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Identification and comparison of volatile carbonyls in different malts and barley using low pressure extraction with HPLC-DAD-MS/MS

Master thesis

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Abstract:
The malt is one of the key entering materials needed for the production of beer, which provides specific organoleptic characteristic to the final product. Nowadays there are many types of malts with various flavours and aromas. These qualities malts acquire during malting of raw barley seeds, especially during the process of roasting, where based on used conditions organoleptic characteristic is formed. Volatile carbonyls, present in high concentration in malts, are among others responsible for these specific malt qualities. The main aim of this thesis was to optimise conditions for the analysis of volatile carbonyls and to figure out if present carbonyls can be analysed by the low pressure extraction with the solution of derivative 2,4-dinitrophenylhydrazine and subsequent identification and quantification by hyphenated technique HPLC-DAD-ESI-MS/MS. Beside that the aim was to compare signals of carbonyl found in different samples with typical organoleptic properties of each malt provided by malt producers. In the next step we were focused to compare presence and intensity of signals of identified carbonyl compounds among all available samples and to find some compounds, which can be used as typical markers for some kinds of specialty malts. The presence of these markers relate on conditions during the roasting process.

Keywords: barley, malt, volatile compounds, carbonyls, low pressure extraction, HPLC-DAD-ESI-MS/MS
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Abstrakt:
Slad je jednou z klíčových surovin při výrobě piva, která finálnímu produktu dává specifické organoleptické vlastnosti a v pivním průmyslu je v současnosti používáno velké množství různých sladů, které se liší svou chutí a vůní. Tyto typické organoleptické vlastnosti získává slad během sladování původního ječmene (případně jiné vhodné obiloviny) a obzvlášť v kroku pražení, kde se podle zvolených podmínek vyvíjí právě organoleptické vlastnosti sladu. Mezi látky zodpovědné za tyto vlastnosti patří mimo jiné také těkavé karbonyly, které jsou ve sladu přítomné ve velkém množství. Cílem této práce bylo optimalizovat podmínky pro analýzu těkavých karbonylů, zjistit, zda přítomné karbonyly lze analyzovat pomocí zvolené extrakce za sníženého tlaku za použití roztoku 2,4-dinitrofenylhydrazinu a následné identifikace a kvantifikace pomocí spojené techniky HPLC-DAD-ESI-MS/MS. Kromě toho jsme porovnávali signály karbonylů nalezených ve vzorcích s typickými organoleptickými vlastnostmi jednotlivých sladů uváděných výrobci těchto sladů. Následně jsme se zaměřili přítomnost a sílu signálů identifikovaných karbonylů mezi jednotlivými vzorky a hledali sloučeniny, které by mohly sloužit jako typické markery pro některé typy speciálních sladů. Přítomnost a vznik těchto markerů závisí na podmínkách pražení při výrobě sladu.

Klíčová slova: ječmen, slad, těkavé látky, karbonyly, extrakce za sníženého tlaku, HPLC-DAD-ESI-MS/MS
Počet stran: 79
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Table of contents

I. Aims of the thesis

1) Optimization of an extraction methodology followed by HPLC-DAD and HPLC-ESI-MS analysis for determination of volatile compounds from barely and malt
2) Comparison of volatile profile of barley and different types of malt with different degrees of roasting
3) Study the influence of roasting and caramelization of the development of aroma compounds in different malt tissues (husk and remain grain)

II. Introduction

In this final thesis I will describe and compare the presence and intensity of signals standing for various present volatile carbonyl compounds inherent in a barley and different types of malt samples (black, caramel, carared, chocolate, münich, pale ale, pilsner and wheat). Reasons of having different carbonyl compounds profiles in different samples of malts and barley based on conditions of malting process for these samples will be also discussed.

Typical aromas and flavours of malts are mostly evolved during the malting process (especially in the final kilning/roasting step), in which conditions differ in relation with the used barley or with the type of required final malt product. It is mostly a temperature responsible for these differences which has the significant impact on the final malt properties like its moisture, colour or even the overall typical flavour. Organic compounds like esters and carbonyls are mainly responsible for these unique flavours and aromas. For this work only volatile carbonyl compounds were chosen to show the relation between organoleptic characteristics of these observed compounds and flavours and aromas typical for each available malt.

In this work I will try to confirm, that even slight nuances in conditions during the malting process for different types of malt may stand for bigger or smaller differences in the overall profile of volatile carbonyl compounds present in the malt. However the main aim of this work is to find specific volatile marker compounds with some significant differences when comparing some kinds of malt samples in connection with different conditions during the malting process. The correlation between real properties of compounds present in the sample and expected properties will be discussed, too.

To create the complete profile of aroma compounds in all malt samples and the barley, optimised method of low pressure extraction was used to extract volatile carbonyls from samples and subsequently bind them into the solution of 2,4-DNPH derivative. Then HPLC-DAD technique was used for indirect detection of present 2,4-DNPH derivatives of volatile carbonyl compounds. Finally, to obtain all needed information, HPLC-ESI-MS/MS connected technique was used for the final identification of present volatile compounds in all studied samples.

III. Theoretical part

1. PROCESS OF BEER MAKING

1.1. Introduction

The beer is very traditional and nowadays a very popular worldwide spread and produced alcoholic beverage. In some works it is presented, that beer has been produced since thousands years B.C. in many places. This tasty beverage comes from a fermentation process of a wort, in which hops, the malt (mostly made from barley grains), a yeast and of course water in needed quality take its place.

1.1.1. History of the beer production

The very first mention about the beer brewing comes from 6 - 4 thousand years B.C., when old earthen tables set up the first law for prescribing brewing taxes coming in a former Babylon empire. In fact it was probably made in that area about 7 thousand years B.C. It is also actually proved, that beer was made all around the world from empires in China and Egypt to the European nationalities like Greeks, Germans or Slavs. (Hornsey, 1999; Kosař et al.; 2013) The first mention about hops in connection with the brewing of the beer in Bohemia lands comes from the year 1088 with following documents from 11th and 12th century about a production of hops, brewing, beer taxes and even about export laws. Despite that it is very probable, that beer was brewed in the Czech countries before these first official documents. (Kosař et al., 2013)

Even though the Czech Republic is currently considered to be one of the world's most traditional beer producers, it was necessary to overcome a very deep prostration start from 17th century due to a war conditions and having the biggest crisis in the 18th century. Luckily the edification saving brewery tradition came with two big personalities. Namely it was at the end of 18th century the brewer F. O. Poupě who improved the process of brewing and established the first school for brewers in the Czech lands. The second big name was the professor of technics K. N. Balling in 19th century explaining the closer

relations and chemical changes during the brewing process. After getting over that crisis thanks to science and progress it was possible to understand the mechanism of the brewing and to start an industrial beer production which successfully continues in the Czech republic till nowadays. (Kosař et al., 2013)

1.1.2. Beer production and consumption

China is nowadays with almost 40 billion litres of produced beer in year 2018 the biggest beer producer worldwide with a huge gap from the second biggest producer United States with only approximately a half of the China's production. (Figure 1) Czech republic is in this ranking on 21st place with the production of beer at almost 2 billion litres in that year. Global production of the beer, despite of a decrease in production in China and US, was in 2018 over 191 billion litres of this alcoholic beverage. In overall it stands for 0.6% increase of the production in comparison with the previous year and 4.8% increase in comparison with the year 2008. (from www.kirinholdings.co.jp)

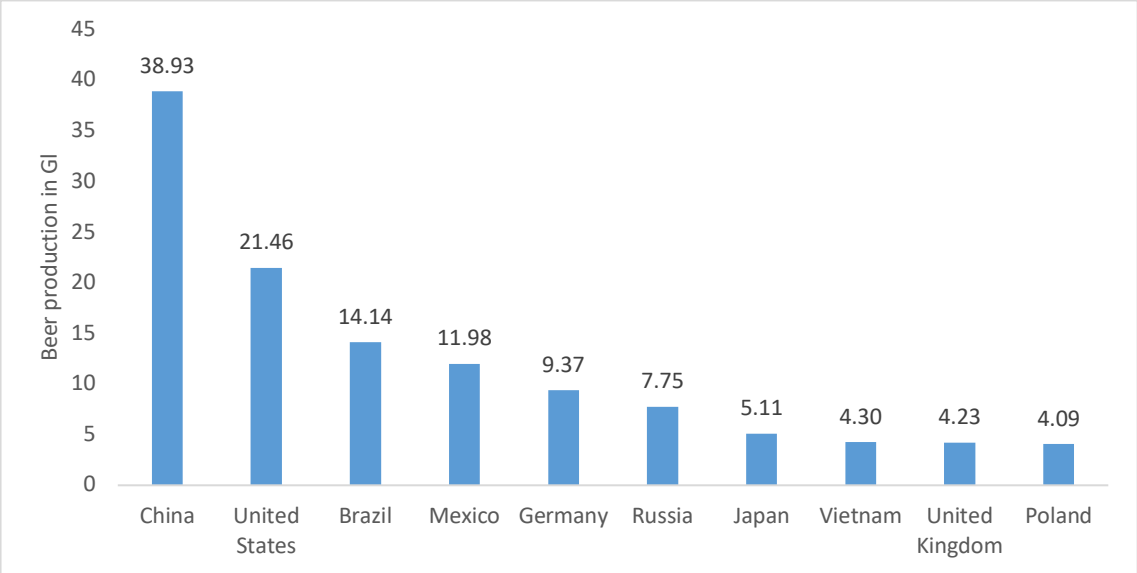


Figure 1: Top 10 worldwide beer producers in gigalitres of beer produced in 2018 (based on data from www.kirinholdings.co.jp)

Although the biggest popularity of beer is set in European countries which represent 8 countries presented by the latest data world's top 10 chart of the biggest beer consumers,

getting through consumed litres of beer per one capita per year (Figure 2). In this chart based on the latest data from 2018 the Czech republic stands on the first place with the consumption little more than 143 litres per capita. On the other side United States, the second largest beer producer, is ranked with 74.8 litres of the drunk beer per capita on 21st place. Despite an enormous popularity in Europe the beer is drunk all around the world. (based on data from www.worldpopulationreview.com)

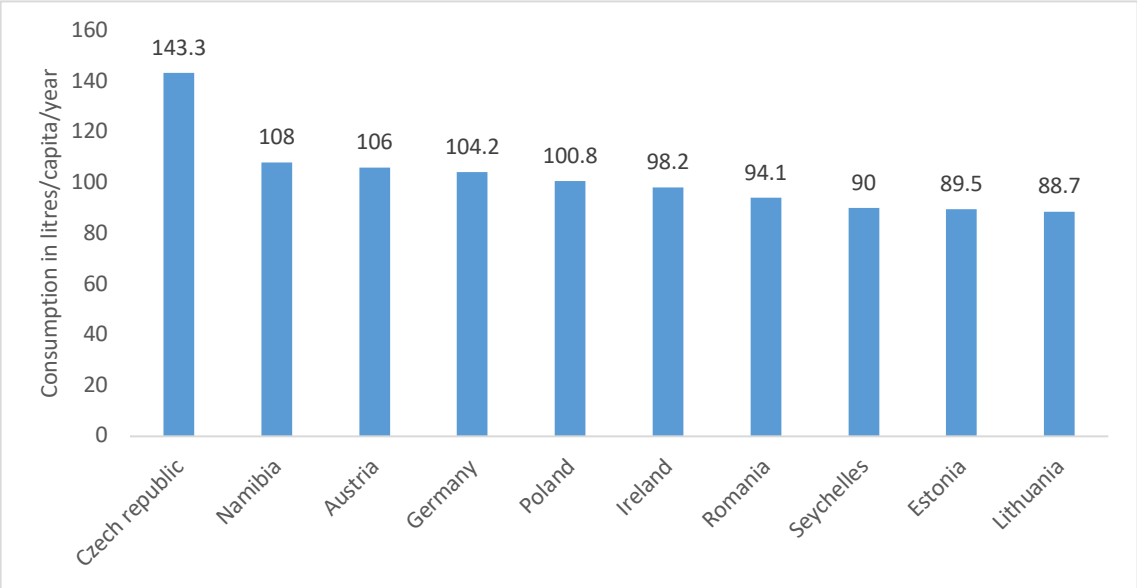


Figure 2: Top 10 country consumers of beer per capita of beer worldwide in 2018 (based on data from www.worldpopulationreview.com)

1.2. Brewing process

A brewing stands for a very complex process consisted of many particular steps as malting, milling, mashing, fermentation, maturation, filtration and packaging. (Figure 3) Raw seeds, hops, yeast and water come in different stages of production as entering ingredients providing to the beer its typical attribute as the taste, bitterness, the volume of alcohol and carbonation with carbon dioxide. At the end of brewing, the packed bottled beer comes as a final product of the whole process. (Encyclopaedia Britannica Inc.)

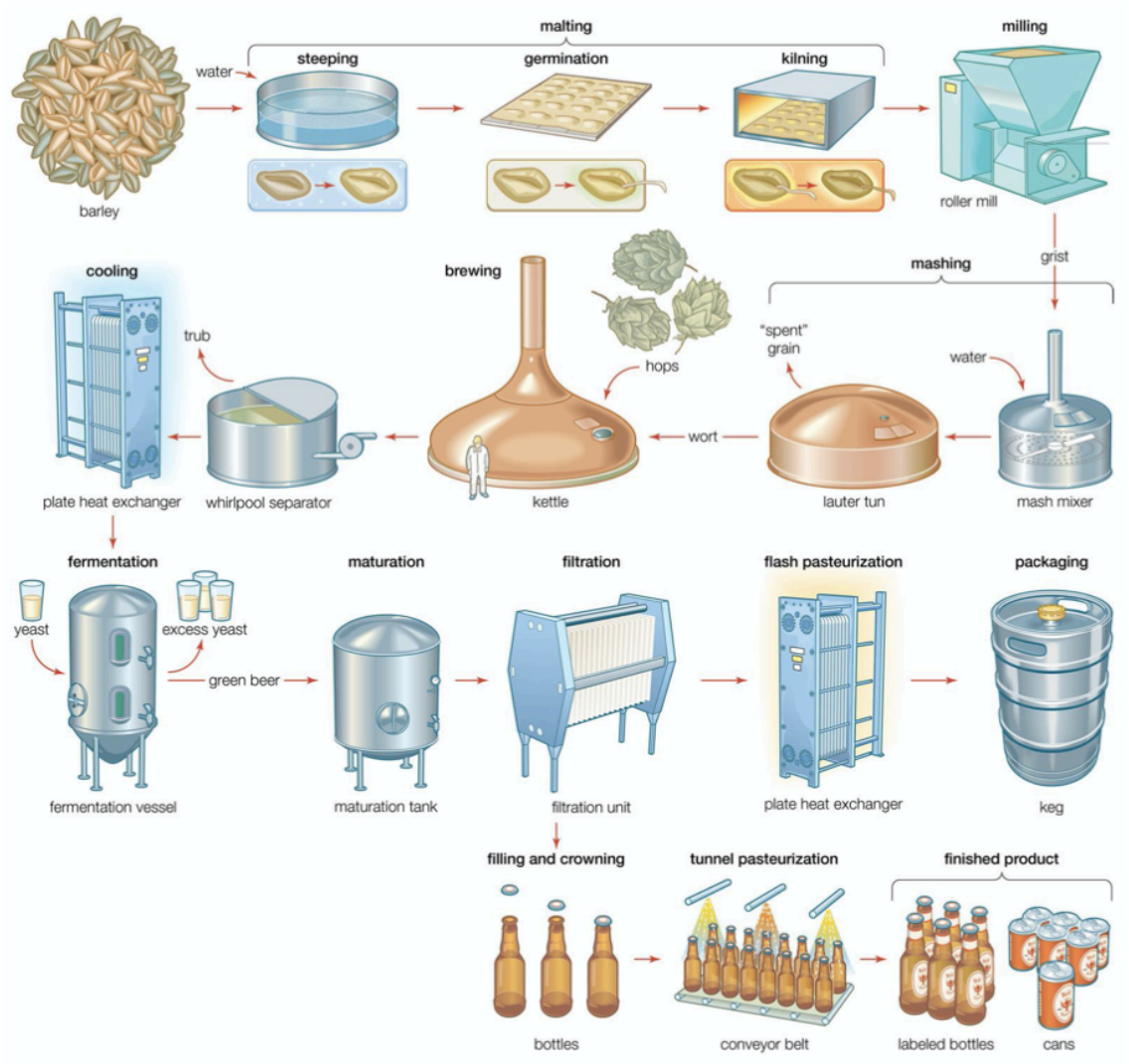


Figure 3: Scheme of the brewing process step by step (from Encyclopaedia Britannica, Inc.)

1.2.1. Malting

In the malting process a raw barley or other less often used cereal species (wheat, rice, etc.) seeds are processed to become a sugar enriched malt, which is mainly responsible for the main colour and aroma of the beer. (Hornsey, 1999) Besides that antioxidant properties of malt it is also important for the oxidative stability of beer. (Carvalho et al., 2016b)

As the malt and malting process is the main content of this work, I will further focus on this important step in its own separated chapter.

1.2.2. Milling, mashing and separation of the wort

During this step the malt from malting process is milled and then mashed with hot water to provide a wort for further fermentation. As the result of malt milling is a destruction and separation of the outer husk and the endosperm which contains all the saccharides and enzymes.

After this separation the mashing with a water comes. In this particular step reductive enzymes (proteases) and enzymes converting polysaccharides into fermentable sugars (α-Amylase and β-Amylase) are activated.

