UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY

Matjaž DEŽELAK

BEER-LIKE GLUTEN-FREE BEVERAGES FERMENTED FROM BUCKWHEAT AND QUINOA

DOCTORAL DISSERTATION

Ljubljana, 2014

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BREZGLUTENSKE PIVU PODOBNE PIJAČE FERMENTIRANE IZ AJDE IN KVINOJE

DOKTORSKA DISERTACIJA

Ljubljana, 2014

This doctoral dissertation is the completion of an interdisciplinary postgraduate study program Biosciences, scientific field Biotechnology. The research work was performed mainly at *Slovenian Institute of Hop Research and Brewing*, *Department of Agrochemistry and Brewing,* and partly at *Technische Universität München, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Lehrstuhl für Brau- und Getränketechnologie* and *University of Ljubljana, Biotechnical Faculty, Department of Food Science and Technology, Chair of Biotechnology, Microbiology and Food Safety*.

Based on the Statute of *University of Ljubljana*, and by decisions of *Senate of Biotechnical Faculty* and *University Senate* dated September 13th, 2010, it was confirmed that the candidate fulfils all the criteria for matriculation. Assist. Prof. Iztok Jože KOŠIR, Ph.D., as supervisor and Prof. Peter RASPOR, Ph.D., as co-advisor, were confirmed.

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Member:	Assist. Prof. Tomaž POŽRL, Ph.D. University of Ljubljana, Biotechnical Faculty, Department of Food Science and Technology
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Date of the defense: 14th of November, 2014

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Mentor:	doc. dr. Iztok Jože KOŠIR
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AB Typical beer contains gluten which makes celiac patiens deprived of the pleasure of beer drinking. This research was aimed to prepare gluten-free beer-like beverages from buckwheat and quinoa and explore their physical, chemical, and sensory properties. Three yeast species were used for the preparation of bottled beverages, which were analyzed fresh and force-aged. In addition, bottom-fermenting yeast *Saccharomyces pastorianus* TUM 34/70 was successively used for eleven times to explore the influence of serial repitching on yeast karyotype and protein profile and on fermentation medium. Analytical work included a determination of brewing attributes, metal cations, fermentable carbohydrates, proteinogenic amino acids, saturated and unsaturated fatty acids, aldehydes, ketones, esters, and higher alcohols. In addition, final beverages were sensorily evaluated. Compared to barley, buckwheat malt, wort and beverages showed pretty similar brewing characteristics, whereas quinoa differed from barley significantly. Particularly, total fermentable carbohydrates were lower where glucose was the predominat, all metals were higher, amino acids in wort were higher with their poor assimilation during fermentation, fatty acid, aldehyde and ketone contents were higher, and selected volatile compounds were lower. Yeast *Saccharomycodes ludwigii* TUM SL17 produced low-alcoholic beverage only in the case of barley. The organoleptic perception of buckwheat beverages was generally better than that of quinoa ones, although all showed a good general acceptance. Serial repitching of *Saccharomyes pastorianus* TUM 34/70 revealed that from the yeast biochemistry point of view, both pseudocereals are suitable as a barley substitute in brewery fermentation for at least eleven fermentations. However, the analysis of fermentation medium suggested that besides barley, serial repitching is appropriate only for buckwheat, which in some cases seems even more suitable. Serial repitching of the quinoa wort showed a drastical distinction from barley; poorly controlled metal uptake, low amino acid assimilation, and low beer aroma compound production. In conclusion, from the brewing point of view, buckwheat can represent a gluten-free substitute for barley beer whereas quinoa shows many unique properties which are welcomed when preparing special beverages of choice, but definitely not for the preparation of beer in the proper sense of the word.

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DK UDK 663.4:664.236:633.1:543(043.3)=111

KG celiakija / brezglutenske pivu podobne pijače / ječmen / ajda / kvinoja / *Saccharomyces pastorianus* TUM 34/70 / *Saccharomyces cerevisiae* TUM 177 / *Saccharomycodes ludwigii* TUM SL17 /senzorična analiza /aromatične spojine / kariotip / proteinski profil / zaporedna fermentacija / aminokisline /fermentabilni sladkorji / kovinski kationi / pivovarski atributi / maščobne kisline / aldehidi / ketoni / validacija metode

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AI Običajno pivo vsebuje gluten zaradi česar je kot živilo neprimerno za ljudi s celiakijo. Namen te raziskave je bil pripraviti brezglutenske pivu podobne pijače iz ajde in kvinoje in raziskati njihove fizikalne, kemijske in senzorične lastnosti. Za pripravo ustekleničenih pijač smo uporabili tri različne vrste kvasovk in analizirali tako sveže kot pospešeno starane pijače. Poleg tega smo preučili vpliv enajstkratne zaporedne uporabe kvasovke spodnjega vrenja *Saccharomyces pastorianus* TUM 34/70 na spremembe kvasnega kariotipa in njenega proteinskega profila ter na kemijske spremembe fermentacijskega medija. Analitsko delo je vključevalo določitev klasičnih pivovarskih pokazateljev kakovosti, kovinskih kationov, fermentabilnih ogljikovih hidratov, proteinogenih aminokislin, nasičenih in nenasičenih maščobnih kislin, aldehidov, ketonov, estrov ter višjih alkoholov. V primerjavi z ječmenom so ajdov slad, pivina in pijače pokazali primerljive pivovarske karakteristike, metdem ko se je kvinoja bistveno bolj razlikovala od ječmena. Pri kvinoji je bila vsebnost fermentabilnih ogljikovih hidratov nižja pri čemer je glukoza prevladovala, vsi kovinski kationi so bili višji, aminokisline v pivini so bile višje in slabo asimilirane tekom fermentacije. Vsebnost maščobnih kislin, aldehidov in ketonov je bila višja, vsebnost izbranih hlapnih snovi pa nižja. Kvasovka *Saccharomycodes ludwigii* TUM SL17 je proizvedla pijačo z nizko vsebnostjo alkohola le pri ječmenu. Organoleptično so bile pijače iz ajde ocenjene bolje kot tiste iz kvinoje, čeprav so vse pokazale zadovoljivo splošno sprejetost. Zaporedna fermentacija s kvasovko *Saccharomyes pastorianus* TUM 34/70 je pokazala, da sta z vidika kvasne biokemije obe psevdožiti ustrezni za vsaj enajst fermentacij. A ne glede na to je iz analize fermentacijskega medija bilo moč sklepati, da je za zaporedno fermentacijo poleg ječmena primerna le ajda, v nekaterih primerih je pri slednji primernost celo večja. Po drugi strani pa je zaporedna fermentacija kvinojine pokazala ogromne razlike v primerjavi z ječmenom. Privzem kovinskih kationov je bil slabo nadzorovan, asimilacija aminokislin je bila močno zmanjšana in tudi tvorba hlapnih spojin je bila bistveno zmanjšana. Zaključna ugotovitev je, da je ajda primerna za uporabo v pivovarske namene kot brezglutenski nadomestek ječmena, medtem ko kvinoja kaže veliko edinstvenih lastnosti, ki so dobrodošle pri pripravi specialnih pijač za posebne namene, vsekakor pa ne ječmenovemu pivu podobnih pijač.

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ABBREVIATIONS AND ACRONYMS

AA	amino acid
AT_{50}	the percentage of attenuation time needed to produce/consume a half of total produced/consumed attribute of interest
CV	the coefficient of variation
DP2	sugars with two degrees of polymerization, dissacharides
DP3	sugars with three degrees of polymerization, trissacharides
DS	steady decrease,
DU	unsteady decrease
EI	electron impact ionization
F1-F11	the numbering of particular successive fermentation
FA	fatty acid
FAN	free amino nitrogen
FC	fermentable carbohydrate
FID	flame ionization detector
FLD	fluorescence detector
Fmoc	fluorenylmethyloxycarbonyl chloride, a secondary amine derivatization reagent
FR	rapid fluctuation
FS	slow fluctuation
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
HMW	high-molecular weight
HPLC	high-performance liquid chromatography
HPLC-FLD	high-performance liquid chromatography-fluorescence detection
IS	steady increase
IU	unsteady increase
KI	Kolbach index
LMQ	low-molecular weight
MMW	middle-molecular weight
NAD^+	nicotineamide adenine dinucleotide
NC	negative correlation
NSC	non-significant correlation
OPA	ortho-phthalaldehyde, a primary amine derivatization reagent
PC	positive correlation
RID	refractive index detector
RSD	relative standard deviation
TSN	total soluble nitrogen
TUM 177	the name of *Saccharomyces cerevisiae* yeast strain
TUM 34/70	the name of *Saccharomyces pastorianus* yeast strain

TUM SL17	the name of *Saccharomycodes ludwigii* yeast strain
VC	volatile compound
$\Delta_{max-min}$	the difference between the maximal and minimal value

1 INTRODUCTION

1.1 AIM OF THE STUDY

The realization that a healthy lifestyle, including proper nutrition, reduces the risk of disease and increases health and well-being has received a huge amount of publicity in the past two decades (Arendt and Dal Bello, 2008). The concept of "functional foods" comes from Japan after its introduction during the 1980s, describing special foods which aid health and decrease the risk of diseases (Arendt and Dal Bello, 2008). In November 1995 the European Commission introduced concerted action on "Functional Food Science in Europe" (FUFOSE), which aimed to establish a science-based approach for concepts in functional foods science. As an outcome of this work a new definition of functional food has been established (Arendt and Dal Bello, 2008):

> *"A food can be regarded as functional, when it has proved satisfactory that it influences positively one or several physical functions beyond a nutritive value, in a way that it has relevance for the well-being or the reduction of disease risks."*

A functional drink should contain biologically active substances in order to bring about real additional benefits. Currently, six groups of biological food substances are known (Elmafada, 1998): secondary plant substances, prebiotic carbohydrates, omega-3 fatty acids, conjugated linoleic acid, peptides from milk protein, and Maillard products. According to the above definition, gluten-free beverages based on pseudocereals could already be considered as functional drinks in the broader sense (Arendt and Dal Bello, 2008).

Historically, beer is one of the world's oldest prepared beverages, possibly dating back to the early Neolithic when cereals were first farmed (Hornsey, 2003). Since the introduction of the beer purity law (ger. *Reinheitsgebot*) in 1516, barley has been traditionally used as the main ingredient of beer (Arnold, 1911). To facilitate consumer requirements, other cereals (rice and maize) and pseudocereals (buckwheat, quinoa, and amaranth) have been investigated as brewing ingredients because of the absence of gluten and the presence of compounds that are claimed to have positive effects on health (Zarnkow *et al*., 2005; Kreisz *et al*., 2005).

Celiac disease prevalence has been estimated to be 1 in about 100 people worldwide (Hamer, 2005; Sollid and Khosla, 2005). Such a rate establishes celiac disease as one of the most common food intolerances known. This disease is caused by an immune-mediated response in the small intestine triggered by the ingestion of gluten in genetically-susceptible individuals (Fasano and Catassi, 2001). The only effective treatment is a strict

adherence to a diet that avoids ingestion of cereals (wheat, spelt, triticale, rye, and barley) that contain gluten and their products throughout the patient's lifetime (Ellis *et al*., 1990).

In the case of buckwheat, results collected so far strongly suggest that with the aid of commercial enzymes, buckwheat malt has the potential for replacing barley malt as a gluten-free material (Arendt and Dal Bello, 2008). Still more extensive work is required to optimize fermentation performance and beer characteristics (e.g. flavor, aroma, and foam development) (Arendt and Dal Bello, 2008). Regarding quinoa, extensive research has been carried out worldwide mainly on the agricultural aspects of quinoa (Sigstad and Garcia, 2001), but little has been done on a physiological level or its malting and brewing potential. With regard to the use of quinoa as a brewing ingredient, Kreisz *et al*. (2005) performed malt analysis on optimally malted quinoa and found a slightly higher extract than barley malt.

Since customers are looking for innovative products and additional health benefits while consuming food or drinks, the nutritive properties of buckwheat and quinoa make attempts to investigate these raw materials for malting and brewing purposes worthwhile. But when predictions about the suitability of a cereal for brewing purposes are being made, it is useful to compare its malt properties with others, in particular barley malt. Barley (*Hordeum vulgare* L.) and barley malt have historically been shown to be exceptionally suited for beer brewing purposes and malted grain is still the single most important raw material for beer production nowadays.

Therefore, focus on the brewing potential of the malted buckwheat and quinoa was given at first. Brewing with the 100% malted buckwheat (*Fagopyrum esculentum*, Moench) and 100% malted quinoa (*Chenopodium quinoa*, Wild.) was performed with the use of different brewing yeast species. In particular, besides the bottom-fermenting strain TUM 34/70 (*Saccharomyces pastorianus*, E. C. Hansen) which is commonly used for the industrial lager beer brewing, the bottom fermenting yeast for the production of the low-alcohol beer (*Saccharomycodes ludwigii*, E. C. Hansen) and the classical top cropping strain used for the production of Kölsch beer TUM 177 (*Saccharomyces cerevisiae*, Meyen) were used. Existing malting, mashing and fermentation, conditioning and bottling methods known from the literature, with some modifications, were followed. Worts and the final bottled, force-aged and/or naturally aged beverages were assessed for brewing attributes, metals, fermentable carbohydrates, amino acids, aroma compounds, fatty acids, aldehydes, and ketones. In addition, the final beverages were sensorily evaluated by a trained evaluators. To assure a reference for a useful evaluation of brewing potential of buckwheat and quinoa malt, the same procedures were used for the brewing with 100% malted barley malt.

Next point of our research interest was the influence of a successive application ("serial repitching") of the brewing yeast (*Saccharomyces pastorianus*, E. C. Hansen) strain TUM 34/70 on its karyotype and protein profile and on the nutritional properties of the fermentation medium when fermenting worts derived from the malted buckwheat and quinoa. Individual cells of the brewing yeast exhibit a finite replicative lifespan, which is widely believed to be a function of the number of divisions undertaken. Furthermore, during fermentation yeast is liable to many stress conditions of the environment such as oxidative stress, osmotic stress, change in pH, anaerobic conditions, toxicology of the ethanol, shortage of feeding mater, low temperatures, etc. As a consequence, yeast cells undergo constant modifications in terms of physiology, morphology, and gene expression. In particular, the gradual loss of telomeric sequences (Chiu and Harley, 1997) and differential expression of proteins involved in yeast flocculation (Boulton and Quain, 2001) were already observed.

Such characteristics play an important role in the performance of yeast during alcoholic beverage production, influencing sugar uptake, alcohol and flavour production, and also the flocculation properties of the yeast strain. However, although yeast fermentation performance is strongly influenced by the condition of the yeast culture employed, until recently cell age has not been considered to be important to the process. For a long time appropriate number of successive yeast application was determined only empirically. In that manner, eleven successive fermentations were performed followed by the study of chromosomal size changes the qualitative and quantitative cell wall protein profiling. In addition, chemical attributes, such as some metal cations and other important nutrition, flavor, and stalling compounds were followed to ascertain the effect of replicative cell age on the fermentation performance. On this wise, a better insight was given into a co-dependence between genetic and biochemical changes of the yeast and chemical characteristics of the fermentation medium. The limit successive fermentation in which yeast is still capable to produce beer, satisfactory for the end user, was proposed for barley, buckwheat and quinoa malt, respectively.

To the best of our knowledge, no such detailed examination of buckwheat and quinoa-derived gluten-free beer-like beverages has been yet performed.

1.2 HYPOTHESES

Amino and fatty acid content of the buckwheat and quinoa beer-like beverages is higher as compared to common, standard barley beer, and aldehyde and ketone content does not differ significantly.

Functional yeast cell age results in changed metabolism in a sense that it becomes less suitable for the fermentation of the buckwheat and quinoa wort.

The fermentation profile of organic compounds and metal cations vary significantly between individual yeast batches and these variations are specific for malted barley, buckwheat, and quinoa products, respectively.

Non-conventional malt sources have no impact on cell-wall protein profile, chromosomes length, and flocculation capacity but they vary significantly between individual yeast batches.

2 LITERATURE REVIEW

2.1 BEER AND BEER BREWING

Beer is a traditional beverage and one of the most charming foodstuffs of mankind. It is produced worldwide and for the majority of societies it is a beverage of choice. Traditionally, it is prepared from the barley malt although the addition of other malted or non-malted raw materials is common. Usually, at least one of the ingredients presents a source of gluten which triggers the autoimmune reaction in genetically predetermined individuals (Shan *et al*., 2005). Although beer is a highly diluted barley product, the analyses of beer from barley with hordein deletion and commercially available beers confirmed that all barley-based beers tested still contained hordeins (Colgrave *et al*., 2012). On the contrary, there are some rare studies reporting the gluten content in commercial beers below the limits of Codex Alimentarius Standard (20 ppm) (Guerdrum and Bamforth, 2011), but a precaution is still needed since clinical sensitivity toward gluten differs substantially from patient to patient and the gluten content of the same beer type could vary significantly between batches (Guerdrum and Bamforth, 2001). All that make celiac patients deprived of the pleasure of beer drinking.

Ever since the first human civilizations started to develop, beer was considered as an important foodstuff on the one hand and an essential cultural tie on the other hand. Right from the beginnings, barley was selected as a main brewing component although the addition of other cereals was not uncommon (Hornsey, 2003). General brewing technology procedures have not essentially changed (Figure 1) but in the past decade, research was focused on the use of alternative raw materials for brewing purposes not only to satisfy different dietary requirements of consumers but also to widen the food choices in the market. Among them, gluten-free beer-like beverages are the subject of rising interest (Zarnkow *et al.*, 2005) since the occurrence of celiac sprue is increasing dramatically (Evans and Sanders, 2012).

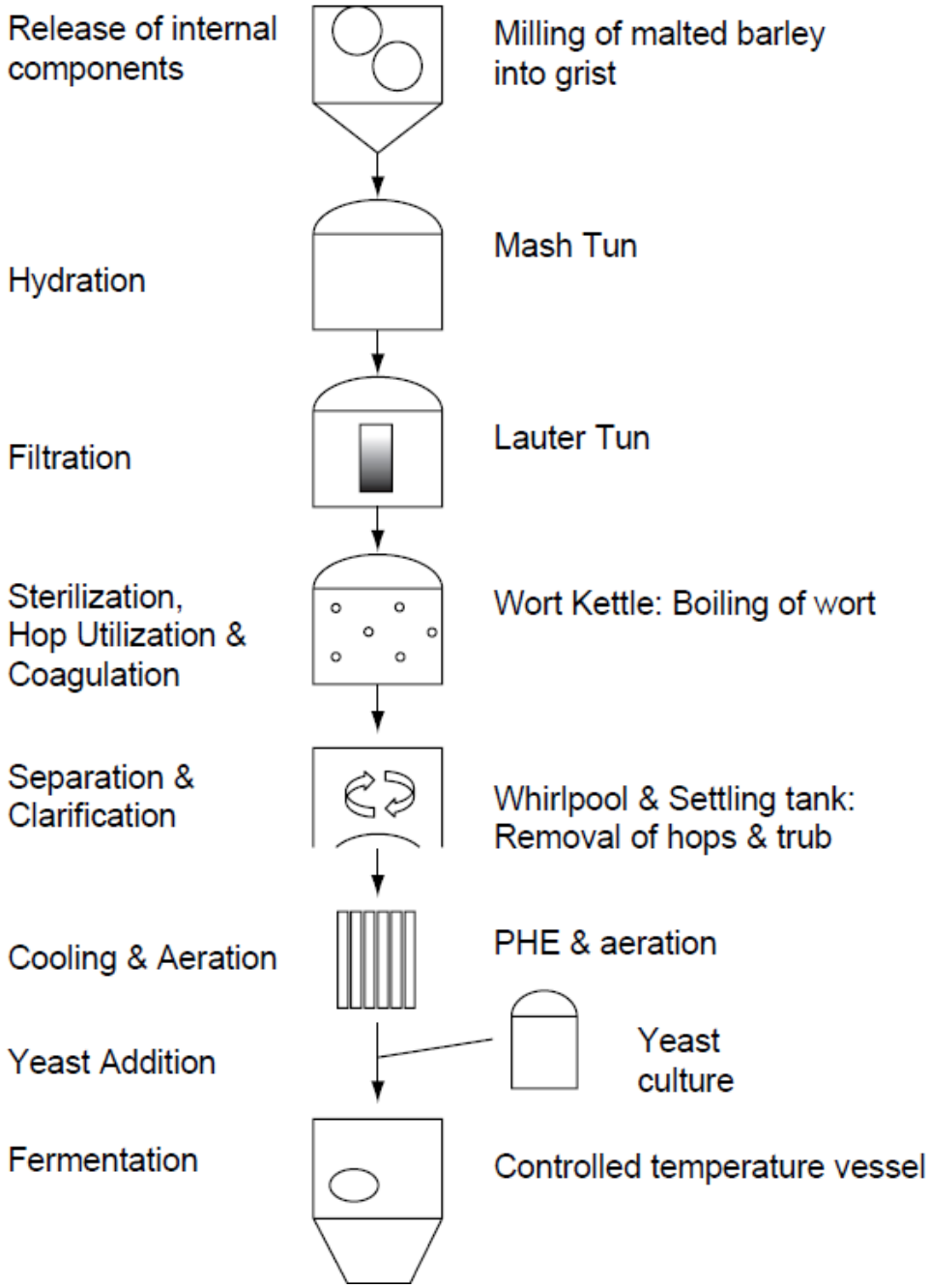

Figure 1: The flow diagram of the brewing process (Arendt and Dal Bello, 2008)
Slika 1: Shematski prikaz pivovarskega postopka (Arendt in Dal Bello, 2008)

The only acceptable solution for celiac patients is a strict life-long elimination of gluten from the diet. However, concerns have been raised over the long-term strict dietary habits as results indicated an unbalanced intake of main nutrients and a limited intake of certain essential nutrients (Alvarez-Jubete *et al*., 2010). The scarce gluten-free food choices available in the market and their poor quality may represent major determinants in the delicate nutrition. Although the gluten-free diet is a well-established solution for celiac disease, nowadays it has been proposed to be also used for the prevention and treatment of

other diseases, such as rheumatoid arthritis (Hafström *et al*., 2001), type 1 diabetes mellitus (Mojibian *et al*., 2009), obesity (Pires Soares *et al*., 2013), and insulin resistance (Jönsson *et al*., 2005) also in non-celiac individuals.

Because beer drinking is an important human habit that strengthens cultural ties in the majority of societies and because commercial beer without gluten hardly exists, gluten sensitive individuals often tolerate side effects in order to take part in popular activities. In addition, since gluten-free diet was established also as a precaution step for some autoimmune and metabolic disorders, there is a growing demand for wider choices, higher quality and better tasting gluten-free products also among non-celiac individuals.

2.2 ALTERNATIVE GLUTEN-FREE RAW MATERIALS IN BEER BREWING

In general, a functional food is regarded as a food that is suitable to be consumed as a part of the common nutrition and that contains biologically active components with the ability to increase health and to lower the risk of diseases (Arendt and Dal Bello, 2008). The conclusion of this statement is that the absence of gluten is yet a sufficient reason for beverage to be classified as a functional one. In principle, the designation "gluten-free" only narrows down the possible cereals that are considered as a starting basis for functional beverages (Arendt and Dal Bello, 2008). Thus, the standard procedure is not changed, although it has not yet been decided unanimously what kinds of cereals, beside the pseudocereals, which are basically gluten-free, can be used as a raw material for beverages (Arendt and Dal Bello, 2008).

Almost all standards, analysis, and technological know-how in malting, substrate production as well as fermentation are based on research and experience with barley and barley malt (Arendt and Dal Bello, 2008). No other cereals or pseudocereals have been optimized for malting, substrate production, or fermentation, and their breeding programs are sometimes counterproductive as they are focused on high protein levels and low enzyme activities (Arendt and Dal Bello, 2008). Nevertheless, the general proceedings in evaluating the raw material for the beverage production may be adapted from barley to any cereal or pseudocereal (Arendt and Dal Bello, 2008). From the botanical point of view, buckwheat and quinoa are dicotyledonous plants and thus not cereals (monocotyledonous), but since they produce starch-rich seeds like cereals they are called "pseudocereals". Among quinoa sweet and bitter varieties exist, dependent on the content of saponins. Two varieties of buckwheat are commonly cultivated: common buckwheat (*Fagopyrum esculentum*, Moench) and tartary buckwheat (*Fagopyrum* tataricum, (L.) Gaertn.). Table 1 summarizes the nutritional composition of buckwheat and quinoa (Arendt and Dal Bello, 2008).

Table 1: Biochemical and nutritional composition of buckwheat and quinoa (Arendt and Dal Bello, 2008)
Preglednica 1: Biokemijska in hranilna sestava ajde in kvinoje (Arendt in Dal Bello, 2008)

component	composition [%] (dry weight, range in brackets)	
	Chenopodium quinoa	*Fagopyrum esculentum*
water	12.7	14.1 (13.4-19.4)
protein (N×5.8)	13.8 (12.2-13.8)	10.9 (10.4-11.0)
fat	5.04 (5.01-5.94)	2.71 (2.40-2.80)
starch	67.35	67.2
crude fiber	2.3	–
dietary fiber	12.88	8.62
minerals	3.33 (2.46-3.36)	1.59 (1.37-1.67)

It should be stressed out that most likely it will never be possible to produce an "ideal" beverage containing all potentially beneficial compounds found in pseudocereals and having acceptable organoleptic properties like barley beer. Consequently, when the production of a functional beverage is planned, raw materials and processing steps need to be carefully assessed in order to fulfill the demands of the consumer with regard to taste, aroma, and appearance as well as ensuring that the desired functional properties are available and active (Arendt and Dal Bello, 2008).

When seeking a palatable, high quality, and healthy gluten-free raw material, the nutritive properties of buckwheat and quinoa make aims to research these pseudocereals for malting and brewing purposes worthwhile. The preparation as well as the physical, chemical, and sensory analyses of bottom-fermented beverages from malted buckwheat and quinoa are reported in this doctoral dissertation. Based on the results a critical estimation of their commercial potential has been exposed.

2.3 BUCKWHEAT

Buckwheat originates from Central Asia and was transferred by nomadic people to Central and Eastern Europe. Today, buckwheat is celebrating something of a comeback due to the demand for gluten-free diets, and it is now grown on 2.5 million hectares, producing 2 million tons of grain (Arendt and Dal Bello, 2008). The buckwheat starch shows a characteristic fraction composition in which amylose and amylopectin are found in equal ratios. In general, the buckwheat starch exhibits a higher gelatinization temperature, peak and set back viscosities (Zheng *et al*., 1998) than cereal starches. The water-binding capacity of buckwheat starch is 109.9% which is explained by the small size of buckwheat starch granules (Qian *et al*., 1998). The main components of buckwheat seed proteins are salt-soluble globulins. (Milisavljević *et al*., 2004). Buckwheat proteins have a higher or similar content of all amino acids when compared with wheat proteins, except for glutamine and proline, which are found in lower amounts. Glutamic acid, followed by aspartic acid, arginine, and lysine are the most represented amino acids where the content of the limiting amino acid lysine is 2.5 times higher than that found in wheat flour (Aubrecht and Biacs, 2001). Among lipids, linoleic acid, oleic acid, and palmitic acid

account for 88% of the total fatty acids (Horbowicz and Obendorf, 1992). With typically 80% unsaturated fatty acids and more than 40% of the polyunsaturated essential fatty acid linoleic acid, buckwheat is nutritionally superior to cereal grains (Steadman *et al*., 2001a). Except for calcium, buckwheat is a richer source of nutritionally important minerals than many cereals such as rice, sorghum, millet, and maize but a poorer compared to wheat (Adeyeye and Ajewole, 1992). Much interest is focused in phytochemicals. In a recent study, the total phenolic acids content of buckwheat husk and flour were found in the ranges of 30 and 15 mg/100 g, respectively (Gallardo *et al*., 2006). The bran and wholemeal fraction have a high concentration of tannins (0.4 g/100 g non-condensed and 1.7 g/100 g condensed tannins) and total flavonoids (2.42 g/100 g moist mass), respectively (Liu and Zhu, 2007). Rutin, a rhamnoglucoside of the flavonol quercetin, are of particular interest, as they are also used for medical purposes in many countries. Similar amounts of rutin in tartary and common buckwheat hulls have been reported by Steadman *et al*. (2001b).

Buckwheat can be obtained either hulled or unhulled, but recent studies have demonstrated that the use of unhulled buckwheat is advantageous over hulled material, since the water uptake is slower and the resulting malt is improved (Wijngaard *et al*., 2005b). In addition to lowering malting loss, another advantage of using unhulled material is improved filterability. At these moisture levels the malting loss falls within an acceptable range and malt quality is optimized. Optimal enzymatic activity in buckwheat malt can be obtained when buckwheat is germinated for 96 hours at 15°C (Wijngaard *et al*., 2005b). At this time, the grains are sufficiently modified and nutrients have not yet been exhausted. Moreover, rutin, a polyphenol with functional properties, is increased significantly during malting. Wort derived from malted buckwheat showed low fermentability values and high viscosity levels in comparison to wort derived from barley malt (Nic Phiarais *et al*., 2005; Wijngaard *et al*., 2005b). Optimizations of mashing procedures were performed combining rheological tests with traditional mashing experiments (Goode *et al*., 2005). However, all studies performed so far have shown that the enzymatic content of buckwheat and its malt is significantly lower than that of barley malt (Nic Phiarais *et al*., 2005, Wijngaard *et al*., 2005b; Zarnkow *et al*., 2005). These problems can be overcomed by the addition of commercial enzymes (Bajomo and Young, 1992). It was found that the addition of increasing levels of α-amylase to the buckwheat mash increased color, extract levels, wort filtration, fermentability, and total fermentable extract, along with decreasing viscosity values. Furthermore, the addition of increasing levels of amyloglucosidase to buckwheat mashes resulted in corresponding increases in fermentability and total fermentable extract, along with increases in total soluble nitrogen, free amino nitrogen, and Kolbach index.

2.4 QUINOA

Quinoa was major crop for the pre-Colombian cultures in Latin-America. Since it has been shown that both grains show good nutritional properties, the interest in them has risen

again. The main component of carbohydrates in quinoa is starch. It has higher gelatinization temperatures and higher pasting viscosities than cereals (Arendt and Dal Bello, 2008). Furthermore, the low amylase content is responsible for a high water-binding capacity, high swelling power, high enzyme susceptibility, and excellent freeze–thaw and retro-gradation stabilities (Atwell *et al*., 1983; Qian and Kuhn, 1999a). The amounts of sugars are small; at most few percent (Arendt and Dal Bello, 2008). The quinoa proteins are mainly globulins and albumins. The amino acid profile of each protein fraction showed a balanced content of essential amino acid, with a high level of lysine (4.5-7.0%) (Watanabe *et al*., 2003). Quinoa protein is close to the FAO recommended pattern in essential amino acids (Prakash and Pal, 1998). The degree of unsaturation of the fat is over 75% (Przybylski *et al*., 1994) or, according to Ando *et al*. (2002), even higher than 87%. The content of minerals in quinoa seeds is approximately twice as high as in cereals and the content of vitamins in quinoa is similar to that found in conventional cereals (Arendt and Dal Bello, 2008). Quinoa also contains some phytochemicals. Different tannin contents in quinoa have been reported, with values varying from 0 to 500 mg/100 g (Chauhan *et al*., 1992). The polyphenols present are mainly kaempferol and quercetin glycosides (Zhu *et al*., 2001). Quinoa (whole seeds) also contains between 0.03 and 2.05% of bitter tasting saponins (Cuadrado *et al*., 1995) and 0.1-1.0% phytic acid (Chauhan *et al*., 1992).

To date**,** little research has been carried out on quinoa as a brewing ingredient, and mainly studies on the properties of quinoa starch are available (Atwell *et al*., 1983; Qian and Kuhn, 1999a). Quinoa starch exhibits a much higher viscosity than wheat (Atwell *et al*., 1983) and amaranth (Qian and Kuhn, 1999a). In contrast to maize starch, quinoa starch exhibits a single-stage starch swelling in the temperature range 65-95°C and lower viscosity (Ahamed *et al*., 1996). Quinoa seeds have the advantage of fast germination *in vitro*, although they germinate very poorly in soil (Aufhammer *et al*., 1996). When malted for 36 hours, the α-amylase activity of quinoa increased 4-fold (Atwell *et al*., 1988); however, the starch granules of the perisperm appear not to be extensively degraded by amylase during germination (Varriano-Marston and De Francischi, 1984). While this is not advantageous for the malting process, it may provide some benefit for mashing and brewing. Using 'Response Surface Methodology', Zarnkow *et al*. (2005) optimized the malting conditions of quinoa as follows: steeping time of 36 hours, degree of steeping 54%, and a germination temperature of 8°C for a time of 144 hours. Malting quinoa grain also improves nutrient availability. During germination phytate is reduced by 35-39%, whilst iron solubility under physiological conditions (and *in vitro* estimation of iron availability) increases 2- to 4-fold (Valencia *et al*., 1999).

2.5 CHOSEN YEAST STRAINS

The following information was provided by the supplier of yeast strains.

Bottom-fermenting flocculent yeast: strain TUM 34/70

These yeast strain require a complete trub discharge and a relatively high oxygen necessity in the wort. The flocculent behaviour with the cultures W34/70 is somewhat higher as by the culture W34/78, nevertheless, it is still to be identified as normal. Under well-optimized physical and chemical conditions the results gained from the metabolism from these pure yeast cultures provide a very pure beer. Without an intrusive aroma, nevertheless very mild taste profile. The course of fermentation takes place quickly, whereas certain temperature sensitivity upon cooling is to be monitored. If the beer is to be stored for longer periods the possibility of a yeasty bitter taste should be monitored. This demanding pure cultured yeast strain yield, by good technological guidance, an excellent beer. Raw grain additives of up to 40% accomplish for the yeast by the usage of zinc that supports the metabolism and creates no problems. However, the yeast stress reaction should be monitored. The original wort over 16% should be initially avoided with large tanks.

Kölsch yeasts: strain TUM 177

This is the classical strain used for the production of Kölsch beers, with a light fruity estery taste and character with lower amyl-alcohol contents. Similar to the alt beer yeasts this yeast strain can be fermented with high or low temperatures. The diacetyl degradation is, especially by higher temperatures than 20°C, as good as complete. Therefore, a very clean and very pure beer is to be expected.

Bottom-fermenting yeast for the production of low-alcohol beers: strain TUM SL17

Saccharomyces ludwigii is a special yeast that is used in breweries, that is then used when only a small amount of the wort sugar should be fermented. This is a weak-fermenting style since the maltose and the maltotriose cannot be fermented. Among the wort sugars only glucose, fructose and the sucrose are then fermented.

2.6 NUTRITIONAL AND QUALITY PROPERTIES OF THE FERMENTATION MEDIUM AND FINAL BEVERAGES

Beers are quite similar in most respects but small differences in their composition can greatly affect both appearance and flavour. It is a very complex beverage that contains about 800 organic compounds (Buiatti, 2009). Many of them have such a low level that only those having a flavour active impact can have a real influence on taste and smell perception. Most chemical compounds in beer were either present in the raw materials (malts, hops and water) or they are by-products of yeast metabolism during the fermentation and are responsible for most of the flavour character that is unique to beer (Buiatti, 2009). The main constituents of a beer are shown in Table 2 (Buiatti, 2009).

Table 2: Composition of a typical barley beer (Buiatti, 2009)
Preglednica 2: Sestava običajnega ječmenovega piva (Buiatti, 2009)

substance	concentration	number of different compounds	source or agent
water	90-94%	1	–
ethanol	3-5% v/v	1	yeast, malt
carbohydrates	1-6% w/v	~100	malt
carbon dioxide	3.5-4.5 g/L	1	yeast, malt
inorganic salts	500-4,000 mg/L	~25	water, malt
total nitrogen	300-1,000 mg/L	~100	yeast, malt
organic acids	50-250 mg/L	~200	yeast, malt
higher alcohols	100-300 mg/L	80	yeast, malt
aldehydes	30-40 mg/L	~50	yeast, hops
esters	25-40 mg/L	~150	yeast, malt, hops
sulphur compounds	1-10 mg/L	~40	yeast, malt, hops
hop derivatives	20-60 mg/L	>100	hops
vitamin B complex	5-10 mg/L	13	yeast, malt

2.6.1 Brewing attributes

2.6.1.1 Malt

In the production of malt for brewing purposes after the steeping of grain its germination take place. During this step the progressive degradation of the cell walls of the starchy endosperm takes place, which involves the breakdown of the troublesome β-glucans and pentosans, followed by the partial degradation of the protein within the cells and the partial or locally complete breakdown of some of the starch granules, the small granules being attacked preferentially (Briggs *et al*., 2004). At the desired stage, germination is terminated by the kilning of 'green' malt. It results in the inactivation of some enzymes and changes in flavor and color (Bamforth, 2003). In general, malt extract reflects the extent to which the endosperm has become solubilized during germination of barley and also that insoluble fraction from malt that is released by enzymes during mashing (Bamforth, 2003). The time in minutes taken after the mash has reached 70°C for samples to stop giving a positive iodine test for starch is recorded as the 'saccharification time'. This is really a rough measure of the time taken for the starch to be dextrinized, and is largely dependent on the α-amylase content of the malt. Normally, pH value of pale barley malt ranges between 5.80 and 5.95 (Eßlinger, 2009). Bertholdsson (1999) advocated that total protein content should be lower than 11.5% since higher values are undesirable because of the reduced fermentable extract. Kolbach index (KI) serve as a approximate measure of grain modification – although it do not always parallel the estimates of physical modification – and a malt with a KI between 36% and 42% is considered as a malt that is adequately modified and suitable for single infusion mashing (Briggs *et al*., 2004). Marked over-modification has many practical disadvantages: (i) malt breakage and losses (as dust) are high, (ii) head retention may be poor, (ii) yeast growth can be wastefully excessive, (iii) the wort may contain finely divided material that is hard to remove by filtration and, (iv) because of the excessive levels of reducing sugars and amino acids present, the wort may

darken too much on boiling, due to the formation of melanoidins, the major products of Maillard reactions (Briggs *et al*., 2004). Free amino nitrogen (FAN) values (chiefly amino acids and small peptides) must be sufficiently high to ensure that lack of nitrogenous yeast nutrients does not limit fermentation.

2.6.1.2 Wort

During mashing, enzymes of malt or, optionally, external enzymes degrade polymeric grain constituents, especially carbohydrates proteins. The most interesting enzymes in brewing are those which catalyse the hydrolysis of starch and dextrins, those which attack hemicelluloses and gums (both β-glucans and pentosans), and those which degrade proteins. Viscosity is caused mainly by dissolved sugars, dextrins, pentosanes and perhaps other materials (Briggs *et al*., 2004) and the range between 1 and 2 mPas is believed not to cause brewing problems also when using raw materials other than barley (Eßlinger, 2009; Klose *et al*., 2011; Zarnkow *et al*., 2005; 2010). While high viscosity of wort can cause problems in wort separation, low extract recoveries, and slow fermentation, no link to foam has been confirmed (Briggs *et al*., 2004). Kunze (1996) stated that normal pH value of barley wort ranges from 5.5 to 5.9. The release of the Maillard products formed during malting or their *de novo* formation during wort boiling have a major impact on the color and aroma as well as on the pH of the wort (Eßlinger, 2009). Inadequate yield of small nitrogenous molecules, i.e. FAN, limits yeast growth and impair fermentation (Briggs *et al*., 2004) thus having a great overall influence on the final product. In principle, FAN should not drop under 150 mg/L (referring to extract of 12%) (Eßlinger, 2009). Iodine reacts with dextrin and starch to form a reddish-blue color which gives a valuable information about the completeness of saccharification (Eßlinger, 2009); however, iodine test has a limited reliability since incompletely degraded starch that is complexed, e.g. with lipids, do not give colour with iodine (Briggs *et al*., 2004).

2.6.1.3 Beverages

The proportion of the wort dissolved solids (extract) which can be fermented is called the percentage fermentability of the wort (Briggs *et al*., 2004). Decrease in extract percentage is mainly due to the uptake of fermentable carbohydrate and nitrogenous nutrients and it correlates with ethanol production and biomass gain. Using the same yeast strain under the same conditions, fermentation performance is a function of wort chemical composition. During fermentation, the transformation of wort into beer is accompanied by a decrease in pH as a consequence of (i) the proton antiport component of other uptake systems, (ii) the formation of carbonic acid derived from the carbon dioxide produced during fermentation, and (iii) the excretion of several organic acids, notably, lactic, citric, pyruvic, malic, acetic, formic, succinic, and butyric acids from fermenting yeast cells (Briggs *et al*., 2004). Namely, yeast assimilates a number of metabolites with the use of proton antiport system and around 30% of media acidity is attributed to ATPase-mediated proton pumping (Walker, 2004). Beer pH values are typically in the range of 4.25-4.60. The total soluble

nitrogen (TSN) needs to be sufficiently high so that the 'body' and mouth-feel of the beverage is adequate, and the foam (or 'head') will be stable. FAN is part of the low molecular nitrogen and mainly covers the proteinogenic amino acids (Briggs *et al*., 2004). Products of Maillard reactions have a major impact on the pH and aroma as well as on the colour of the wort. Additionally, high molecular polyphenols can increase the beer color. In darker worts a cause of concern is often a deficiency in FAN level because it is used up in the formation of the Maillard products (Eßlinger, 2009).

2.6.2 Metals

Besides water, metal ions are single most important inorganic substances in wort and their presence in optimal amounts and bioavailable form is a general prerequisite for a satisfactory viability, vitality and fermentation performance of the yeast. Some metal ions may be precipitated during the brewing process on the break and others may be absorbed by the yeast; that means the inorganic salts present in beer are very different from those present in the brewing water used (Buiatti, 2009). Especially the trace metals explored in this study, i.e. iron, copper, zinc and manganese, play a crucial role, being a part of haem-proteins, cytochromes, redox pigments, enzyme cofactors, and others. Their relevance in the brewing process has been widely reviewed elsewhere (Walker *et al*., 2004). Besides the physiology status of yeast, the uptake rate and dynamics of a particular metal is primarily dependent both on its concentration in wort, as well as on its bioavailability. Since the latter is mainly governed by the solubility capacity of a milieu and the presence of complexing chelators (Chandrasena and Walker, 1997), as well as by the sugar and alcohol content in the fermentation medium (Mizoguchi and Hara, 1998), the differences in iron, copper, zinc and manganese concentrations between raw materials, as well as between successive fermentations, are righteously expected. In general, the uptake of metal ions by yeast is a biphasic process. Firstly, ions are concentrated by attachment to the cell surface, a passive process termed biosorption. Suggested mechanisms for attachment to the cell wall include complexation, ion exchange, adsorption and precipitation (Blackwell *et al*., 1995). The process is independent of temperature, does not require metabolic energy or indeed viability. Secondly, ions are transported across the plasma membrane and into the cell by bioaccumulation. This is an active process involving H^+ symport and K^+ efflux. Once in the cell, metal ions are commonly compartmentalized in the vacuolar system (Briggs *et al*., 2004). The particular distribution of different species is contingent on pH, chemical composition, temperature, and redox potential. Even for barley beer there are only few reports regarding metal nutrition which used the same yeast twice (Kreder, 1999; Mochaba *et al*., 1996a, 1996b) or more (Aleksander *et al*., 2009). All in all, knowing the amount of individual metal in wort and beverage give a valuable information about quality prospects but in complex media like brewer's wort metal-metal interactions also affect bioavailability of the single. Metals may compete with each other for binding sites and they may also act antagonistically toward each other in terms of biochemical functions.

Some studies regarding K, Mg, Ca, and Zn interactive effect had already been made (Chandrasena *et al*., 1997); however, very little is known so far.

2.6.2.1 Iron and copper

Both transition metals, Fe and Cu, play an important role in free-radical reactions and are thus responsible for beer deterioration (Kaneda *et al*., 1992). Being involved in the metal-catalyzed Fenton and Haber-Weiss reactions they give rise to reactive oxygen species. Because of the extremely high levels of Fe in quinoa beverage, it is expected to be prone to off-flavor development causing the shortening of the shelf life.

The presence of iron (Fe) is avoided by brewers because it can have a negative action as pro-oxidant and so accelerating the beer staling. Fe salts above 0.2 mg/L can have a negative effect slowing the saccharification, resulting in hazy worts and reduced yeast activity. If its concentration is above 0.3 mg/L causes greyish foam and an increase in colour. Fe concentrations of more than 1 mg/L weaken yeast and increase haze problems and oxidation of tannins (Buiatti, 2009). The predominant Fe species is ferrous ion which acts as a catalyst in some chemical transformations (Kaneda *et al*., 1992), for example oxidation of tannins (Buiatti, 2009). Over time, redox potential changes together with the fall of ferrous ion level and the amount of non-heme ferric ions increase which are finally responsible for beer haze, grayish foam, and increase in colour (Kaneda *et al*., 1992; Buiatti, 2009).

As iron, the presence of copper (Cu) is avoided for the similar reasons since in concentration as low as 0.1 mg/L it behaves as a catalyst of oxidants causing beer haze formation. When Cu concentrations are above 10 mg/L, it becomes toxic to yeast (Buiatti, 2009). In beer-like beverages even small amounts of Cu (~0.15 mg/L) can cause gushing, noticeably contribute to the oxidation, and impart an unpleasant metallic taste (Mayer *et al*., 2003). On the other side, Cu was reported to reduce sulfury flavour by binding sulfur derivatives (Richter *et al*., 2001) which could improve sensory perception.

2.6.2.2 Zinc and manganese

Trace amounts of zinc (Zn) are essential for yeast growth whereas larger amounts can be toxic. Zn has an important role in yeast metabolism and fermentation process with a positive action on protein synthesis and yeast growth. Its levels between 0.08 and 0.2 mg/L is recommended to have positive effects on fermentation while zinc content above 0.6 mg/L can *vice versa* affect negatively fermentation and colloidal stability of beer. More than 1 mg/L of Zn is toxic to yeast cells (Buiatti, 2009). In brewing process, Zn is particularly important because it acts as activator of the terminal alcohologenic Zn-metalloenzyme, ethanol dehydrogenase, and it can also stimulate uptake of maltose and maltotriose into yeast, thereby augmenting fermentation rates (Walker, 2004). What is

more, its deficiency in wort can occasionally occur (< 0.1 mg/L) since Zn ions can be chelated by wort amino acids, proteins, and phytate and a proportion of these may be removed as insoluble precipitates during the wort boiling (Daveloose, 1987). Besides its chemical and biochemical role in brewing, the red color of the mash can be attributed to the high content of Zn (Whali, 1990). Concentrations between 0.275 and 0.550 mg/L are said to be satisfactory and non-toxic (Walker, 2004). Yeast takes up Zn very rapidly from wort and it seems that a large proportion of it is simply biosorbed (cell wall-bound) (Hall, 2001).

Manganese (Mn) in trace amounts is, similarly as Zn, essential for proper yeast growth since it is an important enzyme cofactor and it acts positively on protein solubilization. This ion can inhibit yeast metabolism and affect negatively colloidal stability of beer. In wort it should be present in the range from 0.11 to 0.22 mg/L (Walker, 2004) but in no case more than 0.5 mg/L (Buiatti, 2009). It is an indispensable enzyme cofactor of several yeast enzymes, such as cytosolic CuZn and a mitochondrial Mn superoxide dismutase (Briggs *et al*., 2004).

2.6.3 Fermentable carbohydrates

Regardless of raw material used, if wort is meant for beer preparation, it practically always contains all the carbohydrates which yeast is able to ferment, i.e. fructose, glucose, sucrose, maltose and maltotriose, but their ratios and absolute concentrations can vary drastically. The uptake of sugars by brewer's yeast has been subject to the closest scrutiny as befits their role in industrial fermentations (Briggs *et al*., 2004). After being pitched, the yeast (in an appropriate physiological condition) immediately ingests the monosaccharides along with the simultaneous cleavage of sucrose by an invertase in the periplasm. The initial glucose concentration in the wort plays a key role in the order of the sugar consumption since it represses the utilization of others by an effect known as 'carbon catabolite repression'. That is why high glucose levels in wort are not recommended because the yeast enzymatic system becomes adapted to the high glucose amounts and reduces or even halts the ensuing maltose and maltotriose intake (Eßlinger, 2009). In addition, fermentation performance is affected negatively if glucose is the predominant carbohydrate in wort (Easlon *et al*., 2007) and yeast exhibits higher viabilities in maltose than in glucose media (Stewart, 2006). Ethanol cannot be produced without significant yeast cell growth and non-growing yeast cells ferment only enough sugar to produce energy for cell maintenance (Walker, 2004). In the case of brewers' wort, the utilization of sugars is an ordered process. Sucrose is hydrolyzed by an invertase that is secreted into the periplasm. This results in a transient increase in the concentrations of fructose and glucose, which are assimilated simultaneously. The predominant sugar, maltose is then taken up. When the maltose concentration falls to an undetectable level maltotriose is assimilated. Longer chain sugars are not utilized by brewing yeasts (Briggs *et al*., 2004).

Brewing strains of *S. cerevisiae* utilize a limited repertoire of carbon sources for growth and differences in the patterns of utilization are strain-specific (Briggs *et al*., 2004). For example, (i) lager strains can grow on melibiose (Barnett, 1981), (ii) they utilize maltotriose more rapidly than ale strains (Stewart *et al*., 1995), and (iii) they utilize mixtures of galactose and maltose simultaneously (Crumplen *et al*., 1993). The initial concentration and spectrum of fermentable carbohydrates control the concentration of ethanol synthesized during fermentation (Briggs *et al*., 2004). The conversion of sugars to ethanol is about 85% of the theoretical (Briggs *et al*., 2004). During fermentative growth, the oxidative pathways are inoperative and NAD^+ has to be regenerated by the reduction of acetaldehyde to ethanol (Briggs *et al*., 2004).

Saccharomyces strains are facultative anaerobes and fermentative metabolism during beer brewing is assured by two distinct mechanisms operating at different time periods. In the initial aerobic phase the glucose catabolite repression (the so called 'Crabtree effect') is of principal importance which ensures that metabolism is fermentative. In the later stages of fermentation, although the glucose repressing signal is usually absent, anaerobiosis ensures that oxidative respiratory metabolism does not develop and other sugars are fermented (Briggs *et al*., 2004). After fermentation maltose and maltotriose are the most abundant fermentable sugars and sucrose levels are very low, whereas glucose and fructose presence is below detection limit.

Glucose and fructose are assimilated by the multiplicity of uptake systems which can be divided into two classes, termed high and low affinity (Briggs *et al*., 2004). Their regulation is not precisely elucidated; however, some of them are subject to 'nitrogen catabolite inactivation' (Busteria and Lagunas, 1986). In general, the affinity of the glucose carrier towards its substrate may not only depend on the availability of glucose but also the presence of oxygen, the growth rate, and energy status of the cell (Walker, 1998).

Maltose is the most abundant sugar in wort and the main member of dissacharides. Its uptake is an energy-requiring proton symport process and a subject to both 'nitrogen' and 'glucose catabolite inactivation' (Briggs *et al*., 2004). Maltose utilization is controlled by a complex series of *MAL* genes on different loci, each consisting of three genes, which encode a maltose carrier, maltase and a post-transcriptional regulator of the carrier and maltase genes. In the presence of glucose, the maltose permease is irreversibly inactivated via the action of a protease (Briggs *et al*., 2004).

Maltotriose is the the principal trisaccharide in wort and the largest sugar molecule that can be assimilated by yeast. Its uptake is accomplished by a constitutive facilitated diffusion carrier (Briggs *et al*., 2004) but once inside the cell it is hydrolysed to glucose units with the same enzyme as maltose (Stewart, 2006).

2.6.4 Amino Acids

During mashing, proteins and peptides in malt are broken down to amino acids thereby continuing the enzymatic degradation started during malting operations. As a consequence the level of nitrogenous compounds that will be available to the yeast later in fermentation is increased. Most of these amino acids will be used by the yeast for its multiplication, apart from proline which is not utilized by yeast in anaerobic conditions and carried out through the beer. The amino compounds found in beer are almost exclusively nitrogenous compounds that were not utilized by the yeast. Some wort amino acids are metabolized by yeast to form higher alcohols (or 'fusel alcohols') which are important flavour compounds in beer. In fact deamination and transamination reactions carried out by the yeast cell are responsible for the presence of several organic acids, aldehydes, alcohols and esters in beer; most of them are cast-out carbon skeleton of amino acids which were in wort (Baxter and Hughes, 2001). Table 3 summarizes amino acids found in wort and beer made from barley (Krüger and Anger, 1992). Undoubtedly, beer made from other raw material (e.g. buckwheat, quinoa) would most likely contain entirely different amounts of a particular amino acid.

Saccharomyces strains do not produce extracellular proteases so neither proteins are utilized nor they utilize nitrate or nitrite. The main sources of nitrogen in wort are amino acids, ammonium ion, and some di- and tripeptides (Briggs *et al*., 2004). The presence of ammonia or glutamine causes the repression of the enzymes required for the catabolism of other amino acidss (a.k.a. 'nitrogen catabolite repression') (Wiame *et al*., 1985). Glutamine is an essential precursor for the biosynthesis of other amino acids, as well as purines, pyrimidines, and N-acetylglucosamine, the latter being a structural component of yeast cell walls (Briggs *et al*., 2004). A number of transporters occur in yeast specific for one or small groups of amino acids and there is also a general amino acid permease (GAP) with broad specificity. Regulation of carriers is complex and dependent on the spectrum and concentrations of amino acids present in the medium (Briggs *et al*., 2004).

The assimilable nitrogenous compounds in wort, i.e. primarily amino acids and to a lesser extent ammonium ion and di- and tripeptides (O'Connor-Cox and Ingledew, 1989), affect brewer's yeast by increasing its biomass, stimulating its fermentation performance and determining the pattern of aroma compounds it produces (Lei *et al*., 2012). The importance of specific amino acids in the formation of off-flavours has already been acknowledged (Basarova and Janousek, 2000). Together with other constituents of residual beer extract, free amino acids positively support fullness and promote the drinkability of beer (Nagao *et al*., 1999) but beer with higher α-amino nitrogen show lower biological stability (Pierce, 1987). The importance of exploring the amino acid metabolism during brewery fermentation is therefore reasonable and essential for the optimization of process parameters and fermentation performance.

Table 3: Amino acids in a typical barley wort and beer (Krüger and Anger, 1992)
Preglednica 3: Aminokisline v običajni ječmenovi pivini in pivu (Krüger in Anger, 1992)

amino acid	concentration (mg/L)	
	wort	beer
alanine	60-200	80-120
arginine	6-200	50-100
asparagine	40-180	30-100
aspartic acid	40-100	<10
glutamine	10-110	<10
glutamic acid	35-130	30-50
glycine	20-60	20-50
histidine	20-120	20-50
isoleucine	50-150	10-50
leucine	100-300	10-100
lysine	60-200	10-50
methionine	20-70	<10
phenylalanine	60-220	10-80
proline	280-800	300-500
serine	40-140	10-30
threonine	40-110	5-15
tryptophan	0-40	1-20
tyrosine	60-200	40-100
valine	80-210	50-100

Yeast possesses many cell membrane-spanning amino acid transporters and most of them have a narrow substrate specificity, often only for a single amino acid (Regenberg *et al*., 1999). It is generally accepted that the amino acid uptake is a sequential process although the exact order of uptake is usually determined by individual strain preferences and particular wort properties. More than forty years ago Jones and Pierce (Jones and Pierce, 1964) proposed the categorization of amino acids into four groups on the basis of their assimilation patterns. Later on this classification has been a subject to many refinements (Basarova and Cerna, 1972; Lekkas *et al*., 2007; Palmqvist and Ayrapamino acid, 1969; Perpète *et al*., 2005; Ramos-Jeunhome *et al*., 1979) and individual amino acids were often moved from one group to another to better fit the experimental data. Such a classification depends on the criteria employed, such as the time needed for yeast to assimilate a half of initial amino acid content, its initial removal rate or the so-called "critical time" (Tc) (Perpète *et al*., 2005). Such classifications were made using mainly malted barley and since other raw materials differ drastically, further studies are needed to elucidate the effect of specific wort composition on the expression of genes involved in amino acid uptake and metabolism. Transcription profiles for any given strain may vary even between fermentations involving the same yeast strain – a phenomenon which has been observed for gene products (Kobi *et al*., 2004). Such studies were already done for barley malt (Gibson, *et al*., 2009) but separate investigations using buckwheat and quinoa are necessary.

It would be supposed that amino acids might be incorporated directly into proteins and other macromolecules. However, in brewing strains of *S. cerevisiae*, growing fermentatively on wort, amino acids are catabolized (Jones and Pierce, 1970). During fermentative catabolism, after an ultimate removal of the amino group the resultant carbon skeletons are not fed into oxidative energy-producing pathways nor they provide precursors for gluconeogenesis. The deaminated amino acids are converted to the corresponding α-keto acid analogue which are precursors of other metabolic by-products, which contribute to beer flavour, for example higher alcohols and esters (Briggs *et al*., 2004).

The nature and relative amount of the amino acids in the final beverage can be related to the wort composition as well as to beer fermentation conditions. During fermentation amino acids are used in different ways by yeasts and may have many effects on the final quality of beer. To name only some of them: (i) the presence of methionine, threonine, and isoleucine, in wort modulates the metabolism of sulphur-containing compounds (Gyllang *et al*., 1989), (ii) the amino nitrogen content influences the flavor profile of beer mainly via higher alcohol and ester formation in a positively-correlated manner (Krogerus and Gibson, 2013), (iii) amino acids present in the final beer could also influence negatively the stability of product promoting haze formation (Fontana and Buiatti, 2009), and (iv) L-alanine and L-tryptophan are associated to sweet and bitter sensation, respectively, thereby contributing to overall sensory perception (Fontana and Buiatti, 2009).

Although proline is the most (or one of the most) abundant amino acid in wort its concentration in barley is around twice as high as in buckwheat and quinoa. This is not surprising since hordein protein fraction, the major storage protein of the barley grain endosperm, is characterized by having high levels of proline (Fontana and Buiatti, 2009). On the contrary, storage proteins of dicotyledonous plants are mainly globulins and albumins. These fractions are reported with lower amounts of glutamine and proline (Arendt and Dal Bello, 2009). If proline is present in final beer it supports the instability of the final product. It has a specific affinity for polyphenols thus promoting haze formation. In addition, the most important browning reaction is believed to take place between the amino acid proline and maltose to 3-hydroxy-2-metyl-4-pyrone (maltol); the level of proline in wort can thus affect color development in beer (Fontana and Buiatti, 2009).

In the past, the amino acid assimilation by brewer's yeast has been investigated as a function of various parameters, such as the yeast strain, fermentation stirring regime, fermentation temperature, vessel type, pitching rate and others (Lekkas *et al*., 2007; Palmqvist and Ayrapamino acid, 1969; Perpète *et al*., 2005). In these studies, the influence of different wort composition was also considered; however, the major raw material was always barley malt. To the best of our knowledge, no such experiment has been done using

the buckwheat or quinoa malt. In addition, the amino acid uptake has not been investigated as a function of serial repitching before. In the past decade, the suitability of these two pseudocereals for brewing purposes is under the detailed investigation (De Meo *et al*., 2011; Phiarais *et al*., 2010; Zarnkow *et al*., 2005).

2.6.5 Aroma compounds

Among secondary metabolites, higher alcohols are of great interest since they are produced by yeast in the highest absolute concentrations (Procopio *et al*., 2011). Higher alcohols achieve maximum concentrations in fermenting wort at a time roughly coincident with the point at which FAN falls to a minimum concentration. Those that contribute to beer flavor include 1-propanol, 2-methyl butanol, 3-methly butanol, and 2-phenyl ethanol. It is considered that they impart a desirable warming character to beers such that they intensify the flavor of ethanol. Several authors have reported that the choice of yeast strain has the biggest impact and that ale strains generally produce more higher alcohols than lager strains (Briggs *et al*., 2004).

There is no firm argument what is the main benefit yeast has from producing higher alcohols. They are the precursors of the more flavor active esters. Additionally, because alcohol dehydrogenases are NAD^+-dependent, the suggestion that higher alcohol biosynthesis represents another mechanism for cellular redox control seems trustworthy (Quain and Duffield, 1985). 1-propanol is formed exclusively via the anabolic route since there is no amino acid from which a corresponding carbon skeleton could derive (Briggs *et al*., 2004). Where the wort has a high content of amino acids the catabolic route is favored. Thus, under these conditions, amino acid synthesis is reduced via feed-back inhibition and the pool of 2-oxo acids is generated largely via the amino acid catabolism. In the reverse situation where the supply of exogenous free amino acid is restricted, 2-oxo acids are formed via *de novo* synthesis from sugars and the anabolic route predominates.

In general, volatile esters have aroma notes characterized as fruity/solvent-like and are important odorants of fermented beverages. As opposed to higher alcohols, they are only present in trace amounts (with the exception of ethyl acetate) but they can affect the flavor of beer well below their threshold values. Usually, their formation is mainly yeast strain dependent although the particular composition of wort may have a noticeable influence. Esters have fruity/solvent-like aromas and flavours. The most abundant is ethyl acetate, which accumulates to concentrations of 10-20 ppm. The concentrations of other esters are usually less than 1 ppm (Briggs *et al*., 2004). The synthesis of esters requires the expenditure of metabolic energy suggesting that ester formation must fulfil some other important metabolic role. Supplementation of wort with the unsaturated fatty acid, linoleic acid (50 mg/L) causes a dramatic decrease in ester formation (Thurston *et al*., 1982) and it is now accepted that ester and lipid syntheses are inversely correlated.

