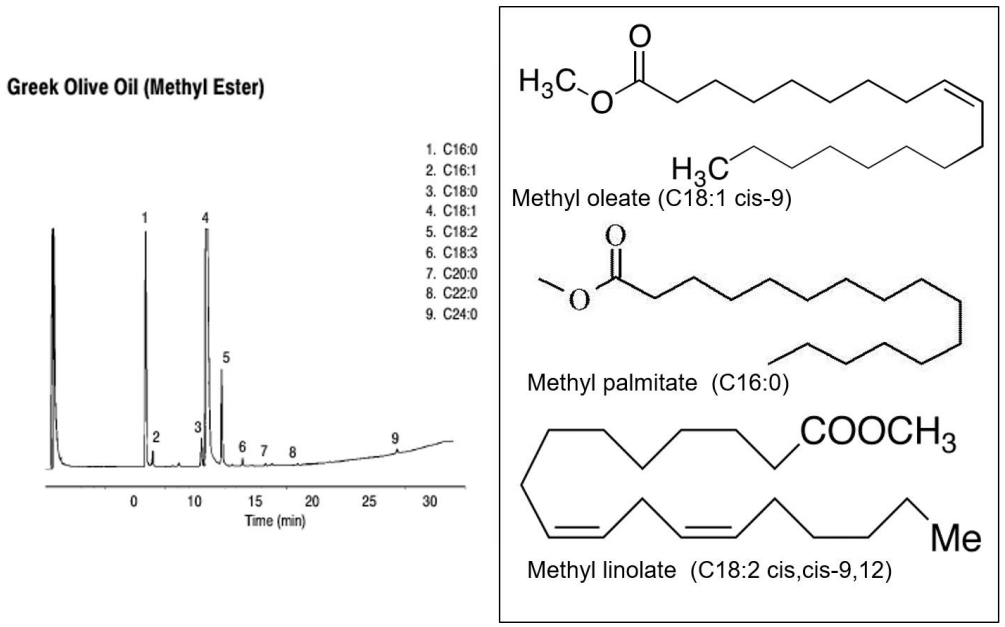
Gas Chromatography

Methyl esters are determined on a Shimadzu GC-8A gas chromatograph connected to a C-R6A Chromatopack integrator. A FFAP type stainless steel column is used for the analysis of olive oil and vegetable oils (suitable for esters). It consists of

adsorption of 10% FFAP (Free Fatty Acid Phase) on Chromosorb W AW 80-100, 3 m long. The carrier gas is pure N_2 with a flow of 20 mL/min. The temperature at the injection port and detector is 250°C. The initial column temperature is 160°C and increases to 250°C by 6°C/min. The internal standard is anthracene at a concentration of 0.7% w/v. 2 μL aliquots of the samples are injected directly onto the column.

Chromatogram data

The chromatogram of the standard solution gives the retention time and area for each peak. The peak of each component can be identified after injection of each component separately and is used for quantitative and qualitative determination of the unknown sample.



Calculations

Quality analysis

The retention time for methyl palmitate is 12.1 min while the oleate elutes in 15.3 min (standard solution). The unknown sample is analyzed by combining the retention times of these compounds in the standard solution and in the sample.

Quantitative analysis

The ratio of the peak area of each methyl ester to the peak area of the anthracene in the standard solution is used to quantitatively analyze the unknown sample (% w/v concentration of the methyl esters). This ratio corresponds to the known concentration of the methyl ester in the standard solution, e.g. 0.2% for methyl oleate and 0.7% for palmitate.

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