Then the filtration to separate the liquid wort extract from the rest of milled grains particles is run. (Carvalho, 2016a)

1.2.3. Boiling of the wort

In this stage of brewing, the wort gained after the extraction is boiled and the hops are successively added to the melange. During the boiling of this mixture enzymatic activity of reductive enzymes in the wort is stopped and at the same time compounds responsible for bitterness and some other typical beer flavours and aromas are extracted from hops.

When the boiling is done, hop residues are separated from the wort. After cooling of the wort, yeast (for brewery the yeast from the family of Saccharomycetacea is used) is added at a proper temperature and extra oxygenation is provided for yeast growth. (Carvalho, 2016a)

1.2.4. Fermentation

The yeast added in the previous step starts to transform fermentable sugars into an alcohol with carbon dioxide as a side product of the process. Fermentation is generally performed in two different ways. Specific yeast types are used for the bottom-fermented or the top-fermented beer and this choice may affect colour and flavour of beer. During this step it is very important to care about the temperature, pH, presence of nutrients and the volume of oxygen to provide the best possible conditions for fermentation and the best possible final product since this step has a significant impact of its aroma and taste qualities.

At the end of this stage inactivated yeast cells are collected and removed. (Carvalho, 2016a)

1.2.5. Maturation

After the first fermentation there comes the secondary fermentation in the maturation step. In this step temperatures are significantly lower and remaining yeast continues to dissolve the rest of fermentable carbohydrates remained in the beer or possible addition of priming sugar. The main purpose of maturation is to remove the residual oxygen, carbonization and maturing of aroma and flavour. (Carvalho, 2016a)

1.2.6. Filtration and bottling

By the filtration all residual yeast and other solid particles are removed. Even proteins and polyphenols responsible for possible sedimentation are removed during the filtration step using amorphous silica gel and polyvinylpolypyrrolidone (PVPP).

After this step is done, beer is carbonated by CO_2 for enhancing its shelf life and also to increase its foaming potential.

Bottling is the last and final step of the whole brewing process. In this step thermal pasteurization of the beer is undertaken to provide microbiological stability of the product. (Carvalho, 2016a)

1.3. Malting process

It is a malt, what mainly gives a typical colour and organoleptic characteristic to the final beer product, and the malting is in general a complex process serving us for creating the needed malt of some quality from original cereal grains. For the brewing industry the mostly used cereal for malting is barley, however sometimes also wheat, oats, maize, rice or sorghum grains can be used for the purpose of preparing malt for beer making. (Hornsey, 1999)

During the malting process some changes appear in the activity of enzymes responsible for hydrolysing a starch into fermentable sugars. During this procedure the structure and the composition of the grain changes as the grain keeps growing. Also, inner transformations of the starch and present proteins connected with a growth of the grain are happening. All of these changes in the grain metabolism are done because of extensive differences in temperature conditions and different humidity obtained in the grain during 3 basic stages of the malting process.

Basically these 3 essential levels of malting process could be presented as steeping (increase of humidity in the grain and start of germination and growth of the grain), germination (continued extensive growth of the grain, enzymes development and starch dismantle with a green malt as a product) and kilning or roasting depending on the temperature used during this step as the final stage (removing the humidity, stopping the metabolism of the grain and its final treatment by using high temperatures). In every stage of this process occur a different temperature and humidity conditions. (Figure 4) These conditions are specific and in some way could differ for every single kind of processing malt depending on the quality of entering barley or other less used cereal. (Hornsey, 1999)

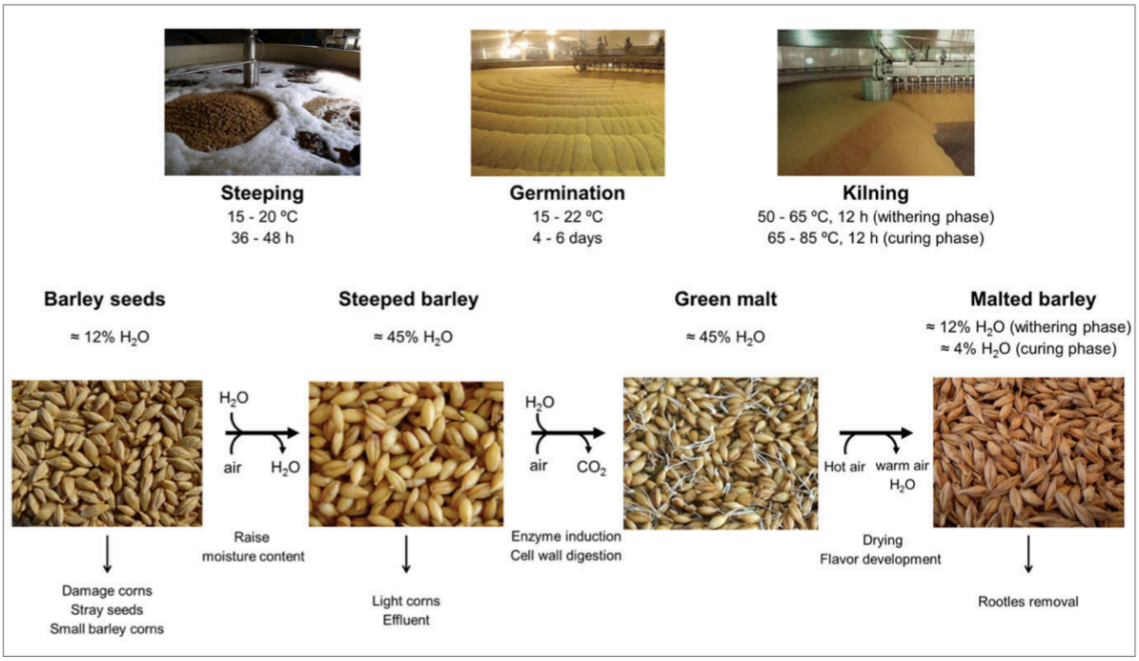


Figure 4: Overall scheme of all of steps in the malting process and pictures of grains at different stage of malting (Carvalho et al., 2016b)

1.3.1. Steeping

Steeping is the first and nowadays considered to be the most important step of the whole malting process mainly responsible for the final quality of made beer. The main purpose of steeping is to increase the volume contain of water in the grains from 12 - 15% to 42 - 44%. The final volume of the water differs depending on different wanted qualities of made malt. The whole steeping process takes place in a big specially developed tanks which are recently usually made from stainless steel. (Kosař et al., 2013)

This increase of water in the grains is mainly made for activating enzymes present in the grains to start typical enzymatic reactions leading to grains germination. The other, and also very important, purpose of the steeping part in the whole malting process is to wash and clean grains from some light dirt and also to extract possible unwanted compounds present on their surface. (Kosař et al., 2013)

Currently the most often used steeping technology is the air steeping. In this technology grains are soaked in three followed-up steps and between each of these steps there is some time for grains to rest called an air break. In every of these three steps a different time of the steeping when the grains are sunk into the water and also a different time of the airy break are used. It is necessary to suck out CO_2 during the airy break to

support breathing of the grains. The suction of CO_2 depends on the temperature in the steeping tank - the higher the temperature, the higher the suction of CO_2 to support the breathing of steeped grains. (Kosař et al., 2013)

Other not that often, but also commonly used technologies are flushing steeping, repeated steeping, shower steeping and classical steeping. Usually choosing of the right technology is determined by the quality of a barley used in a malting process. (Kosař et al., 2013)

1.3.2. Germination

The main aim of germination in the malting process is activating and synthetizing of enzymes, and also from that to achieve some required inner changes in the structure and composition of the grain. To obtain these changes the grains are steeped in a water usually for 4-6 days in a specific humid and temperature conditions. The growth of rootlets is used as the controlling detector of the germination process. The length of the root at the end of germination part is expected to be between 1.5 - 2 times of the original grain size. (Goldammer, 1999)

Phytohormones are mainly responsible for this increase of the enzymatic activity or synthesis of completely new enzymes. For this process it is very necessary for grains to have enough energy for activating the enzymes or synthetizing of the completely new enzymes. Energy for that is obtained from oxidative decomposition of the stocked compounds and with the necessity of enough of the oxygen present.

The most important enzymes taking place in the germination step are the amylases, which are mainly responsible for dissolution of the stored starch. Although β-Amylase is also in its inactive form and less concentration present in the raw barley grain, there is enormous increase of its activity after the second day of the germination process. On the other side α-Amylase is the enzyme completely newly synthetized in the grain since the second day of germination. Normally the longer the germination process is, the higher content of amylases in the grain is. The overall content of amylases in the grain generally depends on the species of used barley and his quality, which can be affected by climatic conditions or the area of the barley plant growth. The content also depends on the water in the grain and on the temperatures used for the germination, when higher temperature

makes the grain to grow faster and also to produce more enzymes. However the lower temperature leads to bigger amount of the enzymes.
Because of that the germination process usually starts at high temperature around 18-20°C to produce and activate enzymes in the grain. After a few days the temperature decreases to a lower temperature e.g. 13-14°C to slowdown the growth of the grain and keep the enzymes active. (Briggs et al., 1981; Kosař et al., 2013)

The starch in the germination is used as the main source of energy for the growth of the germ. During this process 5- 6% of starch is dissolved and the content of present sugars (especially glucose, fructose and sucrose) increase. (Kosař et al., 2013)

At the same time as there are enzymatic processes taking its part, proteins are depleted to build needed enzymes or completely new tissues of the germ. For a purpose of a beer making it is very needed that high-molecular proteins are dissolved thanks to protease (endo and exoproteases) enzymes to smaller soluble low-molecular compounds like oligopeptides or amino acids.

To achieve the best possible results from the germination the process it is very necessary to keep the humidity in the grains same for the whole time (cca 45%). Success of the germination is also affected by using different temperatures and different ratio of present oxygen and carbon dioxide.

At the end the green malt is the product of this stage. This green malt is very unstable and because of all the reactions still going inside the grain it is necessary to transform it to more stable product which will be possible to store. (Kosař et al., 2013)

1.3.3. Kilning/roasting

Kilning is the last and final stage of the malting process. In this step the green malt is taken from the previous step and by using high temperatures it is transformed into a more stable form which is possible to stock. Besides that kilning stage is also responsible for creating specific colour and flavour properties to every single kind of malt based on the temperature used and the overall time of kilning. From these different properties given in this step, the diverse malts are used for different organoleptic needs. (Kosař et al., 2013)

Thanks to high temperature the moisture in the grain is after this process minimalized under 4%. This moisture decrease and high temperature also inactivates present enzymes, which are not able to work in conditions with present moisture below 30% and

temperatures above 60°C. The germination processes in the grain and also the overall growth of the germ in general also stop during the run of this stage.

The kilning is made in two distinct steps. In the first step the green malt is primarily dried out with an air at the temperature approximately at 25°C. After this step moisture content in the grain should decrease to circa 12%. In the next stage grains are dried up with a flow of hot air with temperatures from 60 to 80°C for pale malts (lager) and from 100 to 250°C for dark (special) malts. During this step moisture in the grain is removed to 4%. At the end of this drying step there is a curing step, where the temperature of kilning is increased. This ending stage is responsible for the final differences in flavours and tastes typical for every single malt type. (Kosař et al., 2013)

1.3.4. Impact of roasting and kilning on a final beer product

It is generally known, that during the step of kilning the specific odours, colours and flavours are evolved in a close relations with different time and temperature conditions used in this step of malts preparation. Malts are then classified by their final colour which separates them in general into pale malts group (light colour and flavour) and dark malts group (darker colour, burnt and strong flavours often corresponding with products of Maillard reaction). Differences in the colour of malt based on different kilning temperature can be seen in the Figure 5.

Pale malts are in the process of kilning heated in comparison with roasted ones at lower temperatures from 60 to 95°C and are used as the main ingredient for a beer making. That's because of the richness of fermentable sugars present in these malts which are highly needed for the fermentation process during brewing.

On the other hand dark malts are used in less quantity only for bringing some extra flavour and colour to the beer. Dark malts can be separated into three groups - roasted, caramel and colour brew malts. Roasted malts like chocolate and black malt are mostly made by roasting germinated green or already kilned pale malts for at least 2 hours at high temperatures around 220 - 250°C. This process gives them very strong smoky, burnt flavour closely related with the exact time of roasting. Caramel malts can also come from green or pale malt by roasting them more than 2 hours at the temperature up to 160°C, which gives them characteristic coffee-like, caramel flavour. The third group of colour

brew malts (e.g. münich malt) uses for kilning only green malt and temperatures just around 100°C what gives them typical grainy and malty taste.

Besides the impact on flavours in this stage there is also significant increase of antioxidative phenolic compounds which are responsible for the chemical stability of the beer. This accrual provides around 80% of all phenolic compounds obtained in the beer. In comparison dark malts contain more phenolic compounds than pale ones. (Kosař et al., 2013)

Figure 5: Different colours of various malts depending on different temperatures of roasting/kilning (from blog.distiller.com)

2. GRAIN AND ITS CONSTITUTION

2.1. Structure of grain

In the brewery commonly used barley species is barley NIC9 (Hordeum distichum) with double or six-row composition. Grains from this type of barley are accreted to a spike shank, so an awn is parallel.

The grain, which is the main and only part used for needs of the brewing process, consists of three basic parts - seed coat, endosperm and embryo. (Figure 6)

The embryo is a part, from which a new barley plant will grow, when the grain is placed in ideal conditions (hydration and suitable CO_2 atmosphere for the cell breathing).

The endosperm is the biggest part of the grain. On the top of the endosperm is an aleurone layer. This layer is composed of cells arranged in a row. These cells contain proteins, fats and also amyloid grains which are metabolized when the grain is faced to ideal conditions and the germination process begins. In the inner part of the endosperm cells with thin layer occur. This type of cells contain a stock starch. (Kosař et al., 2013)

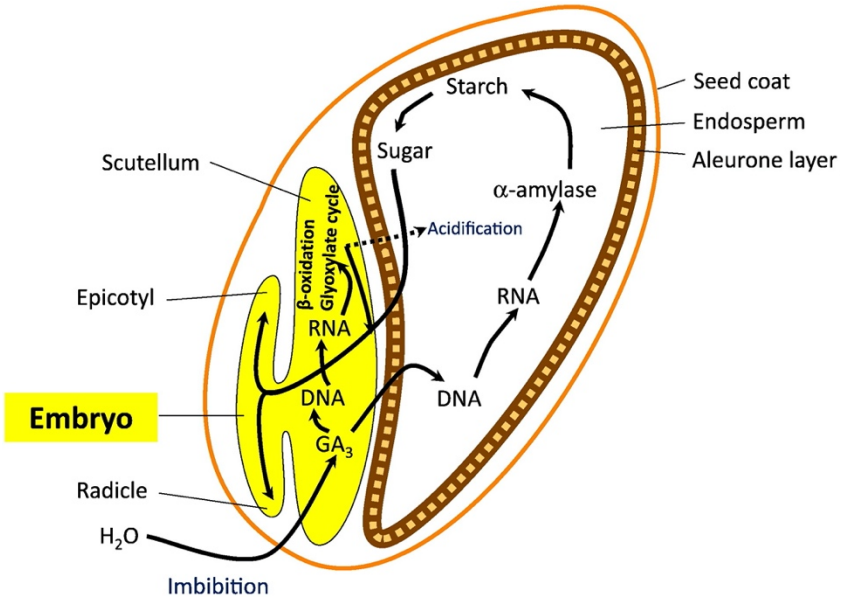


Figure 6: Scheme of the barley grain and inner processes during germination step (Ma et al., 2017)

2.2. General overview of chemical composition of the grain

2.2.1. Inorganic compounds

Inorganic compounds (e.g. P_2O_5, K_2O or SiO_2) are mainly important for the regulation of biosynthesis process of high-molecular compounds and they stand for 2 - 3% of the overall dry mass and are mainly located in the aleurone layer. Beside these compounds inorganic trace elements play also important role as cofactors in the activity of some enzymes complexes creating very active biologic complexes - coenzymes. The most trace elements contained in the grain are for example zinc, manganese, bore or cuprum. (Kosař et al., 2013)

2.2.2. Saccharides

Saccharides are with a 80% of presence mostly contained compounds in barley grains. The starch, used as a basic supply for the growth of the grain, is the major and for malting also the most fundamental polysaccharide (60 - 65% of overall grain weight) composed from together bound molecules of amylose and amylopectin in various ratios depending on the location of the starch storage. Other very important and hardly dissoluble polysaccharides are cellulose which is the main structural material of the glume and hemicellulose responsible for reinforcement of the cell wall. From polysaccharides it is also worth mentioning β-glucane composed from glucose molecules with different types of binding and pentosanes mostly built up from arabinose and xylose placed primarily in the cell walls.

Furthermore the grain also contains less present low-molecular saccharides like sucrose, maltose, raffinose, glucose or fructose which presence mostly depends on the state of the grain maturity. (Kosař et al., 2013)

2.2.3. Fats

Fats, especially present in a form of triglycerides, are in the grain considered to be minor compounds (only 2 - 3% of the grain dry mass) placed mostly in the aleurone layer and the glume. Only around 30% of fats are present in the germ and are used mainly for the substance exchange during the cell breathing in the malting process. Some of these fats remains in the wort and can affect final flavour and foamy characteristic of the beer. (Kosař et al., 2013)

2.2.4. Nitrogen compounds

Nitrogen compounds stand for 10 - 11,5% of the grain dry mass in a form of amino acids, peptides and proteins. However the overall contain of nitrogen compounds is closely related to outer conditions (e.g. cereal variety, fertilization or climatic conditions).