In general, different beer types contain different proportions of the same constituents. Only when novel raw materials are used novel constituents can be found (Briggs *et al*., 2004).

2.6.6 Fatty acids

Free fatty acids are, besides diglyceraldehydes and triglyceraldehydes, practically the only members of lipids found in beer. In fact, the lipid content of brewing raw materials is usually 3-6% (w/w) where lipids are present in the living tissues (embryo and aleurone layer). Most lipids are insoluble and are removed via spent grist thus the lipid concentration is very low, less than 0.1%. Lipases split a small part into glycerine and fatty acids. Especially, non-saturated fatty acids result from reactions with oxygen or enzymatic breakdown into carbonyls by lipoxygenase. They decrease flavour stability already at low concentrations. Milling under inert gas and oxygen-eficient handling avoids these processes. Processes are additionally minimized by mashing at temperatures above 60°C and mash pH value below 5.2 (Buiatti, 2009).

The concentration of free fatty acids, very low at the beginning of the process, decreases further on during the brewing process whereas and the yeast metabolism can affect the presence of these compounds and modify them. Particularly, during fermentation it has been observed that there is an increase in C_6-C_{10} and a considerable decrease in C_{12}-$C_{18:3}$ as consequence of yeast metabolism. The concentration of total fatty acids in finished beer is about 150-300 µg/L and the most abundant are C_4-C_{10} (Bamforth, 2003).

The presence of fatty acids in beer is important because some of them have a high flavour potential, especially short- and middle-chain ones (C_4-C_{12}). Unsaturated ones, particularly linolenic and linoleic acids, are subjected to oxidative degradation resulting in a characteristic aging flavour (Tressl *et al*., 1979). There are two pathways to the oxidation of unsaturated fatty acids: enzymatic oxidation and autoxidation (Buiatti, 2009). Enzymatic oxidation occurs only during mashing and whereas autoxidation take place throughout the brewing process and even after packing. The main cause for the latter are reactive oxygen species which attack susceptible double bonds of oleic, linoleic and linolenic acid, resulting in predominantly 9- and 13-hydroperoxides. These hydroperoxides are unstable and further breakdown of these hydroperoxides into low molecular weight compounds leads to the formation of aldehydes, ketones and/or acids (Section 2.6.7). In particular, a fatty aldehyde, the *trans*-2-nonenal, is a very flavour potent compound, detectable in beer at less than 1 µg/L (Buiatti, 2009). As reduction of these compounds by yeast occurs during fermentation and maturation, no other factor can prevent autoxidation of fatty acids after beer is bottled.

Krauss *et al*. (1972) showed that long-chain fatty acids (C_{12}-C_{18}) have a relationship with beer head retention. The ratio of unsaturated, acting as inhibitor, and saturated fatty acids, acting as promoter, is related to gushing problems (Sandra *et al*., 1973).

In conclusion, the presence of fatty acids is undesirable from the quality and flavour standpoint, especially the unsaturated ones, but they are highly valued from the nutritional point of view. For this reason it is important to reduce the amount of reactive oxygen species, iron and cooper cations to the minimum and favour the presence of antioxidants such as vitamin E. This is a fat-soluble antioxidant that stops the production of reactive oxygen species formed when fat undergoes oxidation (Herrera and Barbas, 2001) and it is found in high quantities both in quinoa (Ng *et al*., 2007) and buckwheat (Zielinski *et al*., 2001) grains. Common fatty acids present in beer are listed in Table 4 (Kaneda *et al*., 1990). All in all, the above-mentioned reasons are more than sufficient to be interested in the analysis of fatty acids in beer.

Table 4: Important fatty acids in a typical barley beer (Kaneda *et al*., 1990)
Preglednica 4: Pomembne maščobne kisline v običajnem ječmenovem pivu (Kaneda in sod., 1990)

fatty acid	concentration (µg/L)
lauric acid (12:0)	5-60
myristic acid (14:0)	8-16
palmitic acid (16:0)	10-50
stearic acid (18:0)	1-30
palmitoleic acid (16:1)	0-3
oleic acid (18:1)	0-5
linoleic acid (18:2)	1-6
linolenic acid (18:3)	0-3

2.6.7 Aldehydes and ketones

After removing yeast from beer before bottling, some yeast-derived enzymes will still be present, influencing the chemical composition of the beer by enzymatic reactions. When finally beer is pasteurized, if at all, the reactions will have a pure chemical origin, ultimately leading to the maximal entropy and minimal enthalpy where the precise time course of their attainment depends on many factors. Some of these chemical alterations will remain unnoticed, but others will lead to significant changes in beer flavour, haze, and foam stability. The myriad of flavour notes changing during staling are due to a broad range of chemical entities; however, organoleptic-active volatile aldehydes, especially those C_7-C_{10} with very low thresholds, are considered to be the most important (Buiatti, 2009). The confirmation of volatile carbonyl compounds being the main ones responsible for the beer taste instability, is the disappearance of the stale off-flavour after addition of carbonyl scavengers to beer, such as aminoguanidine (Bravo *et al*., 2002).

The main beer off-flavours are given by either aliphatic (2-methylpropanal, 2- and 3-methylbutanal, butanal, hexanal and *trans*-2-nonenal) or cyclic (furfural,

phenylacetaldehyde, 5-hydroxymethylfurfural) aldehydes. A significant amounts arise from raw materials or are formed during the early stages of the brewing process; however, most of them are eliminated in different ways, either by the spent grain and hot trub after the whirlpool or by the filtration at final beer clarification. In addition, aldehydes already present in wort and those formed as intermediates in cell metabolism are reduced to corresponding alcohols by yeast during fermentation (e.g. 1-propanol, isobutanol and isoamyl alcohol). The reduction is enzymatically catalyzed by several alcohol dehydrogenases present in yeast, most of which are dependent on the coenzyme NAD^+. After fermentation is completed, the opposite process take place where aldehydes are formed from alcohols through dehydrogenation (oxidation) (Rabin and Forget, 1998) giving their final content in beer of 10-40 mg/L (Table 5).

Several of the aldehydes are of the yeast origin and others are the result of Strecker degradation of amino acids during kettle boil and still others appear to be the result of a random decarboxylation of organic acids. Acetaldehyde (ethanal) is the most common aldehyde in beer and is excreted into the green beer by yeast during the first three days of fermentation and it is responsible for the "green" young beer flavour. In the young beer phase the acetaldehyde content is about 20-40 mg/L and it decreases to 5-15 mg/L in the final product. As beer ages in commercial package, aldehydes may be produced through oxidation of higher alcohols by melanoidins. These aldehydes have a much lower threshold values than the origin alcohols and may be responsible for off-flavours. As mentioned above, the cardboard flavour typical of stale beer is probably due to *trans*-2-nonenal. The furfural content can increase also during pasteurization and storage at 40°C. Usually, its concentration is very low (<15 µg/L) but a level of more than 1843 µg/L was reported by Bernstein and Laufer (1977). In general, the concentrations and mutual ratios of acetaldehyde, 2- and 3-methylbutanal, furfural and 5-hydroxymethylfurfural are generally considered as good markers of beer oxidation where it is not crucial for them that they are separately present in adequate quantities to be sensorily detectable (Buiatti, 2009).

Although aldehydes are present in medium, their flavour can be masked or reduced by some other compounds. For example, sulfite is known to form adducts to staling aldehydes. The strength of the aldehyde bisulfite complexes decreases with increasing chain length and the presence of double bonds. Based on these observations, a staling mechanism was proposed. When the concentration of acetaldehyde rises during beer storage, sulfite will be transferred from staling aldehydes to acetaldehyde, thereby releasing the staling aldehydes (Nyborg *et al*., 1999). Another example is the acetalization of aldehydes. In this instance, a condensation reaction occurs between 2,3-butanediol and an aldehyde like acetaldehyde, isobutanal, 3-methylbutanal and/or 2-methylbutanal, which leads to the formation of cyclic acetals, such as 2,4,5-trimethyl-1,3-dioxolane, 2-isopropyl-4,5-dimethyl-1,3-dioxolane, 2-isobutyl-4,5-dimethyl-1,3-dioxolane and 2-secondary butyl-4,5-dimethyl-1,3-dioxolane, respectively. The equilibrium between 2,4,5-trimethyl-1,3-

dioxolane, acetaldehyde and 2,3-butanediol is reached rapidly in beer. Consequently, during beer aging, the concentration of 2,4,5-trimethyl-1,3-dioxolane will increase similarly to the increase of acetaldehyde (Buiatti, 2009).

Table 5: Important aldehydes in a typical barley beer (Baxter and Hughes, 2001)
Preglednica 5: Pomembni aldehidi v običajnem ječmenovem pivu (Baxter in Hughes, 2001)

aldehyde	concentration (mg/L)	flavour descriptors	flavour threshold (µg/L)[a]
acetaldehyde	2-20	green, paint	25,000
propanal	0.01-0.3	green, fruity	30,000
butanal	0.02-0.03	melon, varnish	1,000
2-methylpropanal	0.02-0.5	banana, melon	1,000
3-methylbutanal	0.01-0.3	grass, apple, cheese	600
hexanal	0.003-0.07	bitter, vinous	350
heptanal	0.002	aldehyde, bitter	50
octanal	0.001-0.02	orange peel, bitter	40
***trans*-2-nonenal**	0.00001-0.002	cardboard	0.11
furfural	0.01-1	papery, husky	150,000
5-hydroxymethylfurfural	0.1-20	aldehyde, stale	1,000,000
phenylacetaldehyde	1-10	pungent, floral, sweer	1600

[a] Baert *et al*., 2012

Ketones belong, as aldehydes, to the group of carbonyl compounds. The most important ketones in beer are diacetyl (butane-2,3-dione) and a related compound pentane-2,3-dione which are produced from yeast metabolites secreted into beer. Both diketones are highly aromatic and considered undesiderable in lighter-flavoured beer. The taste threshold of diacetyl is very low (~0.15 mg/L) and above this value it imparts to beer an unclean, sweetish, butterscotch taste. Since pentane-2,3-dione has a similar effect but with a higher taste threshold (~0.9 mg/L) these compounds are often considered together and referred to as 'vicinal diketones' because both compounds have adjacent carbonyl group. Their breakdown occurs at the same time as other maturation reactions during the beer conditioning process and for this reason their concentrations are considered as the fundamental criterion to evaluate the state of beer maturationr (Buiatti, 2009). Table 6 summarizes the ketones found in highest quantities (Baxter and Hughes, 2001).

The now accepted pathway is that during fermentation of alcoholic beverages, the vicinal diketones diacetyl and 2,3-pentanedione are produced by yeast from intermediates of valine, leucine and isoleucine biosynthesis. These undesirable butter-tasting diketones are formed extracellularly through the spontaneous non-enzymatic oxidative decarboxylation of α-acetohydroxy acids, which are intermediates in the valine and isoleucine biosynthesis pathways. In the latest study (Krogerus and Gibson, 2013) authors claimed that valine supplementation lowered the maximum diacetyl concentration produced during fermentation, but it did not have as large impact on the production of 2,3-pentanedione. Additionally, valine uptake rate negatively correlates with the amount of diacetyl formed

during the growth phase of fermentation, but leucine and isoleucine may be responsible for the decrease of diacetyl production as well.

Table 6: Important ketones in a typical barley beer (Baxter and Hughes, 2001)
Preglednica 6: Pomembni ketoni v običajnem ječmenovem pivu (Baxter in Hughes, 2001)

ketone	concentration (mg/L)	flavour descriptors
3-methylbutan-2-on	0.05	ketone, sweet
heptan-2-one	0.04-0-11	varnish, hops
octan-2-one	0.01	varnish, walnut
nonan-2-one	0.03	ketone, varnish
2,3-butandione	0.01-0.4	butterscotch
3-hydroxy-2-butanone	1-10	fruity, mouldy, woody
2,3-pentandione	0.01-0.07	butterscotch, fruity

2.6.8 Sensory analysis

Both ethanol and carbon dioxide contribute to beer flavour. The latter has a 'mouth tingle' character, whereas ethanol imparts a 'warming' note to beer. In addition, fermentation of wort generates a multitude of other minor products of yeast metabolism (aliphatic alcohols, aldehydes, ketones, organic and fatty acids, esters), many of which contribute to beer flavour. These are formed as by-products of the metabolism of sugars and AAs (Figure 2). The action of yeast on wort also serves to remove some components whose persistence in beer would be undesirable.

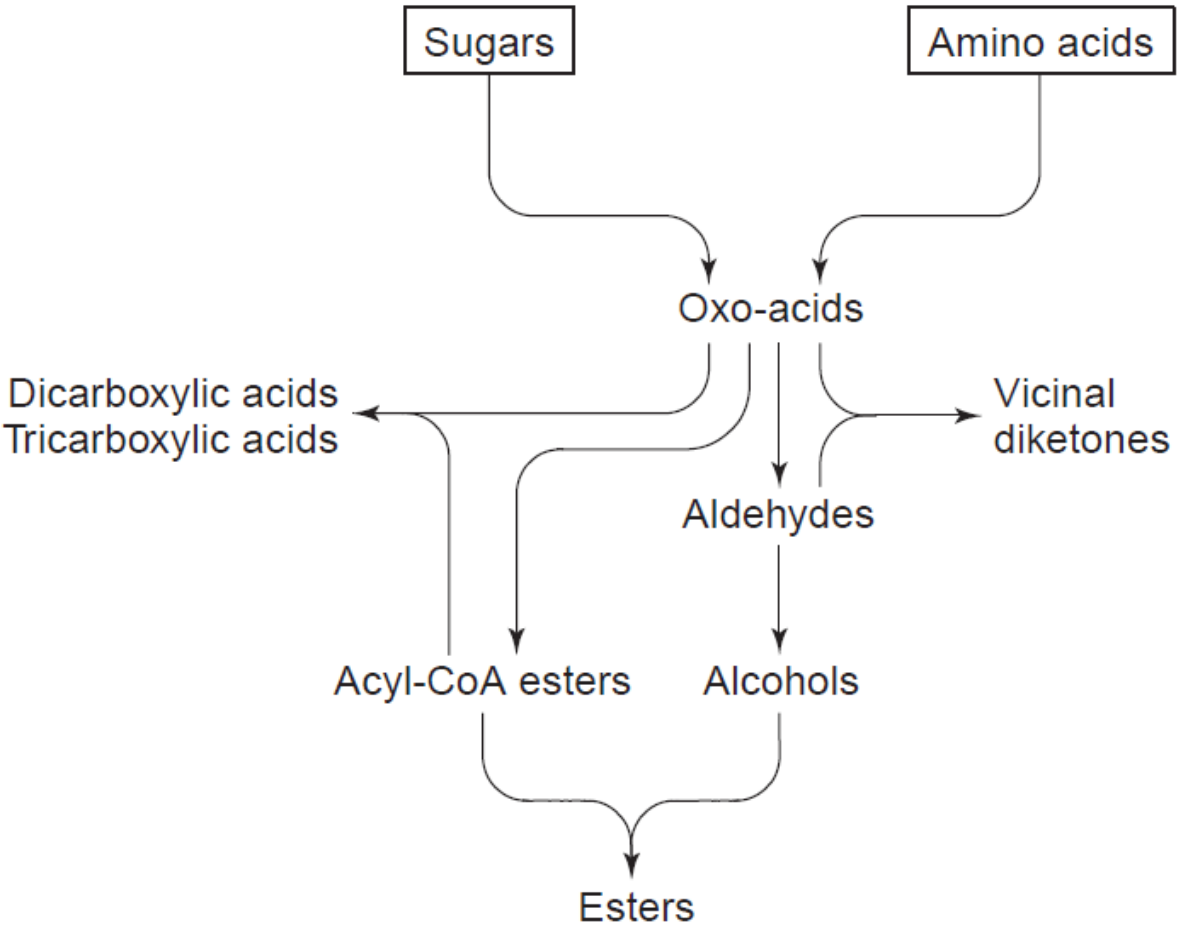

Figure 2: Relationships between the major classes of yeast-derived beer flavor compounds (Briggs *et al.*, 2004)
Slika 2: Povezave med glavnimi razredi aromatičnih spojin, ki so posledica kvasnega metabolizma (Briggs in sod., 2004)

2.7 SERIAL REPITCHING OF THE BREWING YEAST

Attempts to malt, mash and brew using buckwheat (De Meo *et al.*, 2011; Pharais *et al.*, 2006; 2010; Wijngaard and Arendt, 2006) and quinoa (De Meo *et al.*, 2011; Zarnkow *et al.*, 2005, 2007) have already been conducted. Based on these studies, rather high costs are expected during commercial production of beer-like beverages from these two pseudocereals, mainly because of the low intrinsic activity of hydrolytic enzymes and the consecutive need for external enzyme supplementation during mashing. Besides, in comparison to barley, the market price of the buckwheat and quinoa grain is relatively high as well as some modifications of the process technology are required due to the specific nature of the grain.

A yeast propagation plant also represents a relative high cost because it is used only for yeast multiplication without usually producing salable beer. This can be solved by buying starter cultures from specialized companies or by biomass recycle i.e. repitching. Moreover, to reduce the environmental impact of the waste produced at the end of

fermentation, yeast is collected and repitched at the end of beer fermentation. Typical employment of yeast is between 8-15 fermentation cycles where the precise number of times a yeast culture can be reused is dependent on the individual strain (Stewart, 1996), the quality of the cropped slurry (O'Connor-Cox, 1997), the risk of contamination, weakening of fermentation capacity, changes in chemical composition of the final product and in some instances, company policy (Jenkins *et al.*, 2003).

Industrial fermentations performed to produce beer are unique within the alcoholic beverage industry in that the yeast is not discarded after use but is maintained and reused a number of times in a process termed "serial repitching". The number of times a yeast population may be serially repitched is determined largely by a combination of product quality constraints driven by company policy. Beer quality is strongly influenced by the biochemical performance of the yeast during fermentation. The ability of yeast to separate from the beer at the required time, utilise sugars quickly and efficiently, and produce a product with a high yield of ethanol and the correct balance of flavour compounds, is of principal importance. Many intrinsic and extrinsic factors may affect the rate and quality of fermentation and the character of the final product, however replicative ageing has not previously been considered to be important to the process.

The brewing yeast has a limited replicative lifespan. Each cell within a population is only capable of a finite number of divisions prior to senescence and death and the cell mass at division is partitioned unequally between a larger, old parent cell and a smaller, new daughter cell (Ginovart *et al.*, 2011). Studies of the ageing phenotype in both haploid laboratory strains and polyploid brewing strains have indicated that as a consequence of senescence yeast cells are subject to morphological, metabolic and genetic modifications (Jazwinsky, 1990). Such modifications include an increase in size (Bartholomew and Mittwer, 1953) and alterations to the shape and surface appearance of the cell (Mortimer and Johnston, 1959). In addition, generation time is altered (Mortimer and Johnston, 1959), metabolism declines (Motizuki and Tsurugi, 1992), and gene expression (Egilmez *et al*., 1989) and protein synthesis (Motizuki and Tsurugi, 1992) become modified.

Most industrial yeasts are polyploid or aneuploid (Codón *et al*., 1998). While the physical and fermentative characteristics of the working yeast culture may appear consistent, it has been reported that genetic mutations can occur during yeast recycling (Jenkins *et al*., 2003a). Such changes to the population genome may not always be immediately obvious; however, serial repitching may cause certain characteristics to be passed onto subsequent generations (Powell and Diacetis, 2007). Such an event would lead to genetic drift within the culture and is supported by evidence of instability within the brewing yeast genome. Chromosome-length polymorphisms have been reported to occur in a number of commercial lager strains (Casey, 1996; Sato *et al*., 1994) and are most frequently observed in chromosomes containing genes inherently linked to fermentation performance, such as

FLO1, FLO5, FLO9, FLO10, HXK1 and *MAL4*. In addition, the telomere hypothesis suggests that the number of cell divisions is registered by the gradual loss of telomeric sequences (Chiu and Harley, 1997) and may act as a biological clock determining the number of divisions prior to senescence in mammalian systems (Powell *et al*., 2000). Analysis of spontaneous mutants, characterised by increased flocculation potential, have been noted to occur after approximately 6-10 generations in lager yeast strain (Boulton and Quain, 2001). Although changes to flocculation characteristics are perhaps the most frequent type of mutations observed, genetic drift during the course of serial repitching may also be the cause of differences in wort sugar utilisation (Donnelly and Hurley, 1996), diacetyl reduction (Stewart and Russell, 1998), accumulation of petite mutants (Jenkins *et al*., 2003b), and others.

There are many studies regarding both yeast response to serial repitching (Bühligen *et al*., 2013; Vieira *et al*., 2013) and its influence on the final product (Aleksander *et al*., 2009; Vieira *et al*., 2012). Even for barley, studies regarding serial repitching are appearing constantly which clearly shows the practical importance and public interest in this complex field of many co-dependent parameters. Up to now, none of these studies employed buckwheat or quinoa as a main raw material for fermentation. If these pseudocereals are to be used for commercial production of beer-like beverages, serial repitching, a crucial component of commercial brewing, should be well understood in order to avoid potential problems and thus unnecessary time and cost loss.

Therefore, gluten-free beer-like beverages from malted buckwheat and quinoa are somehow close to their commercial production, but rather high expenses are expected due to the relatively high price of grain, some technological adaptations of process and the need for external enzyme supplementation during mashing (Hager *et al*., 2014) and relatively high price of the grain. Serial repitching of yeast biomass represents an easy, efficient and yet common mode for expense reduction, especially in industrial scale. This approach has not yet been studied for the fermentation of buckwheat and quinoa beer-like beverages and a part of our research included a detailed investigation of daily changes of fermentation medium during eleven successive fermentations of barley, buckwheat and quinoa wort.

3 MATERIALS AND METHODS

3.1 THE PREPARATION OF FERMENTED BEVERAGES

The unhulled buckwheat (*Fagopyrum esculentum*, Moench) and quinoa (*Chenopodium quinoa*, Wild) grain of organic growth were ordered from Trouw B.V. (Rotterdam, Netherlands) and Ziegler Naturprodukte (Wunsiedel, Deutschland), respectively. The barley malt "Malt Château Pilsen 2RS" produced by Castle Malting (Lambermont, Belgium) was ordered at Hmezad exim d.d. (Žalec, Slovenia). The commercial enzyme preparations Termamyl™ SC DS (heat-stable α-amylase), Attenuzyme™ Flex (glucoamylase, specific α-amylase), Ondea™ Pro (pululanase, α-amylase, celulase, endo-1,4-xylanase, neutral protease, lipase) and Ultraflo™ (β-glucanase, xylanase) were ordered from Novozymes A/S (Bagsvaerd, Denmark). Pellets of the hop variety Hallertau Hallertauer Tradition (4,5% α-acids) were obtained from Lupex Gmbh (Hallertau, Germany). Final beverages were filtered through 20 x 20 cm depth filters (Seitz K250) of diatomaceous earth (kieselguhr) (Pall Filtersystems GmBH, Bad Kreuznach, Germany). Liquid cultures of brewing yeast *Saccharomyces pastorianus* strain TUM 34/70, *Saccharomyces cerevisiae* strain TUM 177 and *Saccharomycodes ludwigii* strain TUM SL17 were kindly provided by Research Center Weihenstephan for Brewing and Food Quality (Freising, Germany).

3.1.1 Malting

The buckwheat and quinoa malt were prepared in a pilot malt house of a 120 kg capacity based on the procedures described previously (Zarnkow *et al*., 2005) with some modifications. Briefly, the buckwheat and quinoa grain with the germinative capacity of 88.3 and 83.8%, respectively, were soaked into water at room temperature until the final content of moisture 47% (w/w) was achieved, followed by a five-day germination at 20°C (buckwheat) or 15°C (quinoa). Kilning of the germinated grain started at 50°C and the temperature was first raised linearly to 60°C in 16 h, then to 80°C in 2 h and left at that temperature for another 6 h, before it was cooled to room temperature.

3.1.2 Mashing

The malt was ground with a two-roller mill (Winfried Sauer, Frendorf, Germany) at a setting of 0.3 mm. Mashing took place in a pilot brew plant of a 60 L (buckwheat and quinoa) or 30 L (barley) capacity equipped with a mash agitator. An extensive mashing regime was used for each raw material based on the previously optimized procedures (Zarnkow *et al*., 2005) with some modification, as follows:

(i) 13 kg of the buckwheat grist were mixed into 46 L of water at 35°C and let to rest for 95 min. After 40 min, 20 L of mash were transferred to a decoction vessel and heated to 72°C. At this point, 10 mL of Termamyl SC DS were added and let to rest for 45 min. During the next 5 min, the decoction vessel was emptied to the main vessel, together with

3 L of hot water flush, where the temperature was raised to 50°C and let to rest for 80 min. After 50 min, 21 L of mash were transferred again to the decoction vessel and heated to 72°C. At this point, 5 mL of Termamys SC DS were added and let to rest for 20 min. As before, during the next 5 min the decoction vessel was emptied to the main vessel together with 3 L of hot water flush, where the temperature was raised to 65°C, then 20 mL of Attenuzyme Flex were added, and let to rest for 25 min. The temperature was raised to 72°C, kept constant for 25 min, and finally, raised to 78°C for 10 min. Every single heating treatment lasted for 10 min.

(ii) 12 kg of the quinoa grist were mixed into 48 L of water at 50°C and 8 mL of Termamyl SC DS, 24 mL of Ondea Pro, and 8 mL of Ultraflo were added immediately. After a 30 min rest, the temperature was raised to 60°C upon which it was kept constant for 55 min. The mash was heated again to 72°C, followed by a 40 min rest. After the final heating to 78°C and a subsequent 10 min rest, mashing was over. Every single heating treatment lasted for 10 min.

(iii) 5 kg of the barley grist were mixed into 26 L of water at 52°C and let to rest for 30 min. After that the mash was heated to 63°C and let to rest for 35 min, heated again to 72°C and let to rest for 50 min. Then it was heated for the last time to 78°C and let to rest for 10 min. Every single heating treatment lasted for 10 min.

3.1.3 Lautering and wort boiling

In the case of barley and buckwheat the mash was allowed to settle in the lauter tun for 2 to 3 h and the supernatant was transferred to the wort kettle. The sediment was washed twice with hot water to achieve the final wort volume of 64 or 32 L. The sparging water temperature was below 78°C to avoid the danger of washing out the iodine-reactive α-glucans from the spent grain. Regarding quinoa, a mash filter with the use of pressure was used instead of the lauter tun because the quinoa seeds are relatively small and for a successful lautering, a sufficient size of intact husks is needed to form filter cake. The filtrate was washed with hot water until 64 L of wort were collected.

The wort was boiled for 90 min and the total amount of hop pellets added was calculated to achieve the final bitterness of approximately 18 EBC units. 80% of hops were added at the beginning and the rest of them 10 min before the end. At the end, the boiled wort was immediately transferred directly into 19.5 L NC Cornelius steel tanks (Candirect, Duisburg, Germany) and stored in a 0°C room.

3.1.4 Fermentation

Prior to fermentation, the wort and yeast were separately atemperated at 14°C followed by pitching of 100 mL of the liquid yeast culture (200×10^6 cells/mL) into 10 L of the wort in spheroconical fermentors. The fermentation was performed in doublets (n = 2) and when

attenuation was achieved, the fermented wort was filled into NC Cornelius tanks and let to rest at 16°C for 2 days, followed by a rest at 0°C for 12 days.

3.1.5 Filtration and bottling

After conditioning, the fermented wort was filtered using the plates of diatomaceous earth (kieselguhr) with the pore size small enough to retain only yeast and particles of similar size. One day before bottling, the cans containing beverage were put under the pressure of CO_2 calculated to achieve the final CO_2 concentration of around 4.6 g/L. Finally, the beverage was filled into 0.5 L amber bottles and closed with crown caps.

3.1.6 Forced aging

Beer samples were forced-aged a month after bottling by shaking them for 24 h at 200 rpm followed by a 4-day rest at 40°C according to MEBAK protocol (Mitteleuropaischen …, 2013). The exposure to light was not performed since it is a common practice in commercial production to bottle beer in amber bottles, which allow only a minimum amount of light to pass into.

3.1.7 Sampling and sample preparation

The samples intended for the chemical analysis were frozen a week (wort) or three months (beverage) after the production. For the determination of aldehydes and ketones, naturally aged bottled beverages were also used which were kept at 4°C for 2 years. The samples were degassed in the ultrasonic water bath and filtered through a plain disc filter paper (diameter of 150 mm) and cellulose acetate (CA) membrane syringe filter unit (diameter of 25 mm, pore size of 0.45 μm) (LLG Labware, Germany), except in the case of the analysis of important volatile compounds. Chromatographic analysis was performed in triplicates.

3.2 SERIAL REPITCHING OF *Saccharomyces pastorianus* TUM 34/70

For the successive fermentation experiment, the same malt was used as for the preparation of fermented beverages (Section 3.1.1). In addition, the same mashing (Section 3.1.2) and lautering and wort boiling (3.1.3) procedures were followed.

3.2.1 Fermentation and serial repitching

Prior to each fermentation, wort and yeast were separately atemperated at a fermentation temperature of 14°C followed by pitching of an approximately 20,000 × 10^6 cells of *S. pastorianus* strain TUM 34/70 into 10 L of wort in 19.5 L NC Cornelius steel tanks (Candirect, Duisburg, Germany). For the first fermentation, 100 mL of liquid starter yeast culture (~200 × 10^6 cells/mL), was used. During fermentation at 14°C the pressure valve was opened to avoid supersaturation of carbon dioxide (Kruger *et al.*, 1992). Every 24 hours after pitching, the fermentation medium was analyzed for the extract and ethanol content (MEBAK II 2.10.6.1). When wort attenuation was achieved (the last day when a daily change in the extract content was higher than or equal to 0.1%), the particular

fermentation was completed. Yeast propagation and oxygenation followed under aseptical conditions by mixing 0.5 L of fermentation suspension with 0.5 L of fresh wort into an autoclaved 2 L glass bottle (Schott Duran, Germany) and put on an orbital shaker (90 rpm) for 48 h at room temperature with the partly closed cap. Afterwards, the count of viable yeast cells was performed using the methylene blue staining and a Thoma Counting Chamber (Jena Fein-Optik, Germany). The yeast cell concentration was calculated from the average value of three independent yeast cell counts according to the instructions provided by the counting chamber manufacturer. During the counting, samples were carefully inspected under the microscope by experienced personnel for any potential infection or other unusual changes of the yeast suspension. Yeast viability of barley, buckwheat and quinoa samples was > 94%, > 89% and > 85%, respectively. To achieve a final viable yeast count of 2×10^6 cells/mL in the fermentation vessel (excluding the first fermentation), the pitching volume was adjusted considering the particular calculated yeast cell concentration. Throughout the experiment, a special attention was given to assure aseptic conditions.

3.2.2 Sampling and sample preparation

3.2.2.1 Yeast sampling

Samples were taken immediately before the start of an individual fermentation cycle. 50 mL of pitching suspension was centrifuged for 10 min at 4000×g using 50 mL falcon tube. The supernatant was discarded and this step was repeated using the same falcon tube three more times. Samples (approx. 3 g) were frozen immediately and kept at -80°C pending analysis. On the day of analysis, thirty-three samples (11 for each raw material), were first cleaned in order to remove hop remains and other solid particles with the transfer of raw samples onto a double-layered gauze with a 0.5 mm pore size fixed in a 50 ml centrifuge tube (TPP, Switzerland). The subsequent centrifugation step (5 min at 1500×g) efficiently purified the yeast cells. Purified yeast biomass was transferred into 2 mL Eppendorf tubes containing 1 mL of PBS buffer and vortexed. After centrifugation at 4000 rpm for 5 min, the supernatant was discarded and the washing step was repeated until a clear supernatant was obtained.

3.2.2.2 Fermentation medium sampling

The wort samples were frozen a day after production. Samples from fermentation medium were taken every 24 h (± 30 min) and immediately after the sampling, 1 mL of 2 g/L sodium azide was added per 100 mL of sample to stop the fermentation. Sodium azide slows the growth of *Saccharomyces cerevisiae* and induces a lag which increases steeply with the concentration (Wild and Hinselwood, 1956). After 3 h, samples were centrifuged using laboratory centrifuge Sigma 6K15 (Sigma, Germany) for 10 min at 4000×g to remove yeast. Supernatant was collected and frozen until the analysis. On a day of analysis, the samples were defrosted at room temperature, degassed in the ultrasonic water bath and filtered through a plain disc filter paper (diameter of 150 mm) and cellulose

acetate (CA) membrane syringe filter unit (diameter of 25 mm, pore size of 0.45 μm) (LLG Labware, Germany). Reagents and solutions were of appropriate quality and purity, and supplied by Sigma-Aldrich (Germany).

3.3 THE ANALYSIS OF FERMENTATION SAMPLES

Reagents and solutions were of appropriate quality and purity and ordered from Sigma-Aldrich (Germany).

3.3.1 Brewing attributes

The brewing attributes were determined in duplicate (n = 2) according to the standard procedures collected in the current version of either Analytica-EBC (European …, 2010) or MEBAK (Mitteleuropaischen …, 2002, 2006) protocols. The analyses of the malt and wort were done within a week after the production and the analyses of bottled beverage two months after bottling.

3.3.2 Metals

The iron, copper, manganese, and zinc standard solutions "Baker Instra-Analyzed", 1 g/L, were purchased from J.T.Baker (The Netherlands). Iron, copper, and zinc were determined according to the Analytica-EBC (European …, 2010) methods 9.13.3, 9.14.3, and 9.20, respectively, and manganese according to Hoenig and Hoeyweghen (2010) using Perkin Elmer AAnalyst 200 atomic absorption spectrometer (Perkin Elmer, USA) with Perkin Elmer Lumina™ hollow cathode lamp (Cu-Fe-Mn-Zn). The nstrument was controlled with WinLab32 computer software (Perkin Elmer, USA).

3.3.3 Fermentable carbohydrates

Fermentable carbohydrates were determined according to the Analytica-EBC method 9.27 (European …, 2010) using HPLC apparatus (Agilent 1200 Series) equipped with a thermostated autosampler, an ion-exclusion column (Bio-Rad Aminex HPX-87H, 300 x 7.8 mm), and a refractive index detector (RID) (Agilent 1100 Series). The chromatographic conditions were as follows: injection volume: 5 μL, mobile phase: 5 mM H_2SO_4, isocratic elution, flow rate: 0.5 mL/min, optical unit temperature: 40°C, positive polarity, peak width: 0.2-0.4 min, response time: 4 s. Although this method does not offer an opportunity to fractionate different oligosaccharides with the same degree of polymerization, a simple way to distinguish sucrose among other disaccharides has been developed. Two runs of each sample were performed at a different column temperature (25°C or 65°C). At 65°C sucrose hydrolyzes completely to glucose and fructose (Figure 3). From the concentrations of DP2, glucose and fructose [g/100 mL] determined at each column temperature, the sucrose concentration was calculated from the following equation:

$$sucrose\ concentration\ [g/100\ mL] = \frac{(A-B) + \frac{(C-D) + (E-F)}{2}}{2} \quad \ldots(1)$$

A = c (DP2, 25°C) *D = c (Glc, 25°C)*
B = c (DP2, 65°C) *E = c (Fru, 65°C)*
C = c (Glc, 65°C) *F = c (Fru, 25°C)*

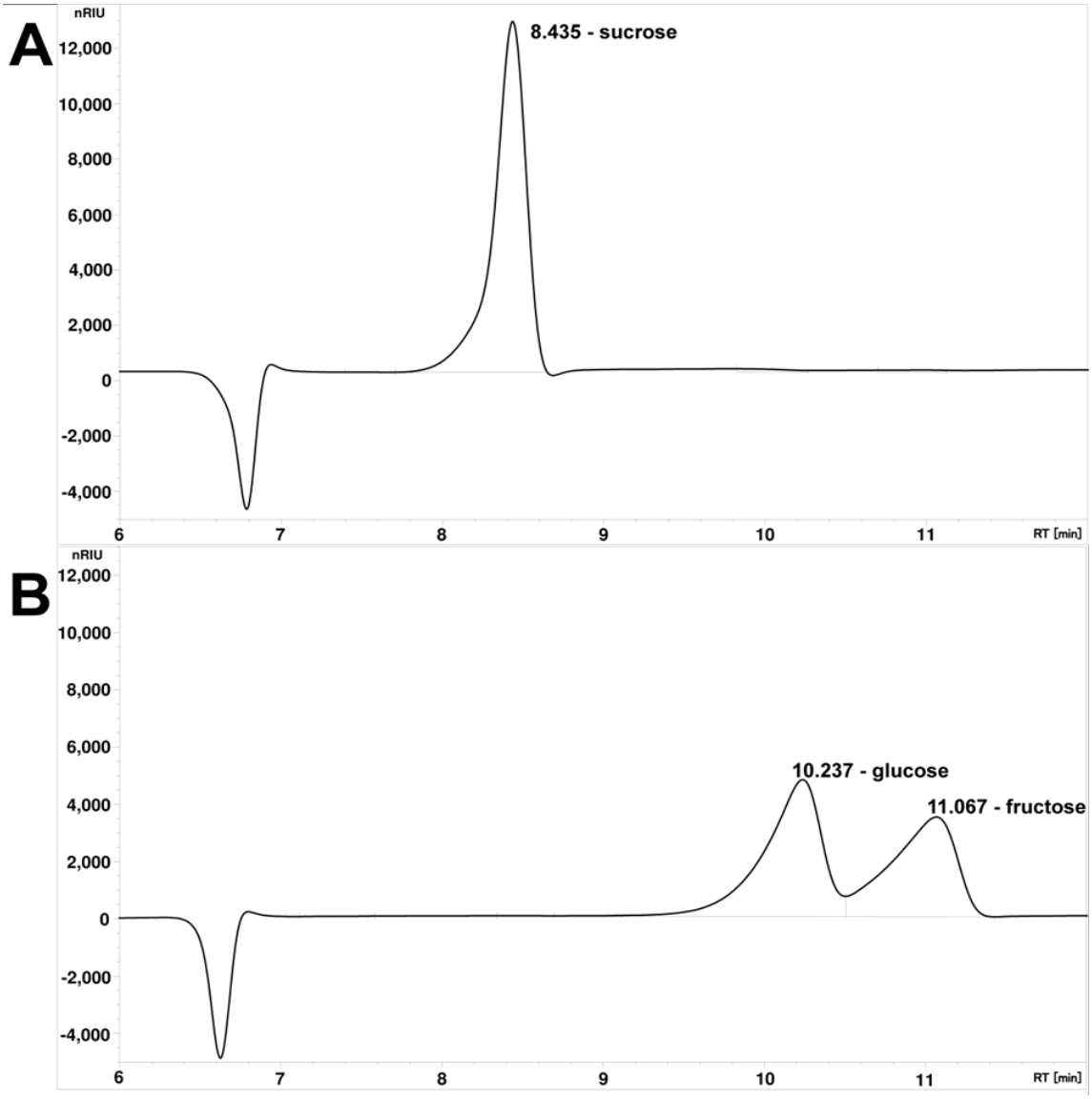

Figure 3: The comparison of HPLC-RID chromatograms of sucrose standard solution (11.5 g/L) at column temperature of (A) 25 and (B) 65°C

Because of the complete hydrolysis of sucrose in the acidic mobile phase at 65°C the calculation of its concentration in samples was possible. The numbers preceding the sugar name indicate its retention time. nRIU – detector response unit.

Slika 3: Primerjava HPLC-RID kromatogramov standardne raztopine saharoze (11,5 g/L) pri temperaturah kolone 25 (A) in 65°C

Popolna hidroliza saharoze v kisli mobilni fazi pri 65°C je omogočila izračun njene koncentracije v vzorcih. Številka pred imenom sladkorja predstavlja njegov retenzijski čas. nRIU – enota odziva detektorja

The HPLC instrument control and analysis of results were done with ChemStation 32 computer software (Agilent Technologies, USA).

3.3.4 Amino acids

Proteinogenic amino acids were analyzed using HPLC apparatus (Agilent 1200 Series) equipped with a thermostatted autosampler, a reverse phase column (Agilent Eclipse XDB-C18, 4.6 x 150 mm), and a fluorescence detector (FLD). The analytical procedure was based on the method optimized by HPLC instrument manufacturer (Henderson and Brooks, 2010). The excitation and emission wavelengths for OPA derivatives were set to 340 and 340 nm, respectively, and for Fmoc derivatives to 266 and 305 nm, respectively. The HPLC instrument control and analysis of results were done with ChemStation 32 computer software (Agilent Technologies, USA).

3.3.5 Volatile compounds

Important beer aroma compounds were determined according to the MEBAK method 1.1.2 (Mitteleuropaischen …, 1996). The unfiltered samples were distilled using Büchi 323 Distillation Unit (Büchi Labortechnik AG, Switzerland) and analyzed by gas chromatography (GC) apparatus Agilent 6890 Series GC System with Autosampler, HP-FFAP Crosslinked column (25 m x 0.2 mm x 0.3 μm), a flame ionization detector (FID), and Chemstation computer software (Agilent, USA). 1 μL of sample was injected (injector temperature: 220°C, split ratio: 25.1 : 1) into the column using helium as a carrier gas at 22°C with the flow rate of 1 mL/min. The column temperature was first maintained at 40°C for 4 min, ramped at a rate of 4°C/min to 60°Cand ramped again at a rate of 15°C/min to 200°C where it was held for 5 min. After that, the column temperature was raised to 240°C instantly, where it was held for 1 min. The detector temperature was 220°C and the data collection rate was 20 Hz at the minimum peak width 0.01 min. The analysis of one sample lasted altogether 24.33 min. Concentrations of compounds in samples were calculated from the calibration curve constructed using external standard solution. Internal standard 1-butanol served only as an indicator of the distillation recovery and its absence in samples was confirmed in preliminary trials (data not shown). The practical analytical procedure was performed by student Natalija Brečko as a part of her diploma thesis work.

In the quinoa samples some chromatographic peaks were observed which could not be identified using standard reagents. The gas chromatography-mass spectrometry (GC-MS) analysis was performed using the Varian STAR 3400 CX (GC) and Varian SATURN 2000 (MS) apparatus, the same column, ion-trap analyzator, and Varian Mass Spectrometry Workstation computer software. 2 μL of a sample were injected (injector temperature: 220°C) manually and carried through the column with helium. The column temperature was first maintained at 60°C and immediately ramped at a rate of 2.5°C/min to 190°C, ramped again at a rate of 50°C/min to 240°C,where it was held for 10 min. The samples were ionized using the electron impact ionization (EI) technique at 70 eV. A detector was

set to pass the fragments with a molecular mass between 46 and 451 g/mol at 1 s/scan and emission current of 20 μA. According to the mass spectra of unknown compounds their identity was proposed by NIST Libraries for Spectrum Hit List.

3.3.6 Fatty acids

The development of the method for determination of free fatty acids in barley beer and beer-like beverages from buckwheat and quinoa was based on reports of Horák *et al*. (2009), Kaneda *et al*. (1990) and Xie *et al*. (2012). We used solid-phase extraction (SPE) of fatty acids from beverage samples followed by precolumn derivatization with 9-chloromethylanthracene (9-CMA) in the presence of tetramethylammonium hydroxide (TMAH). The resultant esters were separated by HPLC and detected using FLD. All reagents, fatty acid standards (hexanoic, octanoic, decanoic, lauric, α- and γ-linolenic, arachidonic, myristic, linoleic, palmitic, oleic, stearic, eicosanoic, arachidic, erucic, behenic, nervonic, lignoceric acid) and other chemicals were supplied by Sigma-Aldrich, Germany.

3.3.6.1 Method optimization and validation

During the method optimization, we tested different organic solvents for the preparation of fatty acid standards, 9-CMA and TMAH stock solutions. In addition, different molar combinations of 9-CMA and TMAH, different combinations of derivatization time and temperature, amount and type of SPE eluent, pH of samples, and different combinations of detector excitation and emission wavelengths were explored. The validation procedure was performed according to the common validation criteria (Rozet *et al*., 2011). In that manner, we investigated method selectivity, linearity, limit of detection, lower and upper limits of quantification, precision, and extraction recovery.

3.3.6.2 Application to real samples

Based on the results of method optimization, the optimal conditions were employed to real samples, as follows. Dimethylformamide (DMF) was used for the preparation of stock solutions of 9-CMA (37.5 mM) and fatty acid standards (1 g/L). TMAH×$5H_2O$ was first dissolved in methanol (10% w/w) and this solution was used for the preparation of stock solution of 30 mM in DMF. SPE cartridges (Supelco Discovery® DSC-18 SPE Tube, 3 mL, 500 mg) were first activated with 6 mL of methanol and washed afterwards with 6 mL of 4% ethanol solution, pH 2 (pH adjusted using the concentrated HCl). 20 mL of filtered (through a plain disc filter paper, diameter of 150 mm, LLG Labware, Germany) and degassed (with the use of ultrasonic bath for 2-3 min) samples were acidified with HCl to pH 2 and gently applied to the wall of SPE column using the automatic pipette. After the extraction, the SPE cartridges were washed with 6 mL of 4% ethanol, pH 2, and dried out with a gentle flow of N_2. Retained fatty acids were eluted with chloroform (3 × 0.5 mL) and the resulting chloroform extract was applied to rotavapor where solvent was evaporated to dryness at 40°C. Isolated fatty acids were immediately dissolved in 1.6 mL

of dimethylformamide (DMF) and stored in glass vials in refrigerator until HPLC analysis. Their derivatization was performed at 75°C for 35 min after adding 200 µL of each TMAH and 9-CMA stock solutions.

3.3.6.3 Chromatography

HPLC separation of fatty acid derivatives was performed using Agilent 1200 Series apparatus equipped with a thermostated autosampler, reverse-phase column (Agilent Zorbax PAH, 100 mm × 4.6 mm, 1.8 µm) maintained at 40°C, and FLD set to excitation and emission wavelengths of 365 and 412 nm, respectively. 10 µL of the derivatization mixture, filtered through polytetrafluoroethylene (PTFE) membrane syringe filter unit (diameter of 25 mm, pore size of 0.45 µm) (LLG Labware, Germany), were injected A combination of the gradient and isocratic elution at a constant flow rate of 0.65 mL/min was used with H_2O (mobile phase A) and CH_3OH (mobile phase B) using the following regime: (i) 0.00-17.00 min, gradient, B = 90→100%, (ii) 17.00-29.00 min, isocratic, B = 100%, (iii) 29.00-29.01 min, gradient, B = 100→90%, (iv) 29.01-32.00 min, isocratic, B = 90%, (v) 32.00 min, STOP.

3.3.7 Aldehydes and ketones

The development of the method for determination of aldehydes and ketones in barley beer and beer-like beverages from buckwheat and quinoa was based on reports of Ali *et al*. (2014), Nakashima *et al*. (1996) and Uzu *et al*. (1990). We used distillation of beverage samples followed by two different separation and detection methods, i.e. (i) The GC separation of aldehydes and ketones in distillate, and their FID detection. (ii) The precolumn derivatization with 4-(N,N-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H) in the presence of trifluoroacetic acid (TFA) followed by the HPLC separation of resultant hydrazones and their FLD detection.

All reagents, aldehyde (acetaldehyde, propanal, butanal, 2-methylpropanal, 3-methylbutanal, hexanal, heptanal, octanal, *trans*-2-nonenal, furfural, 5-hydroxymethylfurfural, phenylacetaldehyde) and ketone (diacetyl, 2,3-pentanedione, acetoin, heptanone, octanone, nonanone) standards, and other chemicals were supplied by Sigma-Aldrich, Germany. Stock solutions of DBD-H (8 mM) and TFA (40% v/v) were prepared in acetonitrile. Stock solutions of aldehyde and ketone standards (~1 g/L) were prepared in H_2O (acetaldehyde, propanal, 3-methylbutanal, furfural, 5-hydroxymethylfurfural, diacetyl, 2,3-pentanedione, acetoin, 3-methylbutanone) or ethanol (butanal, 2-methylpropanal, hexanal, heptanal, octanal, *trans*-2-nonenal, phenylacetaldehyde, heptanone, octanone, nonanone). The validation procedure was performed according to the common validation criteria (Rozet *et al*., 2011). In that manner, we investigated method selectivity (zero signal and matrix effect), linearity, limit of detection, lower and upper limits of quantification, trueness and precision, and extraction

recovery. Standard solutions for distillation were prepared in H_2O with the addition of ethanol (4% v/v).

3.3.7.1 Distillation

To 200 mL of beverage samples (defrosted in refrigerator) in distillation cuvette, 6 g of NaCl and a small drop of anti-foaming agent were added. The distillation was performed using Büchi 323 Distillation Unit (Büchi Labortechnik AG, Switzerland) and 50 mL of distillate were collected.

3.3.7.2 GC-FID analysis

Distillates were first filtered through polytetrafluoroethylene (PTFE) membrane syringe filter unit (diameter of 25 mm, pore size of 0.45 µm) (LLG Labware, Germany) and analyzed with Agilent 6890 Series GC System with Autosampler, HP-FFAP Crosslinked column (25 m x 0.2 mm x 0.3 µm), a flame ionization detector (FID), and Chemstation computer software (Agilent, USA). 5 µL of sample was injected (injector temperature: 220°C, split ratio: 25.1 : 1) into the column using helium as a carrier gas at 22°C with the flow rate of 1 mL/min. The column temperature was first maintained at 50°C for 4 min, ramped at a rate of 4°C/min to 90°C and ramped again at a rate of 15°C/min to 200°C where it was held for 5 min. After that, the column temperature was raised to 240°C instantly, where it was held for 1 min. The detector temperature was 220°C and the data collection rate was 20 Hz at the minimum peak width 0.01 min. The analysis of one sample lasted altogether 24.33 min.

3.3.7.3 HPLC-FLD analysis

To 800 µL of distillate, 150 µL of DBD-H and 50 µL of TFA stock solution were added. Derivatization proceeded at room temperature for 2 h by gentle circular shaking (~30 rpm). Afterwards, derivatization mixture was filtered through polytetrafluoroethylene (PTFE) membrane syringe filter unit (diameter of 25 mm, pore size of 0.45 µm) (LLG Labware, Germany) and analyzed immediately using Agilent 1200 Series apparatus equipped with a thermostated autosampler, reverse-phase column (Agilent Zorbax PAH, 100 mm × 4.6 mm, 1.8 µm) maintained at 25°C, and FLD set to excitation and emission wavelengths of 440 and 450 nm, respectively. 30 µL of the derivatization mixture were injected A combination of the gradient and isocratic elution at a constant flow rate of 1.00 mL/min was used with 0.05% TFA in acetonitrile (mobile phase A) and 0.05% TFA in H_2O (mobile phase B) using the following regime: (i) 0.00-2.00 min, isocratic, B = 55%, (ii) 2.00-9.00 min, gradient, B = 55→30%, (iii) 9.00-11.0 min, isocratic, B = 30%, (iv) 11.00-11.01 min, gradient, B = 30→55%, (v) 11.01-13.50 min, isocratic, B = 55%, (vi) 13.51 min, STOP.

3.3.8 Sensory analysis

The final buckwheat and quinoa beverage were judged for five sensory attributes, i.e. odour, the purity of taste, palatefulness, sparkling, and the quality of bitterness three

months after bottling by a trained panel of eight evaluators according to the MEBAK method (Mitteleuropaischen …, 2013). Each sensory attribute was graded from 1 (worst grade) to 5 (best grade) and the average value of all evaluators represented the final mark.

The overall mark (OM) was calculated from the following equation:

$$OM = \frac{2 \times O + 2 \times P + F + S + B}{7} \quad \ldots(2)$$

O = odour
P = the purity of taste
F = palatefulness
S = sparkling
B = the quality of bitterness

Equation (2) also defines the scale of OM, i.e. from 1 to 5.

3.3.9 Statistical analysis and data representation

Because of the high reproducibility of the HPLC analysis, samples were analyzed only once. Relative standard deviation of each analyte was determined in a preliminary intralaboratory method validation. For all the statistical calculations, the Prism 5 computer software (GraphPad, USA) was used. Statistically significant differences between the data groups of interest were evaluated by one-way analysis of variance (ANOVA) followed by a Tukey's Multiple Comparison Test (differences at $p > 0.05$ were not considered statistically significant). Pearson's r was calculated together with two-tailed p value to quantify the linear dependence between two variables of at least four values for each. Non-linear regression was done using the "log(inhibitor) vs. response - variable slope" model, inherent to Prism 5 computer software. Tables and graphs were constructed using Microsoft Office Excell 2007 and for clarity purposes they contain only mean values.

3.4 THE ANALYSIS OF YEAST SAMPLES

3.4.1 Pulsed-field electrophoretic karyotyping

Yeast chromosomes were isolated by the contour clamped homogeneous electric field (CHEF) technique according to the method described by Carle and Olson (1985) and modified by Raspor *et al.* (2000). Briefly, approximately 50 mg of biomass were resuspended in 1 mL of 50 mM EDTA (pH 7.5) and centrifuged at 4000 rpm for 5 min. Pellets were resuspended in 75 µL of CPES solution (1 : 1 mixture of 1 M EDTA (pH 7.5) and CPESa buffer with a scrap of dithiothreitol) by vortexing. 165 µL of blocking gel at 42°C were added and immediately filled into three block formers and put into a refrigerator for 20 min. Block samples were transferred into 2 mL tubes with 1 mL of CPE solution (1 : 1 mixture of 1 M EDTA (pH 7.5) and CPEa buffer) and incubated at 30°C without

shaking. After 1 h, CPE solution was removed and block samples were washed for 3 × 15 min with 1 mL of 50 mM EDTA (pH 9.0). Afterwards, 1 mL of ETN solution (90 mL of 0.5 M EDTA (pH 9.0), 1 mL of 1.0 M Tris/HCl (pH 8.0), 50 mL of 20% sodium lauroyl sarcosinate, 4 mL of distilled water) with 1 mg/mL of proteinase K (Roche, Basel, Switzerland) was added and incubated overnight at 50°C with gentle shaking. Next day, the block samples were put into 1 mL of 50 mM EDTA (pH 9.0) for 1 h and finally stored in 1 mL of 0.5 M EDTA (pH 9.0). For chromosomal separation, Certified Megabase Agarose (Bio-Rad, Hercules, California, USA) was used. The CHEF-DR III pulsed-field gel electrophoresis (Bio-Rad, Hercules, CA, USA) was set to 6 V/m with a switching time of 100 s for 1 h, 90 s for 8 h , and 60 s for 15 h in TBE buffer (0.5x) at 12°C). The agarose gels were stained with ethidium bromide (0.5 µg/mL) and subsequently documented by the Gel Doc 2000 documentation system (Bio-Rad, Hercules, California, USA). Images were processed and band sizes were quantified based on the CHEF yeast chromosomal marker (Bio-Rad, Hercules, California, USA) by using Bionumerics computer software v7.1 (Applied Maths NV, Sint-Martens-Latem, Belgium).

3.4.2 SDS-PAGE protein profiling

Cellular proteins were analyzed based on the protocol of Zupan *et al.* (2009). Approximately 100 mg of sample biomass were first washed with 1 mL of distilled water, centrifuged for 5 min at 1500 × g and weighted again. According to the pre-weighted wet cell biomass, 70 µL of enzyme reaction mixture [35 µL of protoplast reaction buffer (2x), 2.5 µL Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, Missouri, USA), 30 mM DTT, 2 µg/mL cycloheximide, 15 U Quantazyme ylg™ (Krackeler Scientific, Albany, New York, USA)] was added per 50 mg wet cell biomass and incubated at 30°C and 800 rpm for 2 h. After the reaction, samples were centrifuged consecutively at 1500 × g and 6500 × g at 4°C for 5 min to remove cells and a supernatant fraction, containing the released proteins, was finally collected. We proved the complete isolation of proteins with the control experiments using two samples per raw material (the first and last fermentation). These samples were treated as described above where the only difference was that after the last centrifugation, each sample was divided in two equal parts wherein the supernatant was discarded from the one set of samples. PBS was added to the twelve resulting samples to the final volume of 1 mL and containing biomass was disrupted using Bullet Blender Storm 24 glass bead homogenizer (Next Advance, USA) following the manufacturer's protocol. Samples with previously discarded supernatants showed no protein bands whereas the protein profile of samples with the included supernatant were similar to main samples (data not shown). All protein samples were kept frozen at -20°C until SDS-PAGE analysis.

The isolated proteins were separated by SDS-PAGE using NuPAGE® Novex® Bis–Tris precast gels (Life Technologies, USA). Before SDS-PAGE analysis, protein loads were adjusted for optimal band resolution by the initial measuring of total protein concentrations

with the method of Bradford (Bradford, 1976) using Bradford Protein Assay (Bio-Rad, USA). Gels were stained with fluorescent stain ruthenium II tris–bathophenantroline disulfonate (RuBP) according to the protocol of Lamanda *et al.*, (2004), documented with GelDoc documentation system (Bio-Rad, USA) and analyzed with Bionumerics computer software (Applied Maths, Belgium). Protein concentrations were determined densitometrically with Quantity One computer software (Bio-Rad, USA) as follows. Protein standard Benchmark™ Protein Ladder (Life Technologies, Carlsbad, California, USA) with defined concentrations of protein bands was loaded onto each gel at two concentrations and from densitometric values of bands, which were closest in their size to the particular sample band, a calibration curve was constructed. The concentrations of sample proteins in protein bands (c_p) were finally calculated from the following equation (Zupan *et al*., 2009):

$$c_P = \frac{\frac{m_{S1}}{R_{S1}} + \frac{m_{S2}}{R_{S2}}}{2} \times R_p \times \frac{V_{MIX}}{V_L \times m_B} \; [\mu g/g] \qquad \ldots(3)$$

m_{S1} – the mass of a particular standard protein band at a load of 5µL [ug]
m_{S2} – the mass of a particular standard protein band at a load of 10µL [ug]
R_{S1} – the relative volume of a particular standard protein band at a load of 0.1 µg/µL
R_{S2} – the relative volume of a particular standard protein band at a load of 0.2 µg/µL
R_P – the relative volume of a particular sample protein band
V_{MIX} – the volume of a reaction mixture [µL]
V_L – the volume of a sample loaded on a gel [µL]
m_B – the initial wet cell biomass[g]

Relative standard deviation (RSD) was assessed during the method development (Zupan *et al.*, 2009) and we decided to use average values for eight concentration intervals (from 10 up to 20 kDa: 17.1%; from 20 up to 30 kDa: 13.6%; from 30 up to 40 kDa: 6.9%; from 40 up to 50 kDa: 7.4%; from 50 up to 60 kDa: 7.1%; from 60 up to 70 kDa: 11.1%; from 70 up to 80 kDa: 11.0%; from 80 up to 90 kDa: 15.7%). Based on the calculated molecular mass and RSD values of protein bands, candidate proteins were predicted from the number of amino acid residues calculated from the following equation:

$$AA = \frac{MW - 18}{118.9} \qquad \ldots(4)$$

AA – amino acid residue number
MW – protein band molecular weight [Da]
118.9 – average amino acid residue molecular weight [Da]

This information was then used for searching the *Saccharomyces pastorianus* TUM 34/70 genome (Nakao *et al.*, 2009, Supplementary Table 2) and particular gene information from the Saccharomyces Genome Database (SGD, http://www.yeastgenome.org) (Cherry *et al.*, 2012).

4 RESULTS WITH DISCUSSION

4.1 THE ANALYSIS OF FERMENTED BEVERAGES

Table 7: The maximum relative standard deviation values of determined attributes
The data were obtained from an intra-laboratory validation of methods.
Preglednica 7: Največje vrednosti relativne standardne deviacije raziskovanih atributov
Podatki so pridobljeni z interno laboratorijsko validacijo metod.

sample type	determined attribute	reference	RSD [%]
malt	moisture	MEBAK Rohstoffe 3.1.4.1	4.64
	extract	MEBAK Rohstoffe 3.1.4.2.2	3.07
	viscosity	MEBAK Rohstoffe 3.1.4.4.2	0.36
	saccharification time	MEBAK Rohstoffe 3.1.4.2.4	n.r.
	pH	MEBAK Rohstoffe 3.1.4.2.7	2.31
	total protein	MEBAK Rohstoffe 3.1.4.5.1	3.97
	total soluble nitrogen	MEBAK Rohstoffe 3.1.4.5.1.1	1.08
	free amino nitrogen	MEBAK Rohstoffe 3.1.4.5.5.1	8.07
wort	extract	MEBAK II 2.10.6.1	0.87
	pH	MEBAK II 2.14	1.65
	viscosity	MEBAK II 4.1.4.4	0.53
	free amino nitrogen	MEBAK II 2.8.4.1.1	4.96
	iodine test	MEBAK II 2.3.2	3.00
beverage	extract	MEBAK II 2.10.6.1	0.66
	ethanol	MEBAK II 2.10.6.1	0.48
	pH	MEBAK II 2.14	1.80
	total soluble nitrogen	Analytica-EBC 9.9.1	3.27
	free amino nitrogen	MEBAK II 2.8.4.1.1	2.53
	color	Analytica-EBC 9.28.2	0.30 (≤90) 1.32 (>90)
wort, beverage	iron	Analytica-EBC 9.13.3	7.52
	copper	Analytica-EBC 9.14.3	5.68
	manganese	Hoenig and Hoeyweghen (2010)	8.15
	zinc	Analytica-EBC 9.20	9.10
	trisaccharides	Analytica-EBC 9.27	1.40
	disaccharides		5.44
	sucrose		3.95
	glucose		5.70
	fructose		4.13
	aspartic acid	Henderson and Brooks (2010)	4.97
	glutamic acid		2.89
	asparagine		2.71
	serine		2.82
	glutamine		2.31
	histidine		3.19
	glycine		2.57
	threonine		2.61
	alanine		2.34
	arginine		2.02
	tyrosine		2.74
	valine		2.71
	methionine		2.15
	typtophan		3.42
	phenylalanine		3.02
	isoleucine		2.98
	leucine		2.36
	lysine		4.82
	proline		19.45

…to be continued

…the continuation of Table 7: The maximum relative standard deviation values of determined attributes

sample type	determined attribute	reference	RSD [%]
	acetaldehyde	MEBAK III 1.1.2	6.37
	ethyl acetate		5.48
	methanol		3.63
	1-propanol		4.64
	isoamyl acetate		4.77
	2-& 3-methylbutanol		7.02
	2-phenylethyl acetate		4.20
	2-phenylethanol		5.23

n.r. – not relevant. RSD – relative standard deviation.

4.1.1 Brewing attributes

4.1.1.1 Malt

Moisture, extract, viscosity: The moisture contents of the barley and buckwheat (Table 8) malt were similar to each other and similar to previous reports (Briggs *et al*., 2004). Compared to barley and buckwheat, the quinoa malt contained around 60% more moisture and a similar absolute value had been already reported (De Meo *et al*., 2011). Since the same degree of steeping was achieved for both buckwheat and quinoa together with the same kilning conditions, malting process could not be the reason. It seems that the size and structure of the grain were accountable for this difference (Arendt and Dal Bello, 2008). Compared to barley, extracts of buckwheat and quinoa were a quarter and a half lower, respectively (Table 8). Similar values of the buckwheat and barley malt have been presented elsewhere (Zarnkow *et al*., 2005; Phiarais *et al*., 2010), whereas our extract value of the quinoa malt was up to two-fold lower as reported (Zarnkow *et al*., 2005; 2007). The reason could be kilning with higher temperatures since enzymes are, for the most part, inactivated by heat. Viscosity correlated to the extract and taking this into account, the values of buckwheat and quinoa were both two- and a half-fold higher from that of barley (Table 8). The values of the barley and buckwheat malt were in general consistent with previous findings (Zarnkow *et al*., 2005; Phiarais *et al*., 2010) and the values for all three malts were within the range reported as being satisfactory for brewing purposes, as has been ascertained for quinoa, millet and rice in a previous study (Zarnkow *et al*., 2005).

Saccharification time, pH, nitrogenous constituents: The saccharification time is largely dependent on the α-amylase content of the malt. The results for the buckwheat and quinoa malt (Table 8) showed that they were longer than normally, suggesting the relatively low starch-degrading enzyme activity and the need to use supplementary enzymes. In our case only barley malt was close to the normal pH range (5.80-5.95), whereas the hydrogen ion concentration ($[H^+]$) of the buckwheat and quinoa malt was around an order of magnitude lower and higher, respectively (Table 8). The result of buckwheat malt was significantly lower than those reported by other researchers (Wijngaard and Arendt, 2006). A possible explanation is that because grain was unhulled, some lactic acid bacteria could grow on the

surface during germination. The quinoa malt was supreme in the total protein content over the buckwheat malt and both were supreme over the barley malt (Table 8). These results were expected since similar levels of total protein had also been determined in grain (Kiss *et al*., 2014; Briggs *et al*., 2004). In brewing, the total protein content should be lower than 11.5% since higher values are undesirable due to the reduced fermentable extract. Indeed, only 44% of the quinoa wort extract was fermented. Compared to the barley malt, the soluble protein content of buckwheat and quinoa were both higher (Table 8). Malt with Kolbach index (KI) between 38% and 42% is considered to be adequately modified and suitable for single infusion mashing. KI showed the appropriately modified barley, slightly under-modified buckwheat and significantly over-modified quinoa malt (Table 8). In our case, the over-modification of the quinoa malt caused its crushing to a powder when a series of corns were crumbled between a finger and the thumb. Also, several problems regarding wort separation were encountered and both the boiled wort and the final beverage were of intense black colour. It has been reported that the buckwheat and quinoa FAN values were above standard (Zarnkow *et al*., 2005) but in our case this was true only for quinoa, whereas buckwheat FAN content did not differ significantly from that of barley.

4.1.1.2 Wort

Extract, viscosity, pH, FAN, iodine value: The mashing procedures were designed to give the final extract of 10%. Although the viscosity values of our samples differed significantly (Table 8), they were all within the range (1-2 mPas) believed not to cause brewing problems also when using raw materials other than barley (Zarnkow *et al*, 2005; Klose *et al*., 2011). Interestingly, regarding buckwheat all studies reported viscosity values to be much higher (2.5-6 mPas) than ours, suggesting the appropriate use of supplementary enzymes. The normal pH value of the barley wort ranges from 5.5 to 5.9. In our case (Table 8), this was true for both barley and buckwheat whereas the value of quinoa was more than one order lower. Buckwheat encountered a rise in $[H^+]$ for around an order of magnitude from malt to wort whereas the quinoa wort $[H^+]$ was around two orders of magnitude lower compared to malt. The decrease of pH in the quinoa wort could be interpreted with the release of the Maillard products formed during malting or their *de novo* formation during wort boiling which have a major impact on the colour and aroma as well as on the pH of the wort (Eßlinger, 2009). In principle, FAN should not be lower than 150 mg/L (referring to extract of 12%). All our samples contain a much higher amount of FAN (Table 8), especially the quinoa wort which was obviously because of the addition of enzyme preparation containing protease (Ondea^{TM} Pro). From the results of iodine value (Table 8), it can be concluded that the barley and buckwheat starch was sufficiently degraded and that of quinoa was not. This relatively high iodine value of quinoa wort indicated that either the type/concentration of enzyme preparations or the regime of their supplementation used was not entirely adequate and could be improved further.

Table 8: Brewing attribute values for the barley, buckwheat and quinoa malts and worts
Preglednica 8: Vrednosti pivovarskih atributov za ječmenov, ajdov in kvinojin slad in pivino

brewing attribute	barley		buckwheat		quinoa	
	malt	wort	malt	wort	malt	wort
moisture [%ww]	4.25	n.d.	4.5	n.d.	7.4	n.d.
real extract	80.90% (dw)	10.04% (w/w)	62.80% (dw)	10.36% (w/w)	37.70% (dw)	10.49% (w/w)
viscosity [mPa×s]	1.56	1.13	3.01	2.07	1.87	1.61
saccharification	normal	n.d.	> 20 min	n.d.	> 20 min	n.d.
pH	5.65	5.92	4.57	5.77	6.38	4.49
total protein (TP) [%dw]	10.8	n.d.	13.0	n.d.	16.2	n.d.
soluble protein [%TP]	4.4	n.d.	4.2	n.d.	8.6	n.d.
FAN	0.104% (dw)	133 mg/L	0.096% (dw)	152 mg/L	0.219% (dw)	340 mg/L
Kolbach index [%]	40.7	n.d.	32.3	n.d.	53.4	n.d.
iodine test	n.d.	0.39	n.d.	0.67	n.d.	2.35

ww – wet weight. dw – dry weight. n.d. – not determined. TP – total protein. FAN – free amino nitrogen.

4.1.1.3 Beverages

The fermentation profile, i.e. the extract consumption and ethanol production (Figure 4), was wort type- and yeast strain dependent. Typically, barley wort is pitched with yeast to the final concentration of 15-20 × 10^6 cells/mL. In that way the fermentation of wort with 12% of extract usually lasts five days to achieve attenuation at real fermentability around 85%. In our case, although worts with 10% of extract were used, attenuation was achieved significantly later. This is mainly because 10-times less yeast was pitched. Additionally, in the case of buckwheat and quinoa the influence of matrix to which yeast was not genetically and phenotypically adapted could also be a reason.

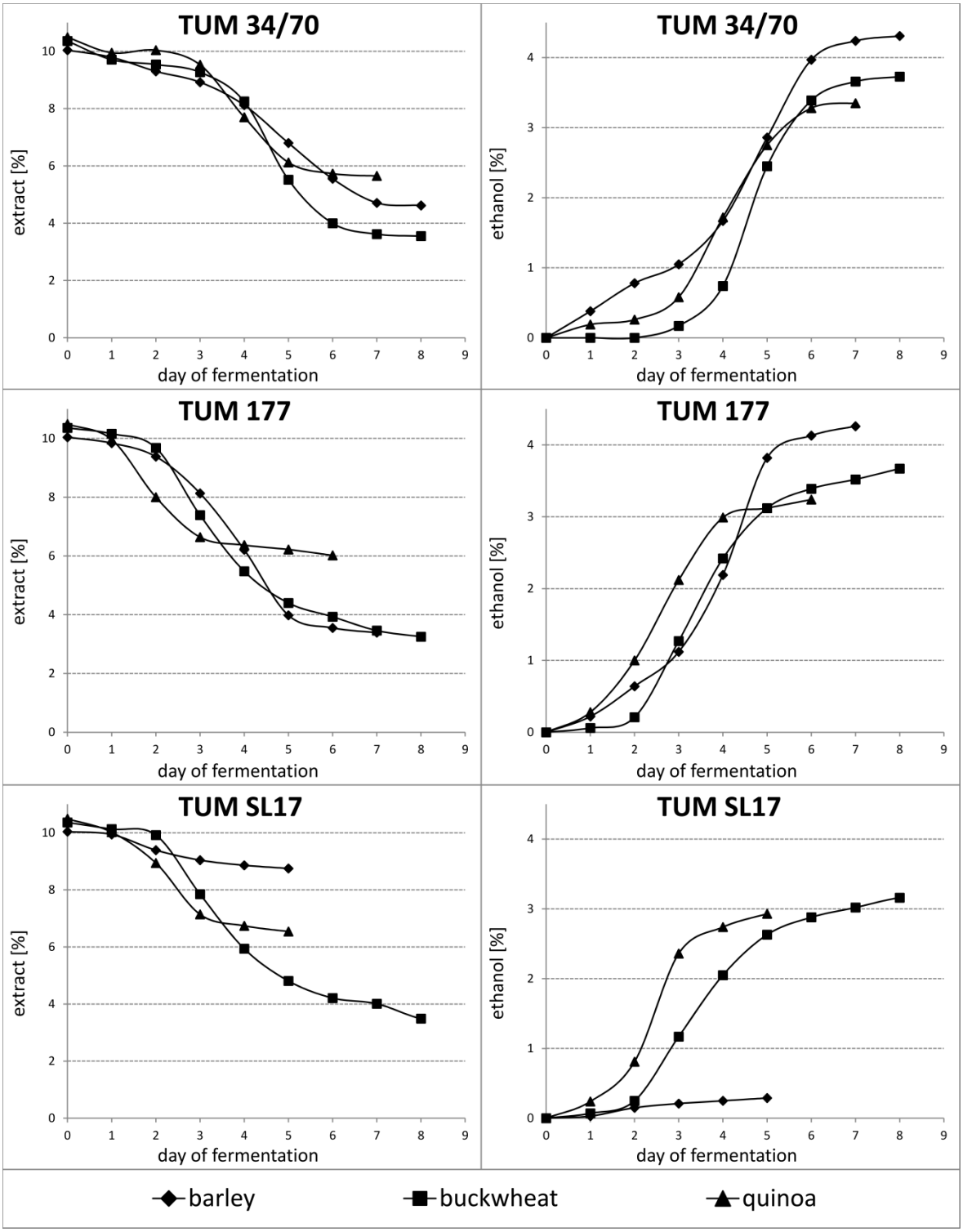

Figure 4: The extract consumption and ethanol production profiles during the barley, buckwheat, and quinoa wort fermentation employing different yeast strains
Slika 4: Profila porabe ekstrakta in tvorbe etanola tekom fermentacije ječmenove, ajdove in kvinojine pivine z uporabo različnih kvasnih sevov

TUM 34/70

The barley and buckwheat wort possessed similar fermentability values (Table 9) whereas less than a half of quinoa extract was fermented. An interesting fact arose when we had calculated the amount of ethanol produced per amount of extract consumed. In that way it can be seen that on average yeast produced 0.67, 0.55, and 0.72% of ethanol when it assimilated 1% of extract in the barley, buckwheat, and quinoa wort, respectively. Conversely, these results suggested that a higher proportion of consumed solutes were shifted to the biomass gain during the buckwheat wort fermentation compared to barley and even higher compared to quinoa. The quinoa beverage was supreme in both FAN and TSN content (Table 9) as well as in their ratio. Namely, compared to the barley and buckwheat beverage (16.9 and 31.2%), in the quinoa beverage FAN represented 64.8% of TSN. These results inversely correlated with the percentage of assimilated FAN by yeast being 83.8, 80.1, and 23.2%. All pH values were somehow typical for brewery beverages. The colour of the barley and buckwheat beverage was within the range normally reported for non-pasteurized bottom-fermented pale lager beer (Table 9). On the contrary, the quinoa beverage was very dark, practically black with the colour value even higher than normally reported for dark beers (50-80 EBC). Both AT_{50} values of barley were comparable with those of buckwheat, whereas lower AT_{50} values of quinoa indicate faster fermentation.

Table 9: Brewing attribute values for the barley, buckwheat and quinoa bottled beverages
Preglednica 9: Vrednosti pivovarskih atributov za ječmenove, ajdove in kvinojine ustekleničene pijače

brewing attribute	barley			buckwheat			quinoa		
	TUM 34/70	TUM 177	TUM SL17	TUM 34/70	TUM 177	TUM SL17	TUM 34/70	TUM 177	TUM SL17
real extract [% (w/w)]	3.59	3.39	8.75	3.42	3.25	3.36	5.91	6.02	6.54
ethanol [% (v/v)]	4.30	4.26	0.29	3.79	3.67	3.19	3.28	3.24	2.93
real fermentability [%]	64.20	65.72	17.99	63.12	63.59	59.77	43.66	44.92	40.34
pH	4.72	4.44	5.12	4.81	4.62	4.80	4.50	4.46	4.47
TSN [mg/L]	128.1	128.7	168.2	97.0	91.2	84.4	402.7	401.5	399.4
FAN [mg/L]	21.6	18.8	49.9	30.2	25.7	29.3	261	252	257
colour [EBC]	10.3	9.6	12.2	16.7	12.0	5.6	129.7	140.7	131.1
AT_{50} [%] (extract)	4.74	3.74	1.98	4.57	3.27	3.43	3.89	1.83	2.19
R^2 [%] (extract)	99.65	99.78	99.36	99.42	99.56	99.43	99.54	99.76	99.78
AT_{50} [%] (ethanol)	4.50	3.92	1.90	4.68	3.45	3.42	4.04	2.52	2.40
R^2 [%] (ethanol)	99.08	99.29	98.78	99.96	99.74	99.71	99.85	99.91	99.74

TSN – total soluble nitrogen. FAN – free amino nitrogen. AT_{50} – the percentage of attenuation time needed to achieve 50% of the total change in the extract or ethanol content. R^2 – the goodness of curve fit.

TUM 177

The situation regarding fermentability, TSN and pH values was practically identical to the situation of TUM 34/70 for all three raw materials (Table 9). On the contrary, colour values of barley and buckwheat were a bit lower and a bit higher for quinoa whereas FAN

values were lower regardless wort type. On average, yeast produced 0.64, 0.52, and 0.72% of ethanol when it assimilated 1% of extract in the barley, buckwheat, and quinoa wort, respectively. These results also do not differ significantly from those of TUM 34/70. Nevertheless, AT_{50} values suggest considerably faster fermentation than in TUM 34/70 although attenuation time do not support that. However, when looking closer to Figure 4 it is obvious that indeed fermentations commenced faster but in the last third they were much longer and slower than in TUM 34/70.

TUM SL17

In the case of buckwheat and quinoa this yeast behaved very similar to TUM 34/70 and/or TUM 177 from all relevant aspects with the exception of colour which was two to three times lower in buckwheat (Table 9). In barley, totally different results were obtained. Fermentability, extract consumption, ethanol production and pH drop were drastically lower than in other two yeasts. TSN and FAN values were higher suggesting the lower degree of yeast proliferation. However, yeast produced only 0.22% of ethanol when it assimilated 1% of extract in the barley wort which means that not the whole amount of carbohydrates yeast is able to ferment was converted to ethanol, suggesting the yeast proliferation took place to some extent.

4.1.2 Metals

4.1.2.1 Iron (Fe)

Fe concentrations between 0.055 and 0.165 mg/L are reported to be sufficient for normal fermentation – if higher, general toxic effects can be observed (Walker, 2004). The levels in the barley (Table 10) and buckwheat (Table 11) wort were lower than this range whereas in the quinoa wort (Table 12) there was an exceptionally high level of Fe, namely around 50-times higher than in the barley and buckwheat wort, respectively. In the quinoa wort fermentation, TUM 177 showed the highest affinity for Fe and TUM 34/70 the lowest one. These results showed that all three yeast strains assimilated ~10-times higher amounts of Fe than needed for proper fermentation, suggesting the probable impairment of yeast vitality in general, especially the yeast growth and fermentation performance.

4.1.2.2 Copper (Cu)

In the buckwheat and quinoa wort, Cu concentrations were two to three times higher than usually found in beer produced from barley malt, i.e. 0.1 mg/L (Walker, 2004). However, yeast possesses several homeostatic mechanisms for maintaining a proper Cu level, therefore, in practice only concentrations over 1 mg/L can literally be toxic (Buiatti, 2009). The Cu assimilation from buckwheat wort was the highest for TUM SL17 and the lowest for TUM 34/70 but, interestingly, this order was inversed in quinoa. In beverages, even small amounts of Cu (~0.15 mg/L) can cause gushing, noticeably contribute to the oxidation, and impart an unpleasant metallic taste (Mayer *et al*., 2003). Only Cu concentrations in buckwheat beverages fermented with TUM 34/70 and TUM 177 were

close to that value. However, why TUM 34/70 and TUM 177 assimilated ~2-times more Cu in quinoa than in buckwheat, is not clear at this moment.

4.1.2.3 Zinc (Zn)

The concentrations of Zn between 0.275 and 0.550 mg/L are thought as satisfactory and non-toxic (Walker, 2004). In our case, only the Zn content of the barley (Table 10) wort fell into this range whereas buckwheat (Table 11) and quinoa (Table 12) had much higher values. In both cases the concentrations were in the range (above 1 mg/L) where Zn is toxic to yeast and inhibits enzymes (Buiatti, 2009). However, both worts were also rich in amino acids and proteins (Table 17 and Table 18) which could chelate surplus Zn but it seems that in quinoa toxic effects still could not be obviated. There are no notable differences in Zn assimilation between different yeast strains. During fermentation, yeast assimilated around 0.3, 1.2, and 1.3 mg of Zn from a litre of the barley, buckwheat, and quinoa wort, respectively, which represents 93, 97, and 69% of the Zn in wort. Yeast takes up zinc very rapidly from the wort and it seems that a large proportion of it is simply cell wall-bound (Walker, 2004). Indeed, a slight negative correlation (Pearson's r ~ -0.81) was observed between the percentage of assimilated zinc and ethanol production/extract consumption ratio.

4.1.2.4 Manganese (Mn)

For proper fermentation, Mn should be present in the range from 0.11 to 0.22 mg/L (Walker, 2004). Both the barley (Table 10) and buckwheat (Table 11) wort fell into this range and once more, the quinoa wort (Table 12) had super-optimal concentration, namely around 12- and 7-times more than barley and buckwheat, respectively. Its assimilation during fermentation by the particular yeast strain was wort type-independent, being the highest by TUM 34/70 and the lowest by TUM SL17. Regarding the latter, only in the case of buckwheat Mn assimilation was significant.

Table 10: The metal content of the barley wort and in fresh and forced-aged beverages fermented with different yeast
Preglednica 10: Vsebnost kovinskih ionov v ječmenovi pivini ter v ječmenovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

metal	barley [mg/L]						
	wort	**TUM 34/70 fresh beverage**	**TUM 34/70 force-aged beverage**	**TUM 177 fresh beverage**	**TUM 177 force-aged beverage**	**TUM SL17 fresh beverage**	**TUM SL17 force-aged beverage**
iron	< LLQ	< LLQ	< LLQ	< LLQ	< LLQ	< LLQ	< LLQ
copper	0.094	< LLQ	< LLQ	< LLQ	< LLQ	0.045	0.046
zinc	0.354	< LLQ	< LLQ	< LLQ	< LLQ	< LLQ	< LLQ
manganese	0.128	0.070	0.076	0.095	0.105	0.127	0.125
sum	0.604	0.129	0.148	0.146	0.176	0.193	0.227

LLQ – limit of quantification.

Table 11: The metal content of the buckwheat wort and in fresh and forced-aged beverages fermented with different yeast
Preglednica 11: Vsebnost kovinskih v ajdovi pivini ter v ajdovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

metal	buckwheat [mg/L]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
iron	0.043	< LLQ	< LLQ	< LLQ	< LLQ	< LLQ	< LLQ
copper	0.267	0.171	0.179	0.137	0.152	0.106	0.088
zinc	1.248	0.041	0.059	< LLQ	< LLQ	0.057	0.063
manganese	0.215	0.160	0.166	0.176	0.213	0.188	0.187
sum	1.773	0.401	0.455	0.369	0.410	0.338	0.347

LLQ – limit of quantification.

Table 12: The metal content of the quinoa wort and in fresh and forced-aged beverages fermented with different yeast
Preglednica 12: Vsebnost kovinskih v kvinojini pivini ter v kvinojinih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

metal	quinoa [mg/L]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
iron	1.954	1.238	1.160	0.847	0.832	0.951	0.983
copper	0.325	0.075	0.050	0.101	0.043	0.132	0.068
zinc	1.934	0.601	0.668	0.405	0.484	0.718	0.711
manganese	1.508	1.060	1.158	1.238	1.236	1.435	1.400
sum	5.721	2.974	3.136	2.091	2.195	3.036	3.162

LLQ – limit of quantification.

4.1.3 Fermentable carbohydrates

The principle carbohydrates normally present in the barley wort are the trisaccharide maltotriose (10-15%), the disaccharides maltose (45-65%) and sucrose (~5%), and the monosaccharides glucose and fructose (~10%) (Eßlinger, 2009). In our case, the barley wort was close to the above mentioned ratio of fermentable sugars, with the exception of high maltose levels (Table 13). The buckwheat (Table 14) and quinoa (Table 15) wort were deficient in maltose but they had around 5-fold more glucose than barley. This result is in agreement with previous research where buckwheat malt gave several times more glucose than maltose across all germination temperatures (Agu *et al*., 2012). Different carbohydrate ratios have been previously shown to affect the fermentation performance negatively where yeast exhibited higher viabilities in maltose than in glucose media (Stewart, 2006). High glucose level in the buckwheat and quinoa worts, both absolute and relative, is thus believed to be − besides the toxic concentrations of some metals − one of a few main reasons for all the principal differences in regard to the barley wort fermentation.

Maltose is the most abundant sugar in the wort and the main member of sugars with two degrees of polymerization (DP2), i.e. disaccharides. The differences in the DP2 content in our wort samples were significant as well as were degrees of its assimilation. Regarding the latter, only 2% of DP2 were left in the barley beverage (Table 13) compared to around 40% in the buckwheat (Table 14) and quinoa beverages (Table 15) after fermentation with TUM 34/70 and TUM 177. For buckwheat, the situation was similar also for TUM SL17, whereas around one-third less DP2 were assimilated in quinoa and not a bit in barley. Due to the high glucose levels, the 'glucose catabolite repression' seemed the major cause for the observed differences in the maltose assimilation in the case of TUM 34/70 and TUM 177, whereas the assimilation by TUM SL17 was impossible in itself (Section 2.5). The sucrose level, whose concentration was calculated from equation (1), was especially low in the quinoa wort (Table 15).

Maltotriose is the principal sugar in the wort with three degrees of polymerization (DP3), i.e. trisaccharide and the largest sugar molecule that can be assimilated by yeast. The absolute concentrations of DP3 in our worts (Table 13, Table 14, Table 15) were usual in brewing but, as opposed to barley, the degree to which DP3 were fermented in the case of buckwheat and quinoa was rather low, similarly as it was observed for DP2 (see above). Namely, around 20% of DP3 were left unfermented in the barley beverage and around 60% in buckwheat and quinoa. In addition, the observations in the case of TUM SL17 were also similar to DP2 as well as are their explanations (see above).

After fermentation, maltose and maltotriose were the most abundant fermentable sugars and sucrose levels were very low whereas the glucose and fructose presence was below detection limit. The overall percentages of assimilated sugars varied significantly, being 95, 85, and 75% for barley, buckwheat, and quinoa fermentation, respectively. Sugar content of forced-aged beverages was not significantly different from fresh beverages (Table 13, Table 14, Table 15). In theory, individual sugar concentrations should not increase during ageing since there is no relevant non-enzymatic mechanism by which sugar molecules could be synthesized.

Table 13: The fermentable carbohydrate content of the barley wort and in fresh and forced-aged beverages fermented with different yeast
Preglednica 13: Vsebnost fermentabilnih ogljikovih hidratov v ječmenovi pivini ter v ječmenovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

fermentable carbohydrate	barley [g/100 mL][a]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
DP3	0.92 (12)	0.19 (55)	0.21 (44)	0.14 (58)	0.18 (60)	0.97 (15)	1.11 (17)
DP2	5.13 (70)	0.11 (30)	0.11 (24)	0.09 (37)	0.11 (37)	5.21 (80)	5.37 (79)
sucrose	0.41 (6)	0.05 (15)	0.05 (12)	0.01 (5)	0.01 (3)	0.35 (5)	0.33 (4)
glucose	0.71 (10)	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
fructose	0.17 (2)	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
sum	7.34	0.35	0.47	0.24	0.29	6.53	6.48

[a] The value in bracket is a percentage of total sugar content. < LOD − below the detection limit. DP3 – sugars with three degrees of polymerization. DP2 – sugars with two degrees of polymerization excluding sucrose.

Table 14: The fermentable carbohydrate content of the buckwheat wort and in fresh and forced-aged beverages fermented with different yeast
Preglednica 14: Vsebnost fermentabilnih ogljikovih hidratov v ajdovi pivini ter v ajdovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

fermentable carbohydrate	buckwheat [g/100 mL][a]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
DP3	0.75 (10)	0.44 (34)	0.42 (36)	0.41 (34)	0.39 (35)	0.46 (31)	0.50 (33)
DP2	2.24 (29)	0.79 (61)	0.76 (61)	0.77 (64)	0.70 (64)	0.93 (63)	0.96 (62)
sucrose	0.30 (4)	0.07 (5)	0.06 (5)	0.02 (2)	0.01 (1)	0.10 (7)	0.08 (5)
glucose	4.29 (54)	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
fructose	0.21 (3)	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
sum	7.78	1.30	1.24	1.20	1.09	1.49	1.46

[a] The value in bracket is a percentage of total sugar content. < LOD − below the detection limit. DP3 – sugars with three degrees of polymerization. DP2 – sugars with two degrees of polymerization excluding sucrose.

Table 15: The fermentable carbohydrate content of the quinoa wort and in fresh and forced-aged beverages fermented with different yeast
Preglednica 15: Vsebnost fermentabilnih ogljikovih hidratov v kvinojini pivini ter v kvinojinih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

fermentable carbohydrate	quinoa [g/100 mL][a]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
DP3	0.98 (19)	0.62 (45)	0.66 (47)	0.64 (47)	0.66 (51)	0.76 (42)	0.88 (46)
DP2	1.61 (32)	0.64 (47)	0.64 (46)	0.70 (52)	0.63 (48)	1.02 (56)	1.02 (52)
sucrose	0.12 (2)	0.10 (8)	0.10 (7)	0.01 (1)	0.01 (1)	0.03 (2)	0.03 (2)
glucose	2.31 (45)	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
fructose	0.10 (2)	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
sum	5.11	1.37	1.40	1.34	1.30	1.80	1.94

[a] The value in bracket is a percentage of total sugar content. < LOD – below the detection limit. DP3 – sugars with three degrees of polymerization. DP2 – sugars with two degrees of polymerization excluding sucrose.

4.1.4 Amino acids

The main sources of nitrogen in the wort are amino acids (AA), ammonium ions, and some di- and tripeptides (Briggs *et al*., 2004). The AA contents in our samples are presented in Table 16, Table 17 and Table 18. For illustrative purposes, an HPLC-FLD chromatogram of the quinoa wort is shown (Figure 5) where preliminary experiments were done using different columns and mobile phase regime to achieve the complete separation of all 20 standard proteinogenic amino acids.

There are quite some reports regarding the AA content in barley beer (e.g. Pomilio *et al*., 2010) but to the best of our knowledge there is no such analysis in the buckwheat and quinoa beverage. For buckwheat, a recent study explored the influence of different malting conditions on the AA release (Agu *et al*., 2012) and it seems that this process is hardly influenced by the malting parameters. Because the AA content and its profile (aminogram) strongly depend on the type of raw material used and the conditions of its cultivation as well as on the particular process technology, any correlations of informative value between different researches are hard to find.

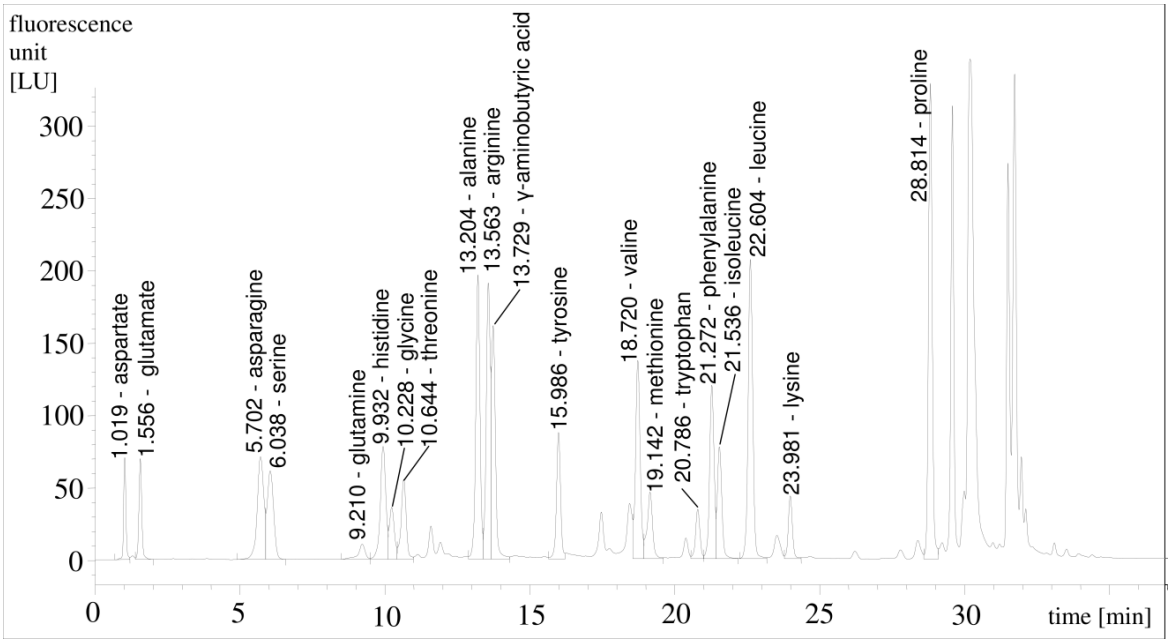

Figure 5: The HPLC-FLD chromatogram of amino acids in quinoa wort
The concentrations of amino acids in samples were calculated from the calibration curve constructed using standard solutions of different concentrations.
Slika 5: HPLC-FLD kromatogram aminokislin v kvinojini pivini
Koncentracije aminokislin v vzorcih so bile izračunane s pomočjo kalibracijske krivulje, narejene na osnovi standardnih raztopin različnih koncentracij.

Regarding worts, total amounts of AAs for barley and buckwheat were similar and both were lower for around 25% than in quinoa. This was expected from the FAN values (Table 8) since AAs represent the majority of the wort FAN fraction. The profiles of the separate AA differed markedly, most likely being a function of both raw material itself and the diverse mashing regimes used. The correlation coefficient showed that all worts have

different aminograms from each other [barley-buckwheat Pearson's r = 0.67 (p = 0.002), barley-quinoa Pearson's r = 0.73 (p = 0.0004), and buckwheat-quinoa Pearson's r = 0.61 (p = 0.006)]. Interestingly, both pseudocereals were more similar to barley than to each other. Regarding the beverages, the positive correlation between barley and buckwheat was almost complete (Pearson's r = 0.99 ($p = 6 \times 10^{-16}$)). Additionally, the coefficient of variation (CV) clearly showed a more equal distribution of separate AAs in the quinoa wort (CV = 0.55) followed by buckwheat (CV = 0.71) and barley (CV = 0.96).

Proline was the most (or one of the most) abundant AA in wort samples its concentration in barley was around twice as high as in buckwheat and quinoa. This was not surprising since hordein protein fraction, the major storage protein of the barley grain endosperm, is characterized by having high levels of proline (Fontana and Buiatti, 2009). On the contrary, storage proteins of dicotyledonous plants are mainly globulins and albumins. These fractions were reported with lower amounts of glutamine and proline (Arendt and Dal Bello, 2008). After proline, arginine and asparagine were the most frequent AAs in the barley wort, both of around 10% of total. In the buckwheat wort, glutamate and arginine were of similar share as proline (around 13% of total). As mentioned above, AAs in the quinoa wort showed very equal distribution; however, only alanine and leucine were found in concentrations around 10% of total, other AAs being far more infrequent. Interestingly, what all wort samples shared, were glycine and glutamine being the rarest AAs. The content of essential AAs was similar in the barley and buckwheat wort and almost twice as high in the quinoa beverage. The profiles of essential AAs were surprisingly similar (Pearson's r = ~0.80 (p = 0.008-0.018) in all cases), with Val, Phe, and Leu concentrations having the highest concentrations.

In brewing yeasts growing on wort, the uptake of AAs is an ordered process, largely independent of the fermentation conditions. Pierce (1987) divided AAs into four classes from A to D based on their order of assimilation from wort during fermentation. The AAs in Group A and B are required for anabolic metabolism, principally protein synthesis. In our case, during fermentation AAs of these two groups were assimilated to a high degree only in barley and buckwheat (86-96%) but not in quinoa (31 and 18%, respectively). Conversely, AAs in Group C are only taken up when the Class A AAs have disappeared and nitrogen catabolite repression is relieved. Our observations were a bit different since after barley and buckwheat wort fermentation a significant amount of Group C AAs were assimilated despite the AAs of the first two groups were still available. Proline is the sole member of Group D. Its oxidation by a mitochondrial oxidase is repressed during fermentation and that is why this amino acid was not utilized during brewery fermentation. However, this classification should not be accepted as a rule. It was made using mainly malted barley and since other raw materials differ drastically, further studies are needed to elucidate the effect of a specific wort composition on the expression of genes involved in AA uptake and metabolism.

TUM 34/70

The assimilation profiles of the AAs in our samples demanded further discussion (proline was excluded). The percentage values of consumed AAs relative to the initial concentration in the wort were different between fermentations, being the most similar between barley and buckwheat (Pearson's r = 0.71 (p = 0.001)).

Table 16: The amino acid content of the barley wort and fresh and in forced-aged beverages fermented with different yeast
Preglednica 16: Vsebnost aminokislin v ječmenovi pivini ter v ječmenovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

amino acid	barley [mg/L][a]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
Asp	59.3 (6.0)	5.1 (3.2)	6.1 (3.5)	5.9 (8.3)	6.6 (7.9)	58.0 (7.8)	32.9 (8.0)
Glu	64.6 (6.5)	6.9 (4.4)	8.1 (4.7)	4.3 (6.0)	4.6 (5.5)	74.5 (10.1)	43.8 (10.6)
Asn	123.2 (12.5)	2.5 (1.6)	2.4 (1.4)	0.6 (0.9)	0.5 (0.6)	47.4 (6.4)	25.9 (6.3)
Ser	46.9 (4.8)	1.8 (1.1)	1.8 (1.0)	1.0 (1.4)	1.5 (1.8)	28.5 (3.8)	17.4 (4.2)
Gln	6.0 (0.6)	6.4 (4.0)	6.8 (3.9)	4.6 (6.6)	5.0 (6.0)	10.8 (1.5)	5.0 (1.2)
His	32.2 (3.3)	6.9 (4.4)	5.7 (3.2)	1.0 (1.5)	2.1 (2.5)	30.5 (4.1)	12.4 (3.0)
Gly	17.4 (1.8)	15.8 (10.0)	17.9 (10.3)	7.1 (10.1)	7.3 (8.7)	18.1 (2.4)	13.9 (3.4)
Thr	33.2 (3.4)	2.9 (1.8)	3.6 (2.1)	1.8 (2.5)	2.5 (3.0)	23.3 (3.1)	13.5 (3.3)
Ala	79.8 (8.1)	19.9 (12.6)	22.4 (12.9)	7.0 (9.9)	8.4 (10.0)	81.1 (10.9)	43.5 (10.5)
Arg	116 (11.7)	30.3 (19.2)	30.9 (17.8)	6.1 (8.7)	6.5 (7.7)	92.4 (12.5)	53.4 (12.9)
Tyr	47.9 (4.9)	10.5 (6.7)	12.0 (6.9)	4.4 (6.2)	4.9 (5.8)	42.3 (5.7)	24.4 (5.9)
Val	71.0 (7.2)	8.8 (5.5)	9.8 (5.6)	6.9 (9.8)	7.8 (9.2)	54.6 (7.4)	33.0 (8.0)
Met	31.9 (3.2)	11.4 (7.2)	13.1 (7.6)	9.3 (13.1)	10.8 (12.8)	21.1 (2.9)	8.5 (2.1)
Trp	31.4 (3.2)	13.3 (8.4)	14.1 (8.2)	1.0 (1.4)	1.9 (2.2)	29.9 (4.0)	17.5 (4.2)
Phe	74.2 (7.5)	6.8 (4.3)	6.4 (3.7)	2.6 (3.7)	3.9 (4.6)	51.4 (6.9)	26.6 (6.4)
Ile	40.4 (4.1)	2.0 (1.3)	3.8 (2.2)	1.5 (2.1)	2.9 (3.4)	26.4 (3.6)	16.3 (3.9)
Leu	72.5 (7.4)	4.5 (2.9)	6.6 (3.8)	3.3 (4.6)	5.0 (6.0)	40.6 (5.5)	22.6 (5.5)
Lys	38.7 (3.9)	2.3 (1.4)	1.8 (1.0)	2.3 (3.2)	2.0 (2.4)	10.3 (1.4)	2.5 (0.6)
sum	986 (100)	158 (100)	173 (100)	70.5 (100)	84.1 (100)	741 (100)	413 (100)

[a] The value in bracket is a percentage of total amino acid content. AA – amino acid.

In barley, buckwheat, and quinoa there were (i) 10, 15, and zero AAs, respectively, the assimilation of which was above 80% assimilation, (ii) 6, 2, and 6 AAs, respectively, the assimilation of which was between 20 and 80%, and (iii) 3, 2, and 13 AAs, respectively, which were assimilated in less than 20% of initial concentration in wort. In all cases, glycine was assimilated to the lowest degree, obviously because of its low content in all wort samples. In all fermentations, yeast showed a special preference for serine, lysine, and asparagine.

Considering the percentage values of assimilated AAs relative to the total assimilated, it can be noticed that during the barley and buckwheat wort fermentation, yeast was using a wider spectrum of AAs whereas in the case of quinoa it has focused on just a few. The correlations between fermentations were very low; the highest between barley and buckwheat has Pearson's r of only 0.57 ($p = 0.011$). Namely, in the quinoa wort fermentation only four AAs fulfilled the 70% of the total need whereas in the case of barley and buckwheat, 8 and 9 AAs were needed, respectively.

Table 17: The amino acid content of the buckwheat wort and in fresh and forced-aged beverages fermented with different yeast
Preglednica 17: Vsebnost aminokislin v ajdovi pivini ter v ajdovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

amino acid	buckwheat [mg/L][a]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
Asp	71.4 (6.8)	4.0 (5.0)	4.6 (5.3)	4.3 (6.7)	5.0 (6.8)	5.0 (3.0)	5.5 (3.0)
Glu	159 (15.1)	3.1 (3.9)	3.5 (4.0)	2.3 (3.5)	2.8 (3.8)	7.5 (4.5)	8.0 (4.3)
Asn	41.8 (4.0)	1.4 (1.7)	1.8 (2.0)	< LOD	< LOD	< LOD	< LOD
Ser	51.9 (5.0)	0.9 (1.1)	0.9 (1.0)	< LOD	0.8 (1.0)	< LOD	0.8 (0.4)
Gln	9.2 (0.9)	0.6 (0.8)	1.5 (1.7)	< LOD	< LOD	1.3 (0.7)	1.5 (0.8)
His	43.7 (4.2)	3.3 (4.2)	1.5 (1.8)	0.9 (1.4)	0.5 (0.7)	3.9 (2.3)	4.2 (2.3)
Gly	11.6 (1.1)	9.3 (11.6)	11.7 (13.3)	4.6 (7.2)	5.5 (7.5)	6.9 (4.1)	6.3 (3.4)
Thr	43.6 (4.2)	2.3 (2.8)	2.8 (3.1)	1.0 (1.6)	1.8 (2.4)	2.3 (1.3)	1.3 (0.7)
Ala	85.7 (8.2)	8.5 (10.6)	8.5 (9.7)	2.0 (3.1)	4.0 (5.5)	6.3 (3.7)	4.8 (2.6)
Arg	154 (14.7)	8.5 (10.6)	9.0 (10.2)	8.8 (13.8)	9.5 (13.0)	39.3 (23.5)	41.8 (22.5)
Tyr	44.1 (4.2)	1.0 (1.3)	1.3 (1.4)	1.0 (1.6)	1.3 (1.7)	3.5 (2.1)	4.3 (2.3)
Val	72.4 (6.9)	20.6 (25.8)	20.3 (23.0)	21.0 (33.1)	20.5 (28.0)	22.0 (13.2)	20.8 (11.2)
Met	38.3 (3.7)	8.0 (1.0.)	9.1 (10.4)	10.0 (15.7)	10.8 (14.7)	11.8 (7.0)	12.8 (6.9)
Trp	29.3 (2.8)	1.0 (1.3)	1.3 (1.4)	1.3 (2.0)	1.0 (1.4)	50.3 (30.0)	62.5 (33.6)
Phe	51.8 (4.9)	3.5 (4.4)	4.3 (4.8)	3.0 (4.7)	4.0 (5.5)	3.5 (2.1)	7.5 (4.0)
Ile	36.4 (3.5)	1.0 (1.3)	1.8 (2.0)	0.5 (0.8)	1.5 (2.0)	1.5 (0.9)	< LOD
Leu	60.2 (5.7)	1.8 (2.2)	2.6 (3.0)	1.8 (2.8)	2.8 (3.8)	1.5 (0.9)	2.5 (1.3)
Lys	44.6 (4.3)	1.3 (1.6)	1.6 (1.8)	1.3 (2.0)	1.8 (2.4)	1.0 (1.0)	1.5 (0.8)
sum	1048 (100)	80.0 (100)	87.9 (100)	63.5 (100)	73.3 (100)	167 (100)	186 (100)

[a] The value in bracket is a percentage of total amino acid content. AA – amino acid. < LOD – below the detection limit.

For barley and buckwheat the relative assimilation of individual AA correlated with the initial concentration in wort (Pearson's $r = 0.97$ ($p = 0.0013$) and 0.99 ($p = 0.003$)) which was different for quinoa (Pearson's $r = 0.40$ ($p = 0.023$)). For instance, in quinoa wort fermentation the most preferred AA lysine contributed as much as 25% to the assimilated AAs, although it was only the sixth most frequent AA in wort and alanine being the most abundant (after proline) was obviously not assimilated at all.

Table 18: The amino acid content of the quinoa wort and in fresh and forced-aged beverages fermented with different yeast
Preglednica 18: Vsebnost aminokislin v kvinojini pivini ter v kvinojinih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

amino acid	quinoa [mg/L][a]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
Asp	83.1 (5.7)	94.3 (7.9)	88.8 (7.6)	90.0 (7.1)	87.1 (7.3)	95.8 (8.2)	94.1 (8.3)
Glu	71.6 (4.9)	88.0 (7.4)	88.5 (7.5)	82.9 (6.6)	81.5 (6.9)	104 (8.9)	103 (9.0)
Asn	65.9 (4.5)	21.3 (1.8)	16.3 (1.4)	22.3 (1.8)	21.4 (1.8)	22.4 (1.9)	19.0 (1.7)
Ser	66.8 (4.5)	20.4 (1.7)	25.1 (2.1)	23.6 (1.9)	22.9 (1.9)	33.4 (2.9)	35.6 (3.1)
Gln	23.3 (1.6)	22.3 (1.9)	19.4 (1.7)	23.0 (1.8)	19.4 (1.6)	20.0 (1.7)	17.1 (1.5)
His	53.7 (3.7)	59.0 (4.9)	55.3 (4.7)	59.9 (4.7)	59.9 (5.0)	59.3 (5.1)	59.4 (5.2)
Gly	30.8 (2.1)	29.2 (2.5)	33.4 (2.8)	31.5 (2.5)	30.0 (2.5)	29.1 (2.5)	29.6 (2.6)
Thr	59.3 (4.0)	23.8 (2.0)	26.8 (2.3)	23.5 (1.9)	21.3 (1.8)	37.0 (3.2)	36.5 (3.2)
Ala	171 (11.6)	185 (15.5)	182 (15.5)	186 (14.8)	180 (15.2)	176 (15.1)	169 (14.9)
Arg	115 (7.8)	106 (8.9)	81.3 (6.9)	118 (9.3)	94.6 (8.0)	107 (9.2)	93.6 (8.2)
Tyr	107 (7.3)	100 (8.4)	106 (9.0)	112 (8.8)	108 (9.1)	97.3 (8.4)	92.8 (8.2)
Val	111 (7.5)	102 (8.6)	103 (8.7)	109 (8.6)	104 (8.8)	88.6 (7.6)	89.9 (7.9)
Met	46.8 (3.2)	38.1 (3.2)	41.3 (3.5)	40.9 (3.2)	39.4 (3.3)	35.3 (3.0)	33.1 (2.9)
Trp	33.4 (2.3)	23.5 (2.0)	19.9 (1.7)	28.4 (2.2)	26.0 (2.2)	24.3 (2.1)	24.1 (2.1)
Phe	103 (7.0)	90.3 (7.6)	88.3 (7.5)	102 (8.1)	95.6 (8.1)	75.4 (6.5)	72.6 (6.4)
Ile	69.6 (4.7)	56.6 (4.8)	62.1 (5.3)	61.8 (4.9)	61.6 (5.2)	49.0 (4.2)	51.5 (4.5)
Leu	154 (10.5)	99.1 (8.3)	105 (8.9)	111 (8.8)	108.1 (9.1)	83.5 (7.2)	85.1 (7.5)
Lys	105 (7.1)	31.5 (2.6)	31.1 (2.7)	37.6 (3.0)	26.1 (2.2)	26.9 (2.3)	29.8 (2.6)
sum	1469 (100)	1191 (100)	1172 (100)	1263 (100)	1187 (100)	1164 (100)	1136 (100)

[a] The value in bracket is a percentage of total amino acid content. AA – amino acid.

TUM 177 and TUM SL17

In general, compared to TUM 34/70 no drastical differences in AA assimilation were observed in the case of TUM 177 and TUM SL17, especially in the quinoa wort fermentation (Table 18) where the AA uptake was absolutely yeast strain-independent. In barley, TUM 177 assimilated all AAs more efficiently, especially Gly, Ala, Arg, Tyr, Trp and Phe. Oppositely, TUM SL17 left all amino acids in excess which is in concordance with higher TSN and FAN values and lower fermentability (Table 9). It assimilated only around a half or less of Asn, Ser, Arg, Val, Met, Phe, Ile, Leu and Lys but not a bit of others. In buckwheat, the AA assimilation by TUM 177 was mainly similar to TUM 34/70 with a bit better final uptake of His, Gly and Ala whereas TUM SL17 differed mainly in two cases. This yeast left around a quarter of initial Arg unassimilated and Trp concentration was even increased by two thirds compared to wort.

4.1.5 Volatile compounds

Figure 6 shows the GC-FID chromatogram of the quinoa beverage fermented with TUM 34/70. Table 19, Table 20 and Table 21 show the concentrations of nine aroma compounds found in the barley, buckwheat, and quinoa wort and beverage samples together with their detection threshold values reported by other researches (Vanderhaegen *et al*., 3003; Procopio *et al*., 2011). Five different higher alcohols were examined and only four distinct chromatographic peaks were differentiated because 2-methylbutanol and 3-methylbutanol coeluted (Figure 6). For the same reason, the information regarding threshold values of 2- and 3-methylbutanol is not relevant. With the exception of methanol they are all fermentation products of yeast thus normally not present in wort. However, some of them were also found in the buckwheat and quinoa wort although their concentrations were very low. Sum concentrations are the highest in buckwheat beverages fermented with TUM 34/70 and TUM 177 ant the lowest in the barley beverage fermented with TUM SL17. The latter was fully expected because of the integrally lower fermentation capacity of the barley wort by this yeast. With the exception of methanol, the levels of other volatile compounds were drastically reduced and also below the usual values in beer thus this beverage was excluded from the further discussion. However, in buckwheat TUM SL17 produced only one third less of the examined volatile compounds in sum whereas in quinoa their sum value was in the range of TUM 34/70. The amounts of a particular volatile compound have generally changed only a little or not at all during forced-aging.

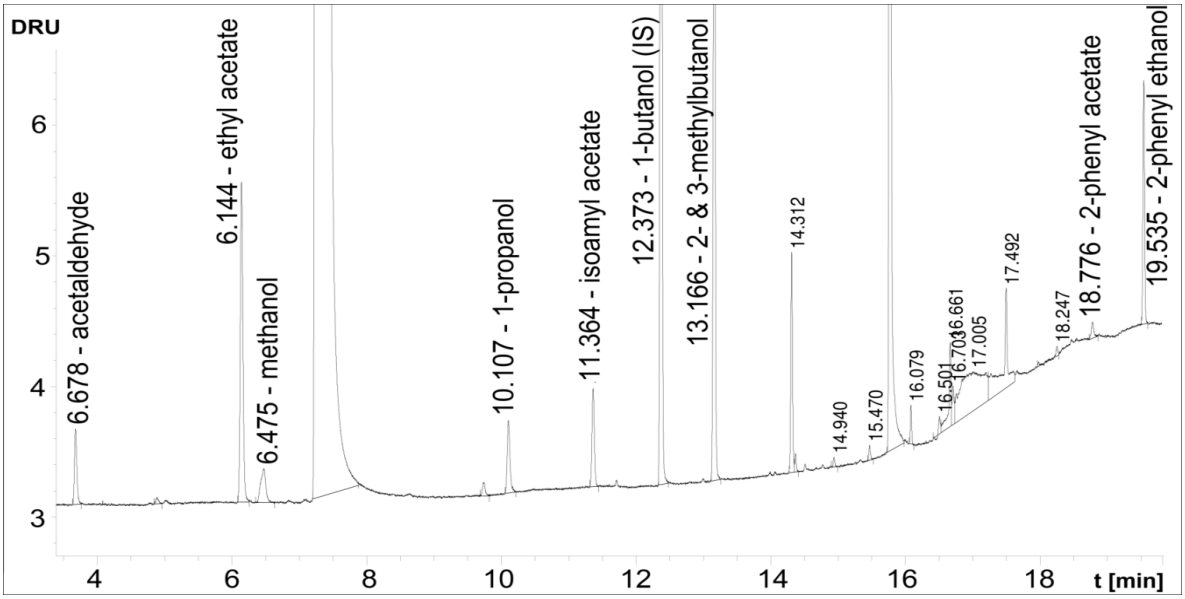

Figure 6: The GC-FID chromatogram of volatile compounds in the quinoa beverage fermented with *S. pastorianus* TUM 34/70

The concentrations of compounds in samples were calculated from the calibration curve constructed using an external standard solution. The internal standard served only as an indicator of the distillation recovery. IS – internal standard. DRU – detector response unit.

Slika 6: GC-FID kromatogram hlapnih spojin v kvinojini pijači, fermentirani s *S. pastorianus* TUM 34/70

Koncentracije hlapnih spojin v vzorcih so bile izračunane s pomočjo kalibracijske krivulje, narejene na osnovi standardnih raztopin različnih koncentracij. Interni standard je služil za izračun izkoristka destilacije. IS – interni standard. DRU – enota odziva detektorja.

Methanol: Methanol is generally undesired in alcoholic beverages due to its toxic effects above a reference dose of 0.5 mg/kg per day (Methanol …, 2014). However, it is practically impossible to suppress its formation during the alcoholic fermentation, thus its concentration in beer normally varies between 0.5-3.0 mg/L (Baxter and Hughes, 2001). The value in the barley beverages was in agreement with this value range (Table 19) whereas buckwheat (Table 20) and quinoa (Table 21) exceed the upper limit two- to four-fold. Interestingly, it seems that the majority of methanol present in beverages originated from wort and only a little increase happened during fermentation. Its emergence during wort boiling is the most reasonable explanation since it would evaporate during this process if its formation would took place in earlier stages. Anyway, regarding an average consumer (~70 kg), even with these relatively high levels of methanol in buckwheat and quinoa beverages, it is possible to consume up to six and ten drinks of 0.5 L, respectively, with absolutely no health concerns (Methanol …, 2014).

1-propanol, 2- and 3-methylbutanol: The content of 1-propanol in our samples differed significantly, being the highest in barley (Table 19) and the lowest in the quinoa (Table 21) beverages fermented with TUM 34/70 and TUM 177. These differences could be fully explained by the fact that 1-propanol is formed exclusively via the anabolic route, since there is no corresponding AA precursor (Arendt and Dal Bello, 2008). Indeed, the higher the FAN value of a wort, the lower the 1-propanol concentration in beverage (and *vice versa*). Its levels in beverages fermented with TUM SL17 were in the range of those fermented with TUM 34/70. Regardless the differences, all values were within the range (3-16 mg/L) thought to be typical for beer (Baxter and Hughes, 2001). The content of 2- and 3-methylbutanol also differed significantly being the highest in buckwheat and the lowest in quinoa beverage, although the values are reported to be normal for beer (40-100 mg/L) (Baxter and Hughes, 2001). 2- and 3-methylbutanol are formed from distinctive 2-oxo acids, which are metabolic intermediates of isoleucine and leucine, respectively (Fontana and Buiatti, 2009). Similarly to 1-propanol, it seems that these two alcohols were also formed predominantly via anabolic route, since the higher the sum of leucine and isoleucine concentration in wort, the lower the 2- and 3-methylbutanol concentration in beverages (and *vice versa*). They both have practically the same qualitative flavour impact, described as malty or solvent-like (Briggs *et al*., 2004). Compared to TUM 34/70, their levels were a bit higher in TUM 177 regardless wort type. On the contrary, TUM SL17 showed wort type-dependent production of 2- and 3-methylbutanol. In particular, their concentrations in buckwheat were around two-fold lower but in quinoa little higher compared to other two yeasts.

Table 19: Selected volatile compounds in the barley wort and in fresh and forced-aged beverages fermented with different yeast
Preglednica 19: Vsebnost izbranih hlapnih spojin v ječmenovi pivini ter v ječmenovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

volatile substance[a]	barley [mg/L]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
acetaldehyde (25)[b]	0.00	21.98	24.54	24.32	24.55	19.84	14.82
ethyl acetate (20)[c]	0.00	23.10	26.38	16.24	16.22	0.61	0.74
methanol	1.21	1.12	1.21	1.37	1.21	1.24	1.35
1-propanol (21)[c]	0.00	12.44	12.92	17.72	17.55	2.56	3.08
isoamyl acetate (1.0)[c]	0.00	1.39	1.66	1.05	1.01	0.00	0.00
2-&3-methylbutanol	0.00	55.22	58.09	62.10	61.76	18.73	23.26
2-phenylethyl acetate(3.0)[c]	0.00	3.03	1.66	2.58	2.13	0.47	0.95
2-phenylethanol (100)[c]	0.00	27.93	17.45	21.38	19.50	10.91	10.19
sum	1.21	146.21	143.91	146.76	143.93	54.36	54.39

[a] Values in brackets express flavour detection threshold [mg/L] reported by [b] Vanderhaegen *et al*. (2003) and [c] Procopio *et al*. (2011).

2-phenylethanol: 2-phenylethanol in beer is normally found in the concentrations from 25 to 32 mg/L (Briggs *et al*., 2004) and its distinctive flavour impact was usually described as flowery or honey-like. Both barley and buckwheat beverages fermented with TUM 34/70 and TUM 177 had comparable amounts of 2-phenylethanol with the values being near the bottom limit of the above mentioned range considered normal for beer. Contrary to 2- and 3-methylbutanol, its levels in TUM 177 were slightly lower than in TUM 34/70 regardless the wort type but again, TUM SL17 showed wort type-dependent production. In buckwheat it produced similar amount of 2-phenylethanol than in barley although fermentation performances differed drastically whereas in quinoa its concentration was two-fold higher, much higher than concentrations produced by other two yeasts. The amounts in the quinoa beverage were in general more than two-fold lower. It was very likely that this low content of 2-phenylethanol in the quinoa beverage impaired the organoleptic perception not only directly but also indirectly since it was found that 2-phenylethanol can suppress the flavour intensity of dimethyl sulphide (Hegarty *et al*., 1995).

Esters in general have fruity/solvent-like aromas notes. Their formation is mainly yeast strain dependent although the particular composition of wort has a noticeable influence. Three major esters were examined (Table 19, Table 20 and Table 21) and in general, barley and buckwheat had similar values of all of them, whereas the quinoa beverage differed significantly. In general, TUM 177 produced a little less esters compared to TUM 34/70 and TUM SL17 produced around two times less of them. Moreover, the concentrations of esters in forced-aged beverages were slightly higher, with the only

exception of 2-phenylethyl acetate in barley. This is not uncommon; some increase in the concentration of ethyl esters takes place also during the beer shelf life (Fix, 2000).

Table 20: Selected volatile compounds in the buckwheat wort and in fresh and forced-aged beverages fermented with different yeast
Preglednica 20: Vsebnost izbranih hlapnih spojin v ajdovi pivini ter v ajdovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

volatile substance[a]	buckwheat [mg/L]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
acetaldehyde (25)[b]	0.00	23.80	23.85	28.56	25.75	21.78	21.86
ethyl acetate (20)[c]	0.00	20.14	21.36	18.99	19.51	12.93	13.27
methanol	8.14	11.66	10.98	18.36	16.64	15.14	14.81
1-propanol (21)[c]	0.92	8.78	9.03	11.73	11.27	9.99	9.95
isoamyl acetate (1.0)[c]	0.00	1.38	1.60	1.22	1.39	0.50	0.53
2-&3-methylbutanol	0.00	70.80	71.71	82.43	77.95	44.84	46.10
2-phenylethyl acetate(3.0)[c]	0.48	2.65	2.78	2.12	2.12	1.24	1.47
2-phenylethanol (100)[c]	0.38	25.13	20.31	19.83	20.38	10.42	10.23
sum	9.92	164.34	161.62	183.24	175.01	116.84	118.22

[a] Values in brackets express flavour detection threshold [mg/L] reported by [b] Vanderhaegen *et al*. (2003) and [c] Procopio *et al*. (2011).

Ethyl acetate, iso-amyl acetate: The amounts of ethyl acetate (which gives a sweet, solvent like flavour to beverage) in barley and buckwheat beverages fermented with TUM 34/70 and TUM 177 were on the upper limit of the interval thought to be usual for lager beer (10-20 mg/L) (Briggs *et al*., 2004). On the other side, the TUM 34/70 quinoa beverage had twice as much of this ester but the TUM 177 had twice as less of it. At least in part, this observation was probably related to the high level of glucose since an increase in ethyl acetate is caused by wort with a high sugar concentration (Walker, 2004). The amount of iso-amyl acetate in beer was relatively low with the normal concentrations being from 0.5-3 mg/L (Briggs *et al*., 2004). On the other hand, it has a relatively low threshold and it is responsible for the fruity, banana-like aroma note in beer. In our case, its concentrations were under the organoleptically detectable limit in all quinoa beverages as well as in all beverages fermented with TUM SL17.