All these compounds are synthetized during enzymatic reactions from ammonia gained from outer sources and organic acids coming from the cleavage of saccharides. Proteins are present in various grain tissues like in aleurone layer, in the exterior side of the endosperm or even in the structures of endosperm cell membranes. (Kosař et al., 2013)

2.2.5. Enzymes

Enzymes are for malting very important compounds responsible for initializing changes in the structure and for the growth process of the germ. Enzymes are in general macromolecular protein molecules serving as the catalyst for various types of chemical reactions in which enzyme-substrate complex is created and the whole reaction is then speeded-up. The speed of creating enzyme-substrate complex can be affected by many factors (e.g. pH and temperature). (Kosař et al.; 2013)

During the malting present amylases are enzymes responsible for the release of fermentable sugars from starch molecule by yeast. (Bertuzzi et al., 2020)

2.2.6. Phenolic compounds

Phenolic compounds are also brought into beer in used cereal grains. However beside grains, hops contain a big amount of these compounds too. It is proved, that the volume of phenolic compounds in the final beer product affects its taste and colour. (Bertuzzi et al., 2020)

Tannins and flavonoids - the main representatives of phenolic compounds are due to their strong redox potential commonly known as antioxidants preventing cells from oxidative stress (Fumi et al., 2010). It is also proven, that these compounds may also have a positive impact on a human's health if the beer is drunk in a moderate level. (Brányik et al., 2012)

2.3. Compounds responsible for diverse flavours in malts

The most significant compounds responsible for differences in the aroma and overall flavour of beer product are considered to be volatile organic compounds like higher alcohols, esters, carboxylic acids, ketones and aldehydes. (Biazon et al., 2009)

Some of these compounds (like carbonyls acetaldehyde or furfural) are already in some less concentration present in the original raw barley grains, but the majority of volatile compounds are evolved, or their concentration significantly increases during the brewing, especially in the specific step of malting process. (Rossi et al., 2014)

Based on this Carvalho and collective (Carvalho et al., 2016b) discovered that most of volatiles corresponding to a typical flavours of the malt are produced during the malting from a lipid oxidation of fatty acids and also from a Maillard reaction. These compounds are generally called MRPs (Maillard reaction products).

This non-enzymatic reaction between sugar's carbonyl group and amino group provided by amino-acids is going on during kilning stage when using high temperatures. Depending on the availability of both reacting groups in the present malt material and used yeast strain various amount of higher alcohols and esters is produced. It was discovered from the research, that dark malts, which have generally richer flavours contain more products coming from Maillard reaction. (Dack et al., 2017)

3. EXTRACTION

3.1. Principle of extraction methods

In general an extraction is a method used for distribution of one or more compounds between two if possible immiscible phases or solvents in the extractor. The separation is based on solubility of each present substance between those different used phases, or on creation of specific bounds between the compound and the absorbing phase.

Nowadays there are many types of different extraction techniques for separating compounds in all possible environments under all possible conditions. For instance a basic liquid-solid extraction commonly known as an elution, in organic chemistry very often used liquid-liquid extraction, or an ion pair extraction for highly polar compounds. Next and also very popular type of extraction is gas-liquid extraction, which is also known as an absorption. For obtaining the best possible result changes of the pressure or the temperature of extraction techniques are very often undertaken. (Hasal et al., 2007)

The effectivity of extractions may be also affected by differences in used temperature, pressure, sample volume and extraction time, so it is often necessary to perform an optimization of extraction conditions. (Welch, 2003)

3.2. Extraction of volatile compounds

Volatile organic compound (VOC) was defined by U.S Environmental Protection Agency (EPA) as ‘‘any compound of carbon, excluding carbon monoxide, carbon dioxide, carbonic acid, metallic carbides or carbonates, and ammonium carbonate, which participates in atmospheric photochemical reactions’’. Possible examples of VOCs are carbonyls, alcohols, halogenated hydrocarbons, olefins, aromatic compounds, ethers and many others. (Li et al., 2020)

It is usual, that for the analysis of VOCs the first step is to transfer these compounds into gaseous form from the sample before the analysis itself begins. A difficulty of this step and the choice of the proper technique depend on a complexity and nature of the sample matrix. For this purpose techniques like static/dynamic headspace extraction, solid-phase microextraction and liquid extraction take place. (Mitra, 2003)

3.2.1. Static headspace extraction

The static headspace extraction is a simple headspace technique running in a special headspace vial normally used for the extraction from solid even liquid samples, which are placed directly into the extraction vial and don't have to be prepared in any way. After the sample is placed into the vial and the concentration of VOCs is in equilibrium in both sample and headspace, it is blown from the vial into the analysing apparatus (for VOCs it is usually GC). (Mitra, 2003)

3.2.2. Dynamic headspace extraction

This type of extraction is very similar to the static version of headspace extraction, but the difference between dynamic and static headspace is in trapping of compounds in the headspace. In dynamic headspace extraction there is no equilibrium between the matrix and headspace concentration of compounds. Instead of that this technique is using a continual flow of inert gas transporting analytes from the headspace to a trap filled with sorbents. This suction of analytes is creating a concentration gradient, which increases the overall effectivity of the extraction. (Mitra, 2003)

3.2.3. Solid-phase micro-extraction (SPME)

SPME is an extraction technique using a sorbent coated silica fibre instead of solvent, which makes it significantly different from previous two methods. The silica fibre is placed in a syringe needle and the sample is taken either directly from the matrix or from the headspace. After the extraction, VOCs are absorbed, or adsorbed onto a fibre and can be thermally desorbed into GC inlet. The coating of the fibre is provided within various polarity depending on the polarity of analysed compounds. (Mitra, 2003)

SPME as the method for extraction of VOCs in malts is very popular among researches focused on these volatile compounds. Volatile organic compounds were in these articles first directly extracted from the malt sample using the head-space extraction on the SPME fibre. (Vandecan et al., 2010; Dong et al., 2013)

After this extraction, compounds bound to the SPME fibre can be then analysed by using effective GC/MS method. The SPME fibre with caught VOCs can be then simply

injected into the insert part of GC column and then pushed byt inert gas (e.g. helium) through the column. The usage of thermal gradient is necessary for the measurement of volatiles in this method. (Vandecan et al., 2010; Dong et al., 2013)

3.2.4. Vacuum-assisted microextraction

Vacuum-assisted headspace solid-phase microextraction (Vac-HSSPME) is a technique using vacuum/low pressure conditions to improve and accelerate the extraction of volatile and semi-volatile analytes from the solid samples to the headspace at a room temperature and within a shorter time period compared to a normal headspace solid-phase microextraction (HSSPME). This extraction process is taking place in a closable container, which is possible to evacuate and to hold the low pressure for needed period of time. (Psillakis, 2019)

Using higher extraction temperature is a possible way to speed up the extraction (Darrouzès et al., 2005), but the increase of the temperature can have an impact on decomposition of the analyte. (Psillakis, 2019) Furthermore by placing a little fan into the evacuated container it is also possible to shorten the overall time and to increase the efficiency of the extraction without undergoing the risk of the analyte decomposition (Santos and Rodrigues, 2020).

4. HPLC-UV/VIS

4.1. HPLC

High Performance Liquid Chromatography (HPLC) with a liquid mobile phase with dissolved sample in it and a solid stationary phase is nowadays the most common elution chromatographic method for all kinds of analysis worldwide. Due to its wide usage this method is used for all of the possible separations of different types of compounds. It is commonly used for separation, quantification or identification of inorganic, or organic molecules, or also for separations of big biomolecule complexes. (Skoog et al., 2019)

Compounds in HPLC are separated due to various polarity or size of analysed compounds. In the first separation type based on the polarity of molecules, every molecule reacts with the stationary phase of used column differently. Some reacts more and some less, or even don't react at all.

The other possibility is the separation by the size of present molecules which makes also differences in a retention between molecules. These two types of molecule retentions in the chromatographic column show why different compounds come out from the column in different times. (Skoog et al., 2019)

4.1.1. Types of HPLC

There are many different types of HPLC based on the chosen stationary phase and the type of the interaction between the analyte and the stationary phase.

Partition chromatography is used to separate analysed molecules based on theirs different mobility through the stationary phase depending mostly on a size of the analyte and the level of the analyte-phase interactions. The bigger the analyte is, the smaller the interaction is and so is its retention in the column. Based on this the retention time for these molecules is shorter than it is for smaller sized ones.

For ions and highly charged molecules an ion-exchange chromatography is used. In this type of chromatography cations and anions are exchanged between the sample and used stationary phase selected based on a form of observed analyte.

The next and very often used type of chromatography is the liquid-solid chromatography. This kind of chromatography is using for the separation a difference in

the adsorption of analysed molecules in the sample on used stationary phase. The efficiency of the separation process depends on the chosen type of the stationary phase (normal/reverse) and on used eluents in the mobile phase.

Beside these mainly used types of chromatographic methods there are many others HPLC methods used for some specific separations based on the nature of separated material. For instance it could be a hydrophobic interaction liquid chromatography (HILIC), size-exclusion chromatography, a chiral chromatography or an affinity chromatography. (Skoog et al., 2019)

4.1.2. Reversed-phase HPLC

Reversed-phase high performance liquid chromatography (RP-HPLC) has been used due to its high selectivity, accuracy and reliability since its invention in 1970s till nowadays. It is a very popular experimental method used for all kinds of measurements from analysing diverse polymer nanoparticles and proteins to separations of neutral compounds. (Surve and Jindal, 2019; Mane et al., 2017; Zhang *et al.*, 2004) Even the RP-HPLC method itself was in 2004 recommended for testing of chemicals by the Organisation of Economic Co-operation and Development. (OECD, 2004)

RP-HPLC uses non-polar alkyl (usually C-18) chains coating the silica layer in the column leading to creating the hydrophobic stationary phase. Hydrophilic mobile phase used in RP-HPLC consists of polar aqueous solutions (e.g. acetonitrile and methanol in water). Analytes in RP-HPLC are separated based on their polarity - hydrophobic molecules tend to retain on the hydrophobic surface of stationary phase, on the other side hydrophilic molecules remain in the mobile phase with less interactions to the non-polar stationary phase and they elute as first. (Molnár and Horváth, 1976)

To elute molecules of low polarity attached to the stationary phase of the column and to speed up the process it is often necessary to also decrease the polarity of used mobile phase by increasing concentration of a non-polar organic solvent and so setting a gradient programme is mandatory for faster elution of all molecules. (Kromidas, 2016)

4.2. UV/VIS spectroscopy detection

Only compounds absorbing UV or visible light can be qualitatively and quantitatively analysed by non-destructive UV/VIS detectors. The absorption of organic molecules is typically measured between wavelengths 180 - 760 nm. This absorption is a result of the interaction between photons and electrons taking part in inner chemical bounds or located in free electron couples of present heteroatoms like oxygen, nitrogen, halogens or sulphur. A unique absorption band of the molecule is created as an effect of a transition of an electron from a base state to one other excited state from many possible rotational or vibrational excited states. (Skoog et al., 2019)

In case of organic compounds absorbance analysis, it is possible to see in the visible area only those compounds, which have incorporated in their molecule unsaturated bonds, or which have unsaturated functional groups (e.g. phenolic compounds like flavonoids). These groups serve as the chromophore absorbing in UV/VIS area of spectra.

The determination of compounds with all bonds saturated, but despite that with a tied heteroatom (e.g. oxygen, sulphur, nitrogen, …) in the structure, it might be tricky to estimate their absorbance since there are many commonly used heteroatomic solvents like alcohols, or halogenated solvents, which are basically used. All of these compounds might be excited by the beaming in the range 180 - 250 nm. (Skoog, 2019)

4.3. Indirect spectroscopic detection

For those compounds, where the absorbing chromophore in their structure is missing, the reagents with included chromophore are used for a derivatization of the original compound. In case the chromophore is missing, it is necessary to choose the suitable derivative reagent pushing the equilibrium of the reaction with observed compounds to the direction of coloured products possible to measure by UV/VIS spectroscopy. (Skoog, 2019)

In general reagents used for spectroscopy detection are divided into two main groups: inorganic and organic reagents creating complexes with either inorganic ions or organic molecules (Skoog, 2019) and the derivatization of analyte is run either before the chromatographic separation (pre-column), or after the separation (post-column). (Rastkari et al., 2013)

For organic compounds there exists a wide range of possible derivatization reagents depending on the presence of various functional groups in the analysed molecule. Especially for the derivatization of highly polar, chemically instable compounds like carbonyls, which even have no chromophore or fluorophore for a purpose of forwarding UV/Vis spectroscopy detection, plenty of reagents were developed. These well-known and deeply studied reagents are for instance 2,4-dinitrophenylhydrazine (2,4-DNPH), o-2,3,4,5,6-(pentafluorbenzyl)hydroxylamine hydrochloride (PFBHA), 2,2'-furil, or 4-(2-(trimethylammonio)ethoxy)benzenaminium halide (4-APC). (Lili et al., 2010; Chen et al., 2018)

The other and for HPLC also very popular possibility to measure compounds without the chromophore is attaching them to a reagent containing fluorophore followed by the fluorescence spectroscopy technique. Bis-(2,4,6-trichlorophenyloxalate) (TCPO), bis-(2,4-dinitrophenyl) oxalate (DNPO) and various analogues of luminol are usual reagents used for luminescence derivatizations of analytes. (Barnett and Francis, 2005)

4.3.1. 2,4-DNPH

2,4-dinitrophenylhydrazine (2,4-DNPH) is a usual derivative compound used for attaching to carbonyl compounds present in a various samples. (Figure 7) It is commonly used in analysis to determine aldehydes or ketones in food, wood-based materials or air samples. Use of this compound is very wide thanks to its fast reaction with carbonyls, stability of created complex, relatively low price and very good detection by various separation techniques like capillary electrophoresis, or liquid chromatography and detectors like mass spectrometry, or UV/VIS spectroscopy. (Chen et al., 2018)

The binding in this complex-making reaction is made between a carbonyl group of present aldehyde or ketone and an amino group from the 2,4-DNPH molecule. (Figure 8)

The most effective observing wavelength commonly used for the UV/VIS spectroscopy detection of 2,4-DNPH hydrazone derivatives with aldehydes and ketones is around 360 nm. (Cordeiro et al., 2018)

Possibility of using tandem MS detection techniques for DNPH - derivatives compared to UV detection is advantageous because of the increased selectivity especially for these molecules. (de M. Ochs et al., 2010)

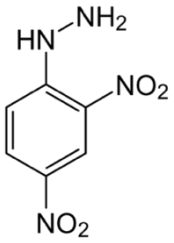


Figure 7: Structure of 2,4-dinitrophenylhydrazine (2,4-DNPH) used for the indirect detection of carbonyl compounds

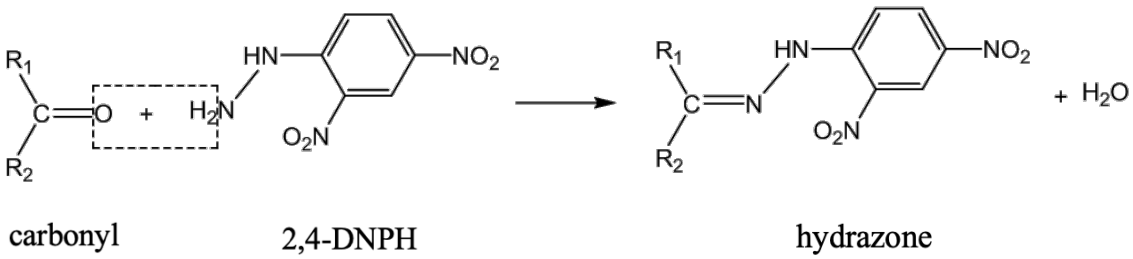


Figure 8: Reaction between carbonyl compound and 2,4-dinitrophenylhydrazine molecule creating a DNPH derivative - a hydrazone

5. MASS SPECTROMETRY

5.1. MS

A mass spectrometry (MS) is a wide spread multifunctional analytical technique for reliable and exact studying of the analysed sample. It provides an important information about the mass of single atoms, or whole molecules. Furthermore it allows us to study the elemental composition of the observed molecule and also sometimes it is possible to find out its exact structure. (Skoog et al., 2019)

The basic principle of the MS technique is firstly the ionization of present compounds in the ion source. The ionization is followed up by the second step, which is the analysis of created ions by their different ratio of the molecule charge and its mass (m/z) in the mass analyser.

It is possible to run the MS analysis of some type for the sample in every state. There are many different sets of ion sources and mass analysers. The choice of these analysers depends on the conditions and the nature of the sample. (Skoog et al., 2019)

Ion sources can run under both evacuated and atmospheric conditions and their aim is to transfer the sample into gaseous form and to ionize present molecules by their bombarding by electrons, photons, ions or other molecules. After the ionization process is done, the sample in gaseous state is transformed into the mass analyser.

The mass analyser can separate present molecule ions by their m/z depending on the type of used mass analyser. Magnetic sector analyser separates ions by their different trajectory in the magnetic field. Quadrupole and ion trap analysers separate or capture ions with using different electric field for each m/z ratio. Ion cyclotron resonance captures specific ions by using exact voltage and magnetic field at the same time. Time of flight analyser is able to separate present ions by their different times of flight through the analysing tube depending on the mass of ions. (Skoog et al., 2019)

5.2. HPLC-MS

Connection of LC with MS is the same as others (e.g. GC-MS, LC-NMR, LC-NMR-MS), a technique connecting the separation technique (HPLC here) with the spectroscopic technique (MS here) with a possibility of connecting more than just two techniques. These combined techniques (so called hyphenated techniques) can be used for both qualitative and quantitative analysis, providing us with a great amount of data about studied analytes. (Rahman, 2018) The combination of HPLC and MS proved itself as a great option for qualitative and quantitative analysis of various complex samples. (Kromidas, 2016)

HPLC as LC separation method is used for its great efficiency to separate present compounds in the HPLC column. HPLC supplies MS with single divided compounds through a three-way diverter. After analysis of peaks from the sample in MS molecular ions provide the information about quality of every analysed compound. For the purpose of analysing more complex molecules (e.g. natural compounds) a tandem mass spectrometry was developed for the possibility of analysing created fragments of the original molecule. After collecting data from the analysis the identification is done by comparing and matching present peaks of molecular ions and their fragments with data in online libraries with MS data. (Rahman, 2018)

Ion sources mainly used in the hyphenated HPLC-MS technique have to be able to evaporate and disperse the sample in present solvent at the same time. For the proper function of transmittal of analytes from HPLC to MS detector sources like electrospray (ESI), atmospheric pressure chemical ionization (APCI) are used. For the analysis of natural products, quadrupole analyser is also commonly used. (Rahman, 2018)

IV. Experimental part

1. METHODOLOGY

1.1. Preparation of used chemicals and samples

1.1.1. Preparation of acetate buffer

The acetate buffer with pH value 4.8 was used for the HPLC analysis. To prepare the buffer the volume of 1 litre was made every time.

Instruments: weight, spatula, 1 l volumetric flask, 50 ml 10 ml pipette, 1 ml micropipette with appropriate tips

Chemicals: ammonium acetate (Sigma-Aldrich, A7262, > 98%), glacial acetic acid Merck, 1.00066.0250), 99.9% acetonitrile (HPLC gradient grade, Fisher, A/0627/17), ultra-pure water (from Direct Q® 3UV equipment)

Process:

1) 0.8 gram of NH_4CH_3COO was weighted
2) Ammonium acetate was then dissolved in the pure clean water and placed into 1l volume flask
3) After addition of approximately 500 ml of ultra-pure water, 10 ml of acetonitrile was added into the solution because of its antibacterial character
4) After that step 600 µl of glacial acetic acid was added
5) then ultra-pure water was added till it reached the guideline
6) Acetate buffer was then stored in the refrigerator at temperature around 4°C

1.1.2. Preparation of the 2,4-DNPH solution

For the indirect detection of volatile carbonyls present in the samples of malts in HPLC analysis 0.3% acidic solution (acetonitrile and 40mM HCl in 1:1, v/v ratio) of 2,4-dinitrophenylhydrazine was used.

Instruments: 25 ml flask with a lit, 25 ml pipette, weight

Chemicals: 2,4-DNPH (Acros Scientific, 117060250, > 30% moisture), 40mM HCl, 99.9% acetonitrile (HPLC gradient grade, Fisher, A/0627/17)

Process:

1) First 0.75 g of 2,4-DNPH was weighted in the flask
2) Then 12.5 ml of 99.9% acetonitrile was added and 2,4-DNPH was dissolved
3) 12.5 ml od 40mM HCl was added into the solution for creating the acid environment for analysis

1.1.3. Standards preparation

To confirm the accuracy of retention times of studied compounds in the column during the HPLC analysis, the standards of hexanal and propanal, both in 10ppm concentration, were used.

Instruments: micropipette, 20 ml pipette, flask

Chemicals: hexanal (Sigma-Aldrich, W255718, > 97%), propanal (Sigma-Aldrich, 538124, 97%), ultra-pure water, 99.9% acetonitrile(HPLC gradient grade, Fisher, A/0627/17), prepared 2,4-dinitrophenyhydrazine solution

Process:

1) First the right counted volume of hexanal and propanal was pipetted into two separated flasks
2) Then a solution of water and acetonitrile (in a 50:50 volume ratio) was added to a both of them to a final 50 ml volume
3) After this step the solution with a concentration of 1.10^{-2} M of both standards was prepared
4) For the analysis it was necessary to dilute both standards to a final 10ppm concentration by pipetting 10 µl of each of the standards into 980 µl of 2,4-DNPH solution

1.1.4. Weighting of samples

Studied commercial samples were donated by Super Bock Group (Porto, Portugal). Samples reported different properties (moisture content and colour) as the consequences of different temperatures used for their roasting were chosen for the measurements. (Table 1)
Black malt was used for all of the optimization observations. Other malts were used almost exclusively for the final measurement followed by comparing compositions of all these malts.

Instruments: weight, spatula, weighting container

Chemicals: commercial malt samples - barley, black, caramel, carared, chocolate, münich, pale ale, pilsner, wheat and barley

Table 1: Overview of used malts and barley for the experiment with their characteristic colour and moisture properties and typical temperatures during the kilning

Sample	Colour (EBC units)	Moisture (%)	Temperature of roasting/kilning (°C)
Black malt	1150 to 1680	< 3.5	Roasting up to 235
Pilsner malt	To 3.5	< 4.5	Kilning up to 80-85
Münich malt	10 to 15	~ 3.8	Kilning up to 100-105
Chocolate malt	900 to 1440	< 4.5	Roasting up to 220
Caramel malt	260 to 320	< 3.5	Kilning up to 100-105
Carared malt	15 to23	< 7.5	Kilning up to 100-105
Pale ale malt	7 to 10	< 4.5	Kilning up to 90-95
Wheat malt	5 to 9	< 10	Kilning up to 80-85
Barley	-	-	-

Process:

1) Malt grains were stored in closed bags or boxes avoiding from any bigger exposure of the air or direct sun light
2) When weighting the seeds the bags or boxes with malt should be opened for the shortest possible period of time
3) Weighting was done in a plastic container with a tolerance of maximum 1% deviation for all of measured masses

1.1.5. Separation of malt tissues

To compare the contain of volatile carbonyls in the husk and remained grain endosperm the caramel malt was chosen as the best one. This decision was made based on relatively easy separation of these tissues in comparison with other available samples and also due to the intensive aroma of this malt.

For the purpose of these measurements over 8 grams of both tissues had to be separated and placed into closable flask to avoid loosing volatile compounds during the separation time. After collecting specified mass of both samples the HPLC analysis was run.

1.2. Low pressure extraction (LPE)

Extraction method to use low pressure conditions was developed from the almost similar but simpler method which is using normal pressure for extraction. The only difference between these two extraction methods is in a used cap and to it connected vacuum pump.

For the normal pressure extraction standard plastic cap was used and the measured time starts just at the time when the cap is tightened.

For LPE a cap with a hole in it is used. The extra apparatus to evacuate the container by the air pump, valves and manometer was connected through the hole in the cap (Figure 9). The time measurement in this setup started just in a moment the valve after the pressure stabilization was closed.

Instruments: 100 ml bottle with an especially developed cap with a hole, pump tubes, vacuum pump (Millipore, model no. WP6122050), 5 ml flask, tweezer, vials

Chemicals: 2,4-DNPH solution, sample

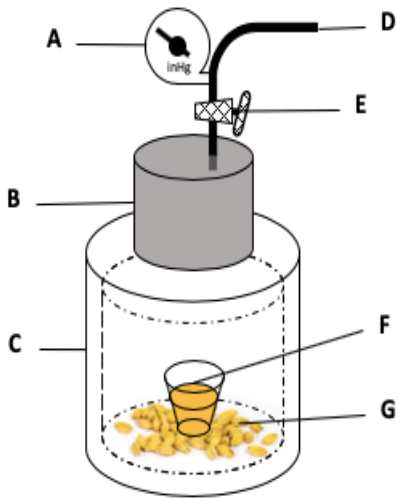


Figure 9: Apparatus used for low-pressure extractions (A- vacuum gauge, B- plastic cap with a drilled hole, C- 100 ml glass flask, D- vacuum pump tubing, E- valve, F- container with 2,4-DNPH solution, G- malt sample grains)

Process:

Setting up the extraction

1) First the right mass of malt grains was put into the container which was before that cleaned by 50:50 solution of acetonitrile and pure-clean water and then dried out.
2) After that a place to insert small flask into the bigger container was prepared and then grains were moved back to surround the inner small flask constantly.
3) Then 50 µl of 2,4-DNPH solution was measured by a pipette into small flask, cap was tightened and vacuum pump for decrease the pressure was turned on.
4) When the pressure was stabilized at the minimum point of pressure achieved by the air pump, all the valves were closed and the measurement of the time started.

At the end of the extraction

1) When the set time was over, the valve closest to the lit was opened as first in order to check, that there was no pressure increase in the flask.
2) If the pressure remained the same, other valve was opened, the pressure fell to atmospheric value and the cap was untightened.
3) By the pipette the 2,4-DNPH solution was taken from the inner flask and put into previously signed vial for next analysis.

1.2. High performance liquid chromatography (HPLC)

The High Performance Liquid Chromatography PerkinElmer system with the pump Jasco PU-2089 Plus and UV/VIS detector Jasco UV-2070 Plus was used as the main method to separate and analyse studied malt samples. The column Gemini 3u C_{18} 110A (ref. 00F-4439-E0) from Phenomenex brand allowed us to achieve a good separation of present compounds with good resolution results in a time of analysis less than one hour. The column was used at the room temperature without any temperature gradient.

Used mobile phase consisted of 99.9% acetonitrile for HPLC use and prepared 10mM acetate buffer in ratio of 50% of each for all the time of separation process. The flow of the mobile phase for the separation was set to 0.45 ml/min with maximal pressure value set at 25 MPa. The time of each run was set to be 55 minutes and in each run 20 µl of the sample was injected into the loop. Thanks to the Jasco AS-2055 Plus autosampler it was

possible to create a sequences of measurements with “on” and “off” method at the beginning and at the end for stabilizing and then washing the column.

The device Jasco LC-Net II/ADC was used to transport data from the chromatograph to the computer and the software ChromPass Data System by Jasco was used for setting up the chromatographic process and also for the interpretation of the results and the measurement of peak areas.

1.3. Mass spectrometry (HPLC-MS-DAD)

To attain all masses and typical mass fragments of separated carbonyl - 2,4-DNPH derivatives the mass spectrometer (Finnigan LCQ Deca XP Plus) with ESI for the ionization of these derivatives and the DAD detector set at wavelength of 360 nm as a detector were used. For the separation process the same column Gemini 3u C_{18} 110A as for the HPLC analysis and also the same mobile phase with acetonitrile and acetate buffer in ratio 50:50 were used.

To detect the masses in range 160-500 m/z ESI was set to negative ion mode with the capillary heated at 325°C. As the sheath gas an auxiliary N_2 was used. Sheath gas was set at 60 (arbitrary units) and auxiliary gas was set at 23 (arbitrary units). The source voltage for ionization was set at 5 kV and capillary voltage at 15 V.

All of these mass spectrometry measurements were carried out by specialized technician responsible for MS analysis at the Department of organic chemistry at the University of Porto.

After the measurements were done, typical wavelengths, masses and mass fragments were interpreted in XCalibur software by Thermo Electron Corporation.

2. RESULTS

2.1. Method try-out and choosing of the best malt for following optimization measurements

As first it was necessary to find out, if the same methodology of low/normal pressure extraction connected with indirect detection of volatile compounds from different malts by binding the carbonyl molecules with 2,4-DNPH molecule can be used as it was invented in the previous research made by Cordeiro and collective at the same working place (Cordeiro et al., 2018). In this work also volatile carbonyl compounds were observed, but in samples with different types of coffee grains.

Beside using the same extraction methodology and binding of carbonyls with 2,4-DNPH, also the same separation and detection technique was used for sample analysis. That means that C_{18} based capillary column for HPLC separation was used with a flow of mobile phase (ACN and acetic buffer in 50:50 volume ratio) set at 4 ml/min and pressure was kept below 8 MPa. The DAD detector was used to detect studied compounds. Each HPLC run was set to 55 minutes, what was enough time for all 2,4-DNPH derivatives to get from the column to the detector.

Three various malt samples with different qualities were used for this first analysis. Black malt was chosen as an typical example of roasted malt with the expected biggest content of aroma compounds. Pilsner malt as a typical kilned standard malt is often used to create the main flavour and the colour of the final beer. And as the third sample was used münich malt as example of a typical kilned Ale malt bringing some extra flavour and smell to the final beer.

All of these extractions were done in triplicates of measurements using both - normal and low pressure conditions. For this first trial measurement was used the same mass (5 g) and time period (15 min) for the extraction as it was previously described in the Santos's article, in which similar coffee grains extraction were performed (Santos and Rodrigues, 2020).

For the decisive comparison of measured samples total peak area numbers provided by the software ChromPass Data System were used.

The low pressure conditions and the sealing of used apparatus were also tried out in this first set of measurements.

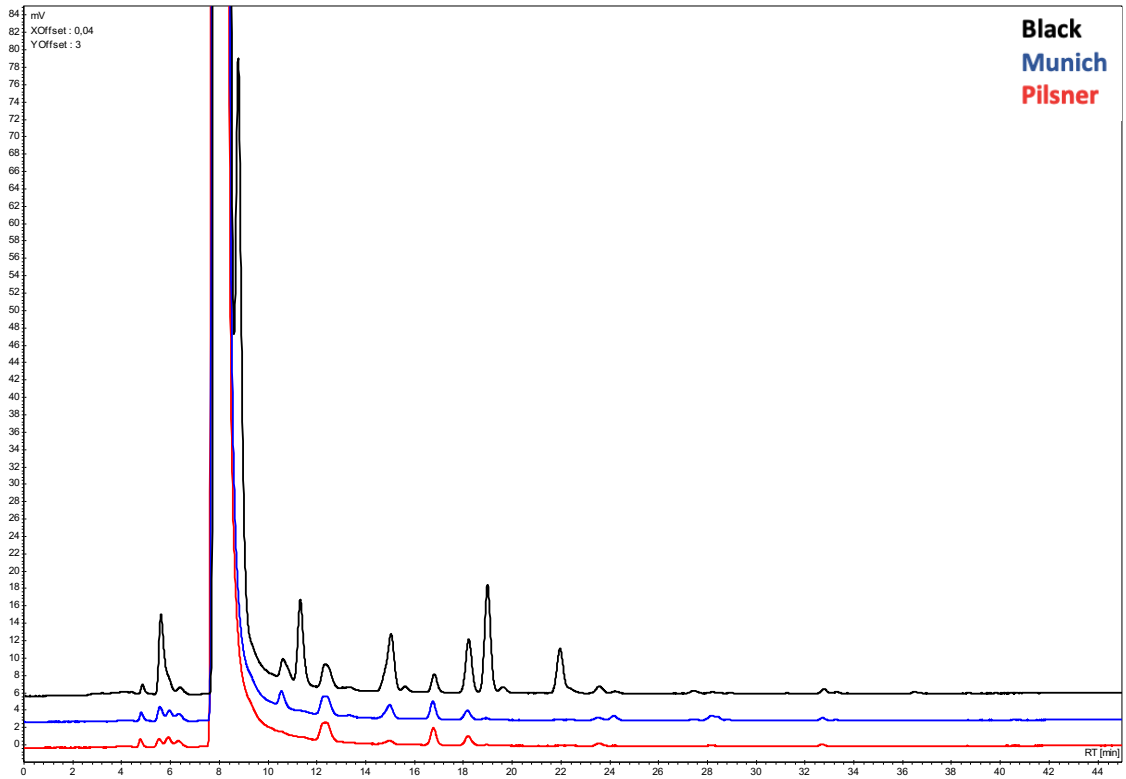


Figure 10: Chromatogram at wavelength of 360 nm comparing carbonyl volatile profiles from münich, pilsner and black malt for the mass of 10 grams and 15 minutes extraction time

Table 2: The comparison of overall peak area means from three measurements measured at λ = 360 nm for every used type of malt in normal pressure conditions

Sample	Total peak area [$mV.min^{-1}$]
Pilsner	3.73 ± 0.2
Münich	5.36 ± 0.1
Black	26.00 ± 0.34

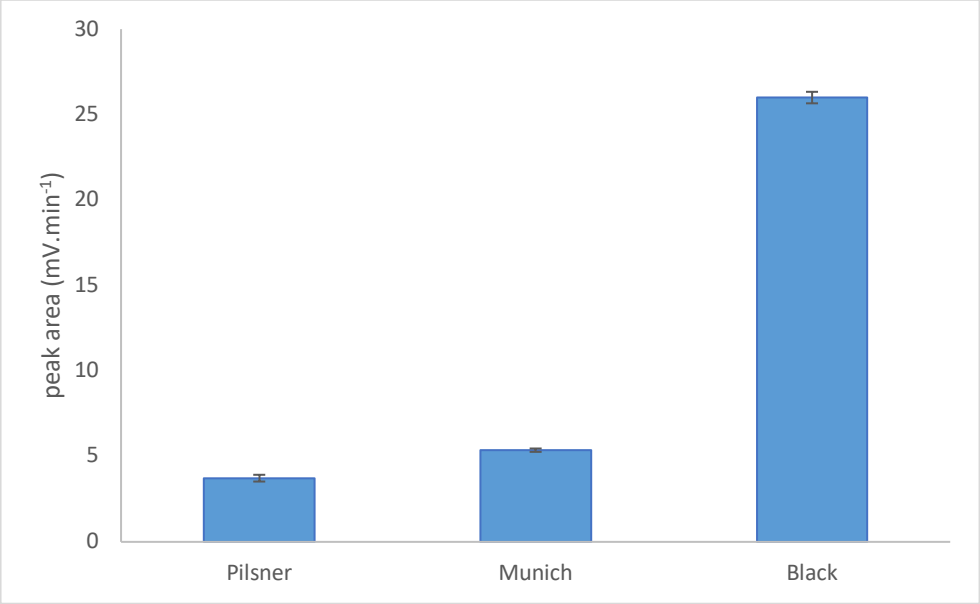


Figure 11: Graph showing the difference in overall peak areas between three different malt samples

After this first series of measurements and after comparison of all chromatograms from all measured samples (Figure 10), black malt was chosen due to its strong signals and approximately five times bigger overall peak area in the chromatographic process as the best malt for the optimization of ideal conditions for this extraction method for volatile carbonyls from malt grains. (Table 2 and Figure 11)

Prematurely it was also possible to see, that the low pressure will probably ensure better conditions for the extraction of volatile compounds from malt grains with signals almost double as big as they were in comparison with extractions using normal pressure.