Table 21: Selected volatile compounds in the quinoa wort and in fresh and forced-aged beverages fermented with different yeast
Preglednica 21: Vsebnost izbranih hlapnih spojin v kvinojini pivini ter v kvinojinih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

volatile substance[a]	quinoa [mg/L]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
acetaldehyde (25)[b]	3.78	38.38	37.05	38.28	35.13	36.80	37.23
ethyl acetate (20)[c]	0.00	43.67	42.91	8.38	7.47	25.85	25.67
methanol	4.53	6.70	6.34	10.55	10.48	14.30	14.22
1-propanol (21)[c]	0.47	5.28	5.06	8.45	8.53	5.53	5.45
isoamyl acetate (1.0)[c]	0.00	0.30	0.30	0.31	0.34	0.12	0.13
2-&3-methylbutanol	0.00	44.99	43.94	49.85	44.10	58.37	58.86
2-phenylethyl acetate(3.0)[c]	0.73	1.47	2.01	1.28	1.63	0.59	0.65
2-phenylethanol (100)[c]	0.34	12.79	10.68	5.97	5.72	18.72	18.64
sum	9.85	153.58	148.29	123.07	113.4	160.28	160.85

[a] Values in brackets express flavour detection threshold [mg/L] reported by [b] Vanderhaegen *et al*. (2003) and [c] Procopio *et al*. (2011).

2-phenylethyl acetate, acetaldehyde: Because of the flowery or honey-like note of 2-phenylethyl acetate, its presence in fermented beverages is desired. It is usually found in beer even in lower concentrations than iso-amyl acetate (0.05-2 mg/L) (Briggs *et al*., 2004) whereas its threshold concentration is around three times higher. All values of our samples were within the normal range or exceeded it but only barley and buckwheat beverages fermented with TUM 34/70 and TUM 177 had the levels of 2-phenylethyl acetate close to the threshold. In quinoa beverages, its concentrations were around two times lower than in corresponding beverages from the other two worts. Acetaldehyde is a major aldehyde in beer with the normal concentrations ranging from 8 to 10 mg/L (Eßlinger, 2009). It is usually reported together with higher alcohols and esters because of its high volatility and possible critical influence on the overall perception of a beverage. The most important observation regarding acetaldehyde content is that its concentrations were truly yeast strain-independent and exclusively wort type-dependent. However, the buckwheat beverages contained only a shade more of acetaldehyde than the barley ones but in the quinoa beverages its concentrations were almost twice as high. Acetaldehyde concentrations in barley and buckwheat beverages were close to the flavour threshold whereas only in quinoa beverages they exceeded it. Regarding the latter, it is possible that the maturation process used has been inadequate since our concentrations were within the range of the young beer, i.e. in the young beer phase, the acetaldehyde content is from 20 to 40 mg/L (Eßlinger, 2009).

Pyrazines, pyridinamines, furans: In the quinoa wort and all beverages some chromatographic peaks were observed during the analysis of volatile compounds (Figure

7) which could not be identified using the usual set of standards. The GC-MS analysis revealed that these "tentative" compounds most likely belong mainly to three groups of chemical compounds, i.e. pyrazines, pyridinamines, and furans (Table 22). However, even using MS detector there were many peaks left unidentified in quinoa wort.

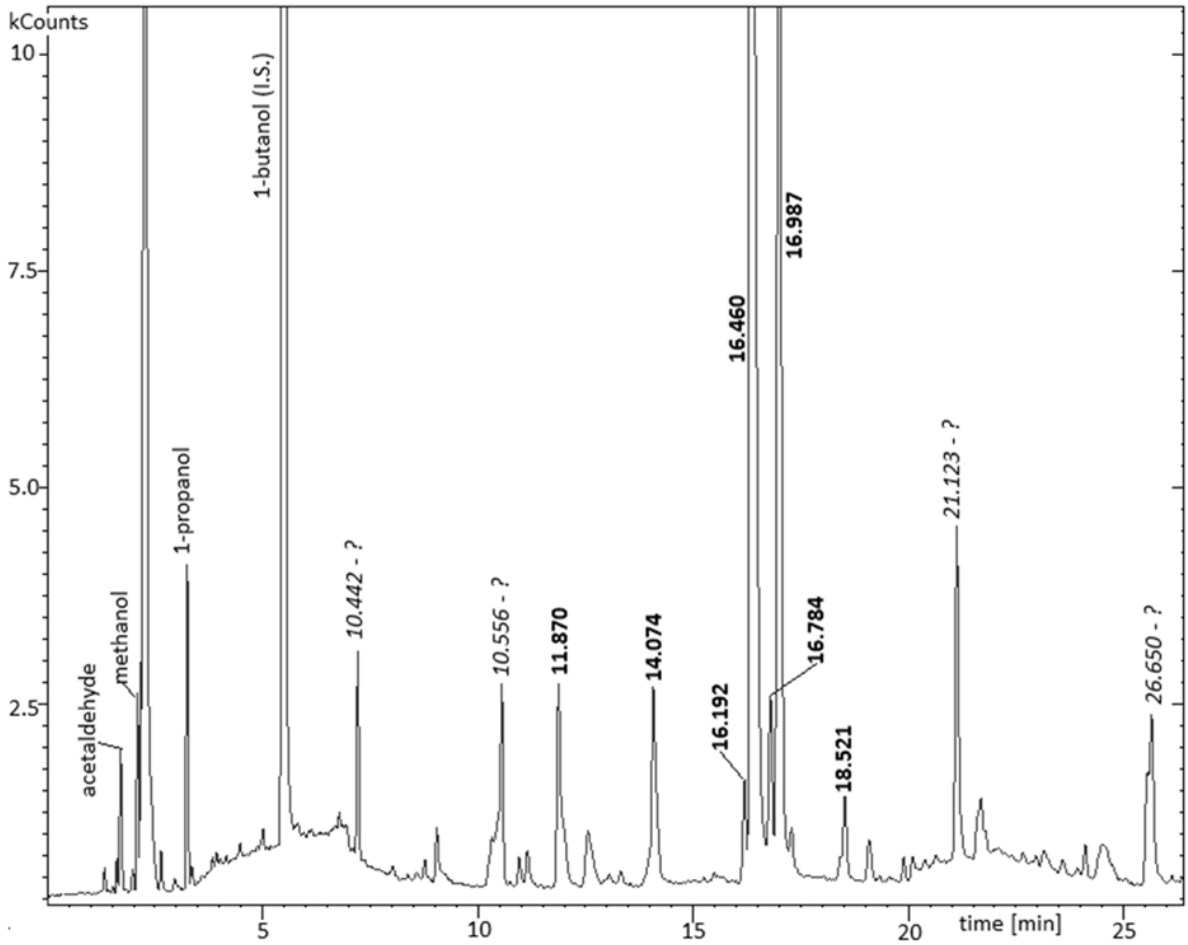

Figure 7: The GC-MS chromatogram of volatile compounds in the quinoa beverage fermented with *S. pastorianus* TUM 34/70
Retention times above peaks in bold represents those identified using NIST Libraries for Spectrum Hit List and retention times above peaks in italics followed by a question mark represent those that gave no results. Compounds identified using standard solutions are also shown.
Slika 7: GC-MS kromatogram hlapnih spojin v kvinojini pijači, fermentirani s *S. pastorianus* TUM 34/70
Retenzijski časi, napisani s krepko pisavo predstavljajo vrhove, ki so bili identificirani s pomočjo baze podatkov 'NIST Libraries for Spectrum Hit List' in tisti, napisani s poševno pisavo s sledečim vprašajem, predstavljajo vrhove katerih identitete ni bilo moč ugotoviti. Spojine, določene s standardnimi raztopinami so tudi prikazane.

Pyrazines generally contain nutty, roasted aromas and these unique sensory properties are important for food industries. According to the mass specter of the quinoa wort the presence of 2,3-dimethylpyrazine and tetramethylpyrazine has been confirmed with a high probability together with some other less probable derivatives (Table 22). In beverage, the later were not identified but 2,3-dimethylpyrazine and tetramethylpyrazine were detected even with a higher probability. Their formation most probably occurred from amino acid

and vicinal diketone since a recent publication reported the unexpected formation of 2,3-dimethylpyrazine and 2,3,5-trimethylpyrazine in addition to the expected tetramethylpyrazine in model system composed of glycine and 2,3-butanedione (Guerra and Yaylayan, 2012). Nevertheless, pyrazines in brewing are not yet so uncommon but they were found in trace amounts only in a roasted barley malt and in wort and beer derived from it (Briggs *et al*., 2004).

Table 22: Volatile compounds of wort and fresh beverage quinoa separated by GC, detected by MS, and identified by NIST Libraries for Spectrum Hit List
Preglednica 22: Hlapne spojine v kvinojini in ajdovi pivini, ločene z GC, detektirane z MS in identificirane s pomočjo knjižnice NIST Libraries for Spectrum Hit List

sample	RT [min]	compound	probability	No. of entries	CAS No.
wort	11.87	2,3-dimethyl pyrazine	77.79	4	5901-89-4
	14.07	2,6-dimethyl-4-pyridinamine	17.85	2	3512-80-9
		2,6-dimethyl-3-pyridinamine	13.32	1	3430-33-9
		2,5-dimethyl-3-(2-methylbutyl) pyrazine	12.81	1	72668-36-1
		2-butyl-3,5-dimethyl pyrazine	10.06	2	50888-63-6
	16.19	2-ethyl-3,5-dimethyl pyrazine	29.05	2	13925-07-0
		5-ethyl-2,3-dimethyl pyrazine	21.09	1	15707-34-3
		2,6-diethyl pyrazine	17.81	1	13067-27-1
		3-ethyl-2,5-dimethyl pyrazine	11.51	5	13360-65-1
	16.46	N-N-dimethyl-O-(1-methylbutyl) hydroxylamine	71.95	1	/
	16.78	3-furaldehyde	71.53	1	498-60-2
		2-furaldehyde	23.97	4	98-01-1
	16.98	tetramethyl pyrazine	65.90	4	1124-11-4
		1,7-dihydro-6H-purin-6-one	14.78	2	68-94-0
	18.52	1-(2-furanyl) ethanone	68.02	4	1192-62-7
		1-(3H-imidazol-4-yl) ethanone	13.19	1	/
		2-ethyl-5-methyl furan	10.37	2	1703-52-2
beverage	11.97	2,3-dimethyl pyrazine	84.65	4	5901-89-4
	14.15	2,6-dimethyl-3-pyridinamine	25.94	1	3430-33-9
		2,6-dimethyl-4-pyridinamine	14.15	1	3512-80-9
		2,5-dimethyl-3-(2-methylbutyl) pyrazine	10.56	1	72668-36-1
	17.01	tetramethyl pyrazine	85.04	4	1124-11-4
		1,7-dihydro-6H-purin-6-one	3.95	2	68-94-0
		3-furan methanol	54.55	2	4412-91-3
	25.54	2-furan methanol	28.13	3	98-00-0
		1,3-butadiene-1-carboxylic acid	5.99	1	626-99-3

Pyridinamines are derivatives of one of the three isomeric amines of pyridine. It seems that two of them, namely 2,6-dimethylpyridine-3-amine (syn. 3-amine-2,6-lutidine) and 2,6-dimethylpyridine-4-amine (syn. 4-amine-2,6-lutidine), are present in quinoa wort and beverage (Table 22). Pyridine and its derivatives might be formed from alkaloids and proteins as has been reported for green tea leafs (Sakasegawa and Yatagai, 2005). 2,6-

lutidine itself has been evaluated for use as a food additive owing to its nutty aroma when present in solution at very low concentrations (Sims and O'Loughlin, 1989).

Furans are derivatives of furan and can be found in heat-treated commercial foods due to the production through thermal degradation of natural food constituents (EFSA, 2011). Among furan derivatives the most common flavor contributors in beer are furfural, 5-methylfurfural, and 5-hydroxymethylfurfural (Hughes, 2008). As opposed to pyrazines and pyridinamines different species of furans were found in wort and beverage (Table 22). In wort, 2-furaldehyde (furfural) (and/or possibly 3-furaldehyde) and 1-(2-furanyl)-ethanone (acetylfuran) were detected. Furfural is a heterocyclic aldehyde with the odor of almonds. Aroma of furfural is described as papery/husky thus not very desired in foods and drinks; however, its relatively high threshold limit (200 mg/L) makes it tolerable to some extent (Hughes, 2008). The beverage obviously contain 3-hydroxymethylfuran (3-furan methanol) which is most likely the reduction product of 3-furaldehyde.

4.1.6 Fatty acids

We decided to use the 9-chloromathylanthracene as a derivatization reagent for fatty acids (FAs) since it easily reacts with a carboxyl group to form the corresponding ester in the presence of a base (such as tetramethylammonium hydroxide). The resultant ester is stable enough to reach the detector without any decomposition under the reverse phase HPLC. It was reported that a fluorescence detection method offers the detection limit of 2 fmol at the excitation and emission limits of 365 and 412 nm, respectively (Korte, 1982).

4.1.6.1 Method optimization and validation

The stock mixture of individual fatty acid (FA) standard solutions (~1 g/L) in their equal volume ratios (1/20) was prepared. The resulting 'stock mixture' was diluted 1:100 and 1.4 mL of it was used for the derivatization with different volumes of 9-CMA and TMAH stock solutions. DMF was added to the final volume of 2 mL and the reaction mixtures were allowed to derivatize at 70°C for 30 min with different amounts of 9-CMA (37.5 mM) and TMAH (30.0 mM) stock solutions. The excitation and emission wavelengths of the FLD detector were set to 356 and 420 nm, respectively. The maximal signal (Table 23, results in bold italic numbers) was achieved at 200 µL of both 9-CMA and TMAH giving their final concentrations of 3.75 and 3.9 mM, respectively.

Table 23: The HPLC-FLD peak areas of fatty acid derivatized with different amounts of 9-CMA and TMAH
Preglednica 23: Površine HPLC-FLD vrhov maščobnih kislin pri derivatizacijah z različnimi količinami 9-CMA in TMAH

reagent	**µL of stock solution**									
9-CMA	150			***200***				250		
TMAH	200	250	300	150	***200***	250	300	200	250	300
FA (~350 µg/L)	**peak area**									
Hex	3169.5	1939.1	1003.0	2080.2	***3854.9***	2790.6	1502.9	1985.3	3558.3	2469.8
Oct	3203.9	1963.6	1008.2	2048.6	***3865.3***	2806.6	1509.2	1949.2	3521.5	2475.2
Dec	2798.1	1718.3	881.2	1760.2	***3376.2***	2454.8	1322.2	1670.7	3046.6	2129.1
Lau	1766.9	1080.5	554.5	1144.1	***2133.5***	1550.9	833.4	1087.9	1984.2	1371.9
α-&γ-Lin	2555.7	1570.0	804.0	1641.3	***3076.3***	2247.6	1206.8	1553.7	2803.9	1947.8
Ara-4	883.5	540.4	273.5	588.4	***1075.3***	781.4	415.5	559.5	938.7	641.6
Myr	1346.8	829.3	424.1	841.9	***1629.6***	1191.3	640.3	793.9	1484.1	1031.7
Lin	1940.7	1169.1	601.7	1193.8	***2285.9***	1673.1	907.1	1138.1	2065.9	1439.1
Pal + Ole	3321.4	2048.8	1066.1	2098.3	***3991.4***	2933.9	1596.0	2395.7	3985.7	3067.7
Ste	1009.2	628.1	323.1	654.2	***1232.6***	906.3	507.4	620.5	1150.5	803.3
Eic	1040.4	634.0	329.0	597.4	***1238.0***	914.9	495.1	569.1	1068.7	758.6
Ara-0	1590.0	981.0	515.3	965.9	***1922.9***	1395.7	757.2	910.9	1710.4	1200.2
Eru	1124.6	695.8	362.4	666.6	***1360.8***	986.2	533.6	618.4	1198.0	878.5
Beh	1199.8	739.9	375.2	709.6	***1441.9***	1052.1	568.8	664.2	1284.2	911.9
Ner	794.2	479.6	240.8	421.5	***941.2***	688.0	366.0	385.9	754.9	580.7
Lig	414.8	40.8	16.9	260.2	***380.3***	275.9	135.7	160.6	300.6	200.0
sum	28159.4	17058.2	8779.0	17672.4	***33806.2***	24649.2	13297.2	17063.7	30856.2	21907.2

FA – fatty acid. Hex – hexanoic acid. Oct – octanoic acid. Dec – decanoic acid. Lau- lauric acid. α-Lin – α-linolenic acid. γ-Lin – γ-linolenic acid. Ara-4 – arachidonic acid. Myr – myristic acid. Lin – linoleic acid. Pal – palmitic acid. Ole – oleic acid. Ste – stearic acid. Eic – eicosanoic acid. Ara-0 – arachidic acid. Eru – erucic acid. Beh – behenic acid. Ner – nervonic acid. Lig – lignoceric acid.

Different derivatization time and temperatures were explored wherein all other reaction parameters were the same as mentioned above. Since a few combinations gave similar results (Figure 8) we made a compromise between high signal, short derivatization time and low derivatization temperature and decided to derivatize at 75°C for 35 min in all further experiments.

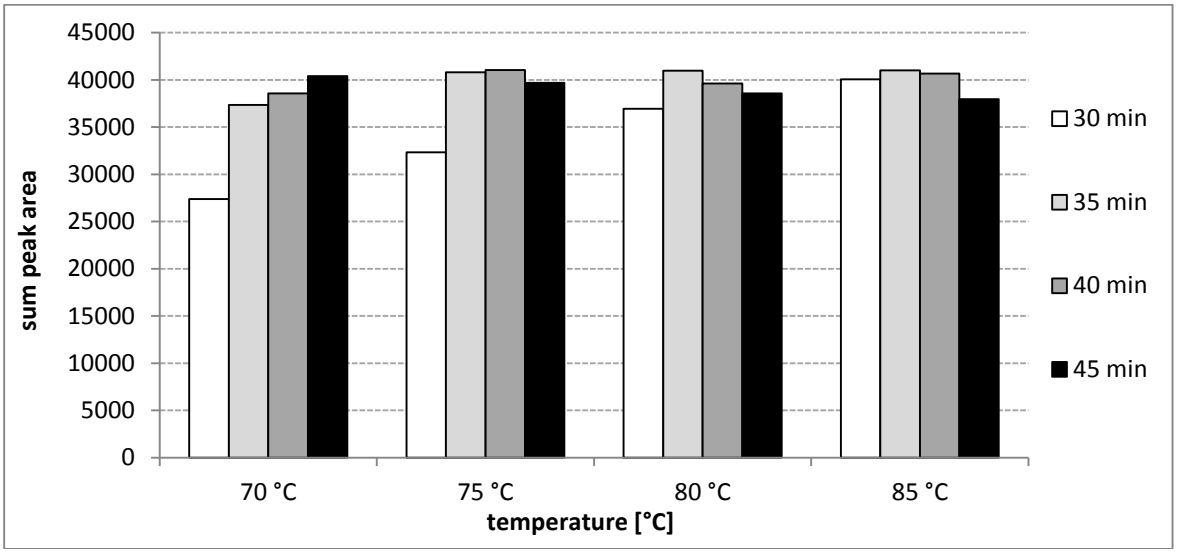

Figure 8: The influence of the derivatization time and temperature on the sum peak area of all fatty acids.
Slika 8: Vpliv temperature in časa derivatizacije na vsoto površine vrhov vseh maščobnih kislin

Combinations of different excitation and emission wavelengths were explored with the use of above-established optimal derivatization time and temperature together with above-mentioned reaction parameters. From Figure 9 it can be easily concluded that the best results were obtained with the excitation and emission wavelengths of 365 and 412 nm, respectively thus this combination was used in all further experiments.

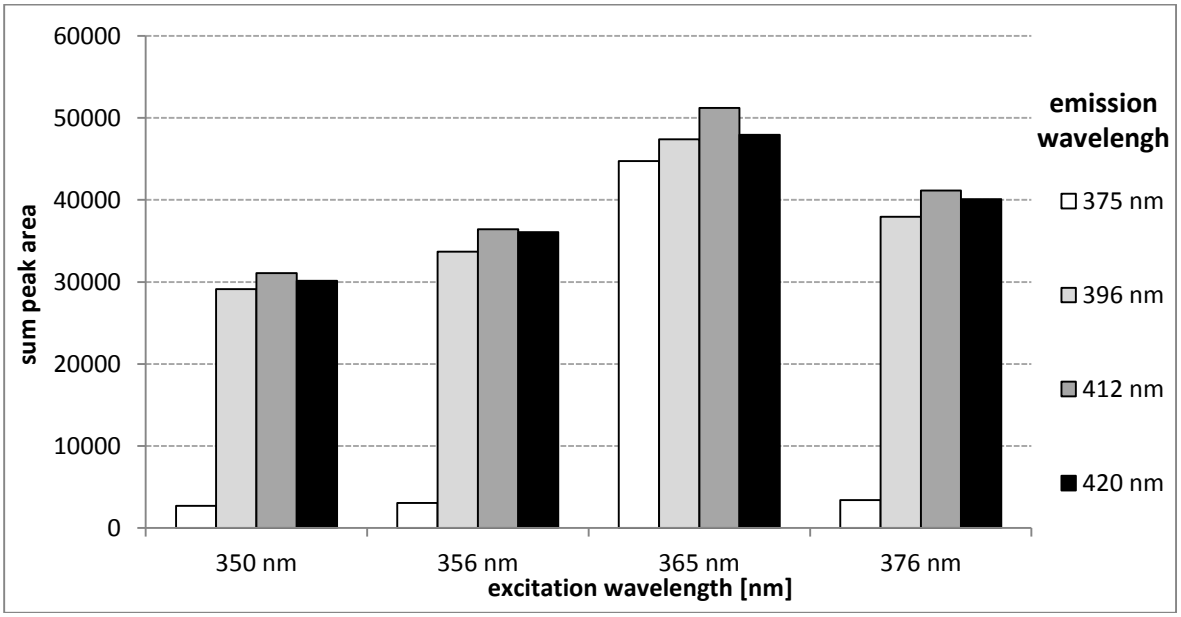

Figure 9: The influence of the fluorescence detector excitation and emission wavelenghts on the sum peak area
Slika 9: Vpliv vzbujevalnih in sevalnih valovnih dolžin detektorja na vsoto površine vrhov vseh maščobnih kislin

When all the relevant parameters were optimized, a special attention was given to assure optimal chromatographic conditions which would enable maximal selectivity, repetability

and sensitivity. However, two the so-called "critical pairs" still coeluted (Figure 10). The retention times and their relative standard deviations of separated chromatographic peaks (Table 24) enabled the appropriate selectivity of analysis.

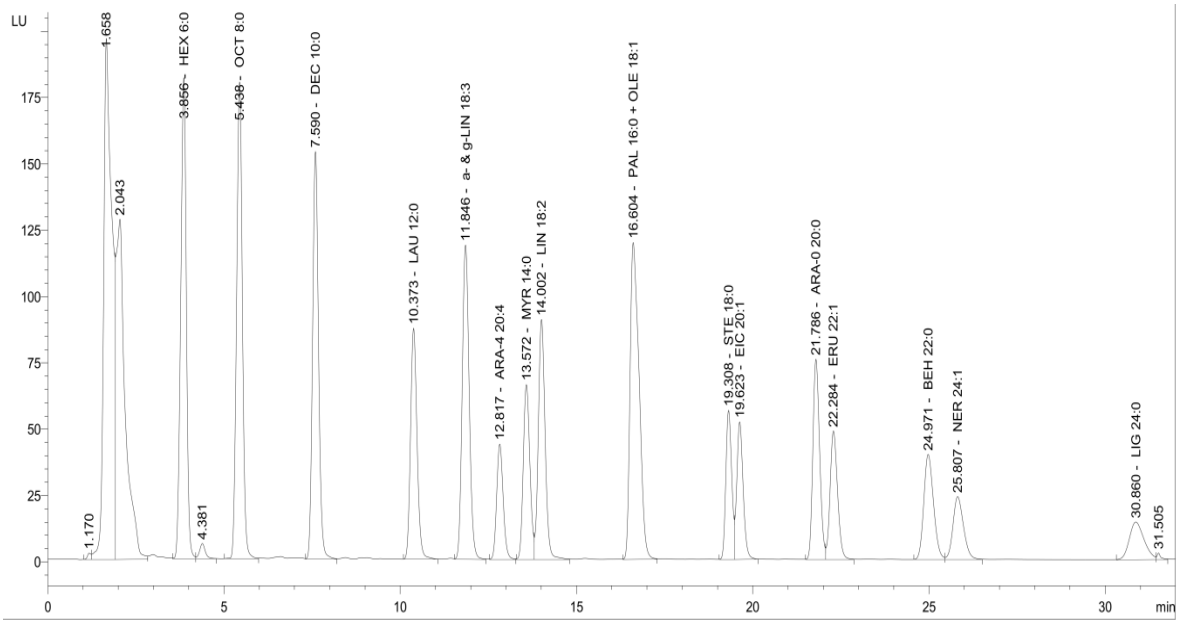

Figure 10: The sample HPLC-FLD chromatogram of fatty acid standard solution
The final concentration of each fatty acid was ~350 µg/L. Hex – hexanoic acid. Oct – octanoic acid. Dec – decanoic acid. Lau- lauric acid. a-Lin – α-linolenic acid. g-Lin – γ-linolenic acid. Ara-4 – arachidonic acid. Myr – myristic acid. Lin – linoleic acid. Pal – palmitic acid. Ole – oleic acid. Ste – stearic acid. Eic – eicosanoic acid. Ara-0 – arachidic acid. Eru – erucic acid. Beh – behenic acid. Ner – nervonic acid. Lig – lignoceric acid.
Slika 10: Primer HPLC-FLD kromatograma standardne raztopine maščobnih kislin
Koncentracija posamezne maščobne kisline je bila ~350 µg/L. Hex – heksanojska kislina. Oct – oktanojska kislina. Dec – dekanojska kislina. Lau- lavrinska kislina. a-Lin – g-linolenska kislina. γ-Lin – γ-linolenska kislina. Ara-4 – arahidonska kislina. Myr – miristinska kislina. Lin – linoleinska kislina. Pal – palmitinska kislina. Ole – oleinska kislina. Ste – stearinska kislina. Eic – eikozanojska kislina. Ara-0 – arahidna kislina. Eru – erukinska kislina. Beh – behenska kislina. Ner – nervonska kislina. Lig – lignocerna kislina.

The solution of 3.75 mM 9-CMA and 3.0 mM TMAH gave no interfering chromatographic signal (Figure 11). Sample was prepared from the stock solutions of both reagents in their final concentrations of 3.75 and 3.0 mM, rescpectively. The chromatogram of 3.75 CMA alone did not differ significantly.

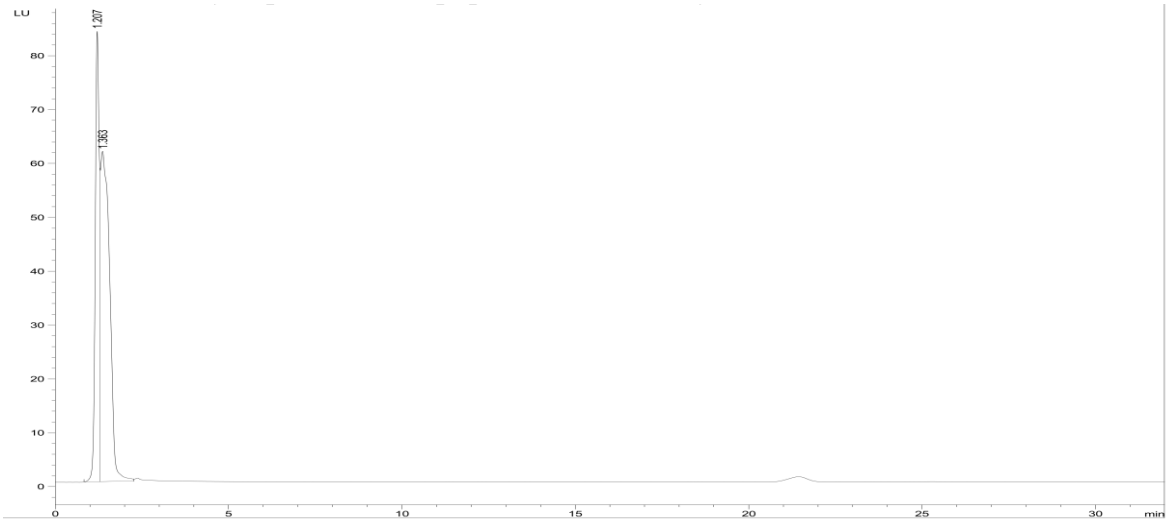

Figure 11: The HPLC-FLD chromatogram of 9-CMA and TMAH
Slika 11: HPLC-FLD kromatogram raztopine 9-CMA in TMAH

Table 24: The average retention time values and their RSD values for a particular HPLC-FLD chromatographic peak (n = 5)
Preglednica 24: Povprečne vrednosti retenzijskih časov posameznih HPLC-FLD vrhov in njihove RSD vrednosti (n = 5)

FA	average retention time [min]	retention time RSD [%]
Hex	3.853	1.44
Oct	5.432	0.93
Dec	7.594	1.22
Lau	10.372	1.42
α-&γ-Lin	11.847	1.41
Ara-4	12.811	1.23
Myr	13.570	2.09
Lin	14.003	1.39
Pal + Ole	16.604	0.98
Ste	19.296	1.64
Eic	19.625	0.87
Ara-0	21.784	1.56
Eru	22.288	1.28
Beh	24.974	1.19
Ner	25.807	1.35
Lig	30.857	1.98

FA – fatty acid. RSD – relative standard deviation. Hex – hexanoic acid. Oct – octanoic acid. Dec – decanoic acid. Lau- lauric acid. α-Lin – α-linolenic acid. γ-Lin – γ-linolenic acid. Ara-4 – arachidonic acid. Myr – myristic acid. Lin – linoleic acid. Pal – palmitic acid. Ole – oleic acid. Ste – stearic acid. Eic – eicosanoic acid. Ara-0 – arachidic acid. Eru – erucic acid. Beh – behenic acid. Ner – nervonic acid. Lig – lignoceric acid.

The linear ranges of HPLC-FLD chromatographic peaks were tested at concentrations of ~5, ~10, ~20, ~50, ~100, ~200, ~500, ~1000, ~2000 and ~5000 µg/L of each fatty acid.

The results in Table 25 were obtained from five independent SPE procedures and subsequent derivatizations and presented as the average values. Excelent linearity was achieved in concentration ranges from around 10 to around 2000 µg/L. The calibration curves (Table 25) were suitable for the quantification of fatty acid derivatives during the intra- and inter-day analysis.

Table 25: The linear ranges, their linear regressions and determination coefficient together with the limits of detection and lower limits of quantification of examined fatty acids for a particular HPLC-FLD chromatographic peak
Preglednica 25: Območje linearnosti, enačba linearne regresije in njen determinacijski koeficient skupaj z mejo detekcije in spodnjo mejo kvantifikacije maščobnih kislin za posamezen HPLC.FLD kromatografski vrh

FA	y = kx+ n	correlation	linear range [µg/L]		LOD	LLQ
Hex	y = 2.48x + 42.03	0.9952	9.27	2060	5.15	17.17
Oct	y = 1.85x + 27.86	0.9968	9.36	2080	5.20	17.33
Dec	y = 1.39x + 27.14	0.9911	9.00	2000	5.00	16.67
Lau	y = 1.35x + 13.30	0.9925	9.00	2000	5.00	16.67
α-Lin	y = 0.90x + 8.52	0.9973	8.91	1980	5.20	17.33
γ-Lin	y = 0.91x + 8.51	0.9962	9.45	2100	5.01	16.70
Ara-4	y = 0.43x + 3.47	0.9977	6.00	3335	3.34	11.13
Myr	y = 1.08x + 14.69	0.9959	9.63	2140	4.74	15.80
Lin	y = 0.96x + 11.42	0.9962	9.36	2080	5.55	18.50
Pal	y = 1.11x + 9.65	0.9946	8.91	1980	4.95	16.50
Ole	y = 0.96x + 16.10	0.9944	10.17	2260	5.65	18.83
Ste	y = 0.79 + 4.06	0.9976	9.63	2140	5.35	17.83
Eic	y = 1.13x + 8.95	0.9953	8.55	1900	4.75	15.83
Ara-0	y = 0.86x + 3.17	0.9979	9.18	2040	5.10	17.00
Eru	y = 0.87x + 3.94	0.9974	9.63	2140	5.35	17.83
Beh	y = 1.02x + 3.82	0.9978	9.09	2020	5.05	16.83
Ner	y = 0.84x + 3.67	0.9972	9.45	2100	5.25	17.50
Lig	y = 0.67x + 4.33	0.9964	10.62	2360	5.90	19.67

FA – fatty acid. LOD – limit of detection. LLQ – lower limit of quantification. Hex – hexanoic acid. Oct – octanoic acid. Dec – decanoic acid. Lau- lauric acid. α-Lin – α-linolenic acid. γ-Lin – γ-linolenic acid. Ara-4 – arachidonic acid. Myr – myristic acid. Lin – linoleic acid. Pal – palmitic acid. Ole – oleic acid. Ste – stearic acid. Eic – eicosanoic acid. Ara-0 – arachidic acid. Eru – erucic acid. Beh – behenic acid. Ner – nervonic acid. Lig – lignoceric acid.

The recovery and precision of the method were assessed by analyzing the low (20 µg/L), medium (200 µg/L) and high (200 µg/L) concentrations of fatty acids. Table 26 shows recovery and precision values for each concentration obtained by five independent analyses of the barley beverage. The values for buckwheat and quinoa samples did not differ significantly (data not shown).

Table 26: The SPE recoveries and RSD values of barley samples spiked with different concentrations of fatty acids (n = 7)
Preglednica 26: SPE izkoristek in RSD vrednosti za ječmenove vzorce z dodanimi različnimi koncentracijami maščobnih kislin (n = 7)

FA	recovery [%]			precision (RSD [%])		
	[20 µg/L]	[200 µg/L]	2000 [µg/L]	[20 µg/L]	[200 µg/L]	2000 [µg/L]
Hex	79.40	86.04	94.77	3.10	4.34	1.41
Oct	80.94	85.90	91.29	3.17	2.02	5.99
Dec	84.79	89.96	93.31	1.96	4.47	4.81
Lau	84.19	88.32	92.07	3.82	3.75	6.00
α-Lin	90.42	93.64	96.25	2.87	3.46	4.12
γ-Lin	92.19	94.35	97.09	2.95	3.30	3.65
Ara-4	78.56	87.51	103.56	3.33	4.23	3.54
Myr	87.76	94.31	95.27	1.38	2.02	2.90
Lin	95.87	96.86	96.36	3.63	3.66	1.09
Pal	83.13	88.25	92.69	2.35	1.42	1.34
Ole	88.91	93.88	97.92	2.32	2.58	1.10
Ste	84.65	90.34	92.77	4.47	3.07	0.84
Eic	86.34	90.46	99.75	2.34	1.07	1.19
Ara-0	92.09	94.21	102.42	2.44	3.35	0.64
Eru	85.05	89.12	104.34	3.54	1.94	1.75
Beh	83.46	89.75	102.72	5.93	5.55	1.58
Ner	89.31	90.61	103.42	5.20	3.43	0.97
Lig	85.53	86.18	101.84	3.04	4.07	2.60

FA – fatty acid. RSD – relative standard deviation. Hex – hexanoic acid. Oct – octanoic acid. Dec – decanoic acid. Lau- lauric acid. α-Lin – α-linolenic acid. γ-Lin – γ-linolenic acid. Ara-4 – arachidonic acid. Myr – myristic acid. Lin – linoleic acid. Pal – palmitic acid. Ole – oleic acid. Ste – stearic acid. Eic – eicosanoic acid. Ara-0 – arachidic acid. Eru – erucic acid. Beh – behenic acid. Ner – nervonic acid. Lig – lignoceric acid.

4.1.6.2 Application to real samples

The sample HPLC-FLD chromatogram of the quinoa wort is shown in Figure 12.

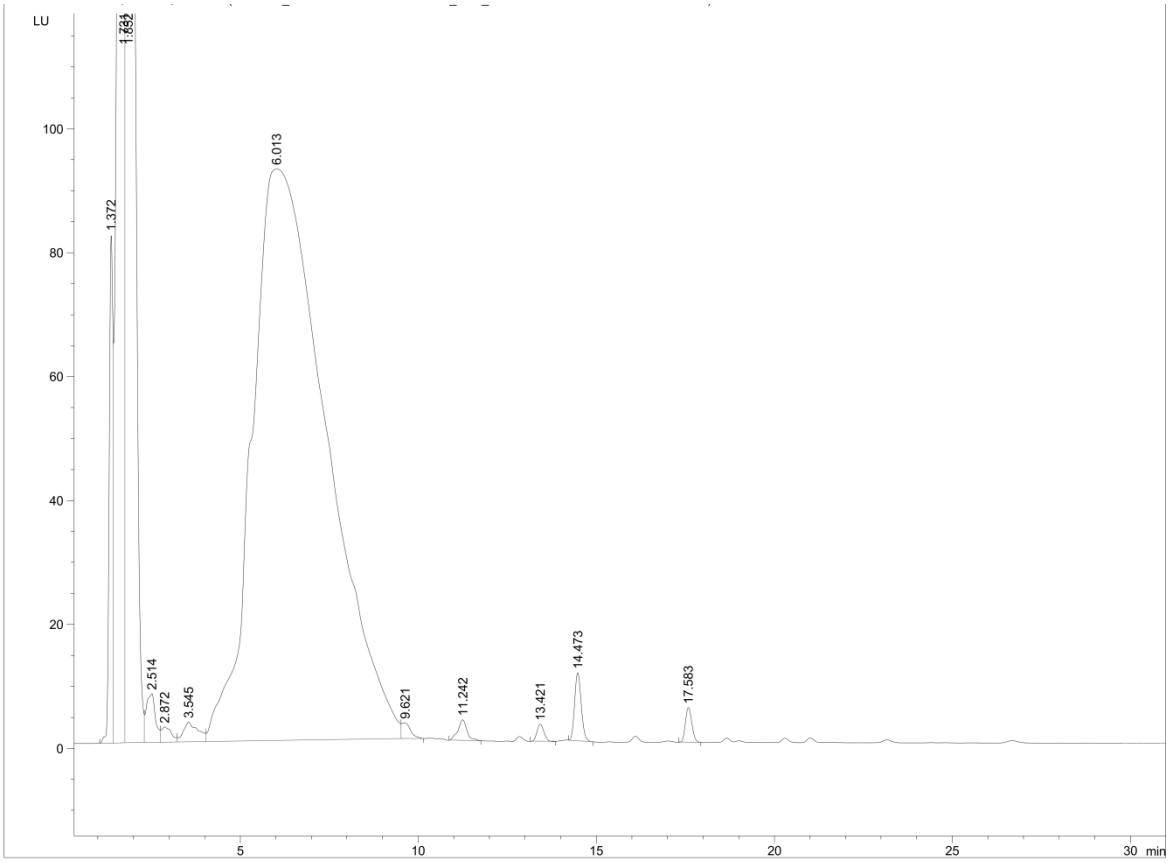

Figure 12: The sample HPLC-FLD chromatogram of fatty acids in the quinoa wort
Slika 12: Primer HPLC-FLD kromatograma maščobnih kislin v kvinojini pivini

The concentrations of 18 examined fatty acids of worts and beverages from the barley, buckwheat and quinoa malt are shown in Table 27, Table 28 and Table 29, respectively.

Regarding beverages fermented with different yeast, some features were recognized that were common either for raw materials, yeast strains or both. Besides Ara-4 and Lig, which were not detected in any sample, we also did not detect Eru, Beh and Ner in any of barley samples and Beh in any of buckwheat samples. The barley and buckwheat worts contained similar amounts of sum FAs as well as similar concentrations of individual FA with the exception of lower levels of Hex and Myr in buckwheat. On the other hand, in the quinoa wort the sum concentration of FAs was around 2.5-fold higher than in barley. Most FAs were present in higher levels, especially α- and γ-Lin (10-fold higher), with the exception of the missing Lau and Ste.

Table 27: The fatty acid content of barley wort and fresh and forced-aged beverages fermented with different yeast
Preglednica 27: Vsebnost maščobnih kislin v ječmenovi pivini ter v ječmenovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

FA	barley [µg/L][a]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
Hex	415.33	138.36	133.16	301.02	320.53	459.78	438.63
Oct	384.08	630.29	368.71	248.98	273.49	804.87	771.73
Dec	1170.42	1146.52	1208.92	221.02	199.38	< LOD	< LOD
Lau	297.36	472.33	319.44	190.9	219.53	215.58	215.03
α-&γ-Lin	203.2	< LOD	< LOD	125.55	99.08	172.73	< LOD
Ara-4	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Myr	142.83	< LLQ	< LLQ	38.57	41.75	184.74	151.95
Lin	254.05	180.18	220.72	243.24	193.69	36.04	76.58
Pal + Ole	16.17	15.91	16.95	58.92	37.71	46.4	51.53
Ste	40.24	41.96	42.41	67.11	61.29	66.09	65.1
Eic	77.46	18.93	18.42	23.73	23.32	53.88	< LOD
Ara-0	< LOD	< LOD	< LOD	26.25	21.01	50.32	< LOD
Eru	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Beh	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Ner	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Lig	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
sum	3001.14	2644.48	2328.73	1545.29	1490.78	2090.43	1770.55

LOD – limit of detection. Hex – hexanoic acid. Oct – octanoic acid. Dec – decanoic acid. Lau- lauric acid. α-Lin – α-linolenic acid. γ-Lin – γ-linolenic acid. Ara-4 – arachidonic acid. Myr – myristic acid. Lin – linoleic acid. Pal – palmitic acid. Ole – oleic acid. Ste – stearic acid. Eic – eicosanoic acid. Ara-0 – arachidic acid. Eru – erucic acid. Beh – behenic acid. Ner – nervonic acid. Lig – lignoceric acid.

Regardless raw material, the sum concentrations of FAs in beverages fermented with TUM 34/70 were always in the range of those in worts. Sum FA concentrations in beverages fermented with TUM 177 and TUM SL17 were comparable between each other and around 1.5-2-fold lower than in worts. In addition, the increase or decrease of a particular FA concentration after fermentation seems primarily FA-dependent. In general, concentrations of saturated short-chain fatty acids usually increased after fermentation and those of unsaturated long-chain fatty acids normally decreased.

Table 28: The fatty acid content of the buckwheat wort and fresh and in forced-aged beverages fermented with different yeast
Preglednica 28: Vsebnost maščobnih kislin v ajdovi pivini ter v ajdovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

FA	buckwheat [mg/L]						
	wort	**TUM 34/70 fresh beverage**	**TUM 34/70 force-aged beverage**	**TUM 177 fresh beverage**	**TUM 177 force-aged beverage**	**TUM SL17 fresh beverage**	**TUM SL17 force-aged beverage**
Hex	205.72	237.69	272.51	439.96	422.15	338.4	325.5
Oct	487.33	364.69	291.42	244.23	267.95	242.37	250.67
Dec	1200.34	1265.53	1534.2	138.71	196.11	117.34	119.58
Lau	371.99	537.16	248.71	226.32	385.81	198.63	270.33
α-&γ-Lin	252.54	< LOD	< LOD	164.94	175.38	156.04	203.72
Ara-4	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Myr	< LLQ	18.44	< LLQ	125.35	< LOD	84.04	106.48
Lin	308.11	261.26	201.8	184.14	144.68	297.3	307.84
Pal + Ole	18.73	19.15	19.43	95.5	44.92	38	37.33
Ste	22.76	75.36	67.38	102.11	106.02	91.99	92.26
Eic	69.63	17.96	20.61	37.04	38.83	41.58	< LOD
Ara-0	< LOD	< LOD	< LOD	31.91	< LOD	41.31	40.33
Eru	< LOD	< LOD	< LOD	< LOD	< LOD	20.93	< LOD
Beh	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Ner	< LOD	< LOD	< LOD	< LLQ	< LOD	< LLQ	< LOD
Lig	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
sum	2937.15	2797.24	2656.06	1790.21	1781.85	1667.93	1754.04

LOD – limit of detection. Hex – hexanoic acid. Oct – octanoic acid. Dec – decanoic acid. Lau- lauric acid. α-Lin – α-linolenic acid. γ-Lin – γ-linolenic acid. Ara-4 – arachidonic acid. Myr – myristic acid. Lin – linoleic acid. Pal – palmitic acid. Ole – oleic acid. Ste – stearic acid. Eic – eicosanoic acid. Ara-0 – arachidic acid. Eru – erucic acid. Beh – behenic acid. Ner – nervonic acid. Lig – lignoceric acid.

Another somehow expected observation was that concentrations of saturated fatty acids practically did not changed after forced aging whereas the content of unsaturated ones were lower, especially in quinoa. This situation can be easily explained by the autoxidation of unsaturated fatty acids (Buiatti, 2009). Another confirmation of this suggestion is that this decrease in concentrations of unsaturated fatty acids was the most prominent in quinoa, less in buckwheat and the least in barley beverages. Furthermore, this is also the falling order of transition metal contents (Fe, Cu) (Table 10, Table 11 and Table 12) which are proverbial to play an important role in free-radical reactions (Kaneda *et al*., 1992).

Table 29: The fatty acid content of the quinoa wort and fresh and in forced-aged beverages fermented with different yeast
Preglednica 29: Vsebnost maščobnih kislin v kvinojini pivini ter v kvinojinih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

FA	quinoa [mg/L]						
	wort	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage
Hex	957.61	1022.78	1102.9	374.58	572.65	537.99	584.68
Oct	1145.81	2023.13	1695.89	526.11	663.74	648.03	718.92
Dec	840.78	743.85	429.14	1755.4	1517.99	< LOD	< LOD
Lau	< LOD	318.52	229.63	121.48	133.33	< LOD	< LOD
α-&γ-Lin	2364.52	933.33	641.78	555.56	< LOD	978.44	744.44
Ara-4	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Myr	182.52	149.03	330.28	104.5	35.41	218.49	< LOD
Lin	335.14	292.34	252.88	215.32	165.77	288.29	238.74
Pal + Ole	438.29	239.58	< LOD	< LLQ	< LLQ	86.11	< LOD
Ste	< LOD	< LOD	< LOD	57.34	61.9	143.59	< LOD
Eic	225.42	115.04	104.6	29.58	38.46	79.79	< LOD
Ara-0	63.63	58.14	69.77	< LOD	< LOD	< LOD	< LOD
Eru	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Beh	126.58	68.63	63.73	< LOD	< LOD	< LOD	< LOD
Ner	44.69	35.71	< LOD	< LOD	< LOD	41.4	< LOD
Lig	68.27	44.78	14.93	< LOD	< LOD	< LOD	< LOD
sum	6793.26	6044.86	4935.53	3739.87	3189.25	3022.13	2286.78

LOD – limit of detection. Hex – hexanoic acid. Oct – octanoic acid. Dec – decanoic acid. Lau- lauric acid. α-Lin – α-linolenic acid. γ-Lin – γ-linolenic acid. Ara-4 – arachidonic acid. Myr – myristic acid. Lin – linoleic acid. Pal – palmitic acid. Ole – oleic acid. Ste – stearic acid. Eic – eicosanoic acid. Ara-0 – arachidic acid. Eru – erucic acid. Beh – behenic acid. Ner – nervonic acid. Lig – lignoceric acid.

4.1.7 Aldehydes and ketones

4.1.7.1 Method optimization and validation

Distillation

The distillation procedure was used mainly to clean samples and to concentrate analytes in some cases. This was necessary since preliminary results with untreated samples gave no results when using the HPLC-FLD technique and there was many interfering compounds when performing GC-FID analysis The stock mixture (1 L) of aldehydes and ketones with the final concentrations of individual analyte of ~50 mg/L was prepared by mixing 50 mL of their stock solutions. The resulting stock mixture was successively diluted 1:10 for four times giving the four 'working mixtures' with concentrations of individual analyte from ~5 to ~5000 µg/L. Using the most concentrated working mixture, i.e. each analyte in concentration ~5000 µg/L, different combinations of the sample volume (100 and 200 mL) and the collected distillate (25, 50 and 100 mL) were investigated by the HPLC-FLD analysis. Derivatization was performed with 100 µL of DBD-H and 100 µL of TFA stock

solutions. Since the maximal signal was retention time-dependent (Table 30), results were critically assessed and a compromise combination of 200 mL sample volume and 50 mL distillate volume (results in bold italics) was used throughout the analytical procedure.

Table 30: The HPLC-FLD peak areas of aldehyde and ketone standard solution when different volumes of the distilled sample and collected distillate were used in comparison with undistilled sample
Preglednica 30: Primerjava površin HPLC-FLD vrhov nedistilirane standardne raztopine aldehidov in ketonov ter njihovih površin po destilaciji različnih količin vzorca in z različnimi količinami zbranega destilata

	volume [mL]						
sample	N/A*	100			***200***		
distillate	N/A*	25	50	100	25	***50***	100
peak retention time [min]	**peak area**						
acetaldehyde	713.9	626.5	762.6	666.7	686.5	***651.3***	668.5
2,3-butandion	425.1	309.6	348.7	342.5	333.8	***390.5***	378.2
propanal	357.3	302.8	328.7	347.1	291.5	***275.0***	300.7
2,3-pentanedione	467.8	338.2	366.5	343.4	417.0	***423.3***	415.9
butanal + 2,3-methylpropanal	424.6	418.9	427.5	371.7	546.0	***495.7***	459.7
phenylacetaldehyde + 3-methylbutanal	495.3	508.9	525.2	436.6	570.8	***569.0***	559.1
hexanal	239.4	254.8	252.1	218.3	332.4	***319.5***	283.6
heptanal	168.9	194.4	192.5	201.0	280.8	***316.2***	258.3
octanal	363.7	394.3	352.0	378.7	446.8	***498.6***	436.6
***trans*-2-nonenal**	27.2	24.9	31.0	42.1	18.6	***24.7***	32.3
sum	3683.2	3373.3	3586.8	3348.1	3924.2	***3963.8***	3792.9

* − the information is not applicable since the undistilled sample was used.

HPLC-FLD analysis

As a derivatization reagent, we used 4-(*N*,*N*-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H) which easily reacts with a carbonyl group to form the corresponding hydrazone. The resultant hydrazone is stable enough to reach detector without any decomposition under reverse phase HPLC. An excellent chromatogram can be obtained by fluorescence detection and a highly sensitive detection can be done because of the strong fluorescence of hydrazones. In addition, since their excitation and emission wavelengths are at longer values, detection has less interference by contaminants.

700 µL of the standard solution of each analyte in concentration ~250 µg/L was used for the derivatization with different volumes of DBD-H and TFA stock solutions. ACN was added to the final volume of 1 mL and the reaction mixtures were allowed to derivatize at room temperature for 2 h with different amounts of DBD-H (8 mM) and TFA (40% v/v) stock solutions. The excitation and emission wavelengths of the FLD detector were set to 450 and 565 nm, respectively, as was suggested by Uzu *et al*. (1990). The maximal signal (results in bold italic numbers) was achieved at 200 µL of DBD-H and 50 µL of TFA giving their final concentrations of 1.6 mM and 2% (v/v), respectively.

Table 31: The HPLC-FLD peak areas of the aldehyde and ketone standard solution when different amounts of DBD-H and TFA were used for derivatization.
Preglednica 31: Površine HPLC-FLD vrhov aldehidov in ketonov pri derivatizacijah z različnimi količinami DBD-H in TFA

reagent	**µL of stock solution**								
DBD-H	150			***200***			250		
TFA	25	50	100	25	***50***	100	25	50	100
aldehyde or ketone (~350 µg/L)	**peak area**								
acetaldehyde	414.3	388.0	387.9	556.3	***588.8***	577.4	501.0	507.9	510.2
2,3-butandion	95.1	109.6	148.7	42.5	***103.8***	39.5	27.2	46.9	78.6
propanal	263.5	252.7	256.6	315.4	***323.3***	315.6	299.3	301.9	306.0
2,3-pentanedione	99.8	118.2	146.5	43.4	***127.0***	75.3	49.9	82.8	119.4
butanal + 2,3-methylpropanal	296.2	283.7	284.1	357.9	***353.2***	352.7	338.5	339.2	339.0
phenylacetaldehyde + + 3-methylbutanal	558.5	583.1	590.4	712.4	***705.9***	704.7	670.4	679.9	681.6
hexanal	209.6	243.7	250.1	298.4	***303.1***	301.8	272.6	288.4	291.7
heptanal	139.6	233.0	250.8	291.7	***302.0***	300.8	249.2	284.4	292.2
octanal	55.0	187.5	220.3	249.5	***268.7***	265.3	180.8	244.4	257.5
***trans*-2-nonenal**	5.7	22.8	32.0	42.4	***44.9***	45.4	2.5	35.4	40.3
sum	2137.3	2422.3	2567.4	2909.9	***3120.7***	2978.5	2591.4	2811.2	2916.5

The optimal derivatization time and temperature were not investigated since it was reported for several times that DBD-H easily reacts with carbonyl group in 0.5-1 h at room temperature (Uzu *et al*., 1990; Uchiyama *et al*., 2001). For this reason we found derivatization at room temperature for 2 h as sufficient. Different combinations of the FLD detector excitation and emission wavelengths were investigated and according to results (Figure 13) the best signal was achieved at 440 and 550 nm, respectively.

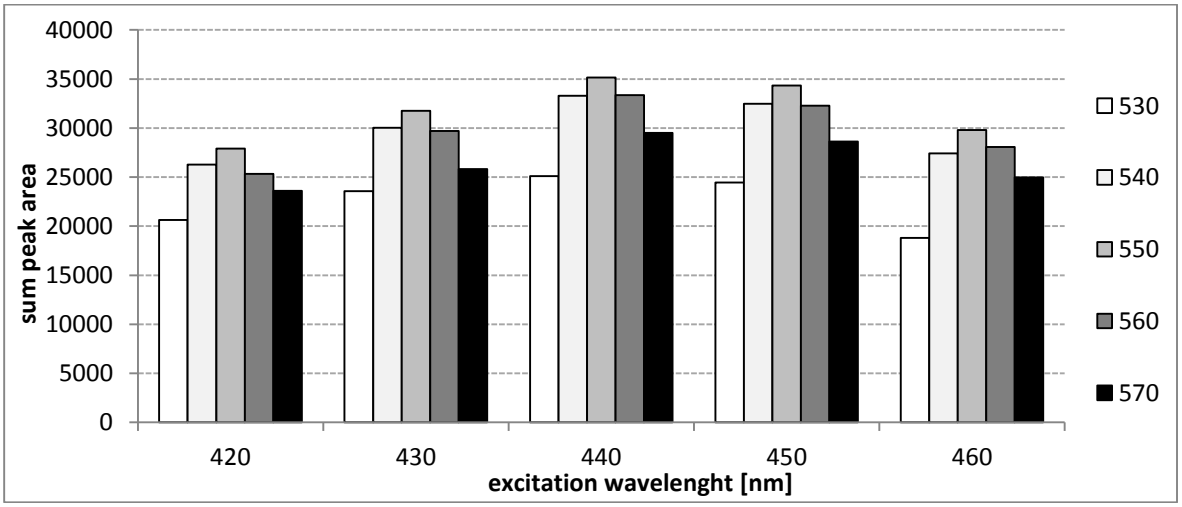

Figure 13: The influence of the FLD detector excitation and emission wavelengths on the sum peak area of examined aldehydes and ketones
Slika 13: Vpliv različnih vzbujevalnih in sevalnih valovnih dolžin FLD detektorja na vsoto kromatografskih vrhov posameznih aldehidov in ketonov

Figure 14 shows the sample HPLC-FLD chromatogram of an aldehyde and ketone standard solution (Figure 14) with the final concentration of each analyte of ~40 µg/L using the previously optimized derivatization and chromatographic parameters. Chromatographic peaks were not symmetrical because we used high injection volumes. In that way we additionally increased the sensitivity of detection whereas its selectivity was not affected.

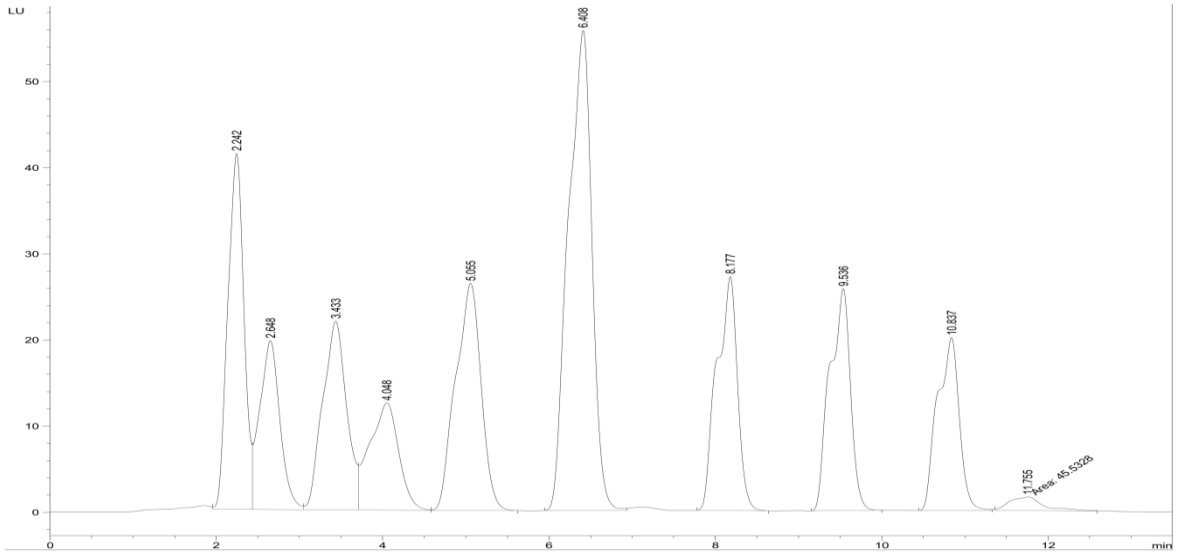

Figure 14: The sample HPLC-FLD chromatogram of aldehyde and ketone standard solution
Slika 14: Primer HPLC-FLD kromatograma standardne raztopine aldehidov in ketonov

The HPLC-FLD analysis proved to be selective since the solutions of DBD-H together with TFA gave no statistical significant interfering chromatographic signal (Figure 15). Sample was prepared from the stock solutions of both reagents in their final concentrations of 1.6 mM and 2% (v/v), rescpectively. The chromatogram of 1.6 DBD-H alone did not differ significantly.

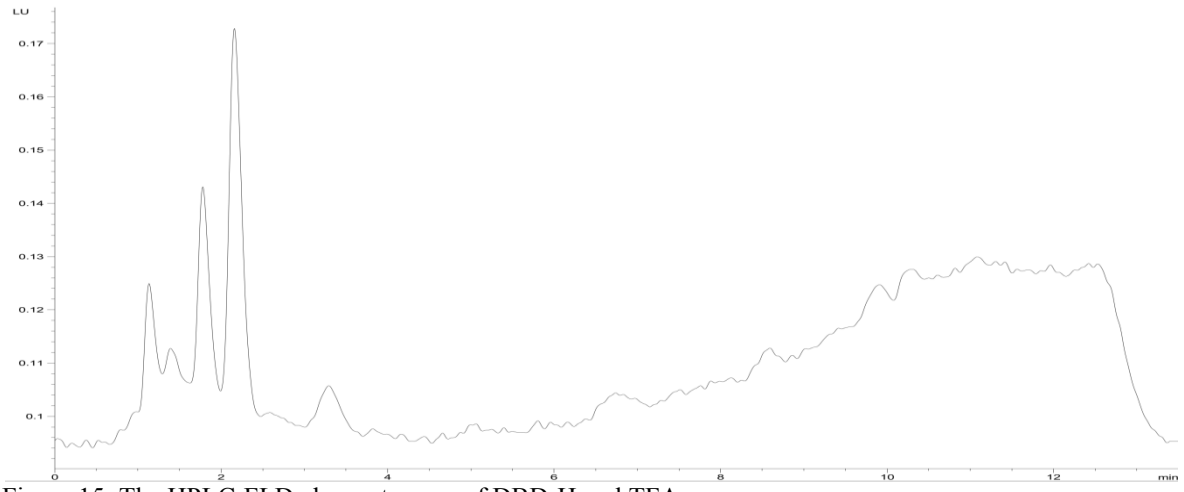

Figure 15: The HPLC-FLD chromatogram of DBD-H and TFA
Slika 15: HPLC-FLD kromatogram raztopine DBD-H in TFA

The HPLC-FLD separation was selective also regarding retention times with the exception of two chromatographic peaks where for each, two aldehydes coeluted (Table 32). Among ketones only vicinal diketones 2,3-butandione and 2,3-pentandione were detected with sufficient sensitivity whereas others gave signal only at concentrations higher than ~5 mg/L.

Table 32: The average retention time values for a particular HPLC-FLD chromatographic peak of aldehyde or ketone and their RSD values (n = 5)
Preglednica 32: Povprečne vrednosti retenzijskh časov posameznih HPLC-FLD kromatografskih vrhov aldehidov ali ketonov in njihove RSD vrednosti (n = 5)

aldehyde or ketone	average retention time [min]	retention time RSD [%]
acetaldehyde	2.242	0.73
2,3-butandion	2.648	1.09
propanal	3.433	0.82
2,3-pentanedione	4.048	1.13
butanal + 2-methylpropanal	5.055	1.76
phenylacetaldehyde + 3-methylbutanal	6.408	1.91
hexanal	8.177	1.86
heptanal	9.536	2.10
octanal	10.837	2.07
trans-2-nonenal	11.755	2.21

RSD – relative standard deviation.

The linear ranges of HPLC-FLD analysis were tested at concentrations of ~5, ~10, ~20, ~50, ~100, ~200, ~500, ~1000, ~2000 and ~5000 µg/L of each analyte. The results in Table 33 are presented as the average value of five independent distillations and subsequent derivatizations. Satisfying linearity was achieved in concentration ranges from around 5 to around 5000 µg/L. The calibration curves (Table 33) were suitable for the quantification of fatty acid derivatives during the intra- and inter-day analysis.

Table 33: The linear ranges, their linear regressions and correlation coefficient together with the limits of detection and lower limits of quantification of aldehydes and ketones in standard solution, determined with HPLC-FLD
Preglednica 25: Območje linearnosti, enačba linearne regresije in njen korelacijski koeficient skupaj z mejo detekcije in spodnjo mejo kvantifikacije aldehidov in ketonov v standardni raztopini, določenih s HPLC-FLD

aldehyde or ketone	y = kx+ n	correlation koeficient	linear range [µg/L]		LOD [µg/L]	LLQ [µg/L]
acetaldehyde	y = 0.073x + 62.7	99.93	5.60	5600	3.59	11.95
2,3-butandion	y = 0.054x + 3.24	99.74	6.52	6520	0.78	2.6
propanal	y = 0.031x + 0.81	99.97	14.27	14270	1.74	5.8
2,3-pentanedione	y = 0.048x – 0.194	99.95	6.40	6400	1.19	3.95
butanal + 2-	y = 0.056x + 4.61	99.98	10.08	10080	1.40	4.65
phenylacetaldehyde +	y = 0.895x - 1.29	99.93	10.22	1222	1.61	5.35
hexanal	y = 0.055x + 0.394	99.94	5.40	5400	1.80	6
heptanal	y = 0.080x – 0.519	99.97	5.59	5590	2.01	6.7
octanal	y = 0.068x + 0.241	99.82	5.42	5420	2.22	7.4
***trans*-2-nonenal**	y = 0.048x + 2.32	99.70	5.63	5630	2.42	8.05

LOD – limit of detection. LLQ – lower limit of quantification.

The recovery and precision results for the standard solution and for barley, buckwheat and quinoa samples are listed in Table 34. For the evaluation of recovery, samples were spiked with aldehydes and ketones giving their final concentrations of 50 µg/L and the results, subtracted for the values of an unspiked samples, were compared with the results of an undistilled standard solution of the same concentration. Distillation recoveries from standard solution were extremely high, especially those of relatively non-polar compounds. Opposite results were obtained for beverage matrices where more polar vicinal diketones were better recovered. Especially for the quinoa matrix, recoveries were generally low.

For the assessment of precision, the same standard solution (each analyte ~50 µg/L) was distilled for seven times and the corresponding RSD values were calculated. The best precision was obtained with standard solution, a little worse with barley and buckwheat and the worst in quinoa; however, from the analytical perspective all the values were satisfactory.

Table 34: The distillation recoveries and precisions of aldehydes and ketones from different matrices (n = 7)
Preglednica 34: Izkoristek destilacije in natančnost določitve aldehidov in ketonov v različnih matriksih (n = 7)

aldehyde or ketone [~50 µg/L]	recovery [%]				precision (RSD [%])			
	standard	barley	buckwheat	quinoa	standard	barley	buckwheat	quinoa
acetaldehyde	110.31	116.29	89.81	91.14	2.39	3.24	3.88	3.53
2,3-butandion	67.76	124.28	147.95	144.79	0.52	0.72	1.05	2.40
propanal	97.74	84.92	85.16	65.32	1.16	1.91	1.61	2.27
2,3-pentanedione	61.26	133.27	155.83	148.85	0.79	0.74	1.28	2.13
butanal + 2,3-	127.57	80.56	83.07	62.18	0.93	1.58	1.34	2.00
phenylacetaldehyde +	111.88	88.72	103.08	81.55	1.07	2.42	2.21	3.87
hexanal	132.78	81.54	86.34	62.30	1.20	2.25	2.08	3.73
heptanal	157.84	72.52	76.82	63.47	1.34	2.09	2.94	3.60
octanal	150.27	70.69	71.66	66.57	1.48	2.07	2.81	3.47
***trans*-2-nonenal**	143.22	69.13	64.52	61.73	1.61	1.96	3.34	3.33

RSD – relative standard deviation.

GC-FID analysis

Figure 16 shows the sample GC-FID chromatogram of an aldehyde and ketone standard solution with the final concentration of each analyte of ~40 mg/L using the previously optimized chromatographic parameters. Selectivity proved as an important added value of GC-FID analysis since among 19 examined analytes only in two cases two of them coeluted (Table 35). In particular, it became obvious that positional isomers, i.e. homologous aldehydes and ketones can not be separated. Besides aldehydes and ketones, the content of 2-pentylfuran was also explored since GC-MS analysis suggested the presence of some furans in the quinoa samples (Table 22).

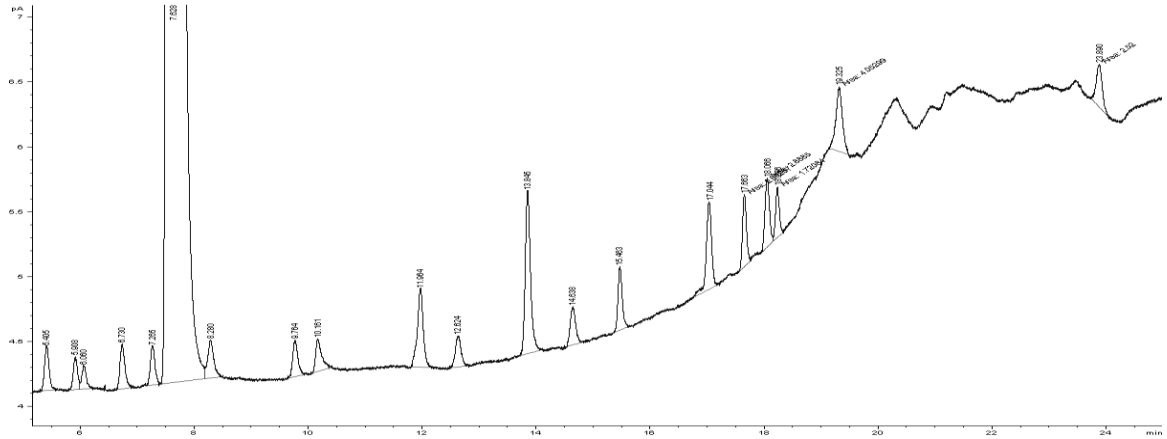

Figure 16: The sample GC-FID chromatogram of aldehydes and ketones in standard solution
pA – detector response unit.
Slika 16: Primer HPLC-FLD kromatograma aldehidov in ketonov v standardni raztopini
pA – enota odziva detektorja.

Table 35: The average retention time values for a particular GC-FID chromatographic peak and their RSD values (n = 5)
Preglednica 35: Povprečne vrednosti retenzijskih časov posameznih GC-FID vrhov in njihove RSD vrednosti (n = 5)

aldehyde or ketone	average retention time [min]	retention time RSD [%]
acetaldehyde	5.405	3.05
propanal	5.907	4.14
2-methylpropanal	6.059	3.50
butanal	6.734	2.17
3-methylbutanal	7.263	2.16
2,3-butandione	8.294	2.39
2,3-pentanedione	9.771	1.93
hexanal	10.172	1.47
heptanal	12.027	1.01
heptanone	12.054	1.54
octanal	13.942	1.08
octanone	13.991	1.38
acetoin	14.638	0.85
nonanone	15.463	1.31
furfural	17.044	0.77
***trans*-2-nonenal**	17.665	1.24
2-pentylfuran	18.065	0.97
phenylacetaldehyde	19.328	1.05
5-hydroxymethylfurfural	23.891	1.14

RSD – relative standard deviation.

The linear ranges of GC-FID chromatographic peaks were tested at concentrations of ~0.1, ~0.5, ~1, ~2, ~5, ~10, ~20 and ~50 mg/L of each fatty acid in a standard solutions. The

results in Table 36 are presented as the average value of five independent distillations. Excelent linearity was achieved in concentration ranges from around 0.5 to around 50 mg/L. The calibration curves were suitable for the quantification of fatty acid derivatives during the intra- and inter-day analysis.

Table 36: The linear ranges, their linear regressions and correlation coefficients together with the limits of detection and lower limits of quantification for aldehydes and ketones in standard solution, determined with GC-FID
Preglednica 36: Območje linearnosti, enačba linearne regresije in njen determinacijski koeficient skupaj z mejo detekcije in spodnjo mejo kvantifikacije aldehidov in ketonov v standardni raztopini, določenih z GC-FID

aldehyde or ketone	y = kx+ n	correlation koeficient	linear range [mg/L]		LOD [mg/L]	LLQ [mg/L]
acetaldehyde	y = 0.15x + 0.15	99.98	0.56	56.00	0.28	0.92
propanal	y = 0.18x + 0.02	99.98	0.58	58.30	0.17	0.56
2-methylpropanal	y = 0.17x + 0.07	100.00	0.53	52.60	0.13	0.44
butanal	y = 0.37x + 0.13	100.00	0.48	48.20	0.20	0.67
3-methylbutanal	y = 0.27x + 0.03	99.98	0.53	52.80	0.20	0.68
2,3-butandione	y = 0.37x + 0.28	100.00	0.65	65.20	0.20	0.67
2,3-pentanedione	y = 0.70x + 0.02	100.00	0.64	64.00	0.14	0.47
hexanal	y = 0.84x + 0.21	99.97	0.54	54.00	0.25	0.84
heptanal + heptanone	y = 0.80x + 0.86	99.51	0.56	55.00	0.35	1.18
octanal + octanone	y = 0.55x + 0.51	97.26	0.54	52.00	0.31	1.02
acetoin	y = 0.89x + 0.05	100.00	0.54	54.10	0.26	0.86
nonanone	y = 1.51x + 0.58	98.75	0.51	50.60	0.41	1.36
furfural	y = 2.60x + 1.32	99.97	0.84	84.40	0.31	1.04
***trans*-2-nonenal**	y = 3.09x + 0.22	99.55	0.56	56.30	0.41	1.37
pentylfuran	y = 0.16x + 0.25	99.85	0.62	61.70	0.36	1.21
phenylacetaldehyde	y = 4.36x + 0.43	99.90	0.69	69.4	0.32	1.05
5-hydroxymethylfurfural	y = 1.43x + 0.29	98.97	0.46	45.8	0.37	1.22

LOD – limit of detection. LLQ – lower limit of quantification.

The recovery and precision results for the standard solution and for barley, buckwheat and quinoa samples are listed in Table 37. For the evaluation of recovery, samples were spiked with aldehydes and ketones giving their final concentrations of 5 mg/L and the results, subtracted for the values of an unspiked samples, were compared with the results of an undistilled standard solution of the same concentration. Interestingly, distillation recoveries from the quinoa matrix were comparable or better of those from standard solution whereas recoveries from the barley and buckwheat matrices were generally lower.

For the assessment of precision, the same standard solution (each analyte ~5 mg/L) was distilled for seven times and the corresponding RSD values were calculated. Precision values were more or less matrix-independent.

Table 37: The distillation recoveries and precisions of aldehydes and ketones from different matrices determined by GC-FID (n = 7)
Preglednica 37: Izkoristek destilacije in natančnost določitve aldehidov in ketonov v različnih matriksih, določenih z GC-FID (n = 7)

aldehyde or ketone [~5 mg/L]	recovery [%]				precision (RSD [%])			
	standard	barley	buckwheat	quinoa	standard	barley	buckwheat	quinoa
acetaldehyde	103.51	96.74	75.75	206.65	1.84	1.23	1.61	2.00
propanal	103.57	96.80	112.05	170.51	1.12	1.39	1.66	2.93
2-methylpropanal	118.89	79.45	73.62	71.34	0.88	0.60	0.33	3.06
butanal	118.66	64.09	75.98	168.38	1.33	2.09	1.85	1.61
3-methylbutanal	110.44	65.19	76.60	213.77	1.35	1.78	2.21	3.64
2,3-butandione	164.02	64.94	61.01	227.44	1.33	2.04	1.75	1.47
2,3-pentanedione	137.63	59.30	83.90	193.29	0.94	2.86	0.72	1.57
hexanal	158.85	60.71	69.79	167.05	1.68	2.68	2.67	1.68
heptanal + heptanone	219.75	70.79	77.23	201.96	2.36	2.50	2.64	1.78
octanal + octanone	206.58	74.06	61.54	181.30	2.04	3.32	2.60	1.89
acetoin	45.54	114.38	89.86	61.17	1.71	1.14	0.57	2.99
nonanone	177.34	68.41	71.11	184.40	3.39	2.96	2.53	2.10
furfural	187.16	104.09	68.21	121.09	2.07	1.78	1.49	3.20
***trans*-2-nonenal**	165.70	90.75	78.07	142.06	2.74	3.60	3.45	2.31
pentylfuran	35.77	215.86	107.09	184.72	2.42	1.42	2.40	3.41
phenylacetaldehyde	177.64	115.55	120.59	150.45	2.10	2.24	3.38	3.52
5-hydroxymethylfurfural	142.51	152.95	61.03	125.14	3.78	2.06	3.29	3.62

RSD – relative standard deviation

4.1.7.2 Application to real samples

For the determination of aldehydes and ketones in our fresh, force-aged and naturally aged beverages from the barley, buckwheat and quinoa wort, we used distillation of samples followed by two different separation and detection techniques, i.e. HPLC-FLD and GC-FID. Both methods have specific pros and cons thus their contemporary application represents an added value.

The biggest disadvantage of HPLC-FLD analysis were two coelutions of two analytes each. In addition, only aliphatic aldehydes reacted with the derivatization reagent sufficiently, whereas very good sensitivity can be regarded as an important added value.

GC-FID analysis suffered from low sensitivity which is mostly unsuitable for the determination of analytes in trace amounts. For this reason, in our case GC-FID analysis proved to be unsuitable for the determination of propanal, 2,3-butanedione, 2,3-pentanedione, heptanal and heptanone, and octanal and octanone. FID detection is not selective but this was not the problem in our case since the good column separation resulted in non-overlaping chromatographic peaks (with the exception of positional isomers of carbonyl functional group).

Acetaldehyde content was already discussed in Section 4.1.5. Propanal was not detected with FID and according to FLD detection it seems that its concentrations were generally scarce, few hundred micrograms at most. Hexanal and *trans*-2-nonenal are both products of fatty acid oxidation (Baert *et al*., 2012). Their concentrations are significantly elevated in force-aged beverages and even more in naturally aged ones. Significant amounts of hexanal were also present in fresh beverages, suggesting its production by yeast or the absence of its reduction.

Furfural and 5-hydroxymethyl furfural can also be discussed together since they are both Maillard reaction products (Baert *et al*., 2012). Both are important markers for the heat load placed on the mash, wort, and beer and also for flavour staling in general since throughout the aging process, their concentrations increase at a linear rate (Baert *et al*., 2012). Our results confirm the increase of their concentrations during aging but in a different way. Concentrations of furfural raised only after force-aging and concentrations of 5-hydroxymethylfurfural only after two years of natural aging.

The so-called Strecker aldehydes, such as 2-methylpropanal, 3-methylbutanal and phenylacetaldehyde, are formed in a series of reactions where transamination can take place between an amino acid and an α-dicarbonyl (Baert *et al*., 2014). They contain one carbon atom less than than the corresponding amino acid from which they were derived. In our case, 2-methylpropanal and 3-methylbutanal concentrations were higher in all aged beverages and the highest mostly in those aged naturally. The same stands also for phenyacetaldehye in the case of quinoa but, interestingly, not for barley and buckwheat beverages.

Butanal content increased during aging regardless raw material but its absolute concentrations were the highest in the quinoa beverages. This increase is, at least partly, due to the oxidation of 1-butanol.

Although six different ketones were investigated, the discussion of three of them is not relevant. In particular, heptanone, octanone and nonanone did not give the FLD signal whereas in GC-FID analysis they coeluted with corresponding positional isomer of aldehydes. Although the chromatographic peak of nonanone seems a result of a single compound – since we did not test nonanal – we are very certain that the latter is the case. 2,3-butandione and 2,3-pentandione, here refered as vicinal diketones, are present in beverages in concentrations 50-300 µg/L. Only in barley beverages their concentrations decreased after aging whereas in buckwheat and quinoa the concentrations increased. Under the given aging conditions, the Maillard reaction was probably responsible for the increase of vicinal diketone concentrations in aged beverages.

Acetoin concentrations in barley and buckwheat beverages were scarce and hardly affected by aging, as opposed to quinoa where absolute concentrations were considerably higher and different aging modes influenced acetoin concentrations differently. In particular, force-aging lowered the initial concentrations of fresh beverages whereas natural aging increased them.

As expected, pentylfuran was detected only in quinoa beverages.

HPLC-FLD analysis

Figure 17 shows the HPLC-FLD chromatogram of the quinoa wort. The concentrations of particular aldehydes and ketones for barley, buckwheat and quinoa samples are presented in Table 38, Table 39 and Table 40, respectively.

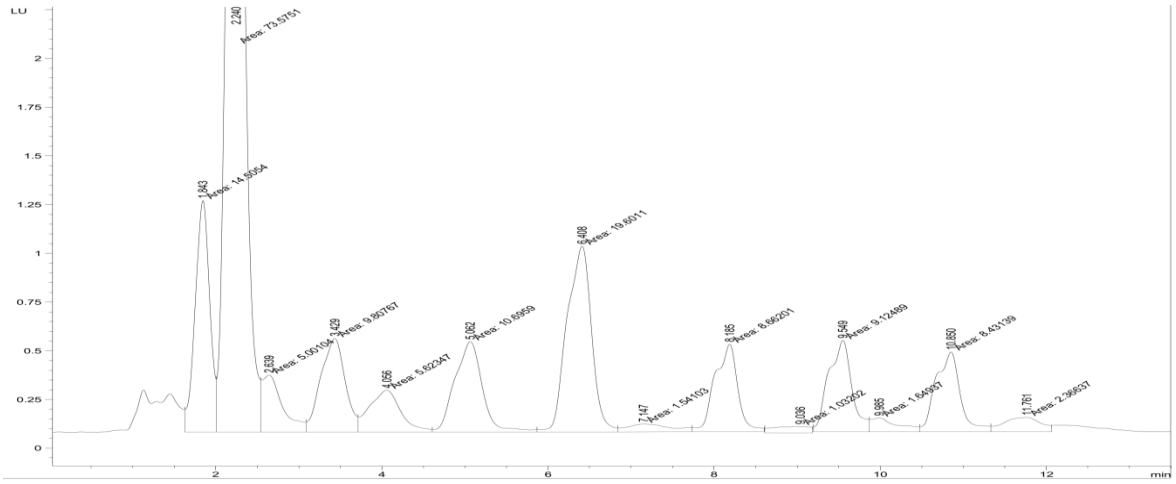

Figure 17: The sample HPLC-FLD chromatogram of aldehydes and ketones in the quinoa beverage fermented with TUM 34/70
LU – detector response unit.
Slika 17: Primer HPLC-FLD kromatograma aldehidov in ketonov v kvinojini pijači, fermentirani s TUM 34/70
LU – enota odziva detektorja.

Table 38: The aldehyde and ketone content of the barley wort and fresh and forced-aged beverages fermented with different yeast, determined by HPLC-FLD
Preglednica 38: Vsebnost aldehidov in ketonov, določenih s HPLC-FLD, v ječmenovi pivini ter v ječmenovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

aldehyde or ketone	barley [µg/L]								
	TUM 34/70 fresh beverage	**TUM 34/70 force-aged beverage**	**TUM 34/70 naturally aged beverage**	**TUM 177 fresh beverage**	**TUM 177 force-aged beverage**	**TUM 177 naturally aged beverage**	**TUM SL17 fresh beverage**	**TUM SL17 force-aged beverage**	**TUM SL17 naturally aged beverage**
acetaldehyde	> ULR	> ULR	> ULR	> ULR	> ULR	> ULR	> ULR	> ULR	> ULR
2,3-butandion	209.02	45.83	53.66	134.58	129.46	58.3	123.76	58.74	29.94
propanal + furfural	285.49	178.73	32.36	307.05	300.48	56.59	288.13	304.92	20.06
2,3-pentanedione	94.31	98.05	81.93	102.53	86.36	87.91	141.93	43.28	26.3
butanal + + 2-methylpropanal	1220.24	3124.78	4489.51	403.88	1142.06	1947.65	1315.55	2979.06	3975.2
phenylacetaldehyde + + 3-methylbutanal	1889.16	3176.89	2967.21	2364.3	2806.84	3417.41	1906.3	2770.04	2451.42
hexanal	550.21	1818.48	3200.98	871.61	250.53	2059.54	3012.31	4847.27	6237.57
heptanal	246.83	422.14	704.76	27.6	23.27	36.74	20.65	26.4	43.56
octanal	33.58	77.77	173.36	181.5	151	409.58	144.79	231.9	323.4
***trans*-2-nonenal**	1.61	106.74	631.9	0.95	19.35	726.49	0.69	5.95	885.2
sum	4530.45	9049.41	12335.67	4394	4909.35	8800.21	6954.11	11267.56	13992.65

ULR – the upper linear limit

Table 39: The aldehyde and ketone content of the buckwheat wort and fresh and forcedaged beverages fermented with different yeast, determined by HPLC-FLD
Preglednica 39: Vsebnost aldehidov in ketonov, določenih s HPLC-FLD, v ajdovi pivini ter v ajdovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

aldehyde or ketone	buckwheat [µg/L]								
	TUM 34/70 fresh beverage	**TUM 34/70 force-aged beverage**	**TUM 34/70 naturally aged beverage**	**TUM 177 fresh beverage**	**TUM 177 force-aged beverage**	**TUM 177 naturally aged beverage**	**TUM SL17 fresh beverage**	**TUM SL17 force-aged beverage**	**TUM SL17 naturally aged beverage**
acetaldehyde	> ULR	> ULR	> ULR	> ULR	> ULR	> ULR	> ULR	> ULR	> ULR
2,3-butandion	26.07	58.07	121.74	35.94	82.86	117.93	49.63	116.13	135.4
propanal + furfural	70.06	58.08	39.56	30.95	21.96	64.87	7.53	16.13	35.4
2,3-pentanedione	147.42	180.78	202.39	119.14	141.06	171.81	70.53	116.13	135.4
butanal + + 2-methylpropanal	3350.18	946.38	742.69	1264.54	2860.06	1244.9	1597.26	1350.66	3471.47
phenylacetaldehyde + + 3-methylbutanal	478.66	3561.83	3188.53	2040.22	> ULR	> ULR	3186.47	> ULR	> ULR
hexanal	49.78	541.68	820.63	816.37	1286.68	2713.81	2732.37	2483.55	4234.72
heptanal	20.26	20.37	31.91	24.58		24.29	19.43	19.68	27.62
octanal	18.43	15.95	30.65	13.7	14.3	26.44	12.66	18.39	23.46
***trans*-2-nonenal**	0.19	2.06	9.96	0.25	2.06	21.46	0.81	2	6.02
sum	4161.05	5385.2	5188.06	4345.69	4408.98	4385.51	7676.69	4122.67	8069.49

ULR – the upper linear limit

Table 40: The aldehyde and ketone content of the quinoa wort and fresh and forcedaged beverages fermented with different yeast, determined by HPLC-FLD
Preglednica 40: Vsebnost aldehidov in ketonov, določenih s HPLC-FLD, v kvinojini pivini ter v kvinojinih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

aldehyde or ketone	quinoa [µg/L]								
	TUM 34/70 fresh beverage	**TUM 34/70 force-aged beverage**	**TUM 34/70 naturally aged beverage**	**TUM 177 fresh beverage**	**TUM 177 force-aged beverage**	**TUM 177 naturally aged beverage**	**TUM SL17 fresh beverage**	**TUM SL17 force-aged beverage**	**TUM SL17 naturally aged beverage**
acetaldehyde	> ULR	> ULR	> ULR	> ULR	> ULR	> ULR	> ULR	> ULR	> ULR
2,3-butandion	152.82	213.54	343.66	223.64	242.97	359.44	193.49	192.69	252.69
propanal + furfural	841.46	256.03	104.31	269.18	264.21	189.03	536.33	235.27	157.5
2,3-pentanedione	121.85	151.07	190.94	132.72	170.83	185.62	174.55	201.54	121.88
butanal + + 2-methylpropanal	158.85	2753.67	4101.35	149.24	2231.12	4168.09	3170.99	> ULR	> ULR
phenylacetaldehyde + + 3-methylbutanal	1357	3571.37	> ULR	2429.42	4569.26	4349.97	> ULR	> ULR	> ULR
hexanal	206.34	523.38	1221.74	180.91	623.09	1622.2	1236.85	4873.83	> ULR
heptanal	28.66	29.13	24.47	22.38	26.46	124.07	25.47	124.79	222.3
octanal	42.55	46.77	28.15	34.82	39.42	126.45	34.69	132.38	220.96
***trans*-2-nonenal**	2.23	27.88	1393.44	1.12	26.79	322.84	3.4	513.64	2115.72
sum	2911.76	7572.84	7408.06	3443.43	8194.15	11447.71	5375.77	6274.14	3091.05

ULR – the upper linear limit

GC-FID analysis

Figure 18 shows the GC-FID chromatogram of aldehydes and ketones in the quinoa beverage fermented with TUM 34/70. The concentrations of particular aldehydes and ketones for barley, buckwheat and quinoa samples are presented in Table 41, Table 42 and Table 43, respectively.

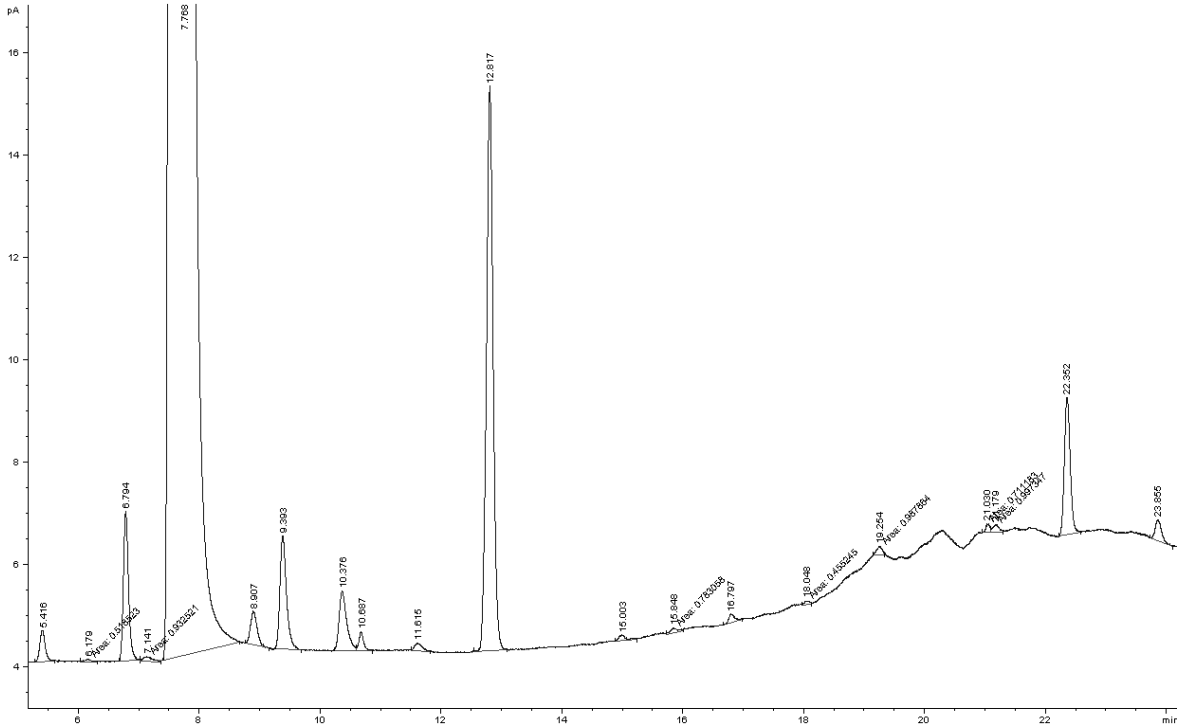

Figure 18: The sample GC-FID chromatogram of aldehydes and ketones in the quinoa beverage fermented with TUM 34/70

pA – detector response unit.

Slika 18: Primer GC-FID kromatograma aldehidov in ketonov v kvinojini pijači, fermentirani s TUM 34/70

pA – enota odziva detektorja.

Table 41: The aldehyde and ketone content of the barley wort and fresh and in forcedaged beverages fermented with different yeast, determined by GC-FID
Preglednica 41: Vsebnost aldehidov in ketonov, določenih z GC-FID, v ječmenovi pivini ter v ječmenovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

aldehyde or ketone	barley [mg/L]								
	TUM 34/70 fresh beverage	**TUM 34/70 force-aged beverage**	**TUM 34/70 naturally aged beverage**	**TUM 177 fresh beverage**	**TUM 177 force-aged beverage**	**TUM 177 naturally aged beverage**	**TUM SL17 fresh beverage**	**TUM SL17 force-aged beverage**	**TUM SL17 naturally aged beverage**
acetaldehyde	25.43	23.75	17.12	19.65	18.44	14.05	8.68	7.99	6.77
propanal	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2-methylpropanal	< LOD	0.87	1.25	< LOD	< LOD	0.43	0.6	2.26	2.79
butanal	0.99	1.63	1.83	0.24	1.46	1.78	1.03	1.01	1.2
3-methylbutanal	1.48	2.6	2.55	1.59	2.56	3.19	1.27	2.45	2.25
2,3-butandione	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2,3-pentanedione	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
hexanal	0.9	1.95	3.83	1.1	1.78	2.5	3.28	4.78	6.72
heptanal + heptanone	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
octanal + octanone	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
acetoin	0.3	< LOD	0.18	< LOD	0.25	0.88	1.16	1.07	0.37
nonanone	< LOD	< LOD	3.37	< LOD	3.5	3.58	< LOD	< LOD	< LOD
furfural	0.35	1.24	< LOD	0.39	0.37	< LOD	0.26	0.27	< LOD
***trans*-2-nonenal**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
pentylfuran	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
phenylacetaldehyde	0.68	0.53	0.65	0.69	0.65	0.47	0.68	0.56	0.67
5-hydroxymethylfurfural	< LOD	< LOD	0.96	< LOD	< LOD	2.61	< LOD	< LOD	0.1
sum	30.13	32.57	31.74	23.66	29.01	29.49	16.96	20.39	20.87

LOD – lower limit of detection. LLQ – lower limit of quantification.

Table 42: The aldehyde and ketone content of the buckwheat wort and fresh and in forcedaged beverages fermented with different yeast, determined by GC-FID
Preglednica 42: Vsebnost aldehidov in ketonov, določenih z GC-FID, v ajdovi pivini ter v ajdovih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

aldehyde or ketone	buckwheat [mg/L]								
	TUM 34/70 fresh beverage	TUM 34/70 force-aged beverage	TUM 34/70 naturally aged beverage	TUM 177 fresh beverage	TUM 177 force-aged beverage	TUM 177 naturally aged beverage	TUM SL17 fresh beverage	TUM SL17 force-aged beverage	TUM SL17 naturally aged beverage
acetaldehyde	22.21	21.76	21.35	32.86	33.14	28	27.12	22.52	17.53
propanal	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2-methylpropanal	< LOD	< LOD	3.36	< LOD	< LOD	1.06	< LOD	< LOD	1.73
butanal	< LOD	1.11	0.97	1.27	2.65	0.99	0.46	1.26	1.81
3-methylbutanal	< LOD	3.06	2.4	1.84	4.2	7.14	2.69	6.24	10.18
2,3-butandione	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2,3-pentanedione	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
hexanal	0.05	0.42	0.92	0.82	1.36	3.25	2.01	2.58	4.68
heptanal + heptanone	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
octanal + octanone	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
acetoin	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	0.44	0.24	0.1
nonanone	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
furfural	< LLQ	< LLQ	< LOD	< LLQ	< LLQ	< LOD	< LLQ	< LLQ	< LOD
***trans*-2-nonenal**	< LOD	< LOD	0.89	< LOD	< LOD	0.84	< LOD	< LOD	0.93
pentylfuran	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
phenylacetaldehyde	< LOD	0.65	0.72	< LOD	0.69	0.6	0.54	0.66	0.63
5-hydroxymethylfurfural	< LOD	< LOD	0.92	< LOD	< LLQ	1.38	< LOD	< LLQ	1.12
sum	22.26	27	31.53	36.79	42.04	43.26	33.26	33.5	38.71

LOD – lower limit of detection. LLQ – lower limit of quantification.

Table 43: The aldehyde and ketone content of the quinoa wort and fresh and in forcedaged beverages fermented with different yeast determined by GC-FID
Preglednica 43: Vsebnost aldehidov in ketonov, določenih z GC-FID, v kvinojini pivini ter v kvinojinih svežih in pospešeno staranih pijačah, fermentiranih z različnimi kvasnimi sevi

aldehyde or ketone	quinoa [mg/L]								
	TUM 34/70 fresh beverage	**TUM 34/70 force-aged beverage**	**TUM 34/70 naturally aged beverage**	**TUM 177 fresh beverage**	**TUM 177 force-aged beverage**	**TUM 177 naturally aged beverage**	**TUM SL17 fresh beverage**	**TUM SL17 force-aged beverage**	**TUM SL17 naturally aged beverage**
acetaldehyde	44.32	41.39	32.82	25.93	27.54	21.81	15.53	14.65	13.89
propanal	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2-methylpropanal	< LOD	2.4	1.23	< LOD	1.77	< LOD	1.21	2.1	1.47
butanal	< LOD	0.77	3.45	< LOD	< LOD	3.93	2.56	3.92	10.01
3-methylbutanal	1.45	2	3.93	2.19	3.11	2.3	4.77	6.52	10.15
2,3-butandione	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2,3-pentanedione	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
hexanal	< LOD	0.66	1.25	< LOD	0.76	1.82	12.61	5.22	10.46
heptanal + heptanone	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
octanal + octanone	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
acetoin	4.05	3.16	5.28	5.44	2.43	7.02	5.8	3.63	8.42
nonanone	< LOD	< LOD	3.47	< LOD	3.55	3.53	3.39	3.53	3.5
furfural	0.71	1.28	< LLQ	0.23	1.21	< LLQ	0.52	1.24	< LLQ
***trans*-2-nonenal**	< LOD	< LOD	1.63	< LOD	< LOD	< LOD	< LOD	< LOD	1.97
pentylfuran	10.31	2.46	5.62	8.72	5.6	1.79	17.37	6.7	4.67
phenylacetaldehyde	0.34	1.53	1.48	0.43	1.51	1.6	0.12	1.45	1.42
5-hydroxymethylfurfural	0.64	< LLQ	1.15	0.45	< LLQ	1.4	0.56	< LLQ	1.15
sum	61.82	55.65	61.31	43.39	47.48	45.2	64.44	48.96	67.11

LOD – lower limit of detection. LLQ – lower limit of quantification.

4.1.8 Sensory analysis

Flavour is defined as "the sum of perceptions resulting from stimulation of the sense ends that are grouped together at the entrance of the alimentary and respiratory tracts". Sensory attributes were organoleptically assessed by a trained panel of eight evaluators. Duplicates of each beverage were assessed separately and the final marks were calculated as an average value (n = 16). Common features od sensory analysis regardless the yeast used were exactly the same marks for odour and sparkling of buckwheat beverages and the same marks for palatefulnes of quinoa beverages (Figure 19).

TUM 34/70

The overall mark, calculated from equation (2), of buckwheat was slightly better (3.83) than for quinoa (3.60) and the marks for particular sensory attribute are shown on Figure 19. Having in mind that the same hoping regime was used, the differences regarding bitterness should be caused by some other bitter tasting molecules, such as polyphenols, proteins, magnesium, sulphate and/or some amino acids (Rousef, 1990). The retronasal odour perception was surprisingly similar, despite the fact that the majority of volatile compounds commonly recognized as pleasant were lower in the quinoa beverage, together with a high level of acetaldehyde and ethyl acetate (Table 19, Table 20 and Table 21). However, the quinoa beverage contained other volatile compounds not found in buckwheat, presumably pyrazines, which could improve its odour perception. The worst grade for the quinoa beverage was granted for its purity of taste and the best grade for palatefulness, which was also the only attribute quinoa scored better than buckwheat. The taste of the quinoa beverage was probably impaired because of the high content of metal cations. High level of amino acids could also impact the taste perception; for instance, L-arginine and L-glutamine are known to give a salty taste (Briggs *et al*., 2004). The buckwheat beverage caused a more variable perception than quinoa, with the exception of the purity of taste (Figure 19). Regarding buckwheat, evaluators were the most consistent about the purity of taste and the least about the sparkling and the quality of bitterness. For quinoa the highest consistency was observed regarding the odour and far the lowest regarding the purity of taste.

TUM 177

The buckwheat beverage fermented with this yeast received practically the same marks of all quality attributes that the beverage fermented with TUM 34/70. The overall marks (3.82) were also very similar as well as were standard deviations of quality attributes. In the case of quinoa, this beverage was rated much better than that fermented with TUM 34/70 which is further supported by a higher overall mark (3.71). Compared to TUM 34/70, evaluators were much less consistent regarding the odour and more consistent regarding the purity of taste.

TUM SL17

From the sensory point of view this yeast proved as the most suitable for the production of beer-like beverages from buckwheat and quinoa. Overall marks of buckwheat (3.89) and quinoa (3.80) beverages were the highest and also comparable between each other. It seems that this yeast positively influenced the perception of the quality of bitterness and the purity of taste regardless the raw material. Compared to other two yeast strains, odour and palatefulness were perceived much more inconsistently in both buckwheat and quinoa whereas the perception of sparkling was much more firm.

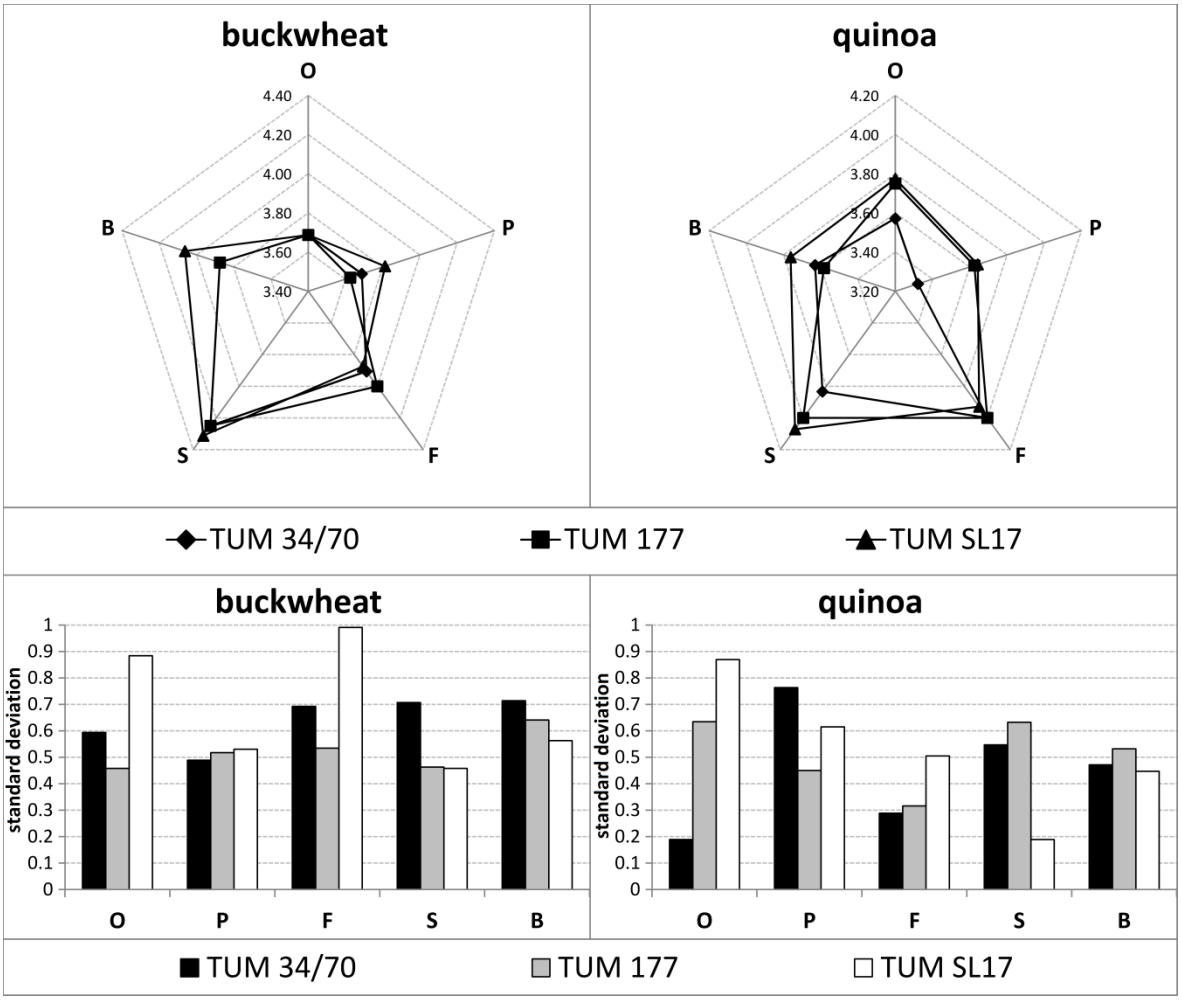

Figure 19: Radar charts of five quality attributes and bar charts of their standard deviation for buckwheat and quinoa beverages
O – odour. P – purity of taste. F – palatefulness. S – sparkling. B – quality of bitterness.
Slika 19: Mrežni diagram petih senzoričnih attributov in stolpični diagram njihovih standarnih deviacij za ajdove in kvinojine pijače.
O – vonj. P – čistost okusa. F – polnost okusa. S – občutek ščemenja. B – kvaliteta grenčice.

4.2 SERIAL REPITCHING OF *Saccharomyces pastorianus* TUM 34/70

4.2.1 The analysis of yeast

Viable yeast cells were counted under the microscope using the methylene blue test and their number per milliliter was calculated. The pitching volume was adjusted to achieve a final yeast cell concentration in the fermentation vessel of 2×10^6 cells/mL (Table 44).

Table 44: The characteristics of successive fermentations of the barley, buckwheat and quinoa wort
Preglednica 44: Značilnosti zaporednih fermentacij ječmenove, ajdove in kvinojine pivine

wort type	viable yeast cell count [×106 cells/mL]	pitching volume [mL]
barley	195.63	102
	88.59	226
	83.59	239
	93.65	214
	75.52	265
	73.02	274
	86.30	232
	68.02	294
	78.02	256
	86.46	231
	84.06	238
buckwheat	199.69	100
	33.44	598
	45.31	441
	62.03	322
	65.78	304
	64.84	308
	91.25	219
	87.97	227
	73.91	271
	76.25	262
	72.97	274
quinoa	204.22	98
	29.38	681
	31.88	627
	33.44	598
	35.78	559
	33.75	593
	39.84	502
	37.81	529
	32.50	615
	41.72	479
	49.69	403

F – the number of successive fermentation (F1F11).

4.2.1.1 Yeast karyotype

We compared the electrophoretic karyotype banding patterns of chromosomal DNA of an industrial allopolyploid yeast *S. pastorianus* strain TUM 34/70 during eleven successive fermentations of barley (Figure 20), buckwheat (Figure 21), and quinoa (Figure 22) wort.

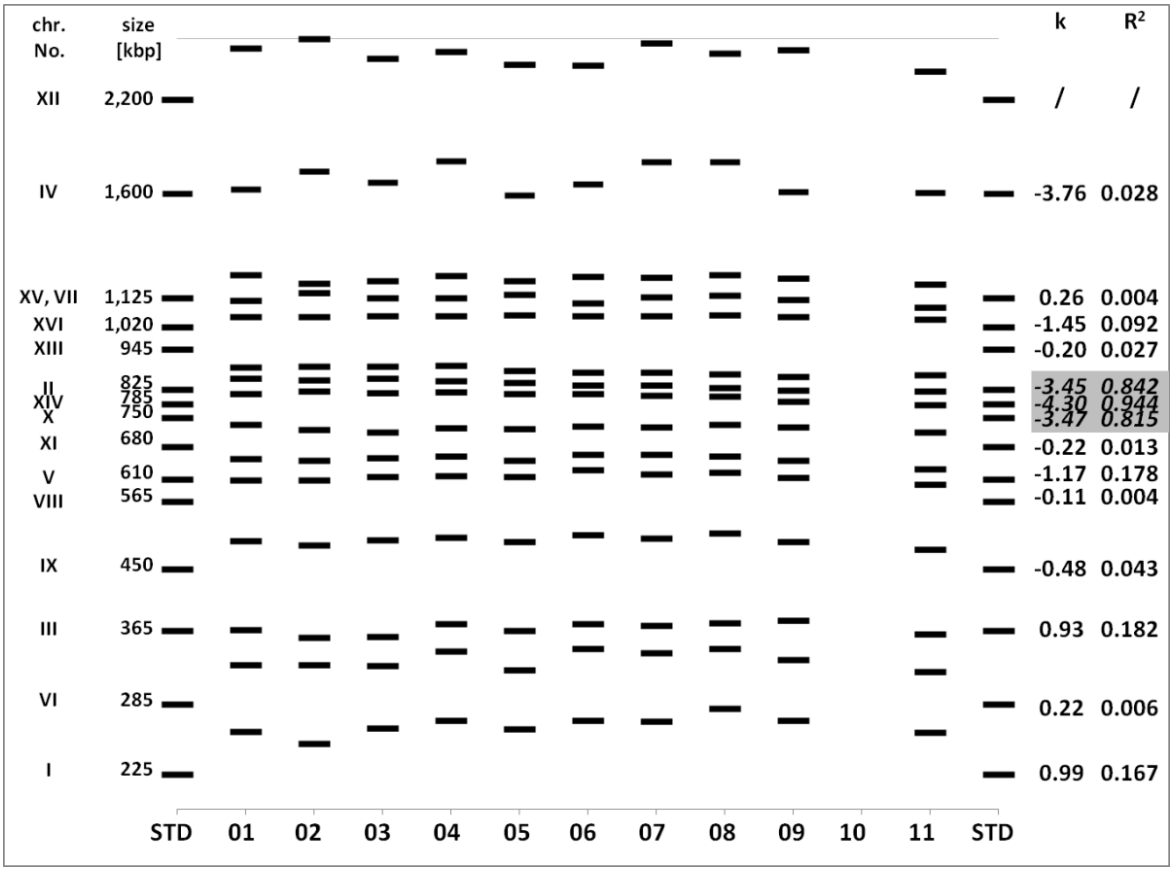

Figure 20: The schematic representation of karyotype banding patterns of *S. pastorianus* TUM 34/70 chromosomal DNA during eleven successive fermentations of the barley wort
STD: CHEF DNA Size Standard (*S. cerevisiae*). 01 – 11: the numbering of successive fermentation cycles. chr. No.: The number of *S. cerevisiae* chromosomes represented by a corresponding band on STD lane. k and R^2: the slope and correlation coefficient of linear regression curve for each chromosome band, respectively. Significant k and R^2 values are written in italics and highlighted.
Slika 20: Shematski prikaz vzorcev kariotipa kromosomske DNA *S. pastorianus* TUM 34/70 tekom enajstih zaporednih fermentacij ječmenove pivine
STD: CHEF DNA velikostni standard (*S. cerevisiae*). 01 – 11: številčenje posameznih zaporednih fermentacij. chr. No.: številka kromosoma *S. cerevisiae*, ki ga predstavlja posamezna črta na STD liniji. k in R^2: naklon in koeficient korelacije linearne regresijske krivulje za posamezen kromosom. značilne k in R^2 vrednosti so napisane poševno in osenčene.

A schematic representation of results was preferred to assure clear and informative data presentation since many PFGE runs of two independent isolation steps were performed and a few photos at different light exposure were took to achieve the maximal resolution for the quantification of every single chromosome band. However, a representative gel photo of barley samples handled with Bionumerics computer software is available (Figure 23).

Among 36 different types of chromosomes of the strain TUM 34/70 (Nakao *et al.*, 2009), 15 distinct bands were separated by pulsed-field electrophoresis which is a common number for such analysis. The band pattern of our samples is close to that of *S. cerevisiae*, however, the average size of sample bands is higher from the latter between for 5 and 10%, similar to findings of Tamai *et al.* (1998). What all three sets of results have also in common is the non-reproducible nature of the band at 2200 kb, a situation that has already been reported by Carle and Olson (1985). In addition, because our samples were collected from real fermentation media, they contained some particulate hop matter and other wort-derived impurities which could influence the quality and resolution of the results obtained. This circumstance was carefully taken into account during the data interpretation.

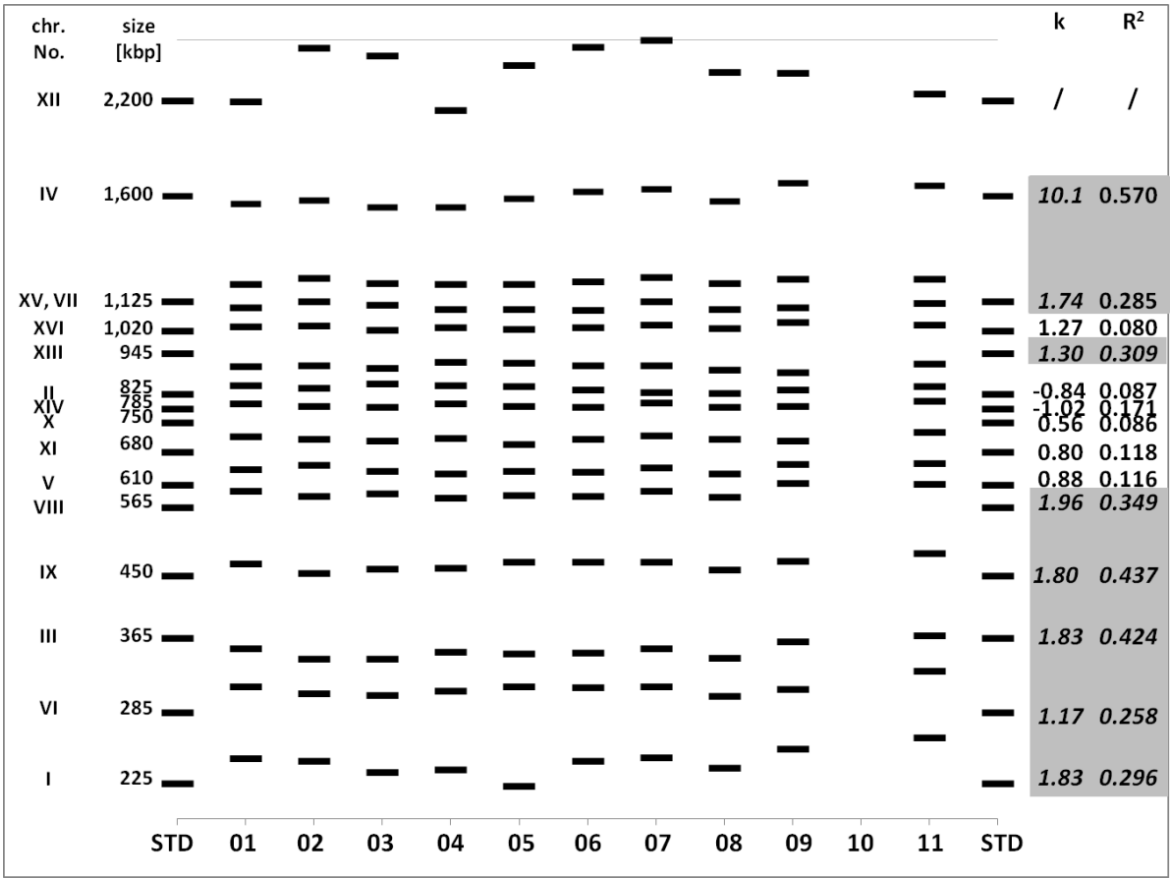

Figure 21: The schematic representation of karyotype banding patterns of *S. pastorianus* TUM 34/70 chromosomal DNA during eleven successive fermentations of the buckwheat wort
STD: CHEF DNA Size Standard (*S. cerevisiae*). 01 – 11: the numbering of successive fermentation cycles. chr. No.: The number of *S. cerevisiae* chromosomes represented by a corresponding band on STD lane. The slope and correlation coefficient of linear regression curve for each chromosome band, respectively. Significant k and R^2 values are written in italics and highlighted.
Slika 21: Shematski prikaz vzorcev kariotipa kromosomske DNA *S. pastorianus* TUM 34/70 tekom enajstih zaporednih fermentacij ajdove pivine
STD: CHEF DNA velikostni standard (*S. cerevisiae*). 01 – 11: številčenje posameznih zaporednih fermentacij. chr. No.: številka kromosoma *S. cerevisiae*, ki ga predstavlja posamezna črta na STD liniji. k in R^2: naklon in koeficient korelacije linearne regresijske krivulje za posamezen kromosom. značilne k in R^2 vrednosti so napisane poševno in osenčene.

In this manner, we were primarily interested in the overall tendency of a single chromosome size change during successive fermentations rather than in chromosome absolute size or in chromosome size difference between adjacent fermentations. Linear regression analysis has been conducted where the slope value (k) revealed the direction and magnitude of chromosome size change and correlation coefficient (R^2) indicated its consistency. Absolute k values below 1.00 and R^2 values below 0.20 were not considered significant. Because of the above-mentioned non-reproducible nature of the high-molecular (HMW) band of 2200 kbp, it was not included in the further analysis.

Average values of k and R^2 showed net decrease in chromosome size in the case of barley ($k = 1.16$, $R^2 = 0.24$) and quinoa ($k = 1.46$, $R^2 = 0.43$) wort fermentation, contrary to the net increase in the case of buckwheat ($k = 1.67$, $R^2 = 0.26$). In barley, only three middle-molecular weight (MMW) chromosomes (~800, ~830 and ~870 kbp) showed significant ($3.45 < k < 4.30$) and highly consistent ($R^2 > 0.81$) size change (Figure 20).

In buckwheat, no significant decrease of chromosome size was observed but there was a slight increase ($1.17 < k < 1.96$) with low consistency ($0.26 < R^2 < 0.44$) of all low-molecular weight (LMW) chromosomes (200-600 kbp) (Figure 21). In addition, one MMW (1,030 kbp) and two (or three) HMW chromosomes (1,190-1,670 kbp) also increased in size with the similar rate ($1.30 < k < 1.74$) and low consistency ($0.28 < R^2 < 0.31$) with the obvious exception of chromosome IV. Its increase rate was the highest in this study ($k = 10.11$) although the consistency of data is medium ($R^2 = 0.57$).

Regarding quinoa, only four chromosomes did not significantly change in size (Figure 22). The increase of three smallest LMW chromosomes (250-400 kbp) was moderate ($1.69 < k < 2.47$) with low data consistency ($0.23 < R^2 < 0.33$). All other seven (or eight) chromosomes (780-1,660 kbp) became smaller with moderate rate ($2.85 < k < 8.45$) with the exception of chromosome XIII ($k = 1.43$). The data consistency of this increase is medium to high ($0.57 < R^2 < 0.83$) with the exception of chromosome IV ($R^2 = 0.36$). Overall, none of the chromosomes significantly changed in size in all three cases and only two chromosomes (V and XI) did not changed at all.

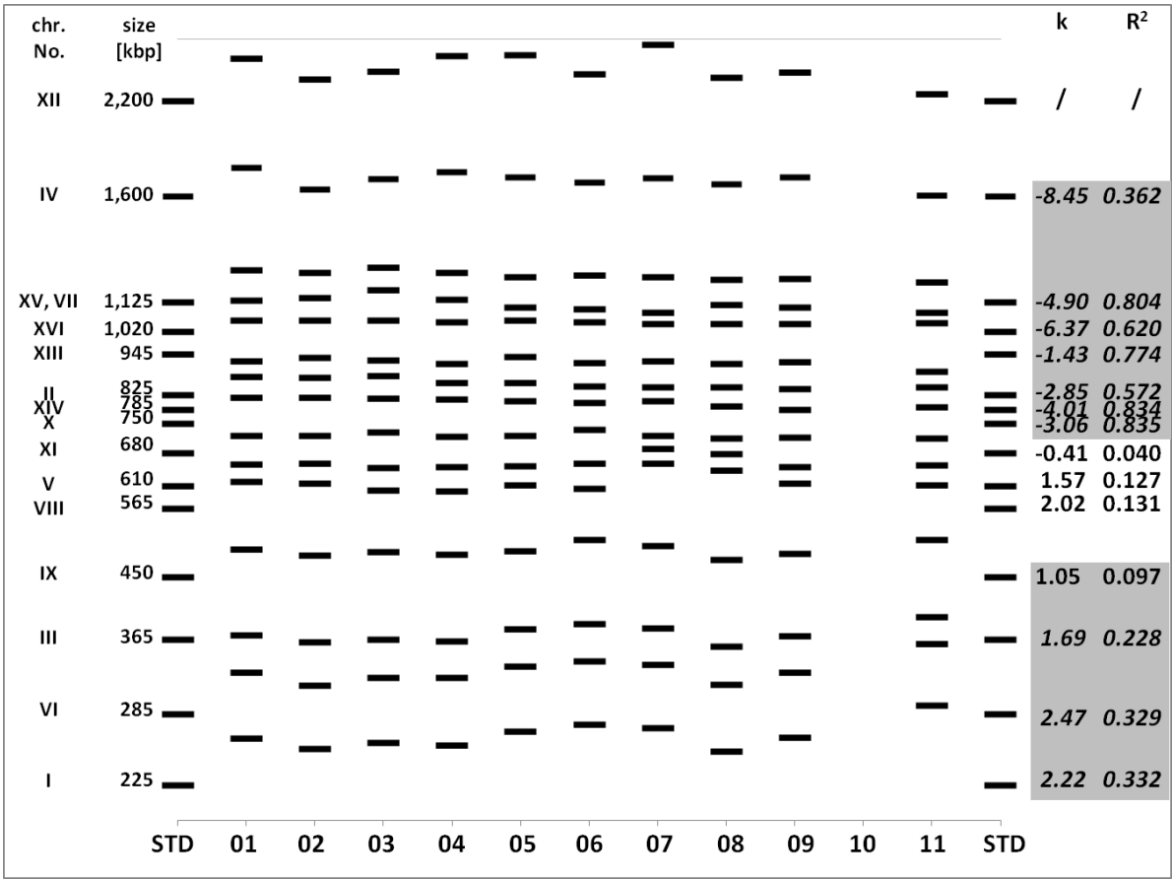

Figure 22: The schematic representation of karyotype banding patterns of *S. pastorianus* TUM 34/70 chromosomal DNA during eleven successive fermentations of the quinoa wort
STD: CHEF DNA Size standard (*S. cerevisiae*). 01 – 11: the numbering of successive fermentation cycles. chr. No.: The number of *S. cerevisiae* chromosomes represented by a corresponding band on STD lane. k and R^2: the slope and correlation coefficient of linear regression curve for each chromosome band, respectively. Significant k and R^2 values are written in italics and highlighted.
Slika 22: Shematski prikaz vzorcev kariotipa kromosomske DNA *S. pastorianus* TUM 34/70 tekom enajstih zaporednih fermentacij kvinojine pivine
STD: CHEF DNA velikostni standard (*S. cerevisiae*). 01 – 11: številčenje posameznih zaporednih fermentacij. chr. No.: številka kromosoma *S. cerevisiae*, ki ga predstavlja posamezna črta na STD liniji. k in R^2: naklon in koeficient korelacije linearne regresijske krivulje za posamezen kromosom. značilne k in R^2 vrednosti so napisane poševno in osenčene.

Up to the present, the influence of different raw materials on the yeast karyotype was practically not considered important and exactly for this reason our results, which show that changes in yeast karyotype are in fact wort-dependent, represents an important contribution to this poorly understood relation between the wort type and yeast karyotype. The chemical composition of wort could have either a direct influence or, more likely, an indirect one, probably via changes in enzyme activity or protein expression in general. Deletions and insertions are the most common reasons for the substantial change in chromosome size but they result in a discrete size pattern which persists for a certain amount of time. On the contrary, gradual decreases (Figure 20 and Figure 22) in chromosome size observed in our case could thus be explained by the telomere hypothesis which suggests that the number of cell divisions is registered by the gradual loss of

telomeric sequences (Chiu and Harley, 1997) and may act as a biological clock determining the number of divisions prior to senescence. On the other hand, eukaryotic organisms possess telomerase enzyme which adds telomeric DNA repeats to the ends of linear chromosomes in order to elongate the telomere. Moreover, telomerase can also bind and extend non-telomeric DNA by adding telomeric repeats *de novo* (Sýkorová and Fajkus, 2009), a situation which could explain the gradual increase in chromosome size we observed (Figure 21 and Figure 22).