After this step also the sealing was found satisfactory when the pressure was stable enough and without significant decrease.

2.2. Optimization of the extraction methodology

Pressure, time and mass optimization steps were performed to find out ideal conditions for all malt and barley samples. (Figure 12) From the previous step the black malt sample was chosen due to its rich number of volatile compounds present in high concentrations, which provide good chromatographic signals.

Every studied optimised conditions (pressure, time period and mass) measurement were done in a triplicates of measurements each time to avoid a risk of possible random mistakes. The obtained results were evaluated by the comparison of total peak areas provided by the software ChromPass Data System during each measured condition.

To keep the same conditions for all optimizations and to avoid possible mistakes resulting from the contamination, there was a new 2,4-DNPH solution prepared for each set of optimization measurements. Beside that before each of these optimization measurements a run of 2,4-DNPH blank coming from the same extraction conditions as sample was always performed.

For the first optimization step the mass was chosen to be 5 ± 0.1 grams and the time period was set at 15 minutes. These conditions were the same conditions that were used in the preliminary step.

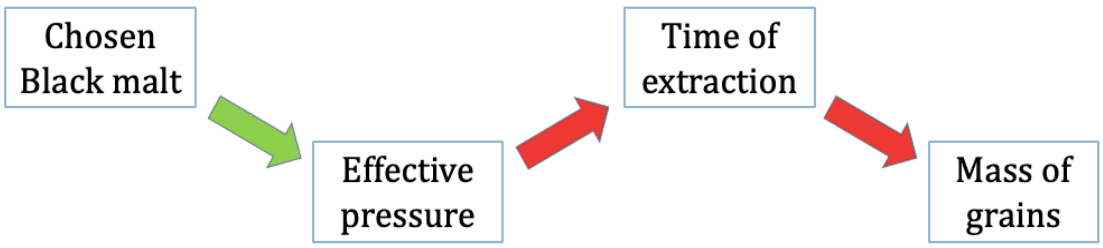


Figure 12: Following steps in the optimization process of the method after choosing the appropriate sample

2.2.1. Pressure optimization

To optimize the pressure there were only two conditions during the extraction studied - normal atmospheric pressure (~ 101 kPa) and low pressure made by the vacuum pump (~ 40 kPa). As it was previously decided the black malt sample was used for the comparison of both pressure conditions.

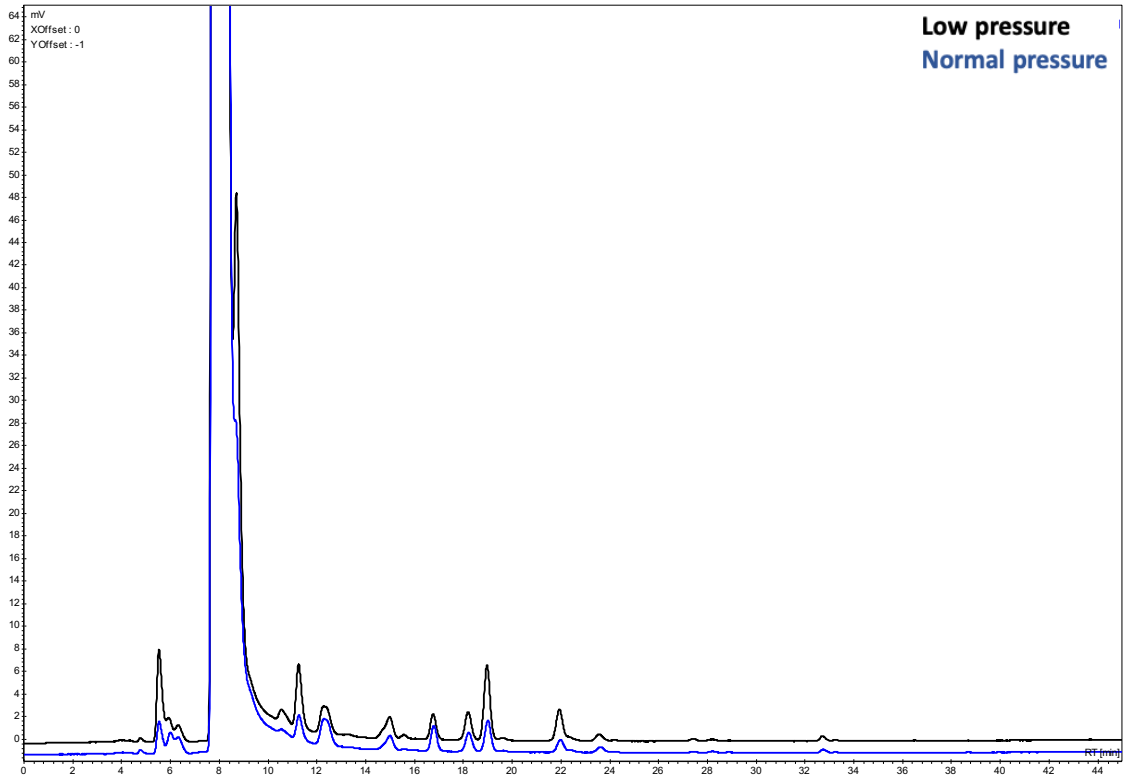


Figure 13: Chromatogram at wavelength of 360 nm comparing carbonyl volatile profiles from sample of black malt using lower and normal pressure for 20 minutes and 5 grams of malt grains

Table 3: Means of overall peak areas measured at λ = 360 nm counted for different pressure conditions (NP - normal atmospheric pressure, LP - low pressure) for the sample of black malt

Pressure	Total peak area [$mV.min^{-1}$]
NP	7.07 ± 0.28
LP	15.57 ± 0.51

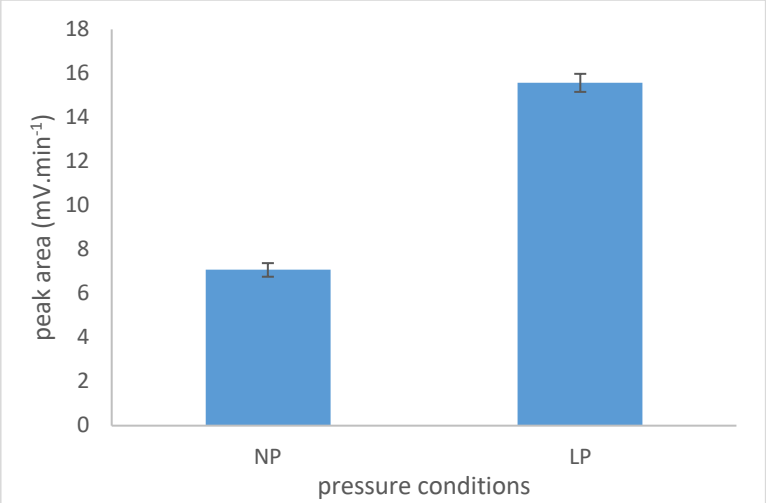


Figure 14: Graph showing the difference for black malt in overall peak area when using normal and low pressure conditions

The study of different pressure conditions showed a significant impact on a growth of overall peak area of present signals. (Figure 13) The increase of studied overall peak area for the sample of black malt was more than twice bigger in all measurements when using the low pressure conditions compared to ones with normal pressure. (Table 3 and Figure 14) After the comparison, low pressure conditions as the best for all further analysis and following optimization steps were chosen.

2.2.2. Time period optimization

After choosing low pressure condition as the one, which will be used in following steps, the next optimization step considering the extraction time was done. For that purpose four different time periods for the extraction procedure were selected: 5.0, 10.0, 15.0 and 20.0 minutes.

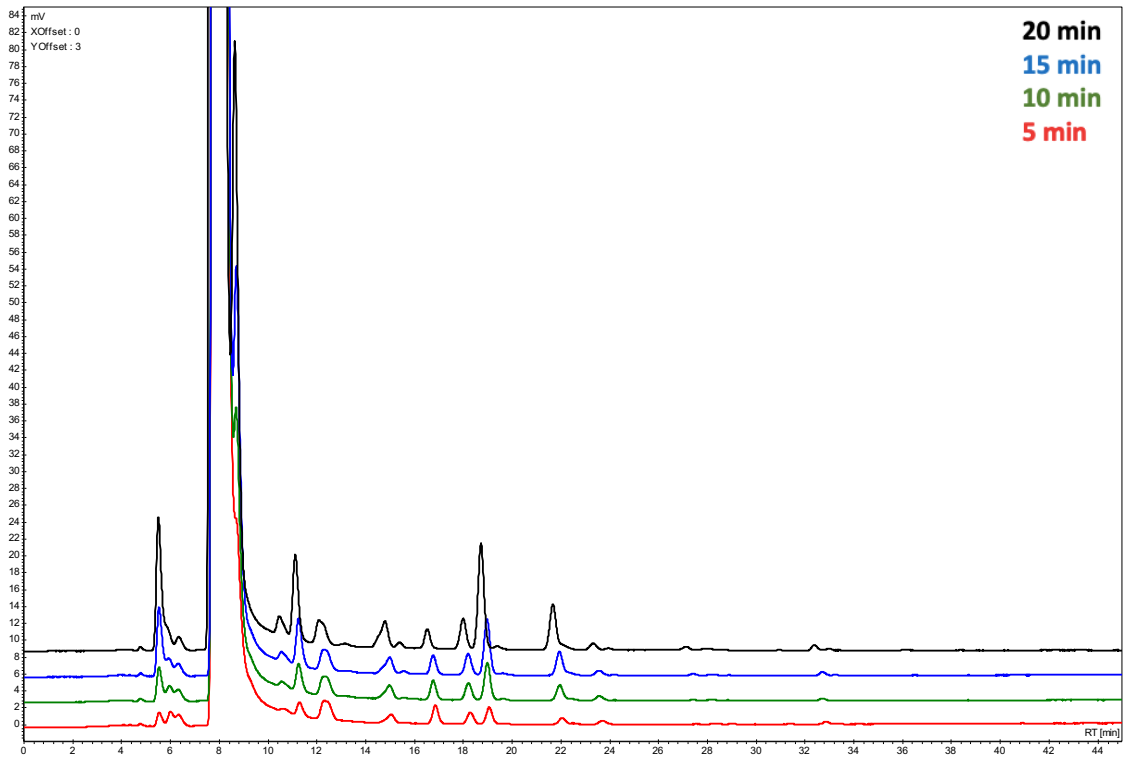


Figure 15: Chromatogram at wavelength of 360 nm comparing carbonyl volatile profiles after using different time periods of extraction for a sample of black malt

Table 4: Means of overall peak areas measured at λ = 360 nm counted for different times of extraction in low pressure conditions

Time [min]	Total peak area [mV.min^{-1}]
5.0	6.09 ± 0.09
10.0	11.18 ± 0.40
15.0	16.69 ± 0.23
20.0	27.15 ± 2.78

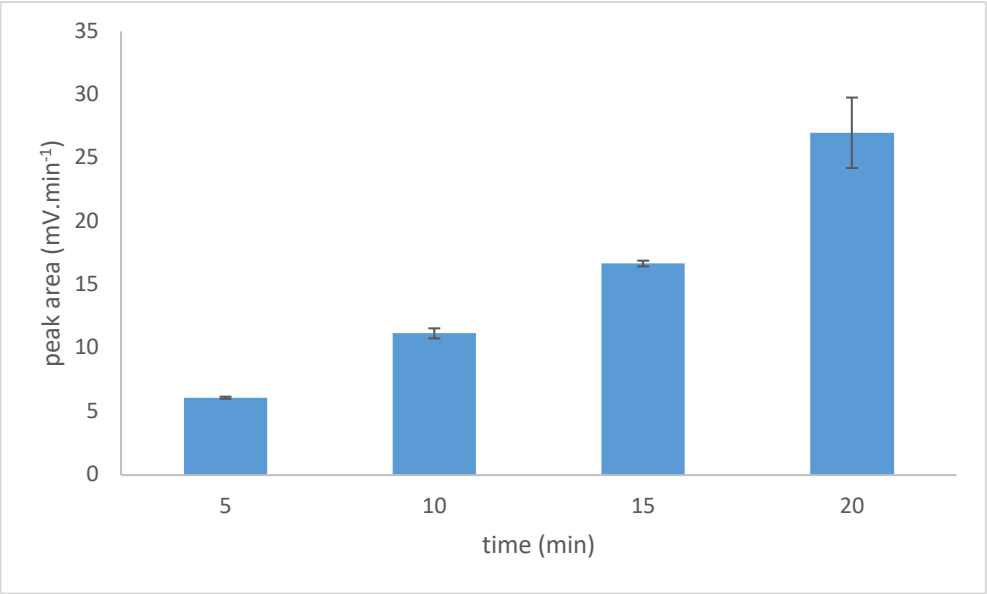


Figure 16: Graph showing relationship between increase of the overall peak area with increase of the time used for extraction with low pressure

Although the extraction with running time of 20 minutes provided us the highest peak area number from all of the measured times, there was no need of prolonging the time of extraction, because all of these peaks that showed at 20 minutes of extraction were also present in 10 or 15 minutes experiments. (Figure 15) It was also very clear, that the deviation rises with the increase of used extraction time (Table 4 and Figure 16), which was not good for the reproducibility and can affect the accuracy of obtained results.

Then after the discussion 15 minutes time period was chosen to avoid possibility of missing some signals in the future measurements of different samples with possible smaller content of some volatile compounds when using shorter period of time.

2.2.3. Mass optimization

After the previous step and choosing the 15 minutes time period as the best extraction time for further analysis, selecting the right mass of malt/barley grains for all the following extractions was needed.

For that reason four more extraction measurements in triplicates were performed for four different masses of malt: 2.5, 5.0, 10.0 and 15.0 grams.

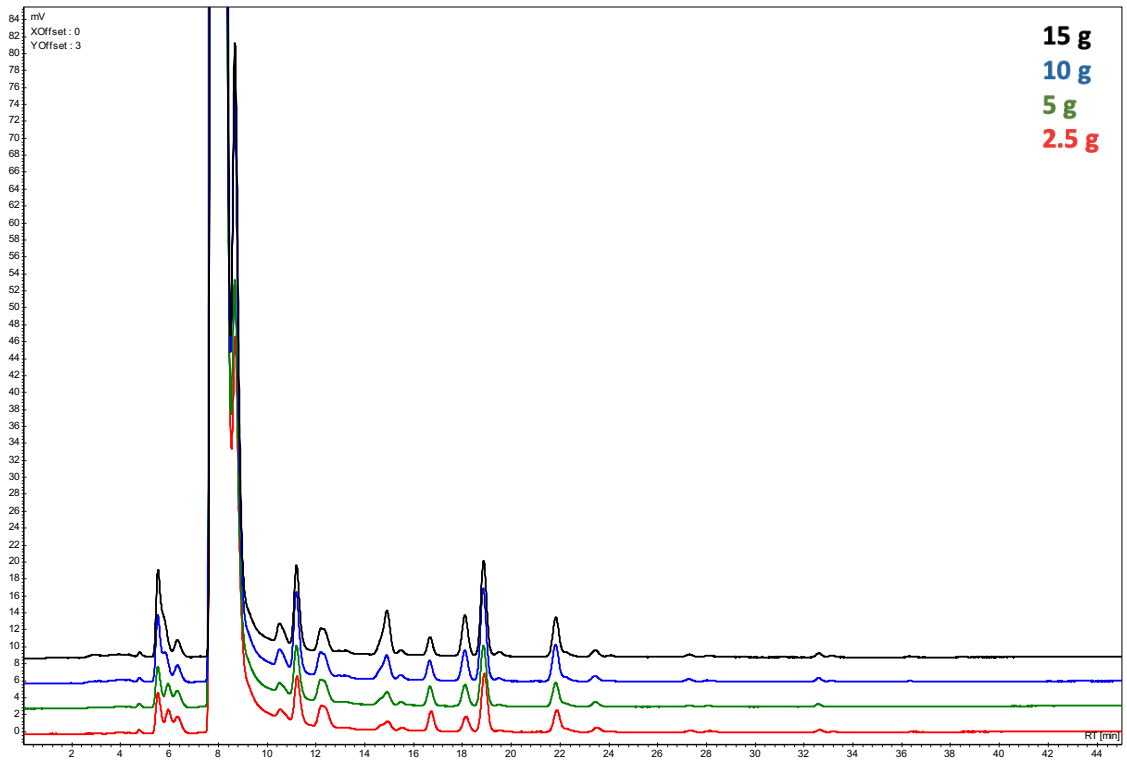


Figure 17: Chromatogram at wavelength of 360 nm comparing volatile carbonyl profiles of different masses used for extraction of black malt in low pressure conditions

Table 5: Means of overall peak areas measured at λ = 360 nm counted for different masses of extracted malt in optimization process

Mass [g]	Total peak area [$mV.min^{-1}$]
2.5	11.29 ± 0.31
5.0	13.40 ± 0.41
10.0	17.19 ± 0.23
15.0	20.35 ± 1.28

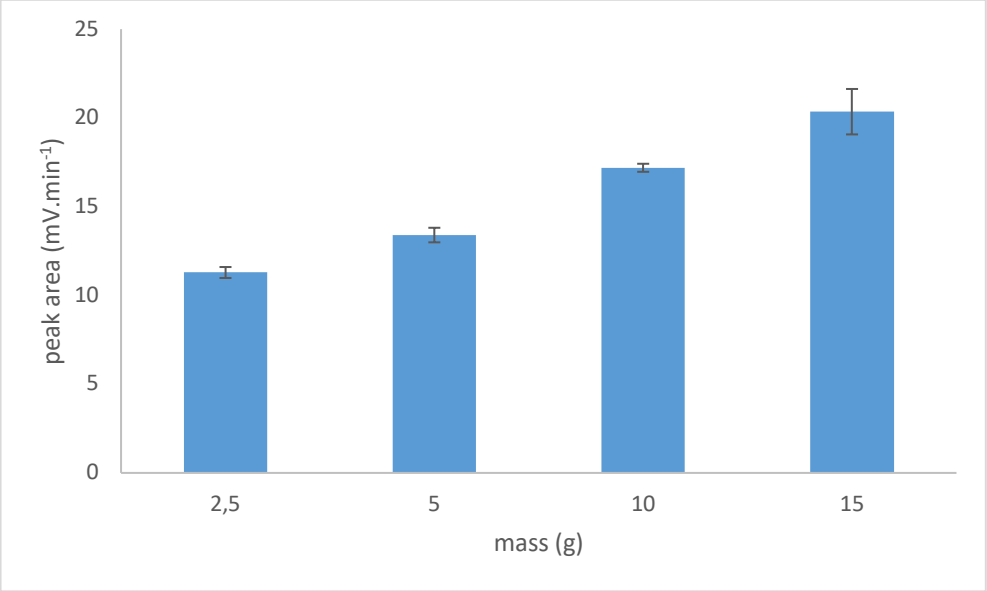


Figure 18: Graph showing relationship between the increase of peak area with the increase of the mass of grains used for extraction with low pressure

As expected when comparing obtained chromatograms from samples with different times of extraction, overall peak area of all present signals increased with every extra added mass of used grains. (Figure 17) As there was a problem with inserting a small flask with 2,4-DNPH solution into a bigger one which was already filled with 15 grams of malt seeds, 10 grams of malt material was chosen as the best option to keep the method as simple and easy to use as possible. Also in the optimization of the mass occurred a similar problem with the increase of the deviation with the increase of used mass. (Table 5 and Figure 18) On the other hand the overall peak area for 10 grams was significantly bigger than for 5 grams used in previous work by Dr. Santos. (Santos and Rodrigues, 2020)

2.2.4. Conclusion of the optimization process

After going through all three optimization steps step by step, ideal conditions for all following sample measurements were set down. Final extraction conditions chosen for further analysis were low pressure, 15.0 minutes as the time period and 10.0 grams of weighted sample grains used for extraction.