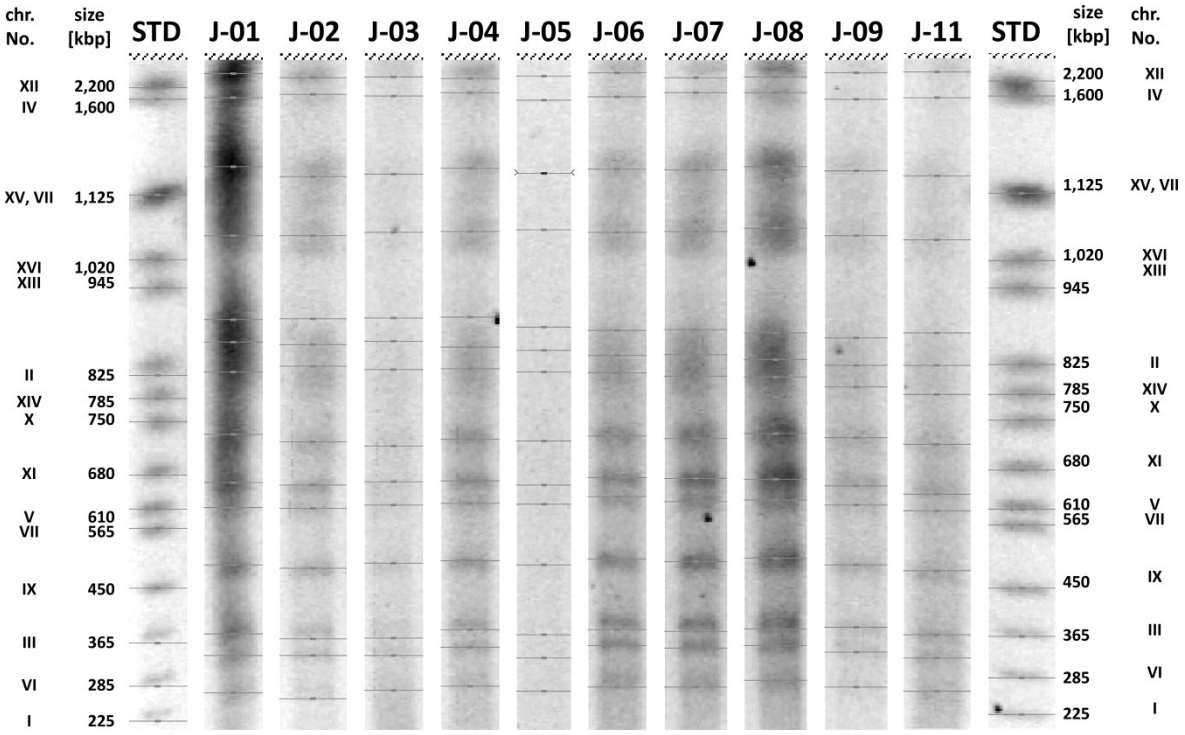

Figure 23: Karyotype banding patterns of *S. pastorianus* TUM 34/70 chromosomal DNA during eleven successive fermentations of the barley wort

The figure is a combination of karyotype lanes obtained from different runs of two independent isolation steps. STD: CHEF DNA Size Standard (*S. cerevisiae*). J01 – J11: numbering of successive fermentations. chr. No.: The number of *S. cerevisiae* chromosomes represented by a corresponding band on STD lane.

Slika 23: Shematski prikaz vzorcev kariotipa kromosomske DNA *S. pastorianus* TUM 34/70 tekom enajstih zaporednih fermentacij ječmenove pivine

STD: CHEF DNA velikostni standard (*S. cerevisiae*). J01 – J11: številčenje posameznih zaporednih fermentacij. chr. No.: številka kromosoma *S. cerevisiae*, ki ga predstavlja posamezna črta na STD liniji.

Any changes in subtelomeric regions are noteworthy since these parts of chromosomes contain many important genes for beer brewing, e.g., genes involved in maltose fermentation, genes of lectin-like protein involved in flocculation (chromosomes I, V, VII and XI), glycolytic enzyme gene HXK1 (chromosome VI), genes involved in sulfite production pathway (chromosomes II, IV, X, XI, XII and XVI), polymeric melibiase genes (Naumov *et al*., 1995; 1996), and others (Cousseau *et al.*, 2012; Nakao *et al.*, 2009).

4.2.1.2 Yeast protein profile

Three SDSPAGE were performed separately for eleven successive fermentations of barley (Figure 24A), buckwheat (Figure 24B), and quinoa (Figure 24C) worts. Although the sample loading volumes were adjusted according to the Bradford assay to reach 7 µg/lane, the total values (Figure 24), calculated from equation (3), differed considerably between particular lanes, i.e. from 2,272 to 13,361 µg/g (barley samples), from 3,064 to 17,045 µg/g buckwheat samples), and from 2,343 to 13,489 µg/g (quinoa samples). In that manner, we calculated the concentration of each protein relative to the total concentration of proteins in a corresponding lane. Poorly separated protein bands were considered as a group and their sum concentrations were presented together with their molecular weight span (Figure 25).

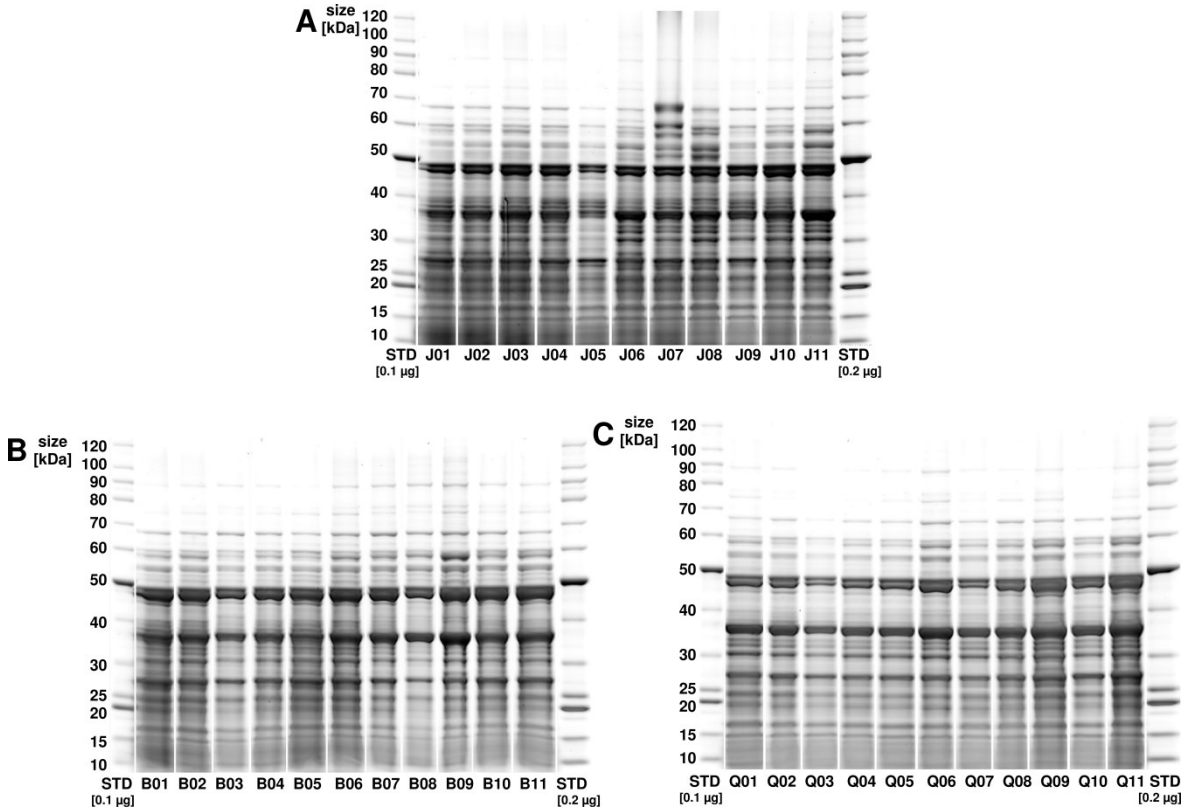

Figure 24: SDS-PAGE protein profile analysis of *S. pastorianus* TUM 34/70 during eleven successive fermentations of the (A) barley, (B) buckwheat and (C) quinoa wort

STD: BenchMark™ Protein Ladder at different loadings. 01-11: the numbering of successive fermentation cycles.

Slika 24: NaD-SPAGE proteinski profil *S. pastorianus* TUM 34/70 tekom enajst zaporednih fermentacij (A) ječmenove, (B) ajdove in (C) kvinojine pivine

STD: BenchMark™ Protein Ladder velikostni standard pri različnih nanosih. 01-11: številčenje posameznih zaporednih fermentacij.

At first we were interested whether there are some general differences comparing buckwheat and quinoa with barley samples (Figure 25). LMW (8.0-14.0 kDa) and HMW proteins (73.8, 76.5 and 87.3 kDa) showed two- to three-fold higher average expression in buckwheat and quinoa wherein the 76.5 kDa protein band was not detected in barley. On the other hand, some MMW proteins were expressed to a lower degree in both

pseudocereals (28.5, 29.4, 37.1, 38.3 and 51.2 kDa) or only in buckwheat (26.3 kDa) compared to barley. The most obvious difference is only ~10% expression of 38.3 kDa protein band in buckwheat in quinoa relative to barley.

Next we were curious whether there are some common patterns of changes in protein profile during successive fermentation cycles (Figure 25). For each protein band the calculation of coefficient of variation (CV), slope of linear regression (k), coefficient of determination (R^2), and Pearson produc-tmoment correlation coefficient (P) has been determined to evaluate the changes during successive fermentations. In all three cases CV increased with the increasing molecular weight in a similar way which means that the larger the proteins of the same size, the more variable were their relative expressions over successive fermentations (from 0.2 to 1.0, data not shown). Besides, the total portion of quantified proteins slowly decreases in barley and buckwheat but markedly in quinoa, which means that total protein synthesis declines during successive fermentation cycles (data not shown).

From the P value for particular protein bands, it is possible to estimate how the tendency of its relative expression is similar between raw materials (Table 45). Protein bands were grouped into three classes: (i) negative correlation (NC, $1.0 < P < 0.3$), (ii) no significant correlation (NSC, $0.3 < P < 0.3$) and (iii) positive correlation (PC, $0.3 < P$ 1.0). Firstly, comparison of barley and buckwheat wort fermentation revealed more or less equal distribution of proteins based on P value (8 NC, 9 NSC and 9 PC). Secondly, comparison of barley and quinoa showed the predomination of NSC protein bands (18), the number of NC protein bands is similar to barley and buckwheat comparison and only one showed a positive correlation. Finally, comparison of buckwheat and quinoa samples also demonstrated the predomination of NSC (18) followed by seven PC and only two NC protein bands. These results clearly show that the relative protein expression in successive fermentation cycles of quinoa wort has practically no positive correlation with barley whereas it has practically no negative correlation with buckwheat.

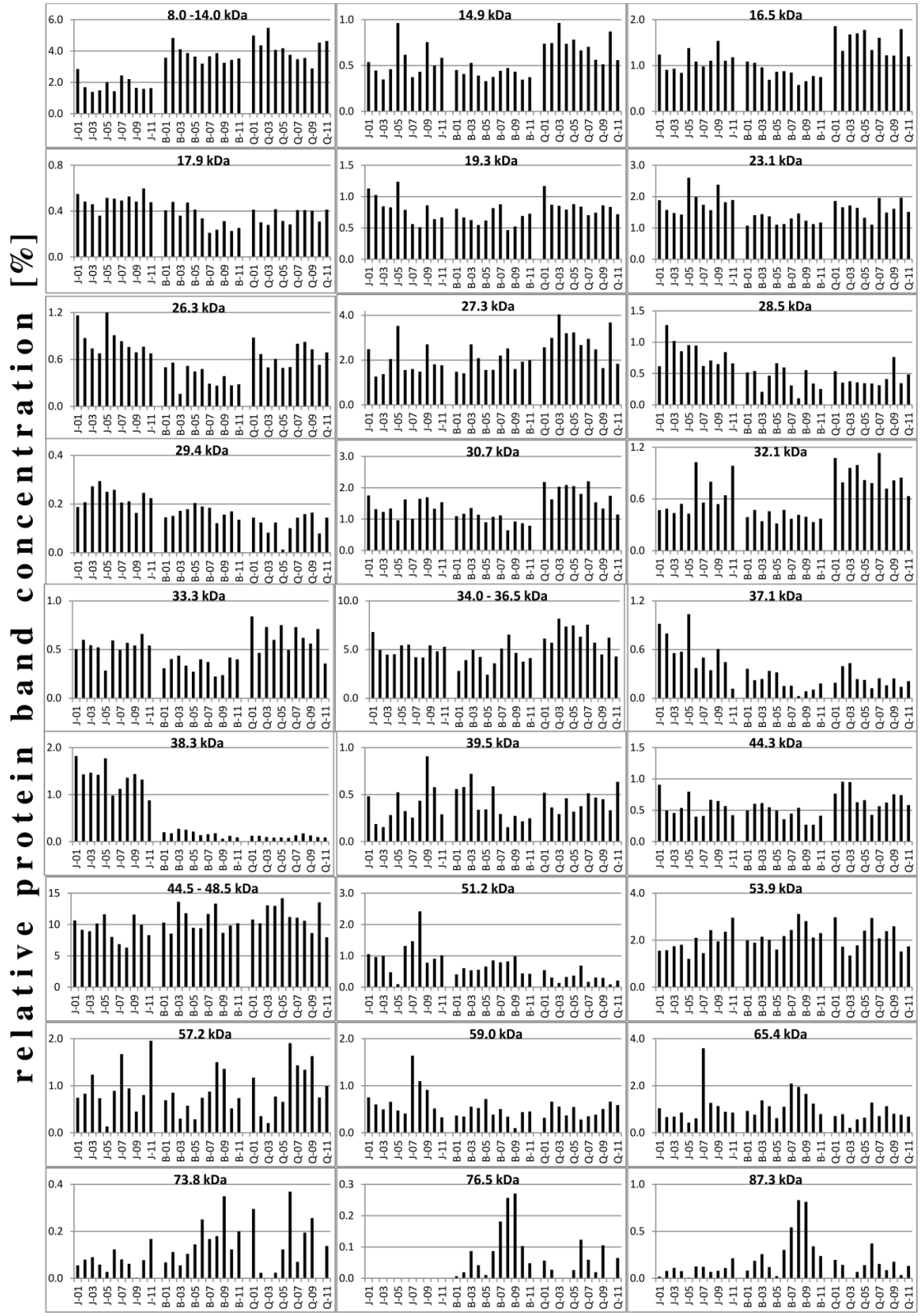

Figure 25: Relative concentrations of protein bands during eleven successive fermentations of different worts J01 – J11, B01 – B11, Q01 – Q11: successive fermentation cycles of the barley, buckwheat and quinoa wort fermentation, respectively.
Slika 25: Relativne koncentracije proteinskih lis tekom enajst zaporednih fermentacij različnimi pivine J01 – J11, B01 – B11, Q01 – Q11: posamezne fermentacije ječmenove, ajdove in kvinojine pivine.

Table 45: The correlation of relative protein expression between different worts derived from barley, buckwheat and quinoa expressed as the number of protein bands that corresponds to the particular P value span.
Preglednica 45: Korelacija v relativni ekspresiji proteinov pri zaporednih fermentacijah ječmenove, ajdove in kvinojine pivine, izražena kot število proteinskih lis, ki ustrezajo določenem velikostnem območju P vrednosti

class name	Pearson product-moment correlation coefficient value span	barley-buckwheat	barley-quinoa	buckwheat-quinoa
NC	(1 < P < 0.3)	8	8	2
NSC	(0.3 < P < 0.3)	9	17	18
PC	(0.3 < P < 1)	9	1	7
	sum	26	26	27

NC – negative correlation; NSC – no significant correlation; PC – positive correlation

On the basis of k and R^2 values, we distinguished protein bands according to their change in relative concentration as increasing ($k > 0$) or decreasing ($k < 0$), both either steadily ($R^2 > 0.40$) or unsteadily ($R^2 < 0.40$), from all the others which showed an uniform but oscillating expression over successive fermentation cycles. For most of the latter, it was possible to determine an oscillating interval (OI) (1, 2, 3, etc.) that expresses the number of successive fermentations after which the relative concentration profile is repeated. Those with the OI 1, 2 or 3 were termed as rapidly changing and the others as slowly changing (Table 46).

In barley, the relative concentrations of six protein bands were decreasing (Figure 25, Table 46). Three of them (19.3, 26.3 and 37.1 kDa) showed a steady tendency of change and three (38.3, 44.3 and 44.5-48.5 kDa) were not. Four were increasing; two (32.1 and 53.9 kDa) steadily and two (39.5 and 87.3 kDa) unsteadily. Other protein bands showed uniform long-term expression although the relative concentration of some oscillated slowly (14.9, 16.5, 23.1, 27.3, 51.2, 57.2, 59.0 and 76.5 kDa) or rapidly (34.0-36.5 kDa). The situation with buckwheat wort fermentation cycles was more dynamic. Four protein bands (16.5, 17.9, 26.3 and 30.7 kDa) were decreasing steadily and five (28.5, 37.1, 38.3, 39.5 and 44.3 kDa) unsteadily. No steady increase of relative concentration was observed but an unsteady increase was noted in two cases (34.0-36.5 kDa and 73.8). Compared to barley, a similar number of protein bands oscillated slowly (14.9, 19.3, 23.1, 51.2, 76.5 and 87.3 kDa) but a lot more rapidly (27.3, 32.1, 33.3, 44.5-48.5, 57.2, 59.0 and 65.4 kDa). Dynamic change of relative concentrations is also present in quinoa wort fermentation. The frequency of unsteady decrease (27.3, 32.1, 33.3, 34.0-36.5, 51.2 and 53.9 kDa) is similar to steady one (14.9, 16.5, 19.3, 30.7 and 44.5-48.5 kDa) and no increase was observed. In addition, rapid changes were also prominent in other protein bands (17.9, 26.3, 29.4, 37.1, 39.5, 44.3, 73.8, 76.5 and 87.3 kDa) whereas, only three protein bands changed slowly over successive fermentations (38.3, 59.0, 65.4 kDa). In overview, not even one protein band was equally characterized in all three cases. Steadily decreasing protein bands were mainly LMW proteins (< 26.3 kDa) and only those between 27.0 and 57.0 kDa were decreasing unsteadily. All slowly oscillating protein bands (OI ≥ 4) were smaller than 28

and larger than 51 kDa (with the only one exception) whereas rapidly oscillating ones were of different molecular mass and only once present in barley wort fermentation.

Table 46: The representation of protein bands categorization based on their oscillating interval (OI), slope of linear regression (k) and coefficient of determination (R^2) together with their candidate proteins
Tabela 46: Razporeditev proteinskih lis na osnovi njihovega oscilirajočega interval (OI), naklona linearne regresijske premice (k) in koeficienta determinacije (R^2), skupaj z njihovimi kandidatnimi protein

protein band size [kDa]	barley	buckwheat	quinoa	candidate proteins*
8.0-14.0	0	0	0	n.r.
14.9	FS	FS	DS	Mtc3p
16.5	FS	DS	DS	Mtc7p
17.9	0	DS	FR	Rtt102p
19.3	DS	FS	DS	n.r.
23.1	FS	FS	0	n.r.
26.3	DS	DS	FR	Nrg1p
27.3	FS	FR	DU	n.r.
28.5	0	DU	0	Iml3p
29.4	0	0	FR	n.r.
30.7	0	DS	DS	Met16p, Thp2p, Cin2p, Oxr1p
32.1	IS	FR	DU	n.r.
33.3	0	FR	DU	n.r.
34.0-36.5	FR	IU	DU	n.r.
37.1	DS	DU	FR	Adh1p, Adh2p, Uth1p, Cdc10p
38.3	DU	DU	FS	Gre2p, Arv1p, Spt2p, Spt3p, Reg2p
39.5	IU	DU	FR	n.r.
44.3	DU	DU	FR	n.r.
44.5-48.5	DU	FR	DS	n.r.
51.2	FS	FS	DU	Pub1p, Bna1p, Gcn5p
53.9	IS	0	DU	n.r.
57.2	FS	FR	FR	n.r.
59.0	FS	FR	FS	n.r.
65.4	0	FR	FS	n.r.
73.8	FS	IU	FR	Mal11p, Kar9pCdc45p
76.5	0	FS	FR	Pso2p, Ufo1p, Bdf1
87.3	IU	FS	FR	Gef1p
DS	3	4	5	n.r.
DU	3	5	6	n.r.
IS	2	0	0	n.r.
IU	2	2	0	n.r.
FS	8	6	3	n.r.
FR	1	7	10	n.r.
0	7	2	2	n.r.

*candidate proteins involved in fermentation performance, yeast stress response, viability or genomic stability. n.r. – not relevant. DS − steady decrease ($k < 0.03$, $R^2 > 0.40$); DU − unsteady decrease ($k < 0.03$, $R^2 < 0.40$); IS − steady increase ($k > 0.03$, $R^2 > 0.40$); IU − unsteady increase ($k > 0.03$, $R^2 > 0.40$); FS − slow fluctuation ($0.03 \leq k \leq 0.03$, $OI \geq 4$); FR − rapid fluctuation ($0.03 \leq k \leq 0.03$, $OI < 4$); 0 – ($0.03 \leq k \leq 0.03$, no OI).

From the brewing technology point of view, proteins that directly influence fermentation properties are of principal importance. They are mainly expression products of afore-mentioned genes related to lager brewing yeast characteristics, i.e. proteins involved in maltose fermentation, proteins of sulfite production pathway, proteins involved in flocculation, and others (Cousseau *et al.*, 2012; Nakao *et al.*, 2009; Naumov *et al.* 1995;

1996). In addition, the expression of any protein involved in yeast viability, vitality, senescence, cell cycle regulation or genomic stability maintenance should also be considered when evaluating the yeast suitability for serial repitching. From the determined molecular size of protein bands, possible candidate proteins were proposed based on the number of amino acid residues, calculated from equation (4), and protein information from the SGD database (Cherry *et al.*, 2012).

Among 1500 possible candidate proteins, the most interesting are listed in Table 46. First, focus has been given to proteins related to lager beer characteristics and those specific for lager brewing yeast (Nakao *et al.*, 2009). Among proteins involved in the metabolism of some sugars (protein products of genes *MAL*, *AGT*, *MPH*, *HXT*) only a 73.8 kDa protein band, which could correspond to the Mal11p protein, showed different expression between raw materials (Figure 25), indicating that sugar metabolism is hardly affected during successive fermentation cyles even using different raw materials. Sulfite production pathway also seems unaffected by serial repitching and the raw materials for the most part since among all proteins of this pathway (Nakao *et al.*, 2009), only Met16p (30.7 kDa) could have differing expressions (Figure 25). From the expression profile of the 30.7 kDa protein band it is likely that Met16p, a methionine-requiring phosphoadenylylsulfate reductase, involved in sulfate assimilation and methionine metabolism, is expressed to a lower and higher degree in buckwheat and quinoa wort fermentation, respectively, compared to barley. In addition, during successive fermentations its expression gradually declines in the case of buckwheat and quinoa but not in barley (Figure 25).

Next, focus has been given to those protein candidates which showed either raw material-specific expression profile or steady increase/decrease in expression during successive fermentation cycles at least in one raw material. LMW proteins (8.0-19.3 kDa) include mainly different enzyme activators and inhibitors, transcription and translational factors, proteins involved in stress response and ribosome subunits. Among them, the inactivation of two protein candidates, Mtc3p, Mtc7p ('Maintenance of Telomere Capping'), could be the reason for observed karyotype differences, because both interact with Cdc13p and it was shown that null mutant show decreased telomere length and transposable element transposition. In addition, Rtt102p ('Regulator of Ty1 Transposition') is a component of two chromatin remodeling complexes and it has a suggested role in chromosome maintenance and regulation of Ty1 transposition. The most interesting protein candidate for 26.3 kDa protein band is the transcriptional repressor Nrg1p which mediates glucose repression. Its presumable different expression in buckwheat and quinoa compared to barley wort fermentation is not surprising due to the high glucose levels in those two worts (Table 14 and Table 15). Protein candidates of 28.5 kDa protein band include Iml3p which establishes pericentromeric cohesion during mitosis. Besides the afore-mentioned Met16p, interesting 30.7 kDa protein candidates include: (i) Thp2p, a subunit of the THO and TREX complexes involved in telomere maintenance, (ii) Cin2p, a tubulin folding factor C

which prevents chromosome loss, and (iii) Oxr1p that is required for normal levels of resistance to oxidative damage. Adh2p, a glucose-repressible alcohol dehydrogenase II, which catalyzes the conversion of ethanol to acetaldehyde and is also involved in the production of certain carboxylate esters, and Adh1p, an alcohol dehydrogenase I, which catalyses the reduction of acetaldehyde to ethanol, are 37.1 kDa protein candidates of principal interest. Both proteins seem pretty believable candidates since this protein band is relatively poorly expressed after buckwheat and quinoa wort fermentation (Figure 25), a likely cause for lower ethanol yield (Table 9) and higher acetaldehyde content (Table 20 and Table 21) in beer-like beverages from these two pseudocereals. Uth1p, involved in cell wall biogenesis, the oxidative stress response, life span during starvation and cell death, and Cdc10p, required in cytokinesis and whose abundance increases under DNA damage stress, are also part of 37.1 kDa protein band. This could indicate that yeast vitality and viability is not affected in the case of buckwheat and quinoa.

The protein band of 38.3 kDa is of great interest since its relative expression in buckwheat and quinoa is dramatically lower than in barley wort fermentation (Figure 25). Protein candidates of interest could explain karyotype changes during successive fermentations of buckwheat and quinoa wort (Figure 20, Figure 21 and Figure 22). In addition, they do not indicate an increased stress response. These candidate proteins are: (i) Gre2p, which is induced by osmotic, ionic, oxidative, heat shock, DNA replication and heavy metal stress, (ii) Arv1p, which is required for normal intracellular sterol distribution and whose null mutant shows decreased telomere length, decreased transposable element transposition and decreased vegetative growth, (iii) Spt2p and Spt3p which are involved in negative regulation of transcription and whose null mutant shows abnormal chromosome segregation, increased mitotic recombination, decreased resistance to stress and starvation, increased chronological lifespan and decreased competitive fitness and (iv) Reg2p, which is involved in glucose-induced proteolysis of maltose permease. Lower relative expression of the 51.2 kDa protein band during successive buckwheat and quinoa wort fermentations (Figure 25) could indicate low stress response and explain karyotype changes since candidate proteins include: (i) Pub1p, whose abundance increases in response to DNA replication stress and its null mutant shows increased chronological lifespan and decreased stress resistance, (ii) Bna1p, which interacts genetically with telomere capping gene CDC13 and (iii) Gcn5p, a catalytic subunit of histone acetyltransferase complexes whose null mutant shows decreased chronological lifespan, increased flocculation and decreased transposable element transposition).

All three largest HMW protein bands show higher relative expression in both pseudocereals, especially in buckwheat (Figure 25). Candidate proteins of 73.8 kDa do not include proteins directly related to fermentation but some of them are involved in yeast propagation, e.g.: (i) Kar9p, a karyogamy protein required for correct positioning of the mitotic spindle and for orienting cytoplasmic microtubules and (ii) Cdc45p, a DNA

replication initiation factor whose repressible mutant shows decreased chromosome maintenance. The protein band of 76.5 kDa is important since it is not expressed in barley wort fermentation. Candidate proteins involve: (i) those related to stress response, e.g. Pso2p, a nuclease required for DNA single- and double-strand break repair whose null mutant shows decreased chromosome maintenance and/or Ufo1p, involved in cellular response to DNA damage stimulus and (ii) those related to regulation of transcription, e.g. Bdf1, involved in transcription initiation and it also functions at TATA-containing promoter. Higher expression of the 87.3 kDa protein band is probably due to Gef1p since it is involved in cellular iron and copper ion homeostasis and it was shown for both buckwheat (Table 11) and quinoa (Table 12) wort that they have higher levels of iron and copper than barley wort (Table 10).

4.2.2 The analysis of fermentation medium

4.2.2.1 Fermentation performance

Figure 26 shows the rate of extract consumption and ethanol production during the eleven successive fermentations (F1-F11) of barley, buckwheat and quinoa wort. The fermentation performance of the first fermentation of a particular wort differed from the fermentation when beverages were prepared (Figure 4). This is most likely because 19.5 L NC Cornelius steel tanks were used instead of the spheroconical fermentors.

Because the attenuation time, i.e. the last day when a daily change in the extract content was higher than or equal to 0.1%, was raw material and successive fermentation-dependent (Table 47), the time axis was relativized. Similarly, because wort from two (buckwheat and quinoa) or four (barley) different brews were used, the dependent attribute was expressed as a percentage of its content in wort. It is obvious that the profile of extract consumption and ethanol production differed among successive fermentations.

These differences were smallest in the case of barley and largest in the case of quinoa. In addition, significant differences were also observed between the same successive fermentations of a particular raw material. In order to quantitatively describe these differences, the non-linear regression was performed fitting the four-parameter symmetrical sigmoidal curve to the experimental data of extract and ethanol content. Three statistics, namely AT_{50} (the percentage of attenuation time needed to assimilate a half of total consumed extract or to produce a half of final ethanol), curve slope (describes the steepness of the curve; higher absolute values belong to steeper curves) and R^2 (percentage of the goodness of fit) were considered for further discussion (Table 47).

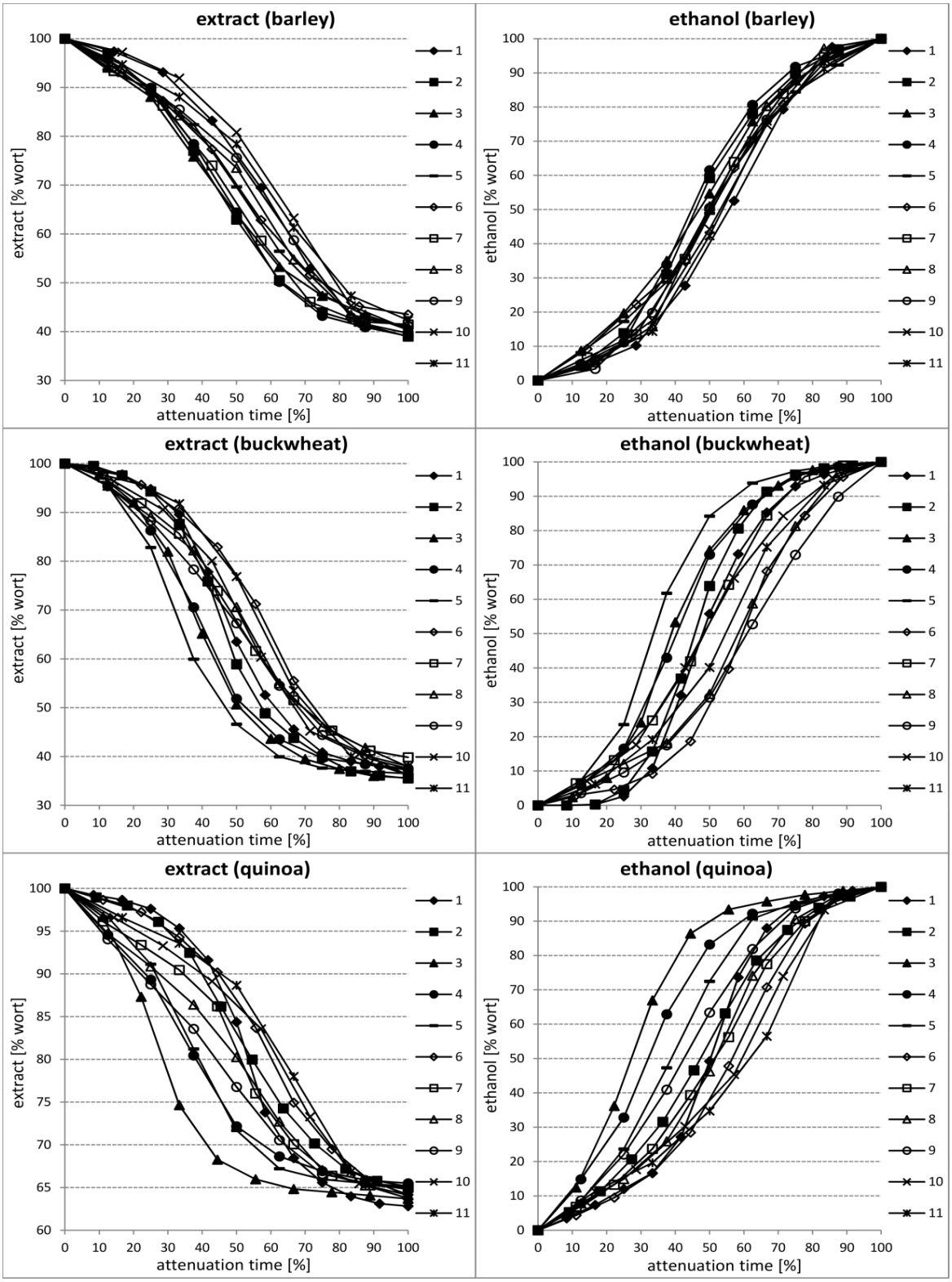

Figure 26: The extract consumption and ethanol production profile for eleven successive fermentations of barley, buckwheat and quinoa wort
01-11: the numbering of a particular successive fermentation.
Slika 26: Profil porabe ekstrakta in tvorbe etanola tekom enajst zaporednih fermentacija ječmenove, ajdove in kvinojine pivine
01-11: številčenje posamezne zaporedne fermentacije.

Table 47: The characteristics of fermentation medium during eleven successive fermentations of the barley, buckwheat and quinoa wort
Preglednica 47: Karakteristike fermentacijskega medija tekom enajst zaporednih fermentacij ječmenove, ajdove in kvinojine pivine

wort type	F	initial extract [%]	final extract [%]	AT [days]	final ethanol [% vol.]	extract AT_{50} [%]	ethanol AT_{50} [%]	extract curve slope [x100]	ethanol curve slope [x100]	extract R^2 [%]	ethanol R^2 [%]
barley	F1	10.31	4.09	7	4.11	57.3	56.5	3.2	3.4	99.88	99.90
	F2	10.31	4.02	8	4.19	43.1	45.9	3.2	3.4	99.98	99.93
	F3	10.31	4.16	8	4.17	41.8	46.1	2.6	2.6	99.93	99.94
	F4	10.34	4.04	8	4.13	44.9	44.6	3.4	3.8	99.94	99.91
	F5	10.34	4.21	8	4.17	49.8	50.5	2.9	2.6	99.78	99.85
	F6	10.34	4.50	7	4.12	48.3	51.1	2.7	2.5	99.84	99.76
	F7	10.13	4.20	7	4.16	45.7	50.6	3.1	3.4	99.76	99.93
	F8	10.13	4.15	6	4.16	52.4	50.4	2.6	3.9	99.64	99.91
	F9	10.13	4.17	6	4.14	56.2	50.4	2.6	3.2	99.65	99.91
	F10	10.16	4.11	6	4.09	61.8	53.4	3.1	3.2	99.84	100.00
	F11	10.16	4.30	6	4.11	59.1	53.4	2.6	3.2	99.73	100.00
buckwheat	F1	10.36	3.86	12	3.80	47.2	48.1	4.6	4.9	99.96	99.84
	F2	10.36	3.68	12	3.82	45.5	45.6	4.7	5.5	99.88	99.95
	F3	10.36	3.69	10	3.77	38.0	39.0	4.3	4.5	99.95	99.85
	F4	10.36	3.89	8	3.63	37.7	39.9	4.1	4.2	99.95	99.95
	F5	10.36	3.78	8	3.74	32.6	33.5	4.7	5.0	99.94	99.89
	F6	10.36	3.89	9	3.70	57.8	60.3	3.4	4.1	99.90	99.93
	F7	10.24	4.08	9	3.72	48.1	48.6	3.0	3.4	99.97	99.86
	F8	10.24	3.88	8	3.64	50.7	60.4	2.8	3.4	99.78	99.72
	F9	10.24	3.70	8	3.66	47.9	64.3	2.4	2.6	99.94	99.97
	F10	10.24	3.89	7	3.69	51.1	48.2	3.8	3.0	99.88	99.98
	F11	10.24	3.71	6	3.71	56.6	54.8	3.8	3.3	99.97	99.85
quinoa	F1	10.49	6.59	12	2.58	52.2	50.5	5.2	5.0	99.90	99.79
	F2	10.49	6.83	11	2.47	51.3	46.7	3.5	2.9	99.97	99.95
	F3	10.49	6.68	9	2.57	25.9	25.6	5.3	4.6	99.92	99.95
	F4	10.49	6.87	8	2.56	32.2	30.1	3.3	3.7	99.84	99.86
	F5	10.49	6.83	8	2.58	35.2	37.7	4.2	3.6	99.96	99.95
	F6	10.49	6.73	9	2.53	58.3	57.5	3.2	3.4	99.92	99.86
	F7	10.59	6.88	9	2.49	48.7	51.8	3.5	2.8	99.27	99.87
	F8	10.59	6.79	8	2.55	44.9	51.8	2.3	3.5	99.48	99.76
	F9	10.59	6.86	8	2.54	34.6	41.4	2.2	2.9	99.66	99.97
	F10	10.59	6.75	7	2.38	61.7	61.2	2.5	2.5	99.30	99.33
	F11	10.59	6.78	6	2.39	63.1	65.2	3.0	2.5	99.43	98.82

F – the number of successive fermentation (F1F11). AT – attenuation time. AT_{50} – the percentage of attenuation time needed to achieve 50% of the total change in extract or ethanol content.

Regarding barley, fermentation time decreased over successive fermentations with the exception of a one-day prolongation after F1 (Table 47). The same stood for buckwheat and quinoa, only that the decrease after F1 was even more prominent and that a one-day increase happened after F5. Yeast was not genetically and phenotypically adapted to the buckwheat and quinoa wort and the first three fermentations seemed to represent a phase during which the major acclimatization to a new environment (e.g. new substrate type) occurred. As expected, this phase was missing in the case of barley. A second phase, which

was characterized by a one-day increase in attenuation time, possibly represented a minor acclimatization event. In a consequent phase the attenuation time gradually lessened to a sixday long fermentation in all three raw materials. Regardless of the attenuation time differences, the extract consumption and ethanol production within a raw material remained very consistent over the whole successive fermentation with the relative standard deviation (RSD) of < 3.90%.

In general, the goodness of curve fit was very high, mostly being above 99.7% (Table 47). The most obvious deviations from this observation can be noticed in the case of quinoa where both the extract and ethanol R^2 values were lower than 99.7% from the sixth and ninth fermentation on, respectively. The successive fermentations of each raw material differed greatly from each other in their AT_{50} values. These differences were raw material-dependent being the smallest in the case of barley (extract consumption RSD = 13.4%, ethanol production RSD = 7.1%), followed by buckwheat (extract consumption RSD = 16.9%, ethanol production RSD = 19.8%) and quinoa (extract consumption RSD = 27.4%, ethanol production RSD = 26.5%). The steepness of fitted curves for barley extract consumption and ethanol production did not show any overall tendency for change, being of 2.9 and 3.2 on average, respectively. In the case of buckwheat and quinoa, the initial absolute values of curve steepnesses were almost twice as higher than in barley and firmly decreased over successive fermentations. That means that, compared to earlier fermentations, the later ones began more intensively with the extract consumption and ethanol consumption profile closer to the straight line. Regarding final ethanol content, it is hypothesized that the lower alcohol production in buckwheat and quinoa are causally connected with lower relative concentrations of the 37.1 kDa protein band in these two pseudocereals (Figure 24 and Figure 25), since this could be due to lower expressions of *ADH1* and *ADH2* genes.

4.2.2.2 Metals

The content of metals in beverages from the barley, buckwheat and quinoa malt and their overall significance were already discussed in Section 4.1.2. Here, the main focus has been given to the uptake dynamics as a function of successive fermentation and raw material type, i.e. barley (Figure 27), buckwheat (Figure 28), and quinoa (Figure 29). The data regarding iron and copper for barley and iron for buckwheat are missing because their contents in the fermentation medium were below the limit of quantification (0.05 mg/L). Table 48 shows the absolute concentrations of metals in wort and final beverage for barley, buckwheat and quinoa. What all three raw materials, regardless of the particular successive fermentation, had in common was a marked uptake of iron, copper and zinc during the first 24 hours although both absolute and relative amounts vary considerably. This was probably the consequence of biosorption which is an immediate and fairly non-specific biophysical attachment of metals to the negatively charged cell wall moieties (Walker *et al*., 2004). On the contrary, manganese was taken up more slowly and to a lesser degree

than other metals, however, the absolute overall uptake was pretty similar between raw materials despite the huge differences in the initial concentrations.

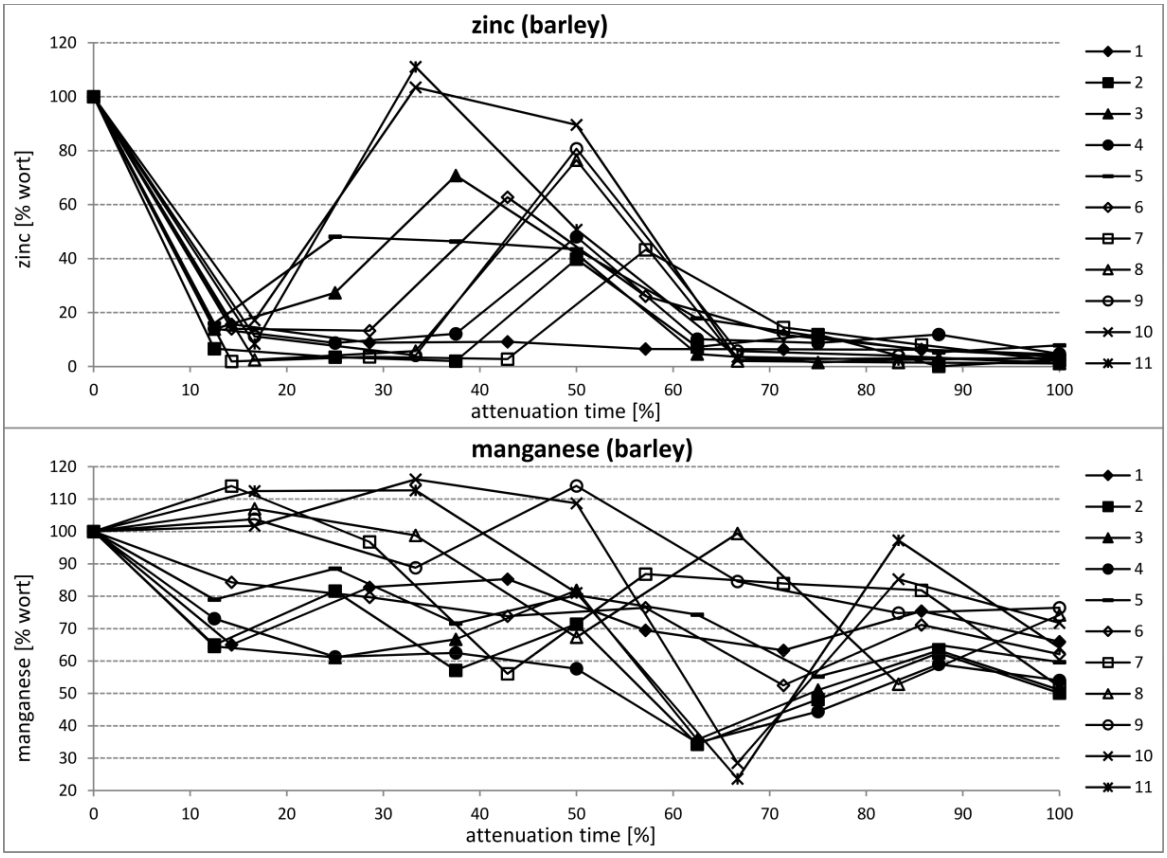

Figure 27: The zinc and manganese uptake and release dynamics for eleven successive fermentations of the barley wort
01-11: the numbering of a particular successive fermentation.
Slika 27: Dinamika v koncentraciji cinka in mangana za enajst zaporednih fermentacij ječmenove pivine 01-11: številčenje posamezne zaporedne fermentacije.

Iron

Quinoa: After the first 24 h, the initial drop of the iron concentration during eleven successive fermentations (Figure 29) differed significantly being the lowest in F1 and F2 (25-35% of the initial), the highest in F3, F6, F8 and F10 (65-75% of the initial), and intermediate in F7, F9 and F11. At the beginning of F1 and F2, the iron content was around 30% of the initial but only at the end of F1 also stayed in that range. The iron concentrations between 0.055 and 0.165 mg/L are said to be sufficient for normal fermentation (Walker, 2004) but it seems that during F1, yeast assimilated almost thirty times higher amount of this metal, most likely not by a controlled active transport. In F2, iron concentration in fermentation medium remained constantly low for the first three days and then it doubled at the end of the fourth day. The dynamics that followed was very harmonious: a three-day interval was being repeated where the relative iron concentrations of the first, second and third day were 80, 70 and 55% of the initial. It seems that no earlier than after the third day of F2 yeast was able to resist the surplus and also probably toxic

concentrations of iron. In the following four fermentations (F3-F6) the iron concentrations of fermentation medium were maintained at relatively high levels with an average iron content of 60-70% of initial. From F7 and forth it seemed that yeast began to augment its iron accumulation to some intermediate level (average iron content 45-55% of initial). It is rather questionable whether this increase in the iron uptake by yeast is due to its increased physiological need for iron, the weakening of yeast vitality, changes of yeast surface, or something else.

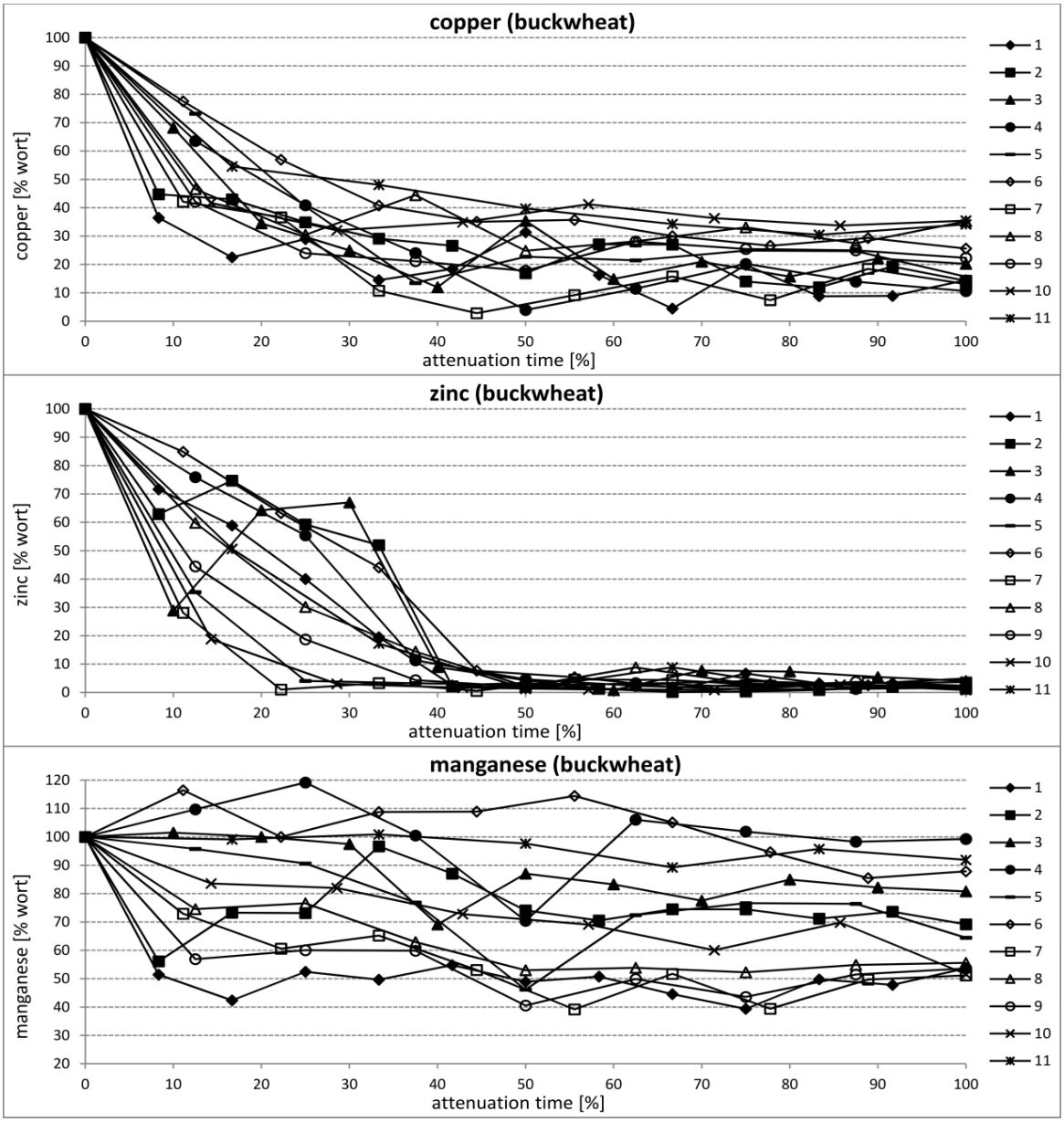

Figure 28: The copper, zinc and manganese uptake and release dynamics for eleven successive fermentations of the buckwheat wort
01-11: the numbering of a particular successive fermentation.
Slika 28: Dinamika v koncentraciji bakra, cinka in mangana za enajst zaporednih fermentacij ajdove pivine
01-11: številčenje posamezne zaporedne fermentacije.

Copper

In general, the assimilation profiles of copper during the successive fermentations of buckwheat and quinoa wort were exceedingly similar (Figure 28 and Figure 29), as well as were their initial and final absolute concentrations (Table 48). Nevertheless, one obvious difference exists, i.e. the initial rate of copper uptake.

Quinoa: The majority of copper was assimilated on the first day (80-90% of the initial) and later on, only minor fluctuations of its concentration occurred which did not exceed day-by-day change greater than 20% of initial concentration. Both the first day uptake and the degree of fluctuations declined with the increasing number of successive fermentation. The final concentrations of copper were very low after F1-F4 (0-10% of the initial) and much higher in later fermentations (20-40% of the initial).

Buckwheat: The firstday uptake of copper is also prominent but lower compared to quinoa (20-40% of the initial). Further uptake gradually declines during the first half of attenuation time. Interday fluctuations are similar to quinoa but the first day uptake of copper declines only from F1 to F6 and then it stabilizes around 50% of initial. Final concentrations were close to each other and general trend of their increase is noticeable.

Zinc

The uptake dynamics of zinc showed that its assimilation by yeast was highly raw material-dependent (Figure 27, Figure 28 and Figure 29) although the influence of successive fermentation should not be neglected. In all cases, zinc was rapidly taken up by yeast at the beginning of fermentation which is in agreement with previous reports that zinc is absorbed by the yeast biomass even before fermentation commences i.e. just after the cells are dispensed into wort (Kreder, 1999; Mochaba *et al*., 1996a, 1996b; Aleksander *et al*., 2009). In our case, the overall absolute uptake seemed primarily a function of the initial zinc concentration except when it was very high, the influence of successive fermentation started to prevail (Table 48).

Barley: The first-day uptake was in the range of the final one (>80% of the initial) which means that no net uptake occurred afterwards. However, only in the first fermentation the zinc content remained at its minimal level throughout the process, whereas in all subsequent fermentations it increased once as the process progressed and it dropped again before the end. In all cases, this increase reached a peak value in the first half of fermentation whereas its intensity was increasing with the process of successive fermentation. This marked release of zinc by yeast was already observed previously (Aleksander *et al*., 2009) and it was interpreted by a drop in pH during the fermentation process, as that drop decreases the apparent stability constant of the zinc metal binders (Lie *et al*., 1975). This happening was presumably more considerable, the more times the yeast slurry was reused.

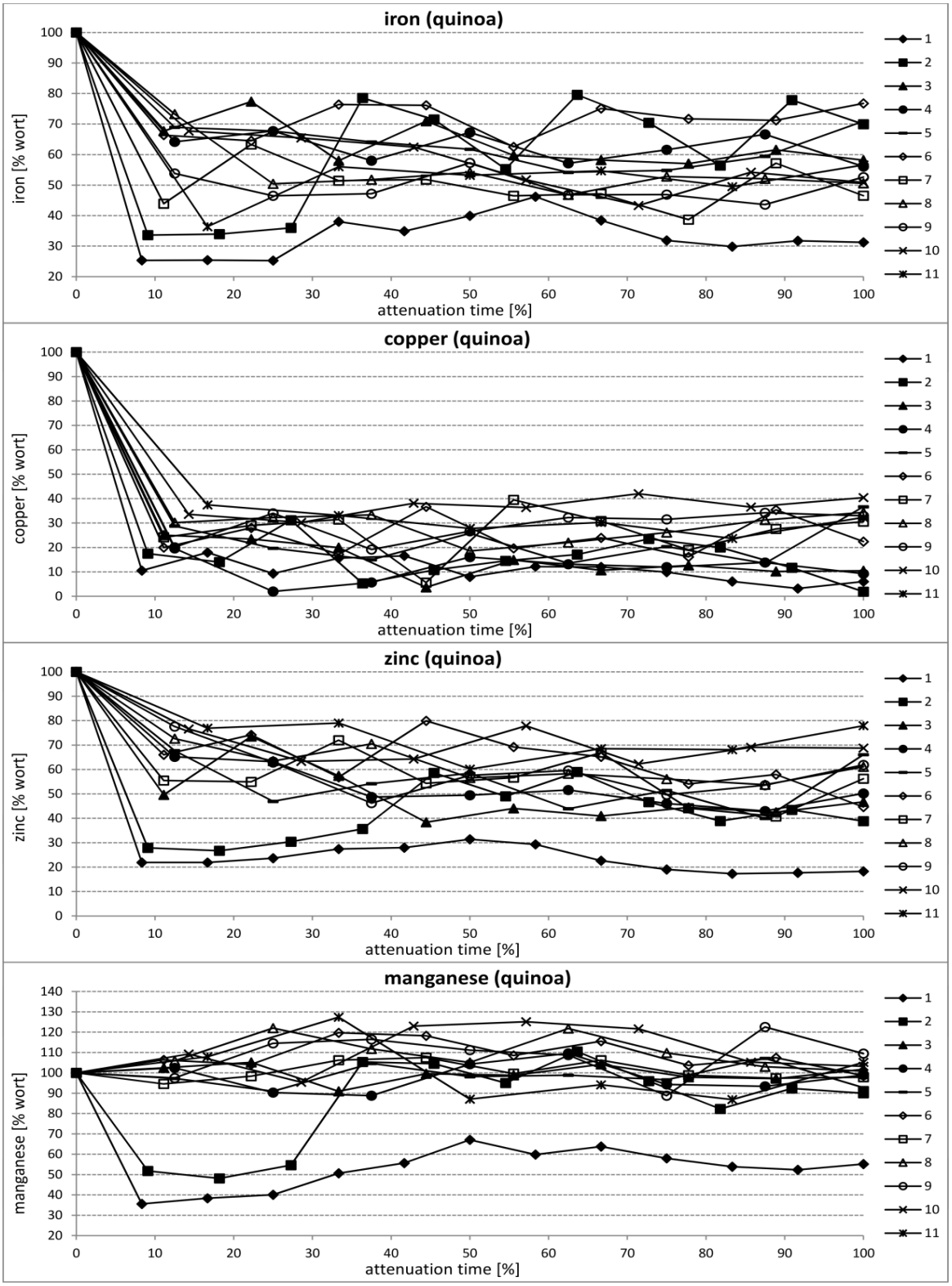

Figure 29: The iron, copper, zinc and manganese uptake and release dynamics for eleven successive fermentations of quinoa wort
01-11: the numbering of a particular successive fermentation.
Slika 29: Dinamika v koncentraciji železa, bakra, cinka in mangana za enajst zaporednih fermentacij kvinojine pivine
01-11: številčenje posamezne zaporedne fermentacije.

It can be concluded that successive fermentation of barley wort influences only the temporary release of zinc during fermentation (i.e. the time and intensity of a peak value) but not the initial and overall uptake. Latter finding is in discordance with other reports (Mochaba *et al*., 1996a; Aleksander *et al*., 2009), most likely because of the low zinc concentrations of our wort.

Buckwheat: The relative uptake of zinc during the first day varied between 15 and 70% of the initial but at the end of fermentation its concentration was below 10% regardless of successive fermentation number. In absolute concentrations, overall uptake represented 5- to 10-times higher values compared to barley (i.e. ~2 mg/L), an amount which could be obviously tolerated by yeast with no excessive harm. Final concentrations were achieved in more or less linear manner between the 20 and 40% of fermentation progression which probably correlated well with the yeast proliferation. As opposed to barley, only during the F2 and F3 the zinc content temporarily increased.

Quinoa: The profile of zinc uptake during the successive fermentation was pretty reminiscent of the uptake profile of iron, especially that of F1 and F2. The uptake of zinc during the first day of fermentation gradually declined from 80% (F1) to 20% (F11), similar to the overall iron assimilation. As for iron, it seems that during the second fermentation yeast became generally able to withstand the toxic zinc concentrations and this ability has been improved continuously in subsequent fermentations. Indeed, the zinc uptake values of later successive fermentations reached the so called "tolerable concentration" of ~2 mg/L, as ascertained for buckwheat above. Furthermore, no temporary single increase of zinc concentration was observed. Instead, fluctuations with no obvious pattern took place.

Table 48: The initial and final concentrations of iron, copper, zinc and manganese for eleven successive fermentations of the barley, buckwheat and quinoa wort
Preglednica 48: Začetne in končne koncentracije železa, bakra, cinka in mangana za enajst zaporednih fermentacij ječmenove, ajdove in kvinojine pivine

		iron		copper		zinc		manganese	
wort type	F	initial conc. [mg/L]	final conc. [mg/L]	initial conc. [mg/L]	final conc. [mg/L]	initial conc. [mg/L]	final conc. [mg/L]	initial conc. [mg/L]	final conc. [mg/L]
barley	F1	0.033	< LLQ	0.087	< LLQ	0.225	0.008	0.137	0.090
	F2	0.033	< LLQ	0.087	< LLQ	0.225	0.006	0.137	0.069
	F3	0.033	< LLQ	0.087	< LLQ	0.225	0.003	0.137	0.070
	F4	0.037	< LLQ	0.095	< LLQ	0.324	0.015	0.183	0.079
	F5	0.037	< LLQ	0.095	< LLQ	0.324	0.026	0.183	0.109
	F6	0.037	< LLQ	0.095	< LLQ	0.324	0.015	0.183	0.114
	F7	0.035	< LLQ	0.092	< LLQ	0.263	0.006	0.155	0.081
	F8	0.035	< LLQ	0.092	< LLQ	0.263	0.003	0.155	0.115
	F9	0.035	< LLQ	0.092	< LLQ	0.263	0.003	0.155	0.119
	F10	0.039	< LLQ	0.093	< LLQ	0.362	0.011	0.144	0.103
	F11	0.039	< LLQ	0.093	< LLQ	0.362	0.012	0.144	0.092
buckwheat	F1	0.051	< LLQ	0.297	0.043	1.446	0.046	0.219	0.116
	F2	0.051	< LLQ	0.297	0.042	1.446	0.016	0.219	0.149
	F3	0.051	< LLQ	0.297	0.060	1.446	0.050	0.219	0.174
	F4	0.051	< LLQ	0.297	0.031	1.446	0.023	0.219	0.214
	F5	0.051	< LLQ	0.297	0.046	1.446	0.062	0.219	0.139
	F6	0.051	< LLQ	0.297	0.076	1.446	0.025	0.219	0.189
	F7	0.048	< LLQ	0.375	0.049	1.999	0.046	0.270	0.138
	F8	0.048	< LLQ	0.375	0.132	1.999	0.043	0.270	0.150
	F9	0.048	< LLQ	0.375	0.084	1.999	0.047	0.270	0.145
	F10	0.048	< LLQ	0.375	0.133	1.999	0.017	0.270	0.139
	F11	0.048	< LLQ	0.375	0.128	1.999	0.033	0.270	0.248
quinoa	F1	4.12	1.29	0.317	0.020	4.934	0.901	1.508	0.832
	F2	4.12	2.88	0.317	0.006	4.934	1.917	1.487	1.357
	F3	4.12	2.40	0.317	0.034	4.934	2.305	1.487	1.535
	F4	4.12	2.31	0.317	0.030	4.934	2.476	1.487	1.492
	F5	4.12	2.92	0.317	0.119	4.934	3.259	1.487	1.399
	F6	4.12	3.17	0.317	0.073	4.934	2.202	1.487	1.488
	F7	5.69	2.65	0.291	0.089	5.072	2.852	1.362	1.333
	F8	5.69	2.88	0.291	0.100	5.072	3.099	1.362	1.374
	F9	5.69	2.99	0.291	0.096	5.072	3.137	1.362	1.491
	F10	5.69	2.90	0.291	0.118	5.072	3.488	1.362	1.406
	F11	5.69	3.22	0.291	0.094	5.072	3.953	1.362	1.432

F – number of successive fermentation (F1F11). AT_{50} – percentage of attenuation time needed to achieve 50% of the total change in extract or ethanol content. R^2 – goodness of curve fit. LLQ – lower limit of quantification.

Manganese

In general, the manganese uptake profile was characterized by relatively high fluctuations during the fermentation process with no distinctive patterns (Figure 27, Figure 28 and Figure 29). Moreover, the overall uptake was always below 50% of the initial manganese concentration.

Barley: The highest first-day uptake concentration, (~35% of the initial) corresponds to F1-F3 and from F4 to F6 initial uptake declined to only ~15%. From F7 on rather steady or increased values were observed. Final concentrations of manganese were 50-80% of the initial.

Buckwheat: Manganese concentrations after the first day were 50-120% and at the end 50-100% of the initial with no explicit trend of their change over successive fermentations.

Quinoa: Relative profiles of manganese uptake for F1 and F2 seemed similar to those of iron and zinc. This was more or less expected since in brewing the recommended values of manganese are in the range of 0.11-0.22 mg/L (Walker, 2004) thus it is believed that, as for iron and zinc, yeast underwent a phenotypical adaptation to excessive manganese concentrations. In none of the fermentations (with the above-mentioned exception of F1 and F2), the manganese concentration was significantly changed after the first day. The same stands for the final concentrations with the exception of F1.

4.2.2.3 Fermentable carbohydrates

The content of fermentable carbohydrates (FCs) in beverages from the barley, buckwheat and quinoa malt and their overall significance were already discussed in Section 4.1.3. Here, the main focus has been given to the uptake dynamics as a function of successive fermentation and raw material type, i.e. barley (Figure 30), buckwheat (Figure 31), and quinoa (Figure 32). The initial content of FCs and their ratios in barley, buckwheat and quinoa wort differed considerably (Table 49), as was expected from the analysis of wort and fresh and force-aged beverages (Table 10, Table 11 and Table 12) where similar values were obtained and discussed in detail. For the purpose of quantitative data interpretation the same principle as in the case of fermentation performance was followed, i.e. the non-linear regression was performed and three statistics, namely AT_{50}, curve slope and R^2 (Table 49) were used to support the discussion. Fructose was excepted from the non-linear regression analysis because of its very low R^2 values (data not shown). Furthermore, in depth analysis of fructose uptake is somehow dispensable since its low absolute values in wort do not influence the fermentation process substantially. All other R^2 values were higher than 95% (99.45% on average).

Table 49: The initial concentrations and AT_{50}, curve slope and R^2 values for DP3, DP2, glucose, fructose and total fermentable carbohydrates during eleven successive fermentations of the barley, buckwheat and quinoa wort

Preglednica 49: Začetne koncentracije ter vrednosti AT_{50}, naklona regresijske krivulje in R^2 za DP3, DP2, glukozo, fruktozo in vsoto fermentabilnih ogljikovih hidratov tekom enajst zaporednih fermentacij ječmenove, ajdove in kvinojine pivine

		DP3				DP2				glucose				fructose	sum FCs			
wort type	F	initial conc. [g/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [g/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [g/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [g/L]	initial conc. [g/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]
barley	1	10.31	56.2	3.9	99.91	60.54	55.49	4.5	99.65	6.14	35.57	5.6	98.67	1.61	78.60	54.12	4.2	99.93
	2	10.31	41.01	3.2	99.84	60.54	46.28	3.1	99.88	6.14	26.05	6.9	98.56	1.61	78.60	43.66	3.1	99.96
	3	10.31	40.72	2.6	99.99	60.54	44.37	2.2	99.36	6.14	32.44	3.9	99.94	1.61	78.60	43.76	2.5	99.74
	4	11.49	44.08	3.7	99.88	58.28	45.72	3.3	99.31	6.01	27.77	6.6	99.06	1.69	77.48	43.72	3.4	99.67
	5	11.49	50.37	3.2	99.81	58.28	54.19	3.5	98.67	6.01	36.62	4.9	99.85	1.69	77.48	52.12	3.4	99.56
	6	11.49	45.04	2.9	99.71	58.28	46.67	1.8	97.95	6.01	31.73	4.1	99.92	1.69	77.48	45.87	2.2	99.14
	7	9.11	44.57	4.6	99.87	56.90	47.13	3.8	99.81	6.30	28.07	8.5	99.87	1.88	74.19	44.31	3.9	99.91
	8	9.11	48.27	4.7	99.65	56.90	50.01	4.1	99.67	6.30	26.43	6.4	99.98	1.88	74.19	47.06	3.8	99.86
	9	9.11	50.48	3.3	99.95	56.90	52.95	3.4	99.7	6.30	30.54	6.1	99.46	1.88	74.19	50.02	3.2	99.92
	10	10.34	55.62	4.3	99.72	53.18	53.64	4.0	99.43	6.86	36.19	5.8	99.51	1.94	72.32	51.93	4.0	99.84
	11	10.34	46.16	3.7	99.73	53.18	46.33	3.4	99.69	6.86	31.68	5.7	99.92	1.94	72.32	45.04	3.5	99.92
buckwheat	1	8.36	53.13	5.7	99.32	25.30	45.82	9.5	99.21	42.86	49.28	5.3	99.89	2.07	78.59	48.1	5.7	99.93
	2	8.36	54.13	6.0	99.06	25.30	44.87	9.8	98.83	42.86	46.90	5.6	99.97	2.07	78.59	46.48	5.9	99.88
	3	8.36	55.99	3.6	99.94	25.30	47.4	4.4	98.7	42.86	46.10	3.4	99.87	2.07	78.59	47.35	3.5	99.78
	4	8.36	43.66	10.7	99.58	25.30	40.56	9.0	98.63	42.86	43.98	4.0	99.9	2.07	78.59	42.89	4.5	99.75
	5	8.36	37.2	8.6	99.92	25.30	38.74	7.0	99.2	42.86	40.8	3.7	99.85	2.07	78.59	40	4.3	99.93
	6	8.36	55.73	8.2	99.68	25.30	48.04	12.1	98.31	42.86	48.21	4.6	99.92	2.07	78.59	48.7	5.4	99.86
	7	8.18	48.79	7.3	99.7	23.83	43.75	8.3	99.25	48.49	48.13	3.7	99.97	1.31	81.99	47.13	4.3	99.91
	8	8.18	49.72	2.6	99.55	23.83	39.06	6.0	98.66	48.49	44.87	3.7	99.91	1.31	81.99	43.63	3.9	99.81
	9	8.18	49.2	2.6	99.35	23.83	33.58	2.0	97.55	48.49	43.11	2.8	99.94	1.31	81.99	42.8	2.7	99.83
	10	8.18	55.14	3.2	96.62	23.83	47.3	8.3	94.82	48.49	43.26	3.2	99.83	1.31	81.99	44.21	3.4	99.47
	11	8.18	56.27	7.2	96.1	23.83	43.23	3.5	97.23	48.49	45.43	3.6	99.61	1.31	81.99	45.77	3.6	99.29
quinoa	1	11.12	68.6	4.7	99.21	17.07	58.25	8.2	99.48	23.14	46.17	6.5	99.83	1.02	52.35	52.11	5.6	99.83
	2	11.12	70.58	4.1	99.80	17.07	63.20	3.8	99.59	23.14	41.13	4.0	99.87	1.02	52.35	50.30	3.1	99.85
	3	11.12	55.12	5.6	99.43	17.07	57.89	4.4	99.46	23.14	40.87	3.0	99.89	1.02	52.35	48.23	3.0	99.97
	4	11.12	50.9	5.4	99.68	17.07	45.66	9.4	99.72	23.14	33.54	3.0	99.89	1.02	52.35	41.28	4.0	99.89
	5	11.12	55.75	4.3	99.86	17.07	49.34	6.5	99.86	23.14	30.02	3.0	99.93	1.02	52.35	40.99	3.3	99.95
	6	11.12	71.64	5.0	99.78	17.07	65.82	5.6	99.66	23.14	42.96	3.5	99.69	1.02	52.35	55.33	3.3	99.87
	7	12.18	56.86	5.3	99.23	20.66	59.34	5.2	99.78	27.68	37.74	3.3	99.85	1.03	61.56	46.44	3.0	99.97
	8	12.18	57.91	4.1	98.99	20.66	60.87	4.9	99.01	27.68	37.38	3.2	99.81	1.03	61.56	45.20	2.6	99.98
	9	12.18	58.09	3.1	98.78	20.66	60.48	3.7	98.49	27.68	35.12	3.8	99.81	1.03	61.56	40.76	2.6	99.91
	10	12.18	71.22	5.8	97.12	20.66	69.87	4.1	98.11	27.68	41.81	2.5	99.81	1.03	61.56	53.31	1.9	99.47
	11	12.18	65.09	5.0	99.66	20.66	65.50	5.9	98.76	27.68	44.00	4.2	99.18	1.03	61.56	51.30	3.3	99.28

F – the number of successive fermentation (F1-F11). AT_{50} – the percentage of attenuation time needed to achieve 50% of the total change in the extract or ethanol content. R^2 – the goodness of curve fit. conc. – concentration. FCs – fermentable carbohydrates.

All three raw materials shared a common characteristic, i.e. after the sixth successive fermentation there was a small (< 5%) but obvious increase in the relative glucose utilization, mainly at the expense of lower DP2 uptake (Figure 33, Figure 34 and Figure 35). On the other hand, there were three minor distinctions observed between different raw materials regarding the total FC assimilation (Figure 30, Figure 31 and Figure 32). Firstly, in the case of barley the most FCs were assimilated overall (80-90%), followed by buckwheat (~80%) and quinoa (65-75%). At this point, the low overall extract consumption and low final ethanol production in quinoa (Figure 26, Table 47) became intelligible. Besides, the low content of FC in quinoa wort (25 and 30% less on average than in barley and buckwheat, respectively), their overall assimilation was also relatively low. Secondly, the curve slope in barley did not change much in the course of successive fermentation whereas in the other two, there is an overall increase from around -0.060 to -0.035. Contrary to barley, in buckwheat and quinoa there was a general tendency over successive fermentations to the faster beginning of fermentation, a situation which can mostly be quantitatively explained by the decreasing steepness of curves.

Glucose

Being the preferred carbohydrate to ferment, the glucose uptake normally begins immediately after yeast is pitched. However, that was not the case in some barley fermentations (Figure 30) where the values were even higher after the first day comparing to wort. It seems that some enzymatic process took place that yielded glucose, e.g. the hydrolysis of sucrose by a periplasm-located invertase (Briggs *et al*., 2004). Most likely, similar occurred in buckwheat (Figure 31) and quinoa (Figure 32) but because of their high initial levels of glucose this phenomenon was unnoticeable. In all cases, glucose was fermented completely but sooner in barley (~60% of the attenuation time) than in buckwheat (~90% of the attenuation time) and quinoa (~80 of the attenuation time). This visual observation is quantitatively supported by average values of AT_{50} for barley (31%), buckwheat (45%) and quinoa (41%). Otherwise, AT_{50} values do not show any trend for change over successive fermentations. From the curve slopes of buckwheat and quinoa it can be concluded that after F2, yeast became able to ferment glucose faster right from the beginning which resulted in less steep fitting curve. Overall, all the differences between barley and both pseudocereals were most likely due to the extreme differences between initial concentrations.

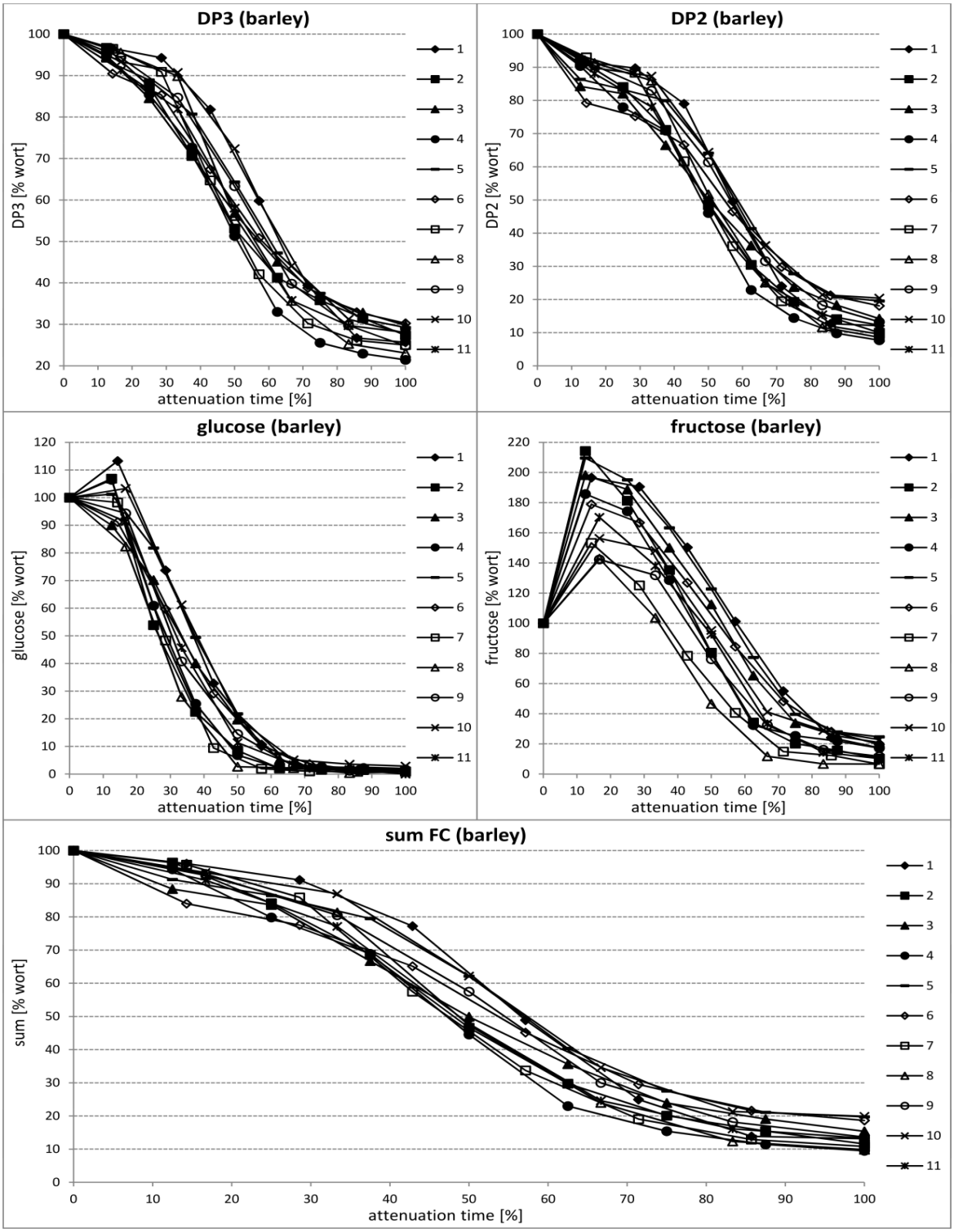

Figure 30: The uptake dynamics of fermentable sugars for eleven successive fermentations of the barley wort 01-11: the numbering of a particular successive fermentation. DP3 – trisaccharides. DP2 – disaccharides. sum FC – the sum amount of DP3, DP2, glucose and fructose.

Slika 30: Dinamika privzema fermentabilnih ogljikovih hidratov za enajst zaporednih fermentacij ječmenove pivine

01-11: številčenje posamezne zaporedne fermentacije. DP3 – trisaccharides. DP2 – disaccharides.sum FC – vsota koncentracij DP3, DP2, glukoze in fruktoze.

It was already hypothesized that the differences in absolute and relative concentrations of glucose between worts are connected to different relative concentrations of the 26.3 kDa protein band (Figure 24 and Figure 25). In particular, this can be due to transcriptional repressor Nrg1p, which mediates glucose repression and negatively regulates a variety of other processes. In addition, high glucose levels may cause extremely lower relative concentrations of the 38.3 kDa protein band in buckwheat and quinoa (Figure 24 and Figure 25), which can be caused by lower expression of Reg2p, involved in glucose-induced proteolysis of maltose permease.

Fructose

The initial fructose concentrations were very low and thus comparable among raw materials (Figure 30, Figure 31 and Figure 32). In almost every fermentation, its concentration dramatically raised after the first day and it stayed above the initial level a substantial amount of time, in some cases even to the end. In buckwheat, fructose is assimilated to a similar degree than in barley whereas in quinoa only 20% of the initial at most. Besides, in quinoa the fructose uptake and release seems pretty chaotic process, as opposed to highly continuous profiles in barley and buckwheat.

DP2 and DP3

The cotemporary discussion of the di- and trisaccharide uptake profile is reasonable since the topologies of their profiles do not differ (Figure 30, Figure 31 and Figure 32). In theory, the uptake of the sugars of two and three degrees of polymerization (DP2 and DP3) should begin just after the majority of glucose has been fermented because it exerts a well-known effect called 'carbon catabolite repression'. Along with barley, this was somehow true also for quinoa wort fermentations since they all have relatively high difference between average AT_{50} values of glucose and DP2/3 (~20%). Oppositely, in the case of buckwheat, the average AT_{50} value of glucose (45.46%) is only a bit higher (50.81%) or even smaller (42.94%) than average AT_{50} values of DP3 and DP2, respectively. This observation means that the fermentation of glucose, DP2 and DP3 extended more or less simultaneously, probably because of a higher expression of the 73.8 kDa protein band in the case of buckwheat (Figure 24 and Figure 25) which raises the possibility that the Mal11p, a high-affinity maltose transporter, is expressed to a higher level.

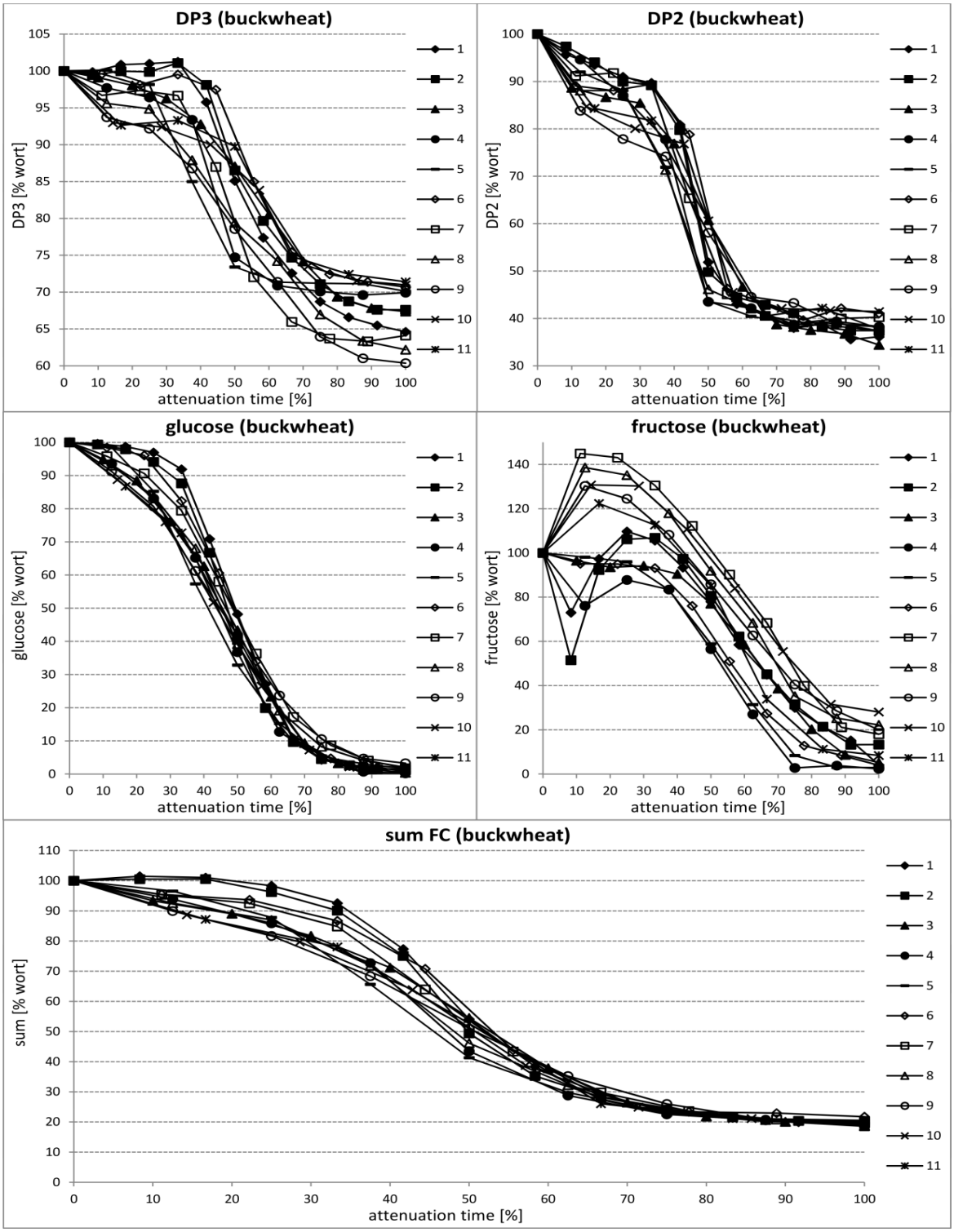

Figure 31: The uptake dynamics of fermentable sugars for eleven successive fermentations of the buckwheat wort

01-11: the numbering of a particular successive fermentation. DP3 – trisaccharides. DP2 – disaccharides. sum FC – the sum amount of DP3, DP2, glucose and fructose

Slika 31: Dinamika privzema fermentabilnih ogljikovih hidratov za enajst zaporednih fermentacij ajdove pivine

01-11: številčenje posamezne zaporedne fermentacije. DP3 – trisaccharides. DP2 – disaccharides.sum FC – vsota koncentracij DP3, DP2, glukoze in fruktoze.

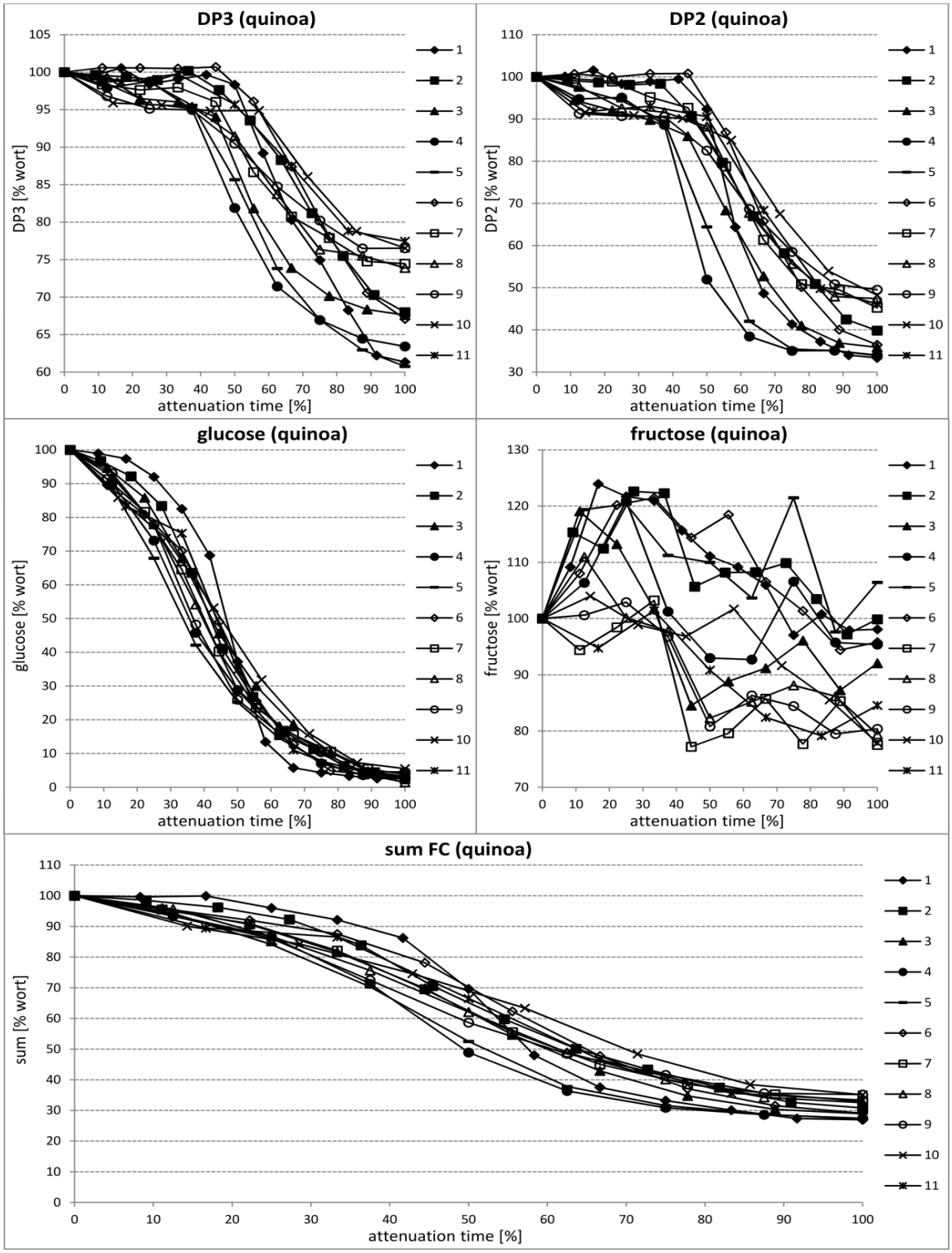

Figure 32: The uptake dynamics of fermentable sugars for eleven successive fermentations of the quinoa wort

01-11: the numbering of a particular successive fermentation. DP3 – trisaccharides. DP2 – disaccharides. sum FC – the sum amount of DP3, DP2, glucose and fructose

Slika 32: Dinamika privzema fermentabilnih ogljikovih hidratov za enajst zaporednih fermentacij kvinojine pivine

01-11: številčenje posamezne zaporedne fermentacije. DP3 – trisaccharides. DP2 – disaccharides.sum FC – vsota koncentracij DP3, DP2, glukoze in fruktoze.

The overall assimilation degree of DP2 and DP3 was two to three-times lower in buckwheat (Figure 31) and quinoa (Figure 32) than in barley (Figure 30). For DP2, overall assimilation rates correlated inversely with initial DP2 concentrations, which was not true for DP3. Moreover, the assimilation in barley started immediately whereas in buckwheat and quinoa, the consumption of DP3 during the first 40% of attenuation time was rather low. Only in quinoa there were remarkable differences between successive fermentations in a sense of both slower rate and lower overall consumption of DP2 and DP3. What was even more important, these differences showed a firm directional trend. Although in the first five fermentations the assimilation capacity of yeast improved, it weakened gradually from then on.

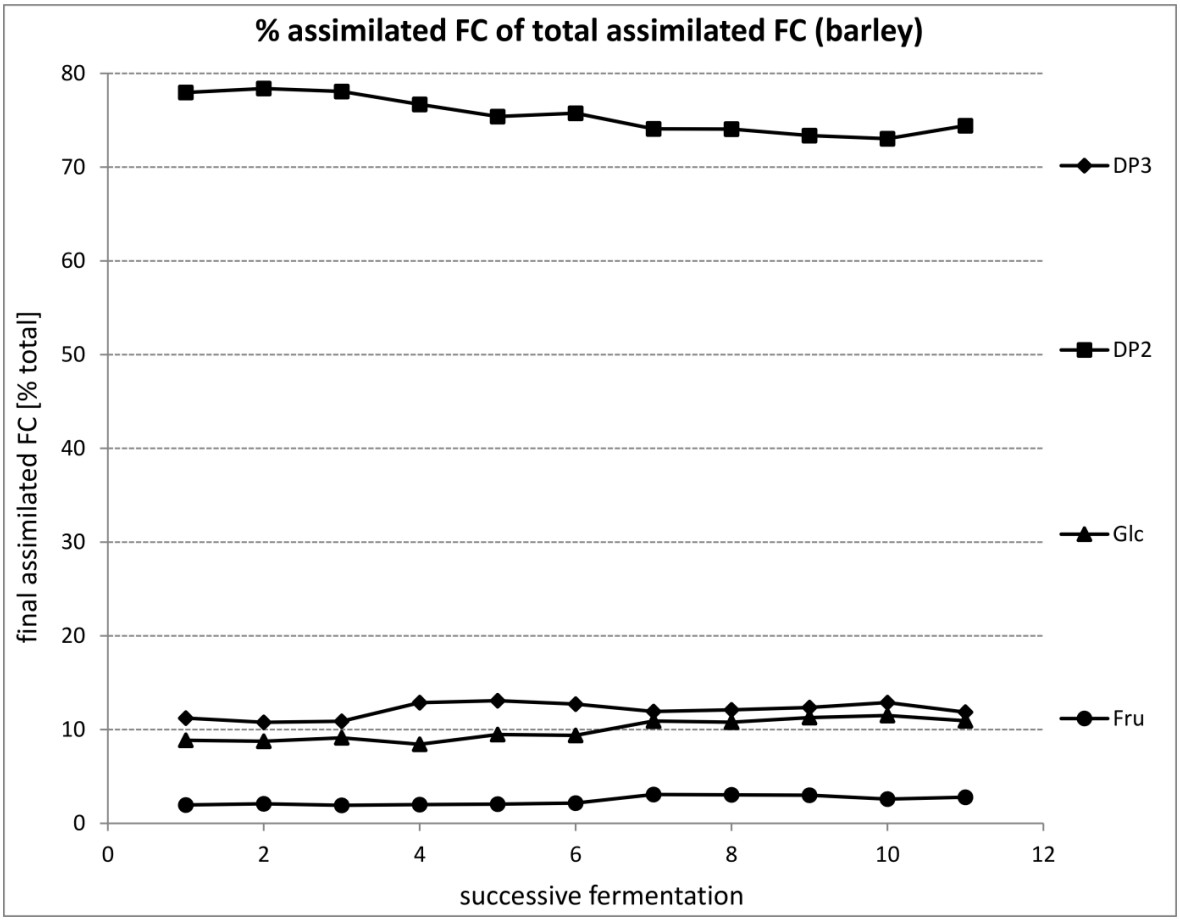

Figure 33: The line chart of the final DP3, DP2, glucose and fructose assimilation for eleven successive fermentations of the barley wort
FC – fermentable carbohydrate.
Slika 33: Črtni diagram končne asimilacije DP3, DP2, glukoze in fruktoze tekom enajst zaporednih fermentacij ječmenove pivine
FC – fermentabilni ogljikov hidrat.

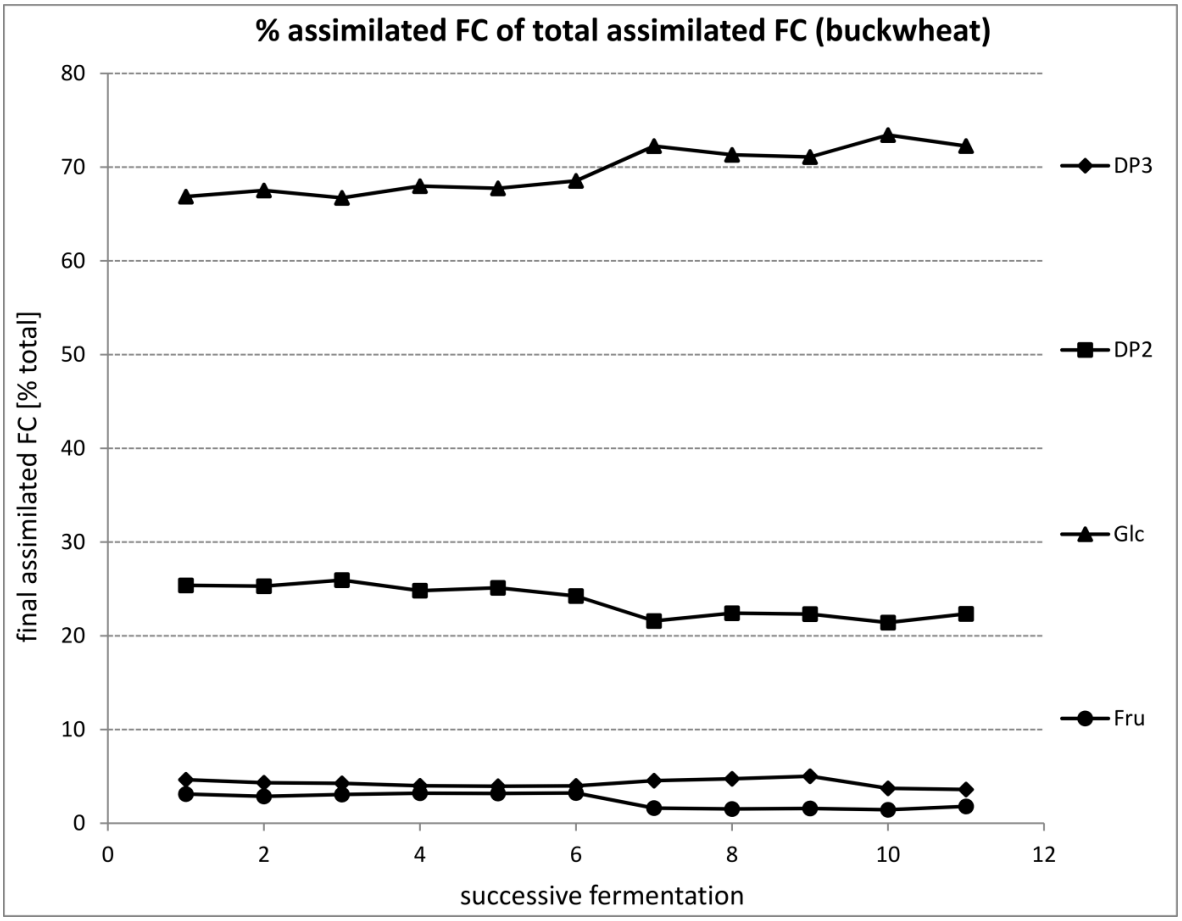

Figure 34: The line chart of the final DP3, DP2, glucose and fructose assimilation for eleven successive fermentations of the buckwheat wort
FC – fermentable carbohydrate.
Slika 34: Črtni diagram končne asimilacije DP3, DP2, glukoze in fruktoze tekom enajst zaporednih fermentacij ajdove pivine
FC – fermentabilni ogljikov hidrat.

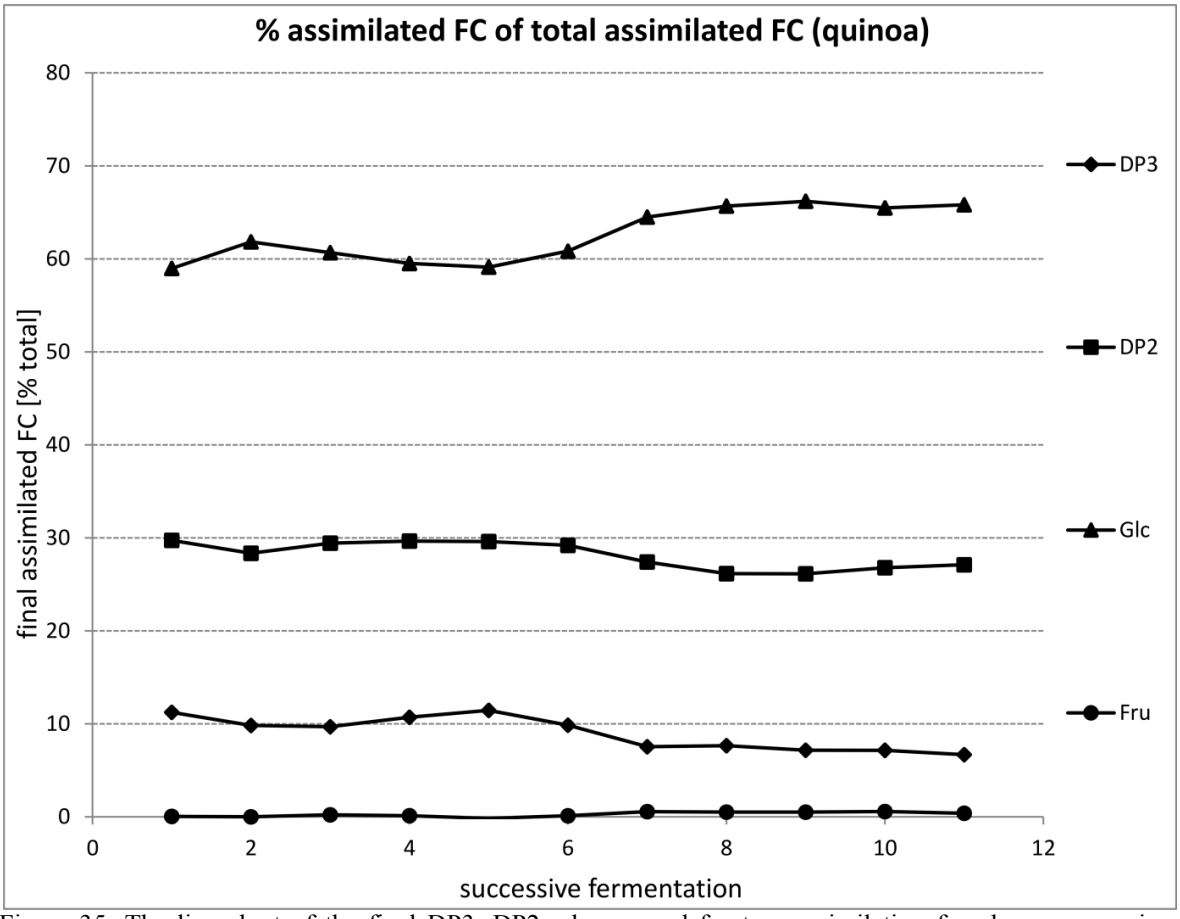

Figure 35: The line chart of the final DP3, DP2, glucose and fructose assimilation for eleven successive fermentations of the quinoa wort
FC – fermentable carbohydrate.
Slika 35: Črtni diagram končne asimilacije DP3, DP2, glukoze in fruktoze tekom enajst zaporednih fermentacij ajdove pivine
FC – fermentabilni ogljikov hidrat.

4.2.2.4 Amino acids

The content of amino acids (AAs) in beverages from the barley, buckwheat and quinoa malt and their overall significance were already discussed in Section 4.1.4. Here, the main focus has been given to the uptake dynamics as a function of successive fermentation and raw material type, i.e. barley (Figure 36, Figure 37 and Figure 38), buckwheat (Figure 39, Figure 40 and Figure 41) and quinoa (Figure 42, Figure 43 and Figure 44). The initial content of AAs and their ratios in the barley, buckwheat, and quinoa wort differed considerably (Table 50, Table 51 and Table 52), as was expected from the analysis of the bottled beverages (Table 16, Table 17 and Table 18) where similar values were obtained. The data regarding proline are not shown because it was excluded from the discussion completely because (i) the proline oxidation by a mitochondrial oxidase is repressed during brewery fermentation (Wang and Brandris, 1987) and consequently this amino acid is not utilized significantly during brewery fermentation, and (ii) the intra-laboratory method validation revealed high relative standard deviation of proline (Table 7), which rendered the reliable data interpretation impossible. Cysteine was excluded from the

chromatographic analysis since it is known to form only a weakly fluorescent derivative with OPA reagent. Although we put quite some effort into the complete HPLC separation of all amino acids, Gly and His derivative coeluted thus the sum of their concentration is given. Nevertheless, the influence of Gly on the properties of the His and Gly sum can be practically neglected since it is present in very low amounts as well as its overall assimilation is scarce (Table 16, Table 17 and Table 18).

Because the attenuation time, i.e. the last day when a daily change in the extract content was higher than or equal to 0.1%, was raw material- and successive fermentation-dependent (Table 47), the time axis was relativized throughout the entire article. Similarly, the dependent attribute was expressed as a percentage of its content in wort because wort from two (buckwheat and quinoa) or four (barley) different brews was used and because uptake profiles based on relative values enabled us to compare different AAs from different source with each other. In order to quantitatively discuss the results, the term "completion time" (t95), here defined as the percent attenuation time necessary for ~95% of the total assimilation, has been introduced. Where appropriate, a designation $\Delta_{max\text{-}min}$ was used to express the difference between the maximal and minimal value. In addition, the non-linear regression was performed fitting the four-parameter symmetrical sigmoidal curve to the experimental data of particular AA. Three statistics, namely AT_{50} (the percentage of attenuation time needed to assimilate a half of total consumed amino acid), curve slope (describes the steepness of the curve; higher absolute values belong to steeper curves) and R^2 (percentage of the goodness of fit) were considered for further discussion (Table 50, Table 51 and Table 52) but only if the R^2 was 98% and higher. RSD values of these three statistics for eleven successive fermentations of each raw material were calculated. Their product, termed "the serial repitching factor" (SRf), was used to support the visual evaluation of the serial repitching and its influence on the AA assimilation profile (greater influence of the serial repitching gave higher SRf values). However, in many cases, especially in quinoa, the uptake profile differed drastically from the sigmoidal curve. In that case, results are described "ambiguous fit" (A.G.), "not converged (N.C.)" or "interrupted fit" (I.R.). Curve fit was expressed as "ambiguous" in the case when it does not nail down the values of all the parameters, "not converged" when it was impossible to converge on a best fit, and "interrupted" if non-linear regression took more iterations than the maximum entered on the diagnostics tab, i.e. 1000. Although these cases of the so-called "bad regression" cannot be quantitatively included into the SRf calculation, they were still considered during the data interpretation in a more holistic way.

Table 50: The initial concentrations and AT_{50}, curve slope and R^2 values of Asp, Glu, Asn, Ser, Gln and Gly+His for eleven successive fermentations of the barley, buckwheat and quinoa wort

Preglednica 50: Začetne koncentracije ter vrednosti AT_{50}, naklona regresijske krivulje in R^2 za Asp, Glu, Asn, Ser, Gln in Gly+His tekom enajst zaporednih fermentacij ječmenove, ajdove in kvinojine pivine

		Asp				Glu				Asn				Ser				Gln				Gly + His			
wort type	F	initial conc. [g/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [g/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [g/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [g/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [g/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [g/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]
barley	1	54.90	52.67	4.69	99.64	56.60	51.52	3.94	99.72	117.47	31.42	5.77	99.66	49.51	32.26	6.54	99.89	33.67	32.84	4.38	99.97	56.35	50.58	1.86	99.42
	2	54.90	45.58	4.92	98.37	56.60	43.63	3.59	99.64	117.47	26.17	11.49	99.91	49.51	25.96	12.48	99.95	33.67	24.57	7.05	98.89	56.35	31.51	2.79	99.42
	3	54.90	53.59	4.54	97.89	56.60	52.36	3.92	99.41	117.47	27.84	7.75	99.59	49.51	30.72	11.87	99.94	33.67	36.06	4.80	98.65	56.35	38.13	1.62	93.59
	4	57.54	44.31	6.86	99.55	60.30	47.06	4.10	99.66	111.43	27.49	14.93	99.85	44.40	27.68	13.44	99.43	29.57	29.42	13.04	99.56	50.43	45.01	7.76	98.79
	5	57.54	59.77	5.24	95.94	60.30	53.27	5.65	99.78	111.43	34.55	4.14	99.61	44.40	37.10	6.48	99.83	29.57	59.19	4.03	96.14	50.43	54.94	2.87	92.33
	6	57.54	55.23	6.33	99.24	60.30	54.31	4.33	98.83	111.43	33.99	5.31	99.76	44.40	33.04	5.60	99.98	29.57	48.01	2.83	96.61	50.43	61.71	3.87	99.22
	7	53.65	42.98	7.27	99.62	52.28	33.76	2.27	98.50	116.98	29.93	11.17	99.59	50.67	29.31	9.40	99.37	28.43	A.G.	A.G.	A.G.	57.32	44.99	3.94	98.45
	8	53.65	43.29	7.70	99.93	52.28	36.63	3.06	98.93	116.98	28.33	6.32	99.86	50.67	26.65	5.76	99.73	28.43	A.G.	A.G.	A.G.	57.32	49.66	3.33	99.51
	9	53.65	52.86	10.23	98.79	52.28	52.71	6.27	98.44	116.98	31.28	6.54	99.84	50.67	30.62	6.26	99.92	28.43	36.68	3.31	91.80	57.32	56.44	5.96	98.58
	10	57.90	59.18	5.74	97.19	61.60	56.00	4.85	99.45	128.38	38.57	4.93	99.78	57.84	39.00	5.22	99.39	47.40	48.76	3.79	97.82	65.67	63.02	5.45	94.28
	11	57.90	A.G.	A.G.	A.G.	61.60	47.82	5.13	98.44	128.38	29.76	4.96	99.06	57.84	29.80	5.65	99.77	47.40	25.47	2.07	99.22	65.67	51.51	3.99	97.15
buckwheat	1	68.20	44.37	9.80	99.58	113.67	47.88	5.93	99.49	41.23	33.24	12.53	99.56	51.06	33.88	12.12	99.19	14.75	33.18	5.54	99.27	55.79	46.48	7.34	99.79
	2	68.20	44.24	12.56	99.83	113.67	47.32	9.09	99.79	41.23	31.58	11.58	99.76	51.06	31.55	8.60	98.95	15.75	29.30	6.80	95.05	55.79	45.62	8.62	99.69
	3	68.20	34.00	13.81	99.10	113.67	35.60	16.37	99.58	41.23	17.33	8.68	99.96	51.06	22.60	10.58	99.13	15.75	36.09	4.40	97.46	55.79	34.45	6.74	99.37
	4	68.20	39.95	21.40	98.52	113.67	40.67	28.10	99.84	41.23	22.80	7.40	99.46	51.06	25.33	9.19	99.59	15.75	28.71	11.22	98.84	55.79	40.57	16.74	98.11
	5	68.20	29.74	18.27	98.80	113.67	28.02	17.01	98.16	41.23	14.96	10.45	99.98	51.06	15.37	9.80	100.0	15.75	25.21	7.22	96.89	55.79	34.46	6.62	98.37
	6	68.20	43.36	12.36	98.90	113.67	42.97	8.94	97.73	41.23	31.69	8.10	99.92	51.06	32.25	8.05	99.94	15.75	28.62	6.86	98.95	55.79	38.71	5.28	99.81
	7	59.52	A.G.	A.G.	A.G.	92.66	47.46	8.82	98.54	43.42	22.72	7.00	99.65	53.61	23.58	7.03	99.67	14.77	29.07	7.59	99.35	57.83	47.48	7.76	98.65
	8	59.52	57.14	11.35	98.64	92.66	57.19	9.81	96.88	43.42	27.78	6.86	99.86	53.61	27.72	10.71	99.55	14.77	31.11	4.34	99.64	57.83	43.08	4.79	99.85
	9	59.52	42.83	5.26	98.69	92.66	41.01	4.39	97.51	43.42	19.19	6.63	99.84	53.61	20.46	6.52	99.75	14.77	28.80	9.58	99.06	57.83	38.91	3.19	99.44
	10	59.52	50.69	8.02	98.47	92.66	45.90	2.88	96.85	43.42	16.32	11.25	99.99	53.61	17.94	8.65	100.0	14.77	22.78	5.98	99.22	57.83	38.54	4.21	99.08
	11	59.52	55.02	13.58	96.56	92.66	A.G.	A.G.	A.G.	43.42	16.37	7.37	100.0	53.61	20.66	6.52	100.0	14.77	29.47	6.83	99.57	57.83	43.95	4.45	98.98
quinoa	1	59.16	A.G.	A.G.	A.G.	56.86	50.32	3.32	95.97	65.93	41.18	4.23	97.90	77.83	44.98	5.49	99.29	23.13	A.G.	A.G.	A.G.	84.71	A.G.	A.G.	A.G.
	2	59.16	N.C.	N.C.	N.C.	56.86	A.G.	A.G.	A.G.	65.93	31.08	2.43	98.76	77.83	34.85	1.87	99.18	23.13	87.33	5.64	30.04	84.71	A.G.	A.G.	A.G.
	3	59.16	A.G.	A.G.	A.G.	56.86	A.G.	A.G.	A.G.	65.93	12.04	5.52	99.97	77.83	19.67	4.60	99.58	23.13	N.C.	N.C.	N.C.	84.71	A.G.	A.G.	A.G.
	4	59.16	58.15	2.68	92.50	56.86	A.G.	A.G.	A.G.	65.93	17.25	3.97	99.35	77.83	24.77	4.65	99.77	23.13	N.C.	N.C.	N.C.	84.71	22.32	2.31	93.00
	5	59.16	32.85	4.50	96.38	56.86	A.G.	A.G.	A.G.	65.93	A.G.	A.G.	A.G.	77.83	26.71	4.91	99.68	23.13	N.C.	N.C.	N.C.	84.71	A.G.	A.G.	A.G.
	6	59.16	80.46	6.34	89.29	56.86	11.10	62.38	87.08	65.93	46.54	3.15	98.15	77.83	48.19	8.17	98.01	23.13	101.70	6.78	39.85	84.71	A.G.	A.G.	A.G.
	7	70.62	N.C.	N.C.	N.C.	57.41	46.14	5.76	75.37	56.40	34.22	3.21	99.39	85.39	34.41	3.47	98.67	24.79	N.C.	N.C.	N.C.	90.48	13.83	4.26	70.78
	8	70.62	58.63	7.16	90.66	57.41	A.G.	A.G.	A.G.	56.40	27.42	2.11	99.54	85.39	31.82	2.76	98.57	24.79	A.G.	A.G.	A.G.	90.48	A.G.	A.G.	A.G.
	9	70.62	A.G.	A.G.	A.G.	57.41	1487.00	0.20	I.R.	56.40	30.11	3.94	99.84	85.39	29.57	3.35	99.71	24.79	62.75	10.26	75.76	90.48	N.C.	N.C.	N.C.
	10	70.62	A.G.	A.G.	A.G.	57.41	N.C.	N.C.	N.C.	56.40	39.40	3.77	99.30	85.39	44.52	3.31	96.71	24.79	A.G.	A.G.	A.G.	90.48	8.95	6.60	63.60
	11	70.62	A.G.	A.G.	A.G.	57.41	N.C.	N.C.	N.C.	56.40	43.57	5.55	99.69	85.39	48.26	3.26	98.53	24.79	A.G.	A.G.	A.G.	90.48	N.C.	N.C.	N.C.

F – the number of successive fermentation (F1F11). AT_{50} – the percentage of attenuation time needed to achieve 50% of the total change in the extract or ethanol content. R^2 – the goodness of curve fit. conc. − concentration. A.G. – ambiguous fit. N.C. – fit not converged. I.R. – interrupted.

Table 51: The initial concentrations and AT_{50}, curve slope and R^2 values of Thr, Arg, Ala, Tyr, Val and Met for eleven successive fermentations of the barley, buckwheat and quinoa wort

Preglednica 51: Začetne koncentracije ter vrednosti AT_{50}, naklona regresijske krivulje in R^2 za Thr, Arg, Ala, Tyr, Val in Met tekom enajst zaporednih fermentacij ječmenove, ajdove in kvinojine pivine

		Thr				Arg				Ala				Tyr				Val				Met			
wort type	F	initial conc. [mg/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [mg/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [mg/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [mg/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [mg/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [mg/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]
barley	1	39.55	37.21	5.89	99.81	95.39	45.93	4.65	99.67	135.04	60.04	8.02	99.80	68.01	56.81	2.56	98.63	74.55	47.57	4.15	99.48	32.39	31.28	5.79	98.58
	2	39.55	A.G.	A.G.	A.G.	95.39	34.83	5.20	99.91	135.04	53.98	3.99	99.89	68.01	36.20	6.67	99.28	74.55	35.89	5.34	99.73	32.39	32.68	3.25	97.86
	3	39.55	A.G.	A.G.	A.G.	95.39	46.16	4.70	98.57	135.04	58.35	10.35	99.94	68.01	59.11	4.85	99.74	74.55	44.85	4.71	99.37	32.39	27.33	7.22	98.22
	4	36.41	27.66	11.13	99.33	87.45	54.75	13.29	99.69	158.70	57.25	9.34	98.21	59.67	44.79	6.31	99.52	70.50	39.73	6.88	99.24	29.86	37.71	5.54	98.97
	5	36.41	36.34	5.91	99.89	87.45	A.G.	A.G.	A.G.	158.70	60.39	16.83	99.27	59.67	59.37	5.41	95.52	70.50	49.28	4.70	98.23	29.86	37.41	3.53	95.90
	6	36.41	33.45	4.47	99.77	87.45	59.70	8.45	99.09	158.70	59.10	6.46	98.87	59.67	51.93	4.44	97.14	70.50	52.83	4.94	99.42	29.86	42.12	3.93	99.75
	7	40.19	30.67	9.80	99.27	94.60	61.06	13.21	98.79	165.26	23.61	8.94	96.36	68.54	43.27	2.96	97.21	73.79	42.94	5.62	99.06	33.32	30.44	3.01	97.98
	8	40.19	28.07	5.30	99.67	94.60	56.19	5.73	99.52	165.26	A.G.	A.G.	A.G.	68.54	44.76	4.89	99.80	73.79	44.54	8.64	99.82	33.32	37.79	4.04	97.90
	9	40.19	32.15	5.55	99.95	94.60	59.76	18.45	99.66	165.26	A.G.	A.G.	A.G.	68.54	50.60	5.72	99.56	73.79	50.41	7.72	99.72	33.32	40.75	4.14	99.51
	10	46.42	44.37	4.34	99.16	108.51	58.49	10.37	99.57	145.53	A.G.	A.G.	A.G.	80.21	57.04	5.70	98.20	83.73	54.07	6.97	99.45	38.36	38.77	5.13	99.79
	11	46.42	27.57	3.88	99.20	108.51	57.28	9.45	99.76	145.53	A.G.	A.G.	A.G.	80.21	42.54	3.53	99.11	83.73	43.97	6.92	99.56	38.36	31.82	3.52	99.45
buckwheat	1	43.91	34.97	12.51	99.66	116.78	44.58	19.96	99.20	85.38	47.06	8.91	99.33	44.03	44.62	8.50	99.84	72.08	41.89	10.04	99.34	28.34	35.92	9.01	99.52
	2	43.91	34.97	13.20	98.72	116.78	43.39	20.36	99.43	85.38	44.75	15.97	99.71	44.03	43.10	10.19	99.79	72.08	39.25	11.97	99.33	28.34	36.14	8.50	99.81
	3	43.91	31.78	10.71	99.61	116.78	39.45	11.25	99.56	85.38	38.71	10.09	99.76	44.03	33.02	13.18	99.82	72.08	31.30	6.65	98.72	28.34	26.47	5.69	99.53
	4	43.91	27.57	8.94	99.71	116.78	40.49	17.54	98.78	85.38	39.61	14.35	99.44	44.03	37.80	7.71	98.63	72.08	31.09	5.02	99.34	28.34	28.23	4.42	99.40
	5	43.91	21.85	6.35	99.18	116.78	39.55	17.52	98.96	85.38	34.71	7.28	99.52	44.03	33.60	6.12	99.06	72.08	29.28	8.68	99.02	28.34	19.18	5.02	98.79
	6	43.91	34.67	8.09	99.91	116.78	45.35	10.64	98.72	85.38	46.86	6.31	99.08	44.03	43.55	5.59	99.61	72.08	41.09	6.05	98.60	28.34	34.82	5.28	99.32
	7	49.76	24.01	6.80	99.29	103.05	46.97	13.46	99.69	79.71	49.91	5.74	99.36	44.16	40.21	4.51	99.74	70.47	37.58	4.72	99.48	27.79	26.91	4.83	99.68
	8	49.76	28.36	13.22	99.80	103.05	46.69	10.55	99.08	79.71	46.57	4.80	99.49	44.16	41.86	4.67	99.82	70.47	44.77	10.63	98.12	27.79	32.62	4.66	99.70
	9	49.76	21.95	6.25	99.83	103.05	45.68	5.66	98.78	79.71	46.67	3.24	99.39	44.16	35.90	4.10	99.64	70.47	29.66	3.43	99.50	27.79	15.25	3.03	99.91
	10	49.76	20.04	5.57	99.87	103.05	48.08	8.41	98.32	79.71	50.27	5.57	98.69	44.16	38.78	3.99	99.18	70.47	29.71	3.15	99.08	27.79	14.94	3.91	99.94
	11	49.76	22.01	5.00	99.94	103.05	42.32	5.70	99.07	79.71	A.G.	A.G.	A.G.	44.16	40.56	3.10	99.06	70.47	37.43	3.29	98.66	27.79	22.19	3.01	99.85
quinoa	1	69.99	44.35	6.77	99.26	155.10	A.G.	A.G.	A.G.	74.26	42.95	3.37	93.99	107.86	A.G.	A.G.	A.G.	81.48	A.G.	A.G.	A.G.	46.98	A.G.	A.G.	A.G.
	2	69.99	39.97	3.19	99.40	155.10	N.C.	N.C.	N.C.	74.26	402.90	0.80	I.R.	107.86	A.G.	A.G.	A.G.	81.48	A.G.	A.G.	A.G.	46.98	A.G.	A.G.	A.G.
	3	69.99	23.42	3.40	98.90	155.10	N.C.	N.C.	N.C.	74.26	19.98	2.53	99.82	107.86	A.G.	A.G.	A.G.	81.48	22.20	83.23	67.98	46.98	A.G.	A.G.	A.G.
	4	69.99	25.97	9.16	99.32	155.10	A.G.	A.G.	A.G.	74.26	9.35	2.89	96.95	107.86	A.G.	A.G.	A.G.	81.48	A.G.	A.G.	A.G.	46.98	A.G.	A.G.	A.G.
	5	69.99	27.18	9.69	99.58	155.10	A.G.	A.G.	A.G.	74.26	10.74	3.57	98.49	107.86	A.G.	A.G.	A.G.	81.48	12.50	79.84	73.53	46.98	A.G.	A.G.	A.G.
	6	69.99	45.91	5.80	99.58	155.10	N.C.	N.C.	N.C.	74.26	43.40	2.20	96.34	107.86	A.G.	A.G.	A.G.	81.48	A.G.	A.G.	A.G.	46.98	A.G.	A.G.	A.G.
	7	80.29	33.72	3.91	98.50	165.59	N.C.	N.C.	N.C.	80.66	32.91	5.17	93.50	111.23	N.C.	N.C.	N.C.	92.70	N.C.	N.C.	N.C.	38.90	22.42	3.84	93.79
	8	80.29	28.60	2.79	99.57	165.59	N.C.	N.C.	N.C.	80.66	A.G.	A.G.	A.G.	111.23	N.C.	N.C.	N.C.	92.70	A.G.	A.G.	A.G.	38.90	19.07	2.11	95.06
	9	80.29	31.06	3.11	99.70	165.59	N.C.	N.C.	N.C.	80.66	34.78	4.42	98.87	111.23	A.G.	A.G.	A.G.	92.70	A.G.	A.G.	A.G.	38.90	A.G.	A.G.	A.G.
	10	80.29	45.73	4.17	98.72	165.59	A.G.	A.G.	A.G.	80.66	45.35	3.93	94.40	111.23	N.C.	N.C.	N.C.	92.70	N.C.	N.C.	N.C.	38.90	A.G.	A.G.	A.G.
	11	80.29	40.37	3.85	99.31	165.59	N.C.	N.C.	N.C.	80.66	65.53	4.19	97.65	111.23	A.G.	A.G.	A.G.	92.70	A.G.	A.G.	A.G.	38.90	A.G.	A.G.	A.G.

F – the number of successive fermentation (F1F11). AT_{50} – the percentage of attenuation time needed to achieve 50% of the total change in the extract or ethanol content. R^2 – the goodness of curve fit. conc. − concentration. A.G. – ambiguous fit. N.C. – fit not converged. I.R. – interrupted.