2.3. MS spectra and identification of present compounds

All of the spectra from mass spectrometry were studied due to identify all present volatile carbonyl compounds bound with the 2,4-DNPH derivative molecule which were successfully previously separated in the same HPLC column, which was also used for chromatographic analysis with DAD detector set at wavelength of 360 nm.

For this work all visible peaks representing carbonyl compound with bound 2,4-DNPH in all samples were analysed using UV/VIS and MS spectra to find their main molecule masses, specific mass fragments and retention time values. After obtaining these information, the identification of compounds present in sample was done. These acquired data were then compared with corresponding data obtained from suitable standards, previous measurements focused on the study of carbonyl compounds bound with 2,4-DNPH done at Department of analytical chemistry at University of Porto, or data from articles dealing with the similar problematic.

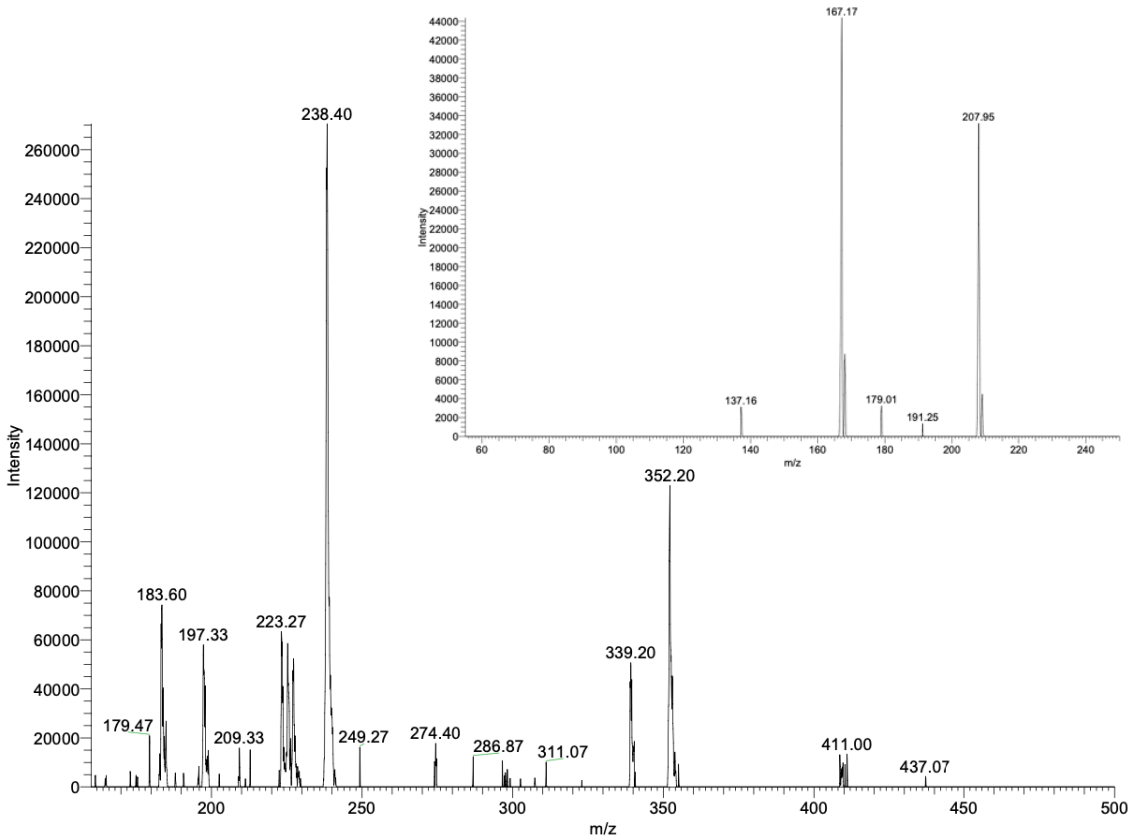


Figure 19: MS^1 spectra at Rt 6.19 of 2,4-DNPH derivate of 2-aminoacetaldehyde showing its parent ion with m/z 238 and MS^2 spectra showing its fragments with m/z values 137, 167, 179, 191 and 208

Overall twenty-six carbonyl compounds bound with 2,4-DNPH derivative were identified in all measured samples after collecting data for each of these compounds from HPLC-DAD-ESI-MS/MS analysis. (Table 6) Twenty-two of these present compounds were identified by comparing MS spectra and retention times with commercial standards. Based on analysis of the properties (R_t, WL, MS and MS fragments) correlating with information reported in literature sources, four remaining compounds were identified (2-aminoacetaldehyde, dicarbonyl oxoacid, 1,2-bis-DNPH and 2,5-dimethylbenzaldehyde).

Two compounds - hydroxyacetone (R_t 9.19 and 11.96) and furfural (R_t 20.10 and 23.38) showed each two peaks in most of sample's chromatograms at two different retention times. These doubled peaks of one compound stand in both cases for two geometric isomers of corresponding hydrazone created by the reaction of these two compounds with 2,4-DNPH molecule.

The peak at R_t 6.19 with a parent ion with m/z value 238 was not possible to identify by using any available commercial standard. Comparing MS fragment spectra it was easily visible, that fragments 167 and 208 appear with higher intensity compared to others. (Figure 19) Although some other fragment signals typical for carbonyl compounds appeared in MS^2 too, these two major signals for 167 an 208 m/z fragments indicate presence of amino group in the compound. After the discussion and information found in the literature (Mizukami et al., 2008) this compound was identified as 2-aminoacetaldehyde which comes as the product from the Strecker's degradation of methylglyoxal during the roasting process. (Mizukami et al., 2008)

The peak at R_t 16.51 had a parent ion with m/z value 475 with secondary fragments m/z 277, 182 and 429. The parent fragment clearly shows that this compound is an oxoacid and secondary fragments are pointing at dicarbonyl compound. After that possible formula was posited to be $C_5H_8O_3$. As no more information were found for more accurate identification of this molecule, this compound was, based on available information, identified as dicarbonyl oxoacid. (Oehme & Dye, 1998)

The peak at R_t 17.82 was due to m/z values 363 and 333 and a secondary fragment with m/z 317 easily identified as 2,4-dinitrophenylhydrazine which is described in many articles (e.g. Oehme & Dye, 1998; Brombacher et al., 2002).

Compound at R_t 35.60 with m/z 313 and three additional fragments with m/z 266, 163 and 178 was identified as 2,4-dimethylbenzaldehyde, which was described in the Grosjean's article (Grosjean et al. in 1999).

Table 6: List of all 26 compounds identified based on their retention times, wavelengths, masses and mass fragments in analysis using data from MS and DAD detector

R_t (min)	Compound	λ (nm)	MS	MS^2	Standard and/or reference
5.70	Formic acid	337	225	182;179; 197; 152	Standard
6.19	2-Aminoacetaldehyde	383	238	167; 208; 137; 179	(Oehme & Dye, 1998)
9.19	Hydroxyacetone	364	253	152; 179; 177	Standard
11.19	Acetoin (Z-isomer)	364	267	152; 177; 179	Standard
11.96	Hydroxyacetone	370	253	179; 152; 177	Standard
12.99	Formaldehyde (blank)	352	209	-	Standard
15.90	Acetaldehyde	364	223	179; 151; 163	Standard
16.51	Dicarbonyl oxoacid	361	475	277; 295; 179	(Oehme & Dye, 1998)
17.37	2-Methyloxolan-3-one	361	279	191; 249; 153	Standard
17.82	1,2-bis-DNPH (blank)	352	363	317	(Brombacher et al., 2002)
19.36	Propanone	367	237	207;179; 151; 191	Standard
20.10	Furfural	394	275	228; 163; 179	Standard
20.83	Propanal	364	237	191; 163; 179; 120	Standard
23.38	Furfural	385	275	228; 163	Standard
23.85	5-Methylfurfural	379	289	242; 163	Standard
25.05	2-Butanone	367	251	221; 152; 205; 189	Standard
25.73	Butanal	364	251	163; 179; 205	Standard
29.88	3-Methylbutanal	364	265	163; 179; 235; 152	Standard
30.08	2-Methylbutanal	364	265	163; 179; 219; 152	Standard
30.52	Pentanal	364	265	163; 191; 235	Standard
30.64	Glyoxal	361	417	182; 234	Standard
34.34	Methylglyoxal	418	431	182;248; 385	Standard
34.88	Hexanal	364	279	163; 179; 152; 191	Standard
35.60	2,5-Dimethylbenzaldehyde	394	313	266; 163; 178	(Grosjean et al., 1999)
38.00	2,3-Butanedione	397	445	182; 364; 399	Standard
39.97	2,3-Pentanodione	400	459	182	Standard

2.4. Comparison of chromatograms

2.4.1. Chromatograms from malts and barley samples

All final chromatograms were used for the overall comparison to show and highlight some interesting differences which occurred between some samples. In all of these chromatograms the biggest peak at 8 minute stands for the 2,4-DNPH molecule. Besides that many other peak signals of various intensities and peak areas for every successfully separated carbonyl compound bound with the 2,4-DNPH derivative occurred. Then each chromatograms were compared step by step and at the end of this comparison were selected some chromatograms with the most interesting differences.

For the first comparison were used all available malts and barley samples. Even from the very first sight it was obvious, that there are some significant differences between aroma profiles at some types of researched malts. (Figure 20) The most obvious differences emerged when the presence or the absence of some peaks were compared. All roasted malts (black, chocolate or even caramel malts) showed very clear and high signals of the most present volatile compounds. On the other hand comparing pale malts (pale ale and pilsner) with raw barley grains showed, that even for pale malts there was significant concentration increase of volatile carbonyl compounds.

The wheat malt sample showed also some distinct differences in the intensity of various present volatile carbonyls in comparison with other malts made from barley. (Figure 23) But since these differences were not anomalous enough, wheat malt was not further involved in the overall malt comparison and based on similar kilning temperatures it was compared only to pilsner malt.

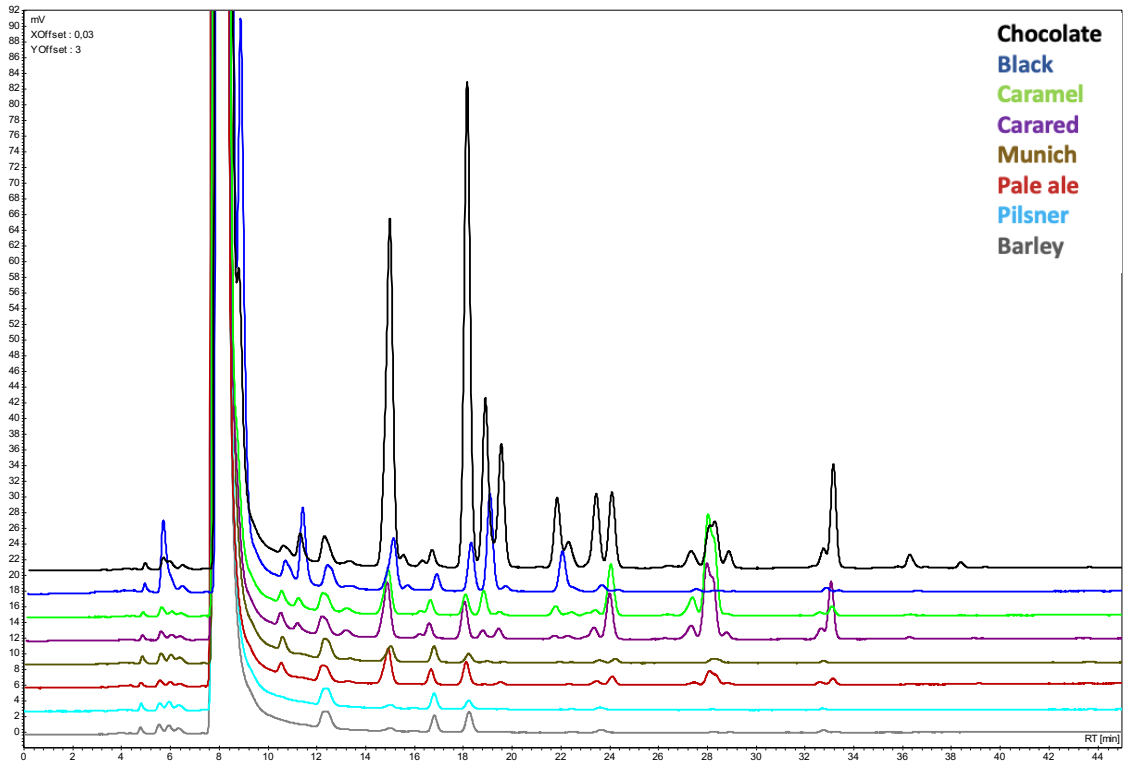


Figure 20: Chromatogram at wavelength of 360 nm showing the comparison of all barley malts and the barley sample showing their different composition of carbonyl compounds bound with 2,4-DNPH

After discussion and detailed comparison of all collected chromatograms from all samples, only five samples were selected for further research. (Figure 24) Each of these five samples represents different temperature conditions during the roasting process and so the different moisture volume in grains. (Table 1) Specialty malts - chocolate and black were chosen because of their very high signals of present VOCs obtained during roasting at high temperatures above 200°C.

Next step was the comparison of caramel malts - caramel and carared in which it can be clearly seen that these two malts have basically the same quality composition with only some less important differences in peak areas of some peaks from present VOCs. (Figure 21) Based on this the caramel malt sample was chosen as a representative for the whole caramel malts group.

For pale malts the pilsner malt sample when comparing with pale ale malt was chosen as the sample representing pale malts with their typically lower kilning temperatures under 100°C. (Figure 22)

It was expected, that wheat malt with kilning conditions quite similar to pale barley malts will show some considerable differences in quality based on different cereal used as the ingredient for this malt, but only some negligible quality differences appeared when comparing with the sample of pilsner malt. (Figure 23)

Although the barley sample showed no important difference in volatile carbonyl profile compared to other barley malts, it was still chosen to indicate increase of observed signals in relation with appended kilning or roasting during the process of malting.

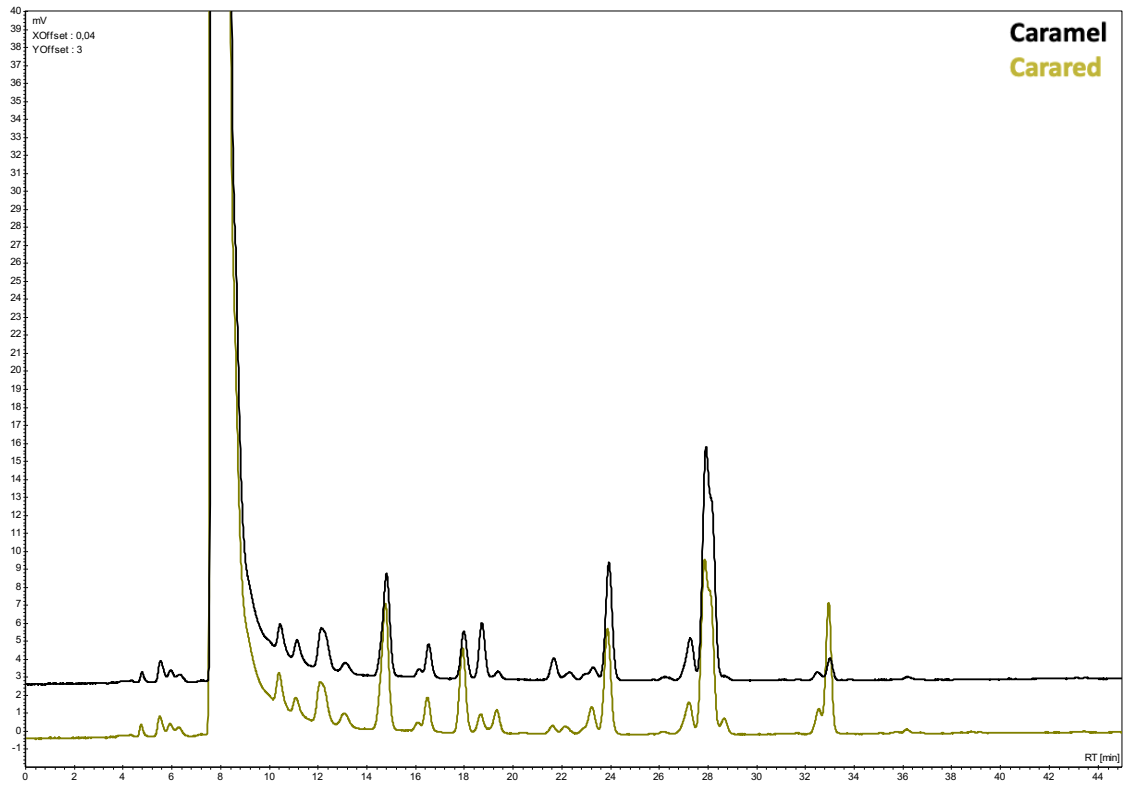


Figure 21: Chromatogram at wavelength of 360 nm showing the comparison of volatile carbonyl profile between caramel and carared malt

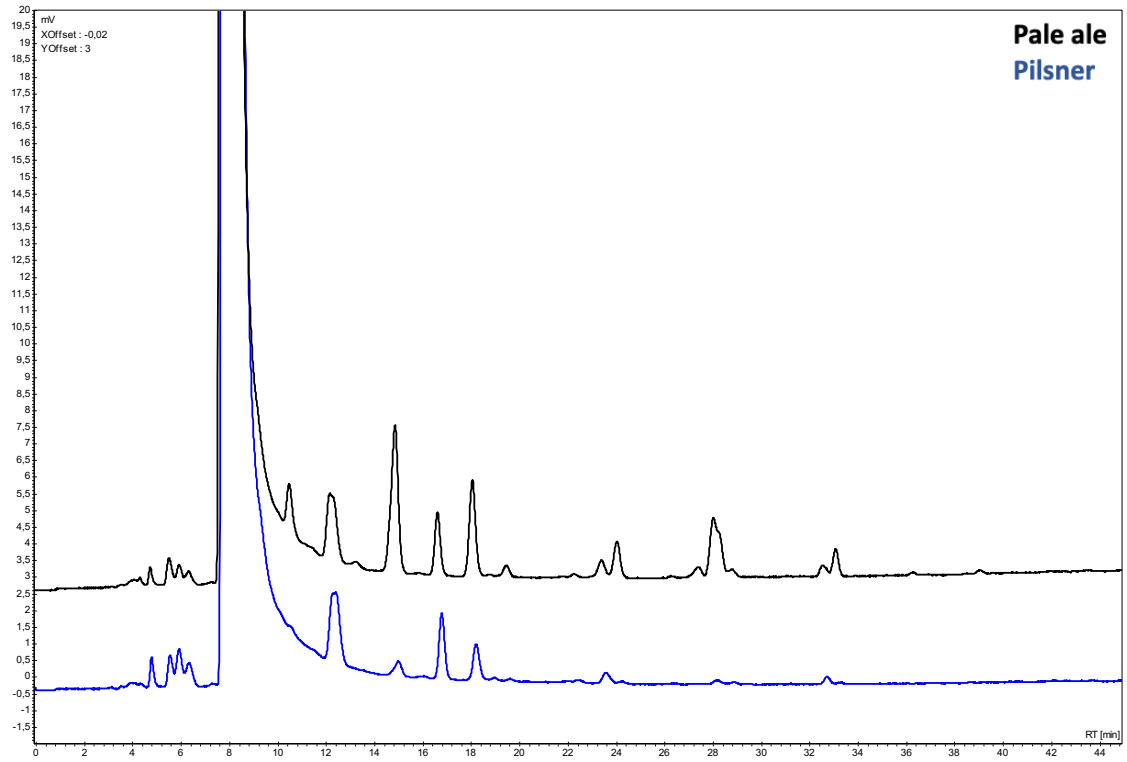


Figure 22: Chromatogram at wavelength of 360 nm comparing volatile carbonyl profile between pilsner and pale ale malt

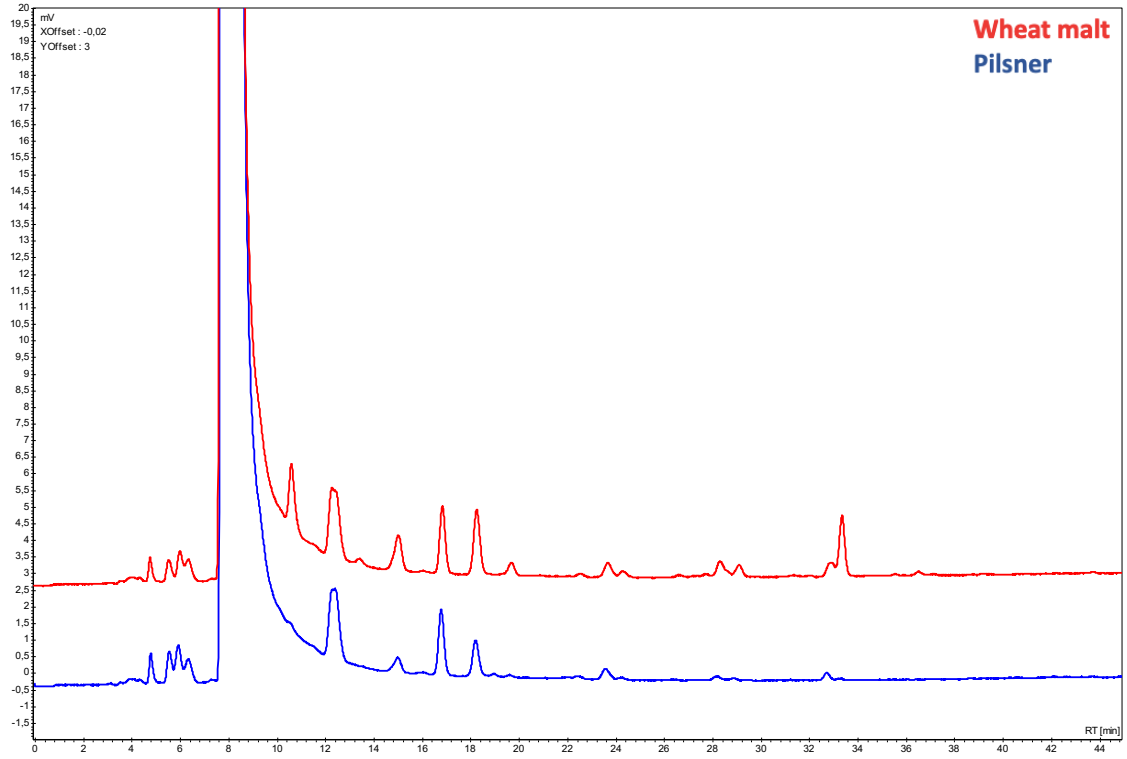


Figure 23: Chromatogram at wavelength of 360 nm comparing volatile carbonyl profile between pilsner and wheat malt samples

After the final selection of further studied samples and the comparison of chromatograms of chosen five samples were done, nineteen present carbonyl compounds with significant intensity differences of showed signals visible by the eye were chosen and numbered. (Figure 24 and Table 7) Then peak areas of these nineteen selected compounds in five studied samples were collected and compared within these samples. (Figure 25)

The main purpose of this step was to possibly find some outstanding compounds, which could be potentially used as markers for compared malts and also to have an opportunity for the possible comparison of these observed samples by using their peak areas. (Table 8)

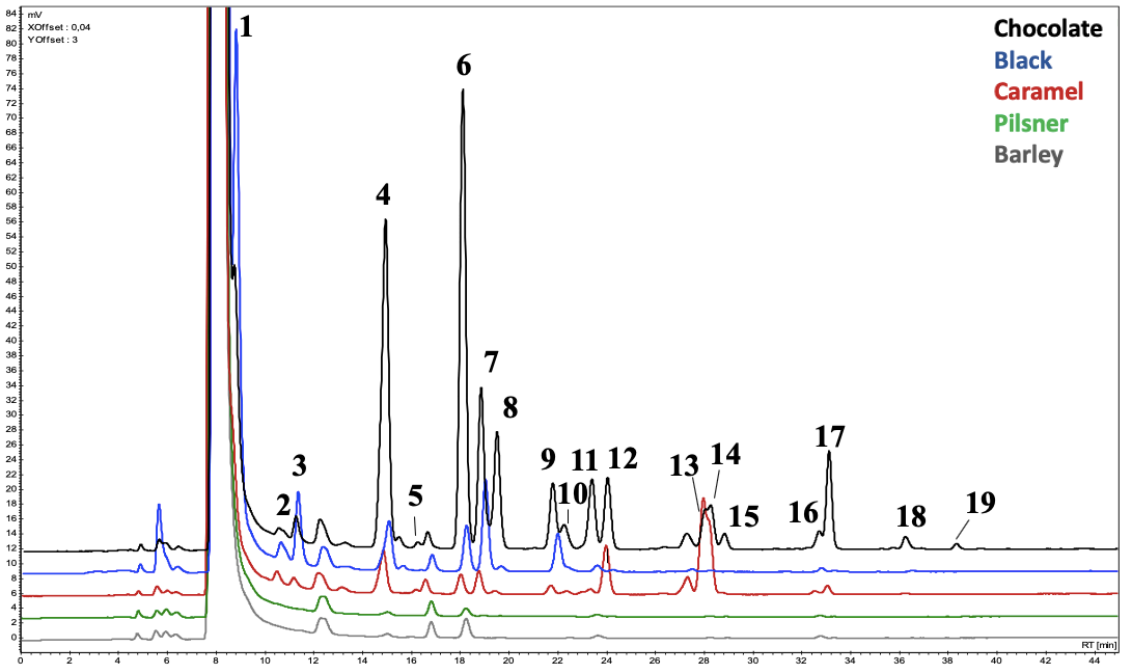


Figure 24: Chromatogram at wavelength of 360 nm showing five chosen samples with numbered peaks standing for selected 2,4-DNPH derivatives from Table 7

Table 7: Numbered selection of nineteen identified compounds selected after the comparison of pilsner, caramel, black and chocolate malt and the barley chromatograms

Rt (min)	No.	Name of compound
9.19	1	Hydroxyacetone
11.19	2	Acetoin (Z-isomer)
11.96	3	Hydroxyacetone
15.90	4	Acetaldehyde
17.37	5	2-Methyl oxolan-3-one
19.36	6	Propanone
20.10	7	Furfural
20.83	8	Propanal
23.38	9	Furfural
23.85	10	5-methylfurfural
25.05	11	2-butanone
25.73	12	Butanal
29.88	13	3-Methylbutanal
30.08	14	2-Methylbutanal
30,52	15	Pentanal
34.34	16	Methylglyoxal
34.88	17	Hexanal
38.00	18	2,3-Butanedione
39.97	19	2,3-Pentanodione

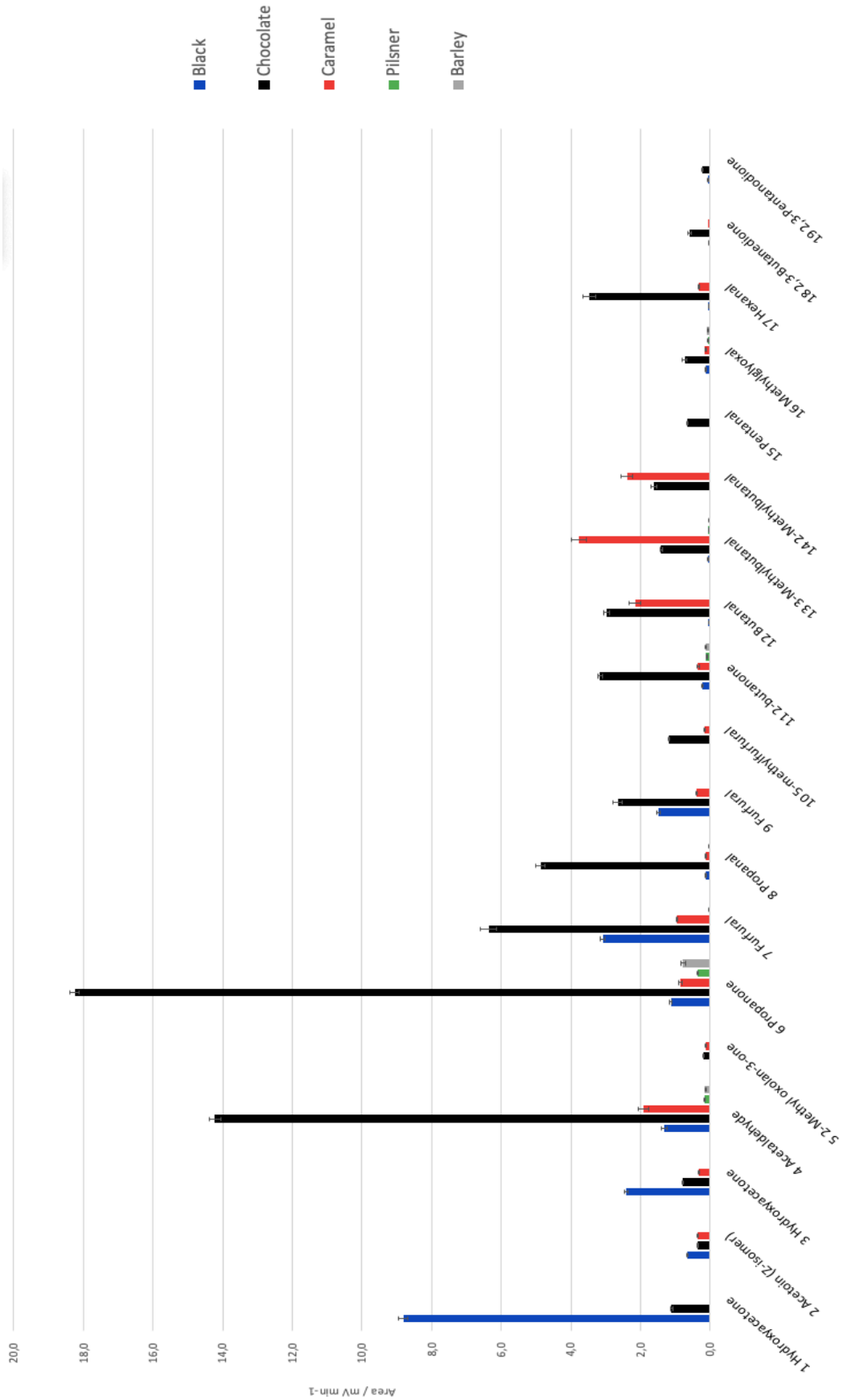


Figure 25: Graphic comparison of collected peak areas of chosen carbonyl compounds for chosen malts and barley

Table 8: Comparison of collected peak areas of chosen carbonyl compounds for chosen malts and barley ordered by their increasing roasting/kilning temperatures

	Barley	Pilsner	Caramel	Chocolate	Black
Hydroxyacetone	0.00	0.00	0.00	1.11	8.82
Acetoin (Z-isomer)	0.00	0.00	0.37	0.35	0.67
Hydroxyacetone	0.00	0.00	0.32	0.79	2.42
Acetaldehyde	0.15	0.16	1.92	14.23	1.31
2-Methyloxolan-3-one	0.00	0.00	0.12	0.20	0.00
Acetone	0.78	0.36	0.86	18.26	1.14
Furfural	0.00	0.04	0.95	6.37	3.08
Propanal	0.03	0.03	0.14	4.88	0.12
Furfural	0.00	0.00	0.39	2.66	1.49
5-methylfurfural	0.02	0.02	0.18	1.18	0.00
2-butanone	0.14	0.12	0.35	3.17	0.24
Butanal	0.00	0.00	2.16	2.98	0.05
3-Methylbutanal	0.04	0.05	3.76	1.41	0.07
2-Methylbutanal	0.00	0.02	2.39	1.63	0.00
Pentanal	0.02	0.00	0.00	0.66	0.02
Methylglyoxal	0.09	0.07	0.16	0.74	0.12
Hexanal	0.02	0.02	0.32	3.46	0.05
2,3-Butanedione	0.00	0.00	0.06	0.60	0.03
2,3-Pentanodione	0.00	0.00	0.01	0.22	0.06

Area ($mV.min^{-1}$)

0.00
0.05
0.10
1.00
5.00
10.00
15.00
20.00

2.4.2. Assignment of marker compounds

After observing and comparing peak signals from collected peak areas of all measured samples from Table 8 and also checking how found compounds are corresponding with real organoleptic characteristics, it was finally possible to select marker carbonyl

compounds typical for chosen samples. Furthermore the point of this step was to find some similarities between measured compounds in chosen samples and their typical flavours and aromas based on characteristics provided by malt producer Weyermann®. (Figure 26)

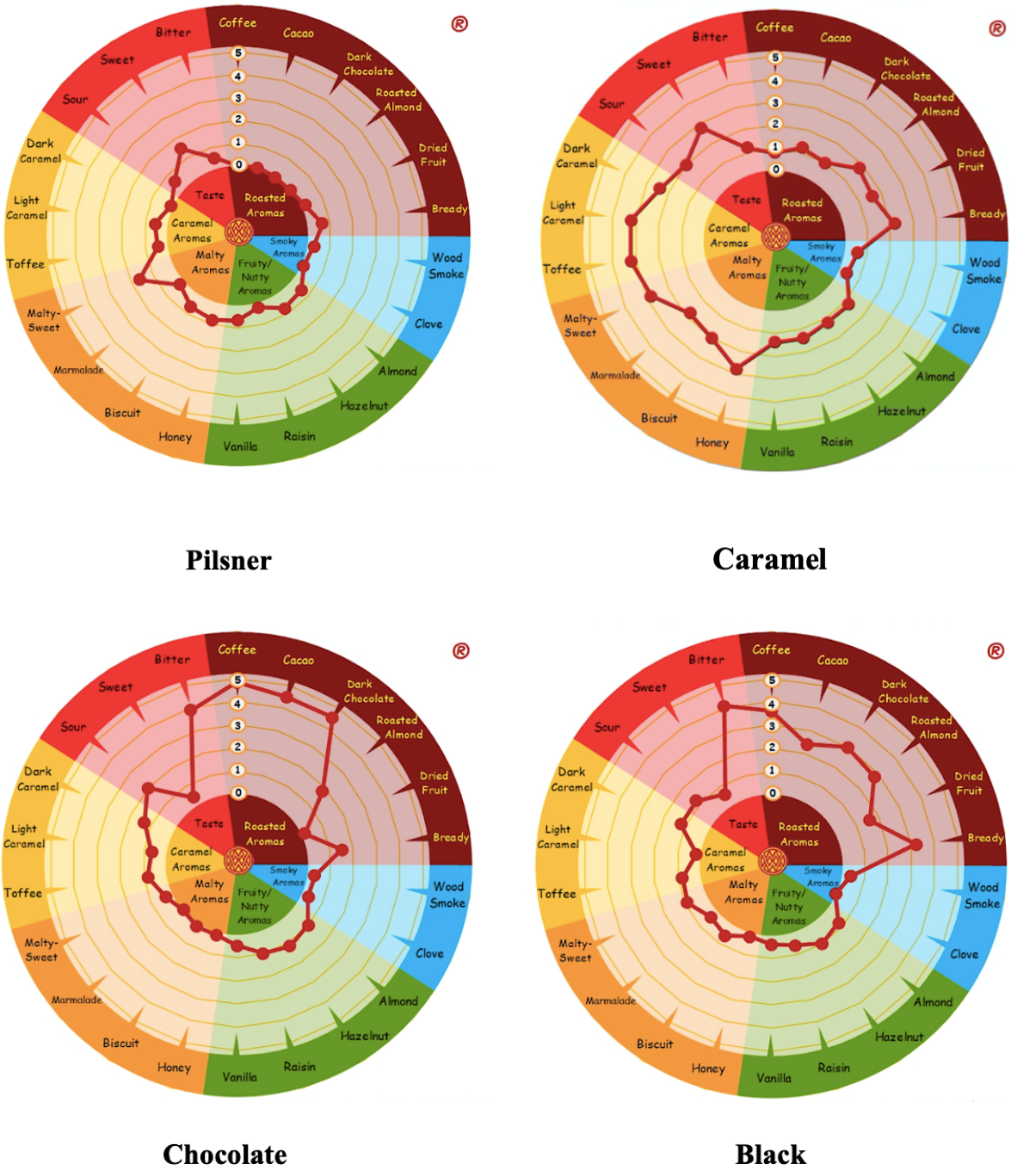
In the process of finding and assigning of these marker compounds, two factors were studied: firstly the peak area of targeted compound compared to other compounds and within samples, and secondly the agreement of compound's flavour with expected flavours in the malt sample. Generally considerable correlations appeared between carbonyl compounds found in samples and already known typical organoleptic characteristics of each malt sample. When the found compounds were compared with aroma wheel graph for every observed sample, some big similarities between given aroma graphs and actual flavour properties provided by found compounds could have been seen. These correlations are also further highlighted in the Table 9.

Two peaks with R_t 9.19 and R_t 11.96 were given by the compound hydroxyacetone, which is typical for its burnt flavour. This compound was due to its highest concentration when comparing malt samples, selected as the marker compound for the sample of black malt. Despite that this compound was also present in less concentration in chocolate malt where it is also corresponding with its sweet, caramel-like flavour.

Acetaldehyde at R_t 15.90 with its characteristic pungent aroma and propanal with R_t 20.83 typical for its possible whiskey, cocoa and nutty flavours were selected as marker compounds for chocolate malt. In this malt were peak areas of these two compounds significantly bigger in comparison with other samples. Although these compounds were chosen as marker compounds and were matching with real aroma flavours, also acetone with its ethereal and apple aromas could serve as the marker due to its great peak area when compared to other samples. Based on aroma wheels (Figure 26) the intensity of these flavours should be more or less the same for all malt samples. Regarding to this flavour mismatch and two more applicable compounds acetone was not involved as the marker for chocolate malt.

Butanal at R_t 25.73, characteristic for its malty and bready flavours, was chosen as the former marker compound for caramel malt sample. In spite of not a clear correlation between cocoa, coffee, fruity or nutty flavours and real flavours/aromas of this malt, compounds 2 and 3-methylbutanal (R_t 30.08 and 29.88) were also chosen as caramel malt's marker compounds.

Pilsner malt sample, as expected, showed signals with lower intensity when comparing peak areas within chosen malt samples. Nevertheless the found present compounds like methylglyoxal, 2-butanone or acetone are well corresponding with organoleptic characteristic of this malt and could be possibly used as its marker compounds in comparison with malts with less intensive volatile carbonyl profiles.



Pilsner **Caramel**

Chocolate **Black**

Figure 26: Flavour/aroma profiles of compared malts in the form of aroma wheel graphs for chosen malts (from www.weyermann.com)

Table 9: Summary of flavours/aromas for each chosen malt or barley sample and presence and intensity of peaks of these compounds in compared samples (according to www.flavornet.org and www.thegoodscentscompany.com)

(+ present peak, ++ high intensity of present peak)

Compound	Typical flavour	Brly	Pils	Crml	Chlt	Blk
Aldehydes						
Hexanal	Grass, fatty			+	++	
Acetaldehyde	Pungent, fruity			+	++	+
Methylglyoxal	caramellic	+	+	+	++	+
Propanal	Whiskey, cocoa, nutty				++	
Pentanal	Almond, malt, bready				+	
Butanal	Cocoa, malty, bready			+	+	
3-Methylbutanal	Chocolate, cocoa, fruity			+	+	
2-Methylbutanal	Cocoa, coffee, nutty			+	+	
Ketones						
2-Butanone	Fruity, sweet	+	+	+	++	+
Acetoin	Buttery, cream			+	+	+
2,3-Butanedione	Buttery, caramellic			+	++	
2,3-Pentanodione	Buttery, caramellic, toasted				+	
Hydroxyacetone	Sweet, caramellic, burnt				+	++
Acetone	Ethereal, apple	+	+	+	++	+
Furans						
5-methylfurfural	Caramellic, coffee, bready			+	+	
Furfural	Bready, almond, sweet, caramellic			+	++	++
2-Methyloxolan-3-one	Bready, buttery, nutty			+	+	

2.4.3. Comparison of chromatograms of separated malt tissues

For the purpose of this additional study, caramel malt sample was chosen. This decision was made after trying to separate these tissues with all available malt samples. In this comparison separating these tissues was the easiest for the sample of caramel malt.

After separation of the husk and the remained grain endosperm it was immediately obvious even by the sensorial analysis by smelling these separated tissues, that there surely are some differences in aroma of these two samples.

Low pressure conditions like for sample extractions were used for the extraction process. Difference was in mass, which was 4 grams for each sample and in time, which was increased to 20 minutes due to available lower masses of samples.

The HPLC analysis was same like for the rest of measured samples using completely same conditions and setting.

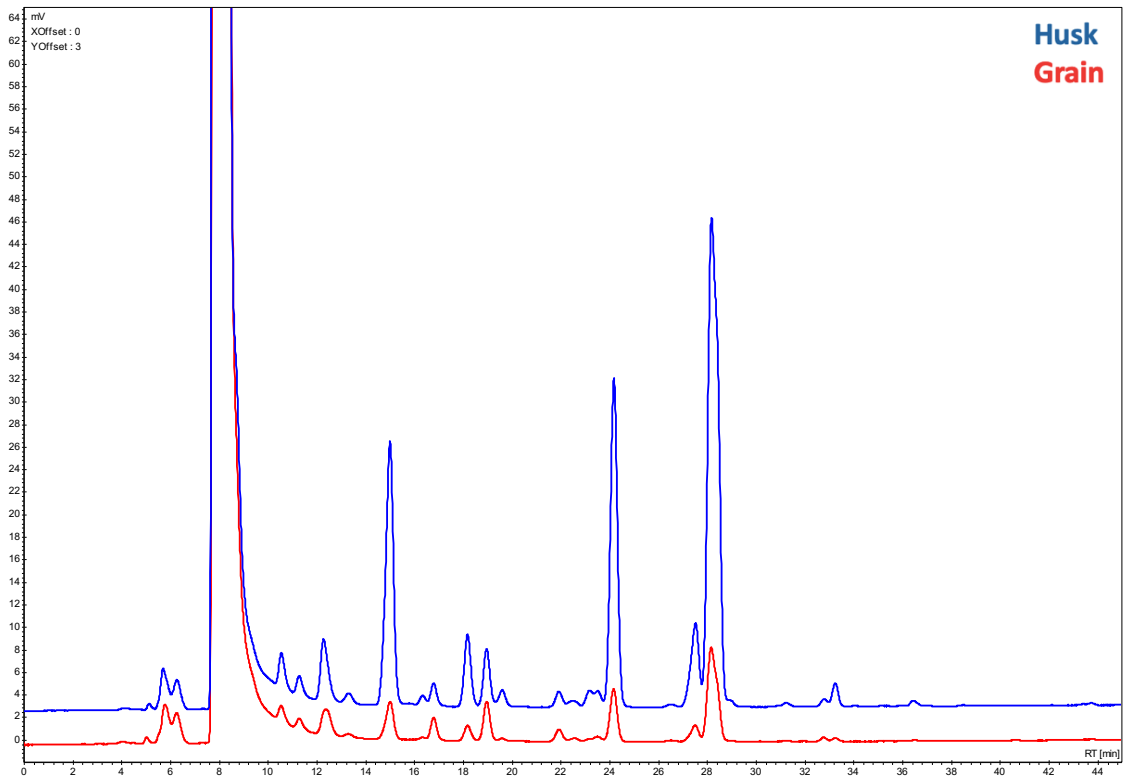


Figure 27: Chromatogram at wavelength of 360 nm comparing volatile carbonyl profiles provided by separated malt tissues samples

Table 10: Comparison of total peak areas of the husk and the grain measured at $\lambda = 360$ nm

Sample	Total peak area [$mV.min^{-1}$]
Husk	58.46
Grain	15.72

When the process of this challenging separation, followed by measurement and comparison of obtained chromatograms was done, it was possible to affirm that the difference between these two samples of different malt tissues was only in quantitative and not qualitative character. (Figure 27)

This tremendous difference in profiles of present volatile carbonyls, therefore in their overall peak areas (Table 10) was very probably caused by very unequal surface of observed samples. Because of that, these results couldn't be considered as valid and further testing focused on comparison of aroma profile of these two tissues has to be done.

V. Discussion

Malt as a traditional basic ingredient for the production of beer has been used in brewing for thousands of years and nowadays is still more often researched for its organic composition and also for its characteristic organoleptic properties. It was discovered, already a long time ago, that differences in used temperature of kilning/roasting and due to the moisture present in malt grains after the malting process are responsible for the creation of various compounds (typically Maillard reaction products) corresponding with different aromas and flavours of the final beer product.

This survey was exclusively focused on carbonyl volatile compounds, which are as a group very important for overall organoleptic properties of the malt, therefore the final beer product. Based on this importance of the malt chemical composition, many studies focused on carbonyl and other present compounds corresponding with typical flavours and tastes in various malts were done during last decades. Usual combination of SPME with GC-MS method was mostly used for these researches studying VOCs in malts. (Fickert and Schieberle, 1998; Dong et al., 2013; Dong et al., 2015) Most of already existing studies were done more as researches uncovering the overall chemical constitution of chosen malts, or connecting antioxidant activities of present compounds, typical flavours or colours of various malts with specific malting conditions. (Wolfenden et al., 2001; Carvalho et al., 2016c)

However, the main aim of this research was to develop the simple working method for the separation and possible identification of particular present compounds and then to assign them with possible specific organoleptic characteristics of beer. After that marker compounds could have been found and attached specifically with studied malt types, which were prepared by using various malting conditions.

Connected HPLC-DAD-ESI-MS/MS method used for the purpose of volatile carbonyl compounds identification used in this work was also already used in similar variation for the analysis of phenolic compounds inherent in different barley and malt samples by Dr. Carvalho. (Carvalho et al., 2015)

The adjusted extraction technique used in this work was combined with already evolved and in articles described techniques. (Cordeiro et al., 2018; Santos and Rodrigues, 2020) These techniques were developed by scientists from the same research group at the Department of analytical chemistry at the University of Porto. The simplicity

of developed extraction technique can be demonstrated by the used apparatus, which consists of just a small glass closable flask connected to the vacuum pump with a little inserted container with 2,4-DNPH solution. Other benefits when comparing this extraction technique with for example SPME is with no doubt affordable price of needed equipment and so low price of run extraction. Another benefits are modesty of needed equipment in the laboratory and big efficiency in the separation of volatile carbonyls from provided samples without necessary heating of the sample. These are main advantages when comparing to other commonly used extraction methods.

VI. Conclusion

The main aim of this work was to set and optimise a methodology of analysis and to deeply study and compare volatile carbonyl compounds in various malt and barley samples. After derivation and extraction of present carbonyls with 2,4-DNPH and then analysing all the samples by HPLC-DAD-MS/MS it was obvious that there were extensive differences in volatile carbonyl profiles between researched samples acquired from different conditions undertaken during the malting process.

During confrontation of all volatile carbonyl profiles the differences in number and intensity of present peaks were compared. The first conclusion proved that barley sample and pale malt samples had poorer volatile carbonyl profile comprised both - number and intensity of peaks, when compared to aroma profiles of roasted specialty malts. Since these distinctions between differently cured malts were observed, it has been more than clear that the number and concentration of present volatile carbonyls has been related with conditions such like the time, temperature and therefore the remained moisture in the grains during the roasting step. After these obvious differences were studied and all data from aroma profiles were collected, it was finally possible to assign some present compounds with known organoleptic attributes within chosen samples and also to set down key aroma compounds for observed specialty malts. Marker compounds were in this study set only for specialty malt samples - chocolate, black and caramel. Since this assigning of marker compounds was successful for these samples, additional studies for other malt samples can be done.

Burnt, toasted, bready and caramel-like flavours can be associated with roasted malts. As the proof of this claim, chocolate malt sample exhibited the richest volatile carbonyl compound profile from all of the roasted malts. Other samples rich on volatiles were caramel malt and black malt. However, the black malt sample was from the very beginning expected to have the richest aroma profile, the intense roasting conditions very probably caused faster volatilization and decomposition of some present compounds and so lowered the intensity of the majority of volatile carbonyls. This revelation just proves, that the temperature used for roasting has a serious impact on the final organoleptic characteristic of the malt.

Next minor aim was to compare separated husk and endosperm tissues to also find possible marker compounds with significant difference in concentration within these two

tissues. However, already the first attempt showed, that the factor of the material surface played an important role when comparing peak areas of these two samples and further studies to eliminate this factor and to find potential marker compounds have to be done.

Used methodology was found efficient enough when considering relatively low RSD values of measured peak areas and the amount of observed volatile carbonyl compounds in malts. It means, that used technique is usable for further analysis of volatile compounds in various malts responsible for different flavours. Used extraction technique showed significant increase of the concentration of volatile carbonyls, too. Moreover, the next benefit of this technique is that the efficiency of the low pressure extraction was sufficient enough, so it was possible to avoid the heating of the sample, which can possibly alter the composition of sample matrices.

The obtained results, simplicity, all referred advantages and the overall efficiency of this extraction technique followed up by HPLC-DAD-MS/MS analysis makes the whole developed method possible to use for more alike studies focused on the analysis of volatiles in malts, or other various food samples in case that the suitable derivative compound is used depending on the disposition of analysed compounds.

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VIII. List of abbreviations

2,4-DNPH - 2,4-dinitrophenylhydrazine

Blk - Black malt sample

Brly - Barley sample

Chlt - Chocolate malt sample

Crml - Caramel malt sample

DAD - Diode array detector

GC - Gas chromatography

HPLC - High performance liquid chromatography

HSSPME - Headspace solid-phase microextraction

MRP - Maillard reaction product

MS - Mass spectrometry

OECD - Organisation of Economic Co-operation and Development

Pils - Pilsner malt sample

PVPP - Polyvinylpolypyrrolidone

RP-HPLC - Reversed-phase high performance liquid chromatography

SPME - Solid phase microextraction

Vac-HSSPME - Vacuum-assisted headspace solid-phase microextraction

VOC - Volatile organic compound