Table 52: The initial concentrations and AT_{50}, curve slope and R^2 values of Trp, Phe, Ile, Leu, Lys and sum of all AAs for eleven successive fermentations of the barley, buckwheat and quinoa wort

Preglednica 51: Začetne koncentracije ter vrednosti AT_{50}, naklona regresijske krivulje in R^2 za Trp, Phe, Ile, Leu, Lys in vsote vseh AAs tekom enajst zaporednih fermentacij ječmenove, ajdove in kvinojine pivine

		Trp				Phe				Ile				Leu				Lys				sum AAs			
wort type	F	initial conc. [mg/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [mg/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [mg/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [mg/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [mg/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]	initial conc. [mg/L]	AT_{50} [%]	curve slope [x100]	R^2 [%]
barley	1	87.05	75.15	14.42	99.43	83.83	42.46	4.14	99.42	47.32	38.03	4.72	99.94	81.39	34.53	5.62	99.92	78.41	34.43	6.56	99.86	1191.43	43.18	3.12	99.94
	2	87.05	103.80	1.70	94.91	83.83	32.98	7.29	99.83	47.32	30.71	6.91	99.80	81.39	27.57	7.66	100.0	78.41	24.54	9.94	99.32	1191.43	31.54	5.15	99.51
	3	87.05	85.06	11.06	97.90	83.83	40.84	5.08	99.53	47.32	36.08	8.04	99.62	81.39	31.73	7.52	99.76	78.41	25.19	5.58	98.87	1191.43	38.39	3.70	99.53
	4	80.19	70.72	11.87	99.39	74.79	37.61	6.67	99.84	43.45	34.66	7.73	99.79	76.00	30.93	8.45	99.82	69.59	30.36	7.24	99.52	1140.28	38.66	3.91	99.44
	5	80.19	A.G.	A.G.	A.G.	74.79	47.85	5.34	99.65	43.45	46.38	6.14	99.32	76.00	40.58	5.06	99.87	69.59	28.88	6.18	99.50	1140.28	46.63	3.95	99.27
	6	80.19	71.42	4.00	95.40	74.79	47.62	4.35	98.75	43.45	44.00	6.11	99.83	76.00	36.09	5.29	99.85	69.59	32.08	7.78	99.53	1140.28	44.93	3.61	99.78
	7	84.29	A.G.	A.G.	A.G.	87.42	36.94	5.24	99.84	47.99	35.81	7.36	99.77	81.48	33.37	8.67	99.79	81.63	26.43	7.65	99.64	1217.84	33.33	3.74	99.17
	8	84.29	70.75	6.72	99.44	87.42	38.65	5.63	99.14	47.99	37.63	7.12	99.15	81.48	34.26	6.87	99.38	81.63	24.70	4.33	99.48	1217.84	35.86	3.02	99.49
	9	84.29	64.67	7.52	98.74	87.42	42.63	3.90	99.52	47.99	41.35	5.08	99.88	81.48	37.35	6.04	99.96	81.63	28.92	4.18	99.28	1217.84	40.90	3.29	99.49
	10	101.05	A.G.	A.G.	A.G.	93.66	44.21	3.72	99.54	54.90	45.63	5.37	99.26	91.08	40.10	4.72	99.78	88.40	28.07	3.47	98.73	1350.64	43.90	3.01	98.86
	11	101.05	A.G.	A.G.	A.G.	93.66	39.84	5.86	99.20	54.90	36.72	5.80	99.03	91.08	35.40	7.12	99.27	88.40	26.67	4.52	99.61	1350.64	35.70	3.03	99.19
buckwheat	1	79.72	51.34	15.88	99.74	47.22	38.55	10.59	99.71	36.96	37.91	12.36	99.84	64.59	34.69	12.68	99.20	44.97	32.27	8.29	99.23	1008.68	41.41	6.90	99.80
	2	79.72	47.64	19.76	99.86	47.22	37.43	10.59	99.85	36.96	36.71	14.13	99.98	64.59	33.63	12.87	99.56	44.97	32.74	17.02	99.94	1009.68	40.74	8.28	99.83
	3	79.72	45.87	19.67	99.90	47.22	35.12	6.28	99.20	36.96	33.22	6.97	99.49	64.59	25.75	11.05	99.73	44.97	25.05	5.99	99.79	1009.68	33.72	6.56	99.66
	4	79.72	42.96	17.10	99.50	47.22	28.31	5.38	99.58	36.96	29.13	6.80	99.50	64.59	A.G.	A.G.	A.G.	44.97	21.72	6.74	99.74	1009.68	35.28	5.90	98.63
	5	79.72	A.G.	A.G.	A.G.	47.22	24.61	5.85	99.81	36.96	26.42	6.08	99.81	64.59	19.96	5.88	99.86	44.97	17.54	5.95	99.51	1009.68	28.11	5.46	99.36
	6	79.72	52.98	14.85	99.79	47.22	38.76	5.46	99.92	36.96	38.18	7.01	99.73	64.59	37.11	7.47	99.51	44.97	31.83	7.60	99.80	1009.68	41.01	5.86	99.64
	7	75.38	53.09	8.62	99.62	47.22	35.16	5.96	99.74	38.17	A.G.	A.G.	A.G.	64.63	30.32	5.19	99.71	46.11	18.75	5.02	99.77	968.26	38.69	4.19	99.42
	8	75.38	54.51	5.99	99.06	47.22	31.70	3.63	99.83	38.17	32.93	4.38	99.55	64.63	30.53	6.24	99.27	46.11	17.14	3.15	99.44	968.26	39.30	3.59	99.53
	9	75.38	57.97	4.00	97.28	47.22	25.08	3.30	99.90	38.17	27.18	4.97	99.77	64.63	23.76	6.40	99.98	46.11	16.20	4.40	99.77	968.26	28.94	2.80	99.88
	10	75.38	A.G.	A.G.	A.G.	47.22	24.18	3.42	99.96	38.17	25.16	5.07	99.92	64.63	18.52	5.40	99.96	46.11	10.81	4.54	99.38	968.26	24.56	2.23	99.37
	11	75.38	A.G.	A.G.	A.G.	47.22	33.44	3.42	98.84	38.17	34.62	3.87	99.28	64.63	26.95	3.85	99.81	46.11	16.60	5.78	99.91	968.26	33.46	2.64	98.92
quinoa	1	53.93	42.73	4.44	91.89	103.49	A.G.	A.G.	90.00	70.59	48.88	7.31	99.71	114.72	49.31	5.25	99.32	105.79	36.49	2.66	97.83	1351.81	38.60	2.88	96.85
	2	53.93	33.05	4.35	91.84	103.49	A.G.	A.G.	88.19	70.59	27.30	1.52	99.43	114.72	38.96	1.75	98.20	105.79	A.G.	A.G.	A.G.	1351.81	A.G.	A.G.	A.G.
	3	53.93	18.53	2.12	96.27	103.49	A.G.	A.G.	99.18	70.59	14.35	4.48	99.66	114.72	22.20	88.57	88.45	105.79	4.75	2.51	99.13	1351.81	14.54	2.39	99.67
	4	53.93	A.G.	A.G.	A.G.	103.49	A.G.	A.G.	83.56	70.59	31.12	4.18	99.28	114.72	25.75	3.50	97.77	105.79	24.77	3.59	98.26	1351.81	11.52	2.70	97.95
	5	53.93	3.87	3.96	99.19	103.49	A.G.	A.G.	98.87	70.59	28.09	4.33	99.72	114.72	26.41	3.84	99.12	105.79	30.56	8.17	99.91	1351.81	16.24	3.58	98.85
	6	53.93	53.50	13.75	96.33	103.49	A.G.	A.G.	95.14	70.59	54.74	3.76	97.80	114.72	56.68	4.75	98.74	105.79	55.19	4.71	97.82	1351.81	41.44	2.04	96.50
	7	63.47	22.70	6.34	74.98	114.95	A.G.	A.G.	89.35	78.96	52.63	2.52	97.97	129.11	39.88	4.24	99.20	121.98	34.14	2.21	99.20	1462.93	26.76	3.58	96.21
	8	63.47	A.G.	A.G.	A.G.	114.95	A.G.	A.G.	83.82	78.96	38.02	5.25	97.81	129.11	44.98	10.76	97.39	121.98	25.90	2.12	96.17	1462.93	17.81	1.98	99.25
	9	63.47	A.G.	A.G.	A.G.	114.95	A.G.	A.G.	94.40	78.96	18.90	0.80	97.55	129.11	39.55	6.16	95.85	121.98	18.67	1.47	96.88	1462.93	20.05	2.11	97.84
	10	63.47	80.19	4.54	79.93	114.95	A.G.	A.G.	85.42	78.96	A.G.	A.G.	A.G.	129.11	56.89	2.18	94.18	121.98	46.56	2.53	97.85	1462.93	36.61	2.06	98.91
	11	63.47	60.96	9.15	96.12	114.95	A.G.	A.G.	60.83	78.96	A.G.	A.G.	A.G.	129.11	50.43	4.06	99.52	121.98	44.07	2.23	98.95	1462.93	45.24	2.22	99.84

F – the number of successive fermentation (F1F11). AT_{50} – the percentage of attenuation time needed to achieve 50% of the total change in the extract or ethanol content. R^2 – the goodness of curve fit. conc. − concentration. AAs – amino acids. A.G. – ambiguous fit. N.C. – fit not converged.

Sum AAs

In the case of barley, the overall assimilation capacity of yeast was influenced by the serial repitching and it declined slowly until F6 but later on it improved again (Figure 38). The total AA assimilation was the most efficacious (~90% of the sum initial AAs) and the slowest (t95 = ~90%) in F1 and F8 and *vice versa* in F5 and F6 (~70% of the sum initial AAs, t95 = ~70%). The initial rate of total AA assimilation gradually increased over successive fermentations.

On the other hand, the serial repitching had no influence on the total assimilation of AAs during the successive fermentation of buckwheat wort (Figure 41). Besides, overall assimilation was very fast (t95 = 50-70%) and efficacious (> 90% of the sum initial AAs). The initial rate of total AA assimilation increased over successive fermentations even more prominently as in barley and the overall AA assimilation became more linear which is expressed by a firm decrease in curve slope (Table 52).

In the case of quinoa (Figure 44), the total AA assimilation was considerably weakened, ranging only from 20-50% of the sum initial. Moreover, the absolute values were also around 50% lower than in barley and buckwheat, despite the higher initial concentration of AAs in wort (Table 52). What was also obvious in the case of quinoa was the influence of the serial repitching where the AA assimilation was faster and more efficacious from F1 to F6; the efficiency of AA assimilation increased after F2 and it reached the initial capacity again in F6. Later on the overall AA assimilation slowed down and the total degree practically halved in F11.

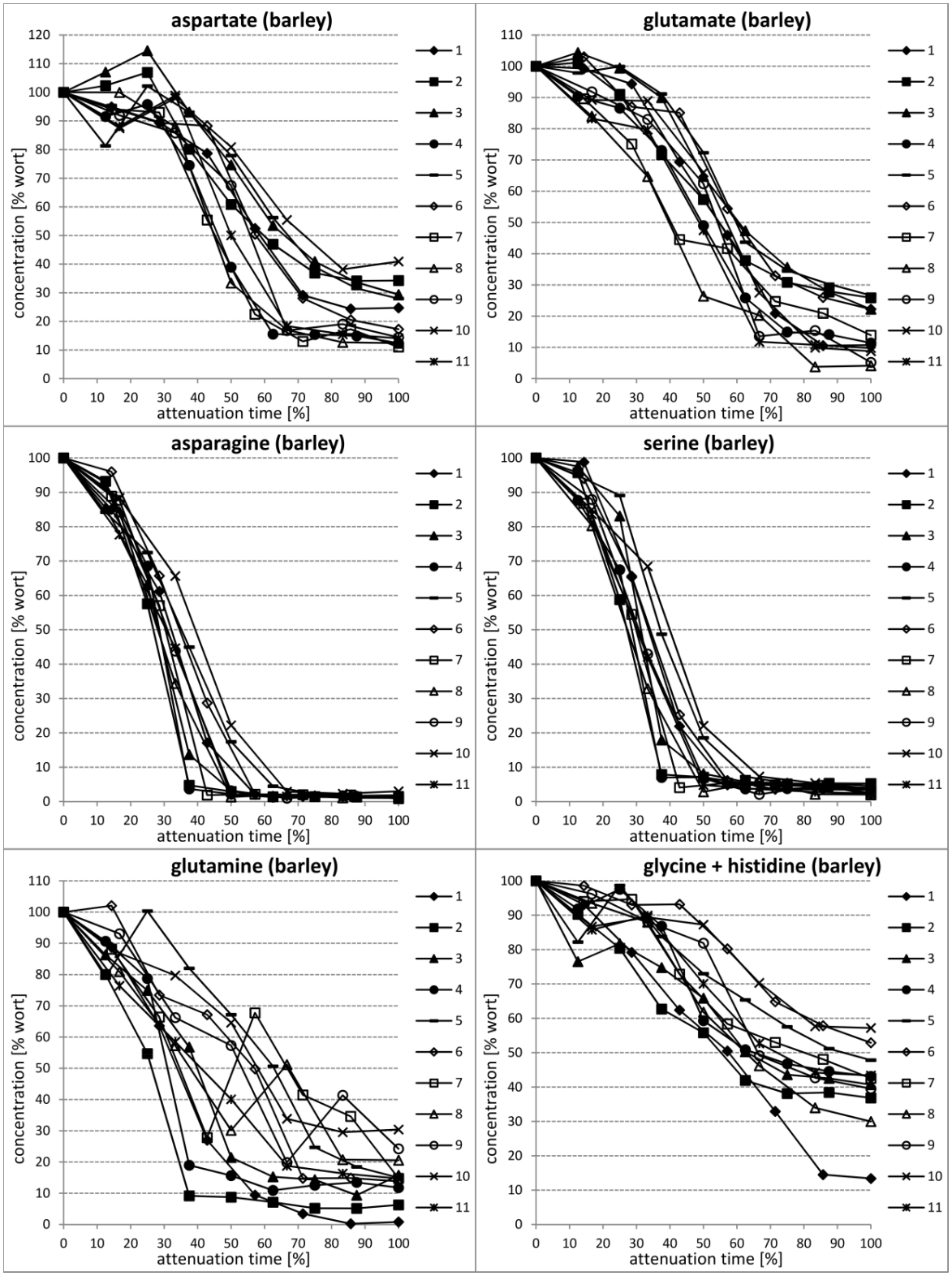

Figure 36: The assimilation profile of Asp, Glu, Asn, Ser, Gln and Gly+His for eleven successive fermentations of the barley wort
01-11: the numbering of a particular successive fermentation.
Slika 36: Profil privzema Asp, Glu, Asn, Ser, Gln in Gly+His za enajst zaporednih fermentacij ječmenove pivine
01-11: številčenje posamezne zaporedne fermentacije.

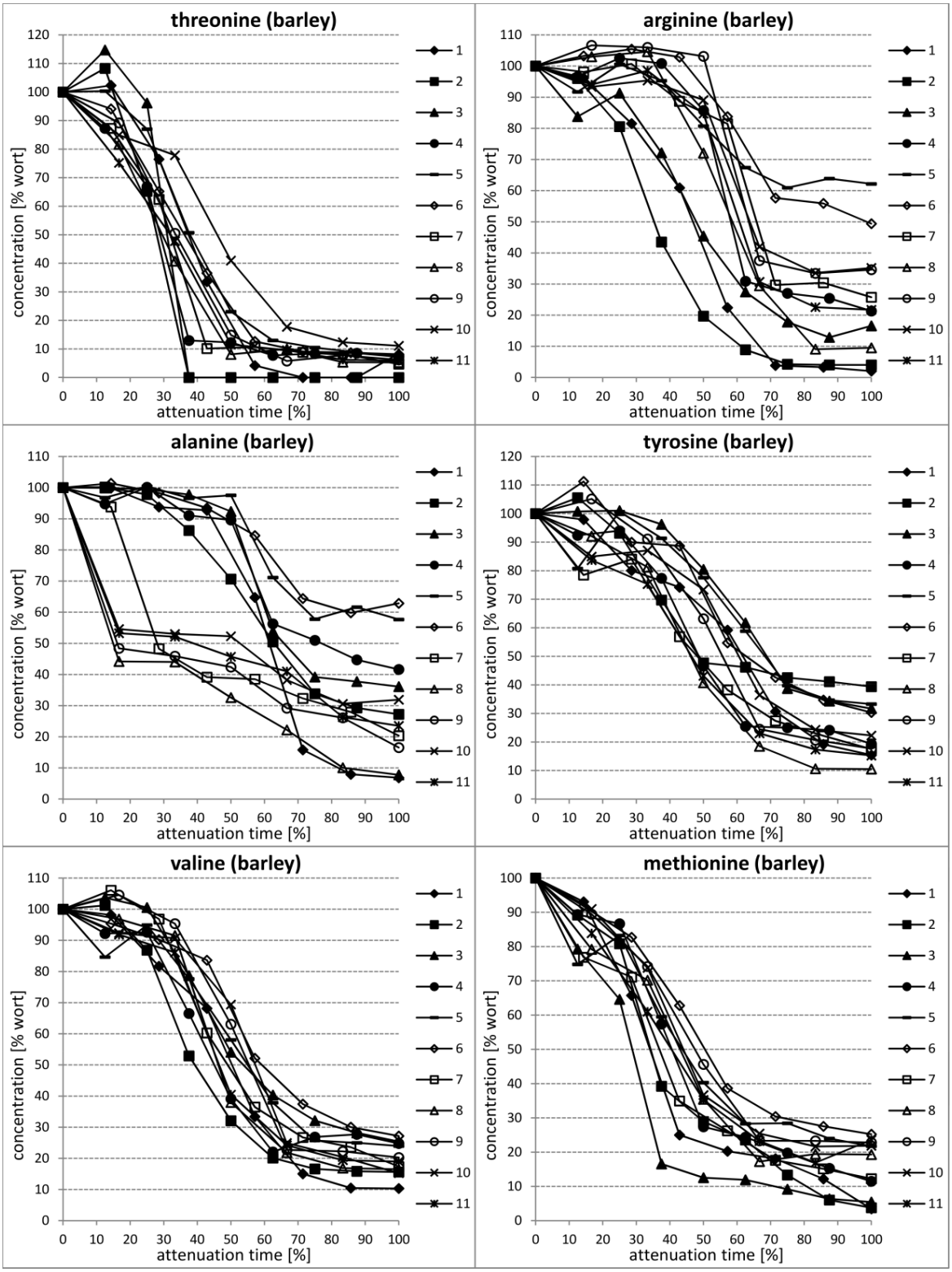

Figure 37: The assimilation profile of Thr, Arg, Ala, Tyr, Val and Met for eleven successive fermentations of barley wort
01-11: the numbering of a particular successive fermentation.
Slika 37: Profil privzema Thr, Arg, Ala, Tyr, Val in Met za enajst zaporednih fermentacij ječmenove pivine
01-11: številčenje posamezne zaporedne fermentacije.

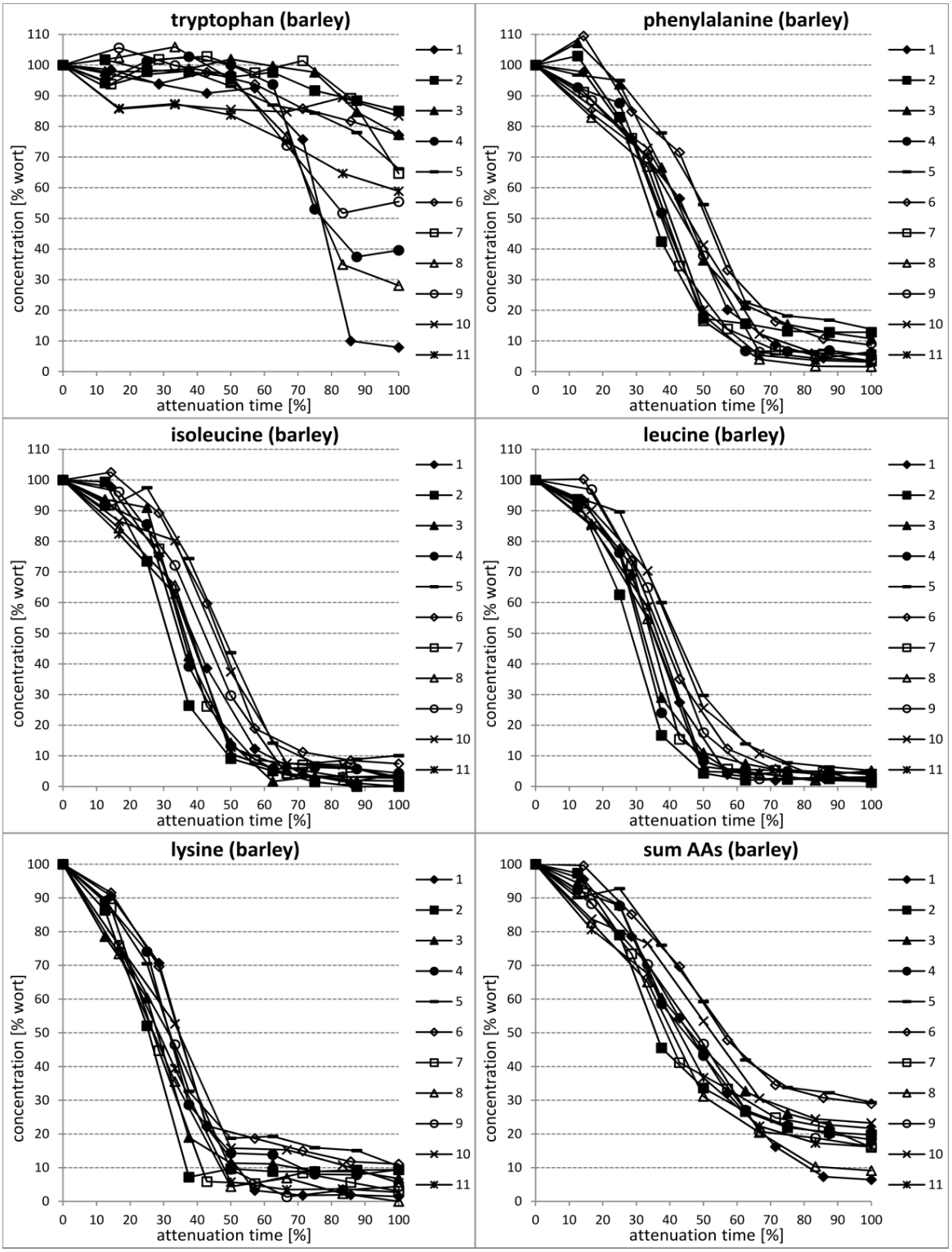

Figure 38: The assimilation profile of Trp, Phe, Ile, Leu, Lys and the total amino acids for eleven successive fermentations of the barley wort
01-11: the numbering of a particular successive fermentation. AAs – amino acids.
Slika 38: Profil privzema Trp, Phe, Ile, Leu, Lys in vsote vseh aminokislin za enajst zaporednih fermentacij ječmenove pivine
01-11: številčenje posamezne zaporedne fermentacije. AAs – amino acids.

Buckwheat wort fermentation

In the case of buckwheat (Figure 39, Figure 40 and Figure 41), the final assimilation was very little influenced by the serial repitching in the case of Gln, Gly+His, Tyr, Val, Met and Lys ($\Delta_{max-min}$ = 10-20%) but practically no influence of the serial repitching on the final assimilation was observed for all the other AAs. On average, Asp, Glu, Arg, Ala, Tyr and Trp were assimilated to a higher degree and only Val to a lower, comparing to barley. Regarding the assimilation profile, it was the least influenced by the serial repitching in the case of Asn, Ser, Arg, Ala and Tyr (SRf < ~200, no bad regressions) and the most in the case of Asp, Glu, Trp and Lys (SRf > ~400, mostly with bad regressions). As in barley, Ala, Arg and Trp were marked by an obvious initial lag phase. No unique properties of fermentation profiles were observed and the overall assimilation dynamics was very close to a typical one observed for barley.

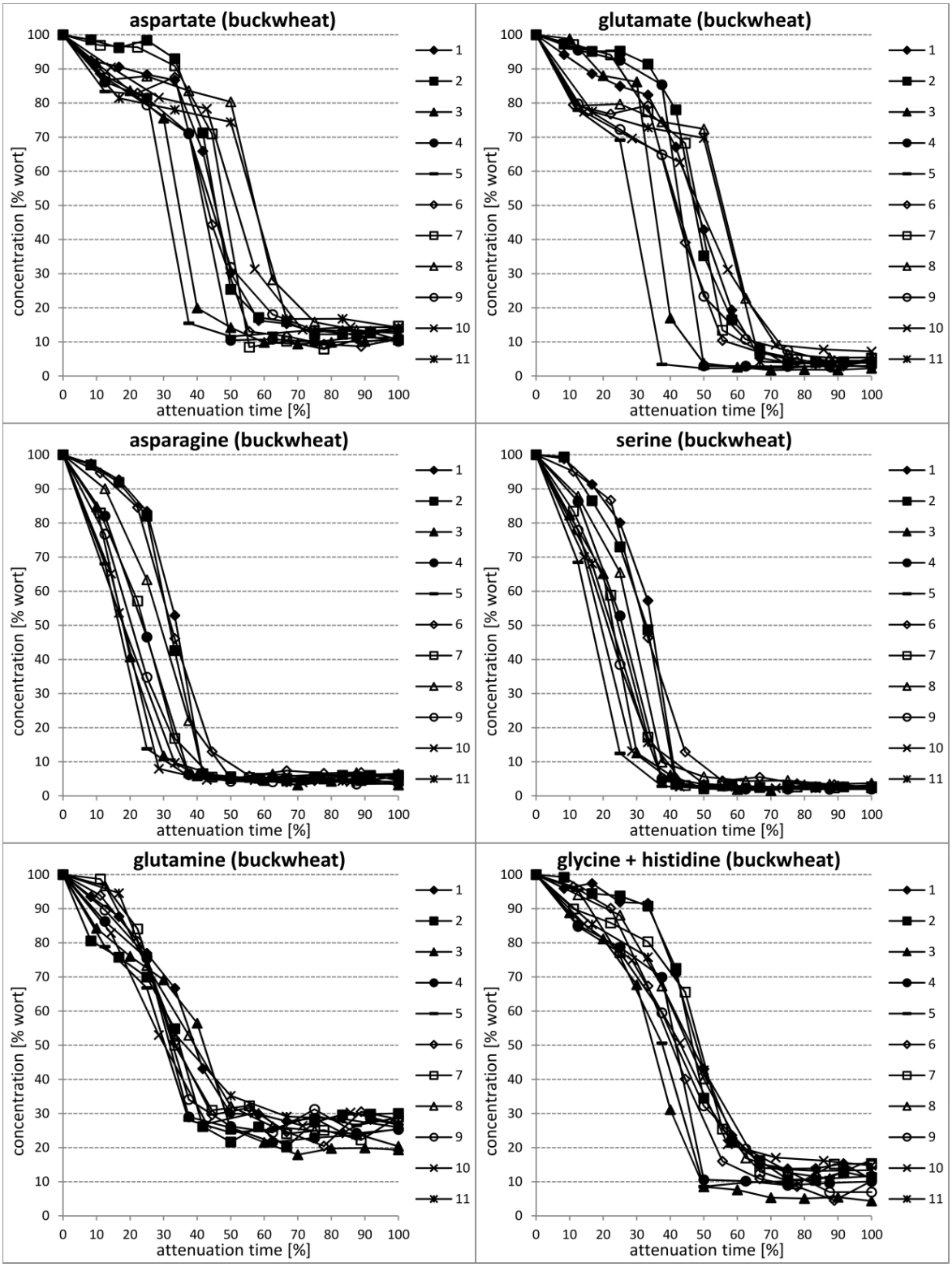

Figure 39: The assimilation profile of Asp, Glu, Asn, Ser, Gln and Gly+His for eleven successive fermentations of the buckwheat wort
01-11: the numbering of a particular successive fermentation.
Slika 39: Profil privzema Asp, Glu, Asn, Ser, Gln in Gly+His za enajst zaporednih fermentacij ajdove pivine
01-11: številčenje posamezne zaporedne fermentacije.

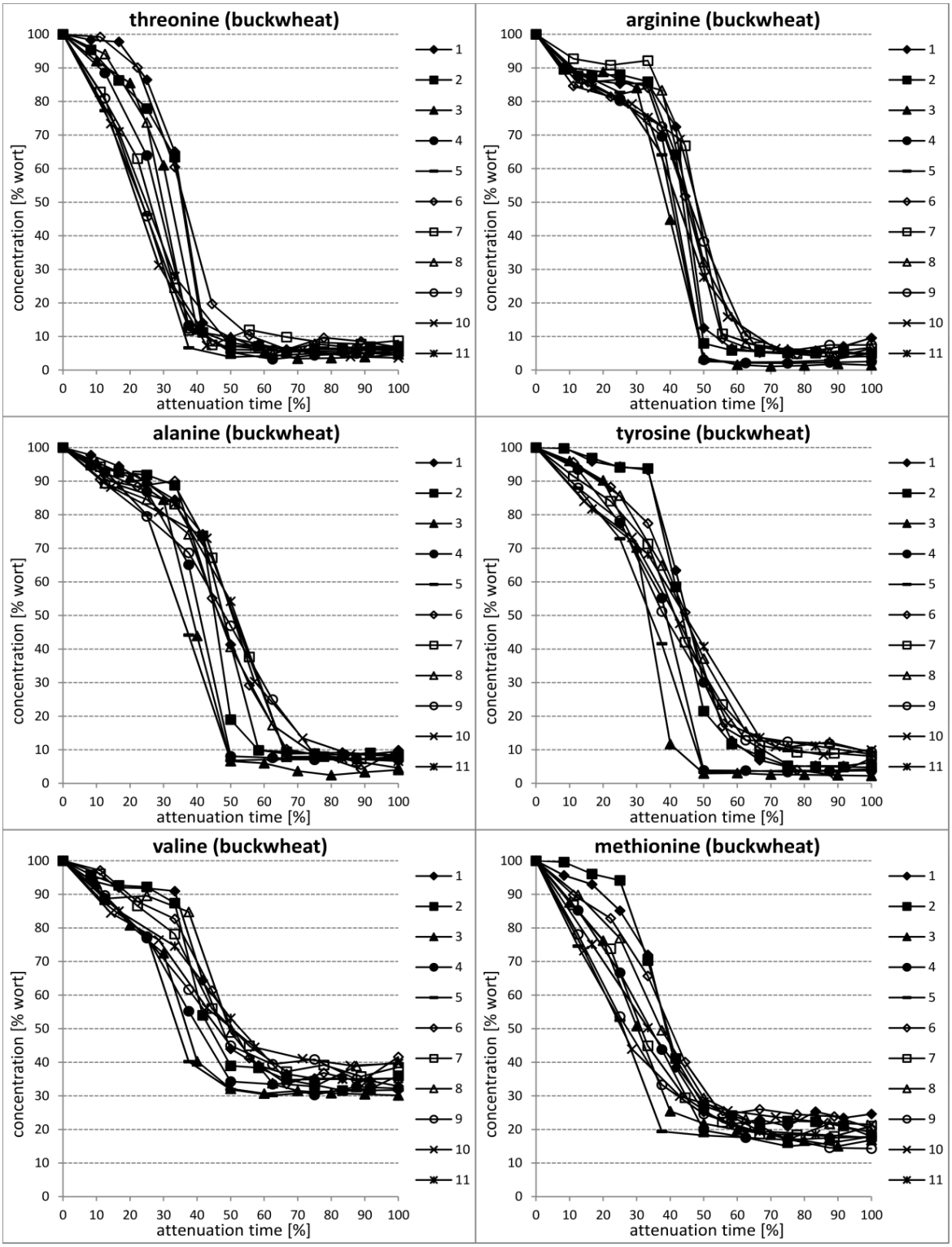

Figure 40: The assimilation profile of Thr, Arg, Ala, Tyr, Val and Met for eleven successive fermentations of the buckwheat wort
01-11: the numbering of a particular successive fermentation.
Slika 40: Profil privzema Thr, Arg, Ala, Tyr, Val in Met za enajst zaporednih fermentacij ajdove pivine
01-11: številčenje posamezne zaporedne fermentacije.

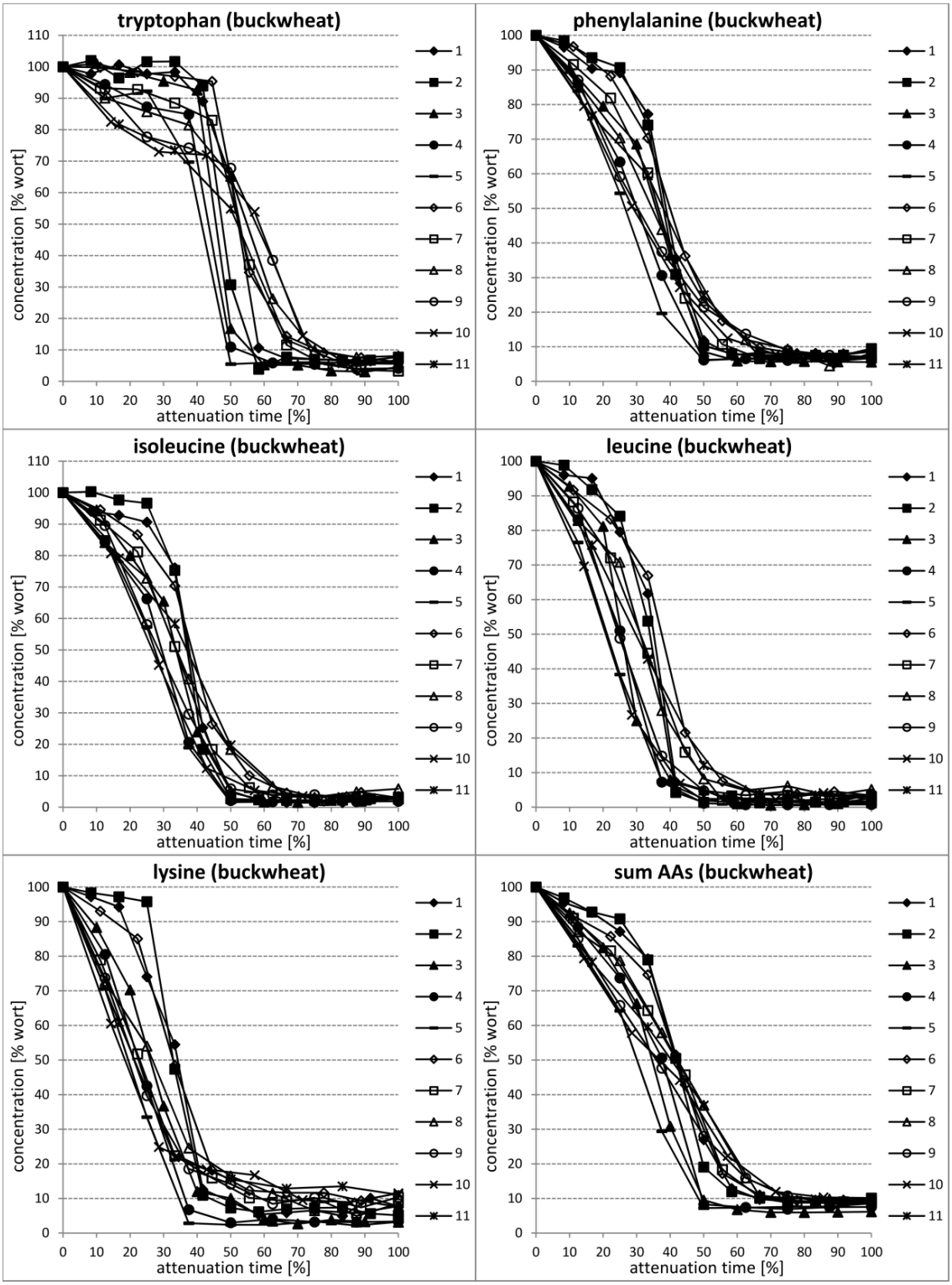

Figure 41: The assimilation profile of Trp, Phe, Ile, Leu, Lys and the total amino acids for eleven successive fermentations of the buckwheat wort
01-11: the numbering of a particular successive fermentation. AAs – amino acids.
Slika 41: Profil privzema Trp, Phe, Ile, Leu, Lys in vsote vseh aminokilsin za enajst zaporednih fermentacij ajdove pivine
01-11: številčenje posamezne zaporedne fermentacije. AAs aminokisline

Quinoa wort fermentation

On the contrary, the AA assimilation in the case of quinoa (Figure 42, Figure 43 and Figure 44) was drastically different and the discussion using non-linear regression statistics was irrelevant. However, Asn, Ser, Thr, Met, Ile, Leu and Lys showed the closest resemblance to a typical AA assimilation profile familiar for barley and these AAs were also the most preferred by yeast. The concentration of other AAs showed more or less fluctuating profile during fermentation with no obvious pattern which obviously indicates the impairment of the uptake/release control. The final assimilation of particular AA was immensely influenced by the serial repitching ($\Delta_{max-min} > 25\%$) with the exception of Arg whose final assimilation was scarce. The same stands for Gln although it was assimilated at the beginning of each fermentation but released later during the second half of fermentation. The net increase of Gln had been already observed before (Gibson *et al*., 2009). Two distinct groups of successive fermentations can be distinguished. (i) F1-F6 were mostly marked by a more efficacious final AA assimilation together with a rapid initial uptake. (ii) In F7 both the final AA assimilation and the initial uptake substantially decreased and they were slowly decreasing even further as successive fermentations proceeded. In F10 and F11 the AA assimilation was the slowest and the least efficacious. Lower amino acid assimilation after F6 somehow corresponds with the decline in relative protein concentration of the majority protein bands (Figure 24 and Figure 25).

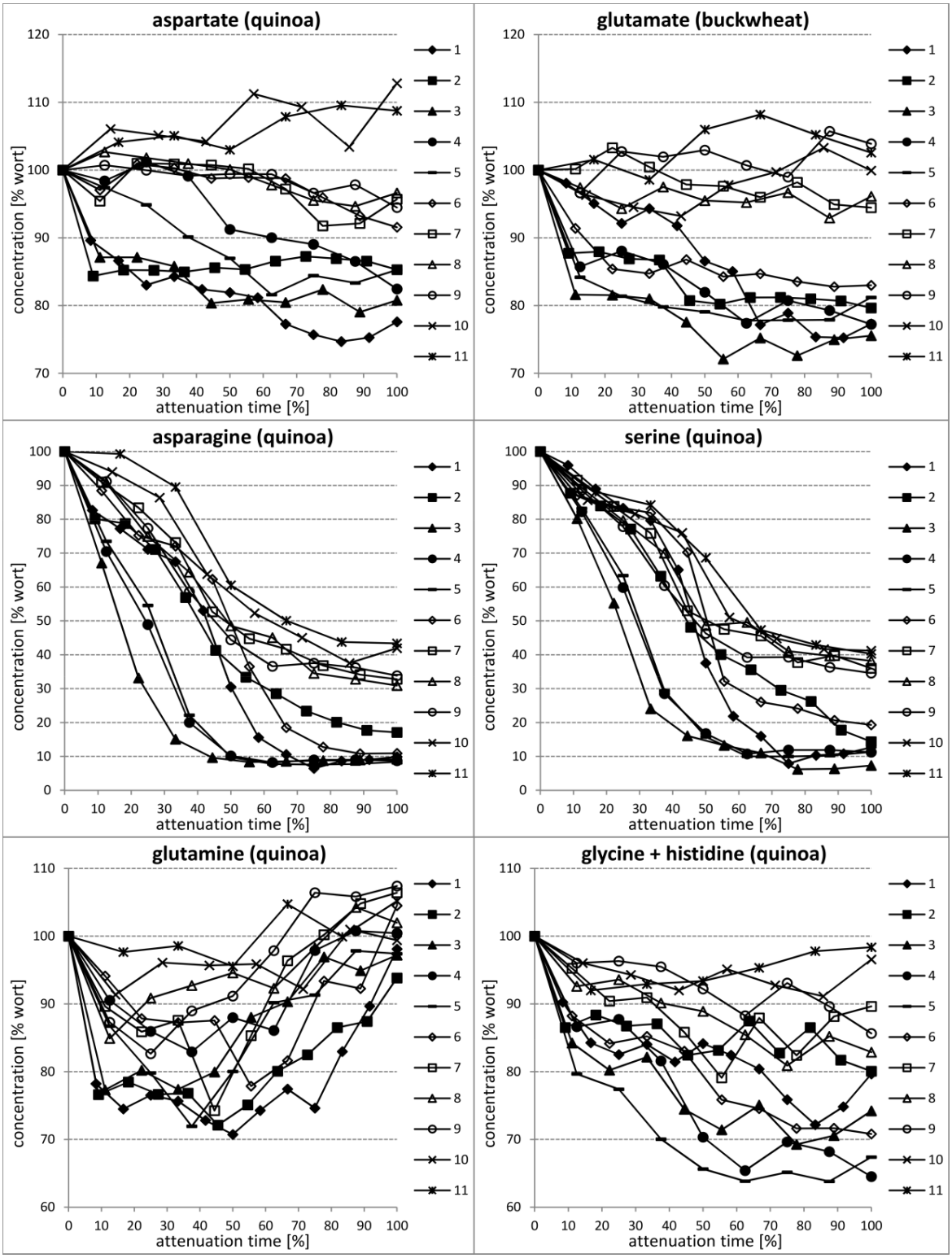

Figure 42: The assimilation profile of Asp, Glu, Asn, Ser, Gln and His+Gly for eleven successive fermentations of the quinoa wort
01-11: the numbering of a particular successive fermentation.
Slika 42: Profil privzema Asp, Glu, Asn, Ser, Gln in His+Gly za enajst zaporednih fermentacij kvinojine pivine
01-11: številčenje posamezne zaporedne fermentacije.

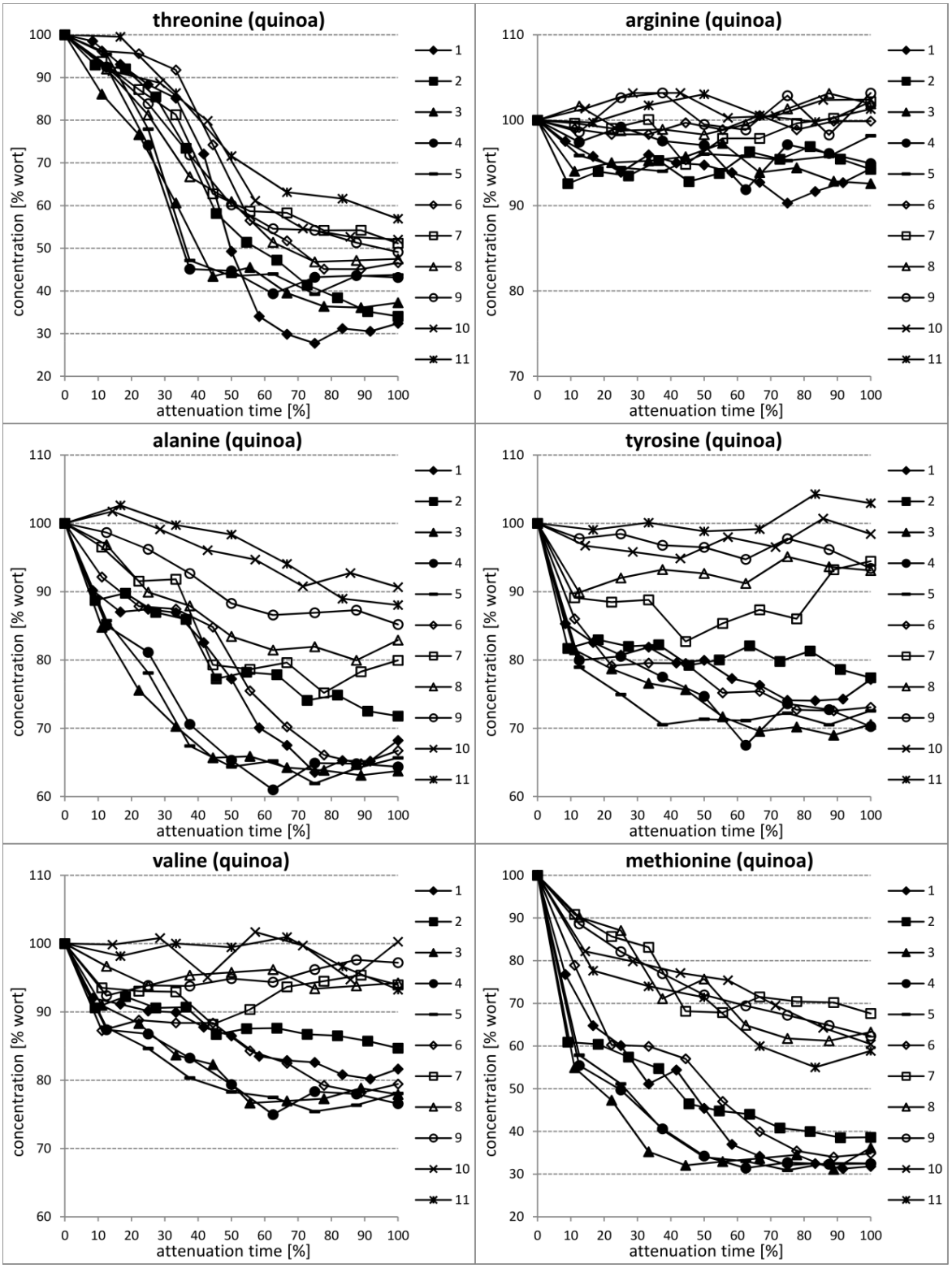

Figure 43: The assimilation profile of Thr, Arg, Ala, Tyr, Val and Met for eleven successive fermentations of the quinoa wort
01-11: the numbering of a particular successive fermentation.
Slika 43: Profil privzema Thr, Arg, Ala, Tyr, Val in Met za enajst zaporednih fermentacij kvinojine pivine
01-11: številčenje posamezne zaporedne fermentacije.

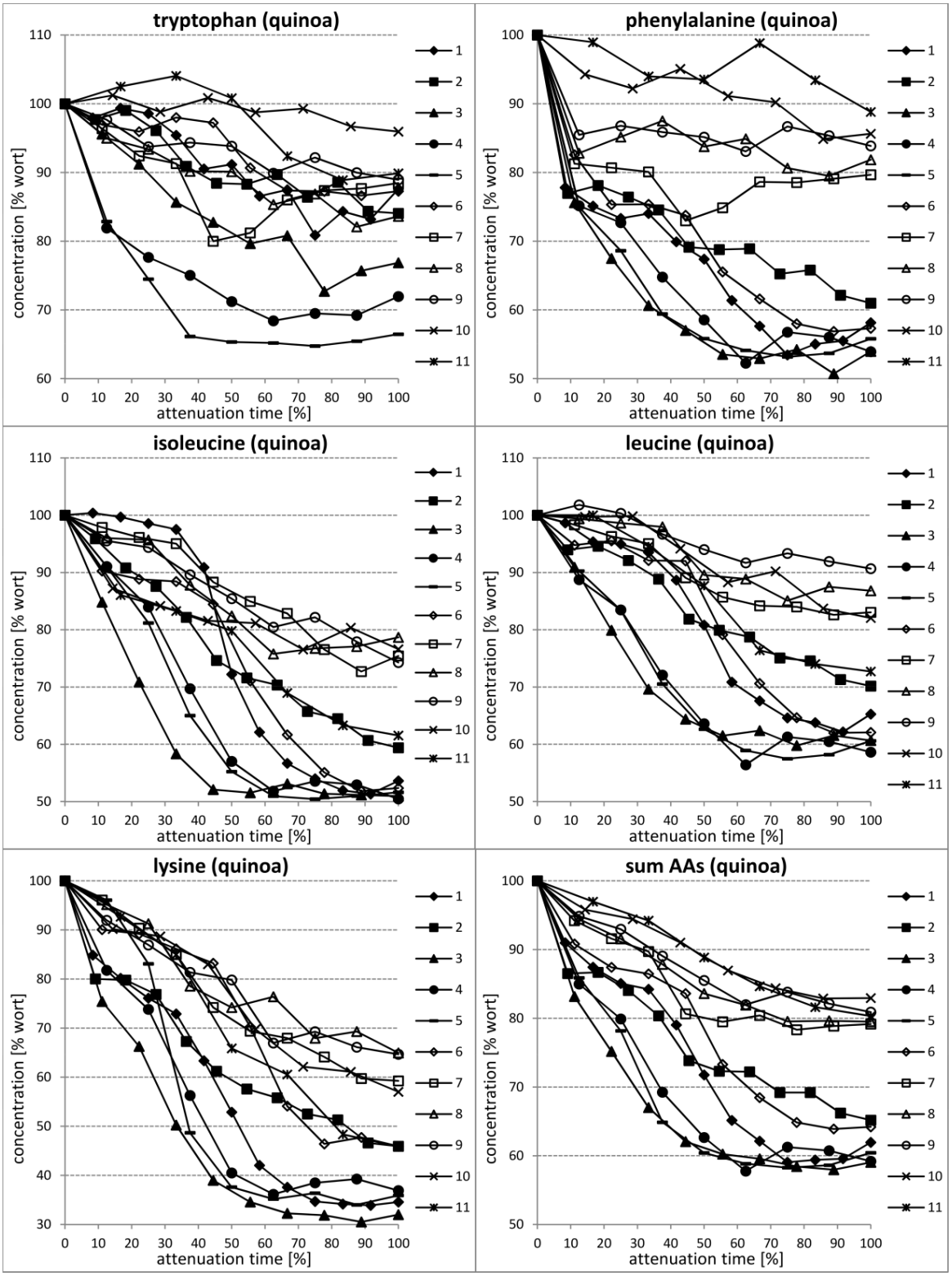

Figure 44: The assimilation profile of Trp, Phe, Ile, Leu, Lys and the total amino acids for eleven successive fermentations of the quinoa wort
01-11: the numbering of a particular successive fermentation. AAs – amino acids.
Slika 44: Profil privzema Trp, Phe, Ile, Leu, Lys in vsote vseh aminokislin za enajst zaporednih fermentacij kvinojine pivine
01-11: številčenje posamezne zaporedne fermentacije. AAs – aminokisline.

The uptake order of amino acids

For the determination of the AA uptake order, none of the criteria used by other researchers proved to be suitable in our case, since they were considerably influenced by the serial repitching and sometimes hard to define. Instead, a more adequate measure was introduced, i.e. the so-called "completion time" (t95) to determine the AA uptake preference of yeast regardless the successive fermentation. At first it was calculated from the experimental data and judged visually afterwards. t95 is somehow similar to Tc (Perpète *et al*., 2005); however, there are some advantages of t95, such as (i) the categorization of AAs is possible using t95 solely and (ii) it can be employed also for AAs that are not assimilated completely. Because of the above-mentioned changeable nature of the AA assimilation during the quinoa wort fermentation, these samples were not included in this analysis.

Table 53: The classification of wort amino acids according to their overall consumption priority by bottomfermenting yeast *S. pastorianus* TUM 34/70
Preglednica 53: Uvrstitev aminokislin v ječmenovi in ajdovi pivini glede na splošen potek njihove asimilacije s strani kvasovke spodnjega vrenja *S. pastorianus* TUM 34/70

	barley		buckwheat	
group	**t95 [%]**	**AAs**	**t95 [%]**	**AAs**
A1	40-60	Asn, Ser, Gln, Thr, Lys	25-45	Asn, Ser
A2	50-70	Met, Leu	35-55	Gln, Thr, Met, Leu, Lys
B1	60-80	Asp, Glu, Gly+His, Arg, Tyr, Phe, Ile	45-60	Asp, Glu, Ile
B2	70-90	Ala, Val, Trp	50-70	Gly+His, Arg, Ala, Tyr, Val, Trp, Phe

The serial repitching influenced t95 to some extent ($\Delta_{max\text{-}min}$ = 15-20% for particular AA) and this influence was similar for all AAs, thus their division into four groups, named as A1, A2, B1 and B2, was possible (Table 53). Because of the above-mentioned influence of the serial repitching on t95, these groups are defined by overlapping t95 value spans. Nevertheless, particular AAs were similarly affected within an individual serial fermentation thus this classification represents no doubt from the successive fermentation point of view. Comparing to barley, the defining t95 value span of each group was lower in the case of buckwheat from 15 to 20%. This range corresponds to the width of groups which means that regarding t95, group N in barley closely corresponds to the group N + 1 in buckwheat by the similar relative attenuation time. As the group names suggest, the first two and the last two possess some common features. It is clear from the Table 53 that, regarding the AAs they contain, the groups are not identical for barley and buckwheat but the whole AA collection of A1 and A2, as well as that of B1 and B2 in barley, coincides entirely with the whole AA collection of homologous groups in buckwheat.

The AA uptake by the yeast strain we used was thus not absolutely an ordered process but it was, at least for some AAs, affected by the wort type. In particular, some AAs were designated to the same group in both cases, such as Asn and Ser (A1), Met and Leu (A2),

Asp, Glu and Ile (B1), and Ala, Val and Trp (B2), but their t95 values differed considerably between raw materials and do not overlap at all. Consequently, the uptake rate of these AAs can be interpreted as raw material-depended to a great extent. On the other hand, when comparing buckwheat to barley, some AAs were apparently "moved" from A1 to A2, i.e. Gln, Thr and Lys, and some from B1 to B2, i.e. Gly+His, Arg, Tyr and Phe. This difference in group classification means that these AAs had comparable t95 values and furthermore, their uptake rate was almost exclusively yeast-depended with negligible influence of the raw material.

The uptake rate of single amino acid

An attention was also given to the final uptake of particular AA in regard to the total AA uptake as a function of serial repitching (Figure 45, Figure 46 and Figure 47). In barley, Asn and Ala represented the highest average share of the total assimilated AAs (> 10% each), followed by Leu, Phe, Lys and Arg (5-10% each), whereas the others represented less than 5% each. However, these values were affected by serial repitching, especially for Trp (RSD = 51%) followed by Arg, Ala, Asp, Gln and Gly+His (RSD = 13-21%). The RSD values of others AAs were below 10%. In general, no obvious pattern of the serial repitching influence can be recognized.

In the case of buckwheat, Glu and Arg represented the highest average share of the total assimilated AAs (> 10% each), followed by Asp, Ser, Gly, Ala, Val, Trp and Leu (5-10% each), whereas the others represented less than 5% each. Contrary to barley, the share of a particular AA was surprisingly unaffected by the serial repitching which was reflected by low RSD values. Except of Glu and Thr (RSD ~ 9%), all the others were below 6%. Some AAs are marked by a small, but sudden change in the share of total assimilated AAs from F6 to F7, whether by an increase (Asn, Thr, Phe, Ile, Leu and Lys) or decrease (Asp, Glu and Arg).

In the case of quinoa, Asn, Ser, Thr and Lys represented the highest share of the total assimilated AAs (> 10% each, ~50% all together), followed by Leu, Phe, Ile and Met (5-10% each), whereas the others represented less than 5%. The share of a particular AA was extremely affected by serial repitching, especially after F6. From F7 onward, the share of Ser, Lys and Thr increased and the share of others decreased.

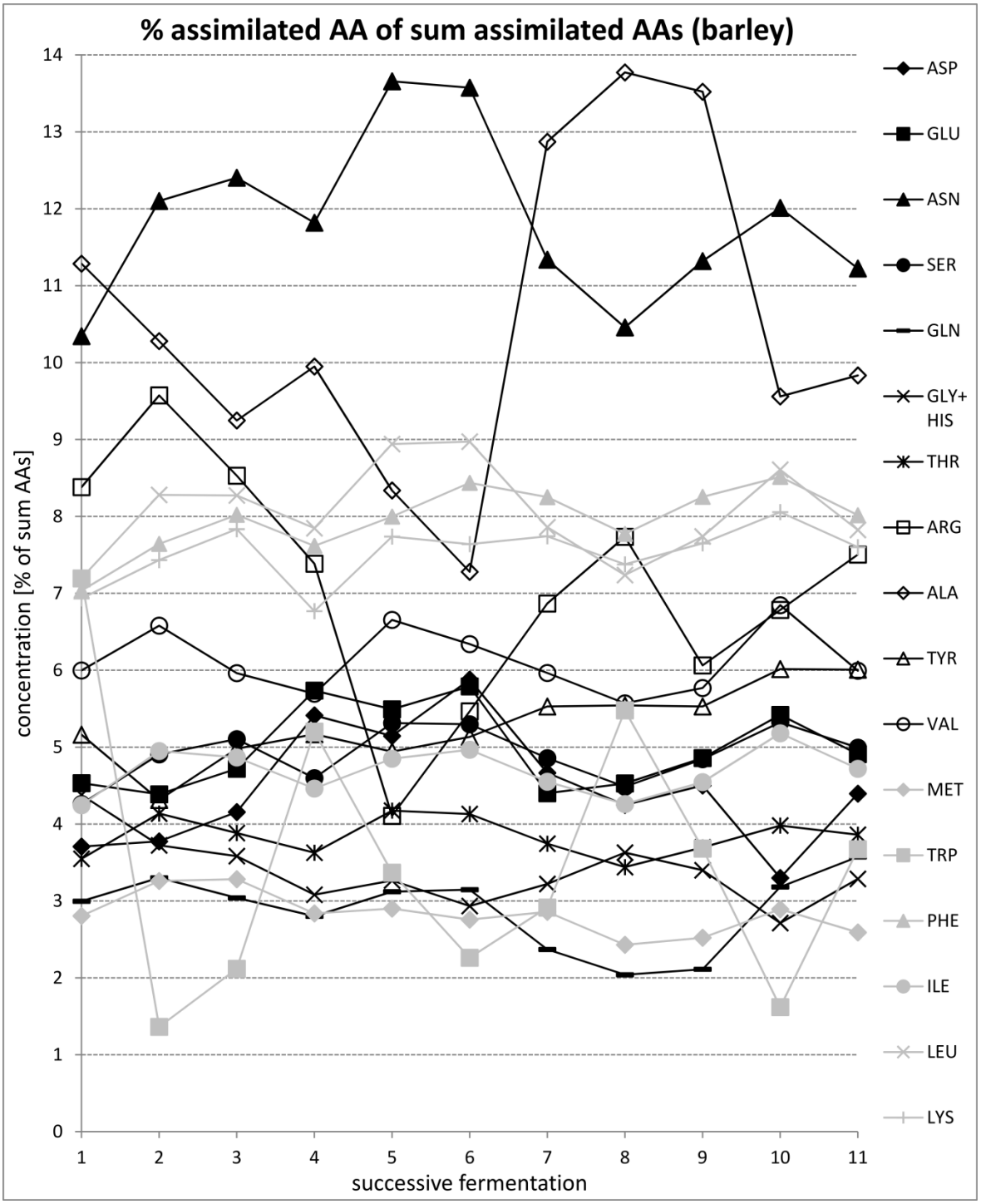

Figure 45: The line chart of the final assimilation of a particular amino acid relative to the sum assimilated amino acids for eleven successive fermentations of the barley wort

Slika 45: Črtni diagram končne asimilacije posamezne aminokisline glede na vsoto vseh asimiliranih aminokislin tekom enajst zaporednih fermentacij ječmenove pivine

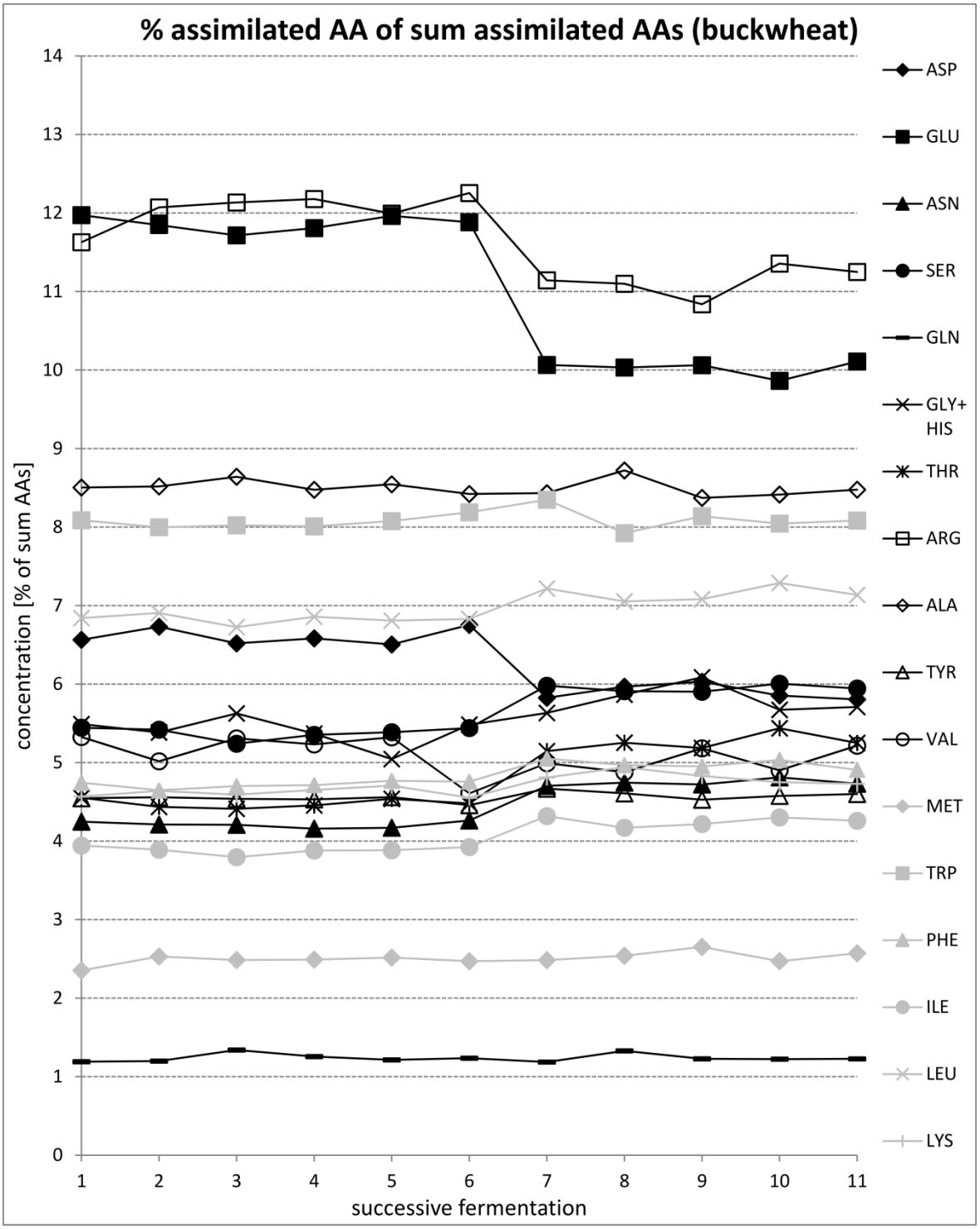

Figure 46: The line chart of the final assimilation of a particular amino acid relative to the sum assimilated amino acids for eleven successive fermentations of the buckwheat wort

Slika 46: Črtni diagram končne asimilacije posamezne aminokisline glede na vsoto vseh asimiliranih aminokislin tekom enajst zaporednih fermentacij ajdove pivine

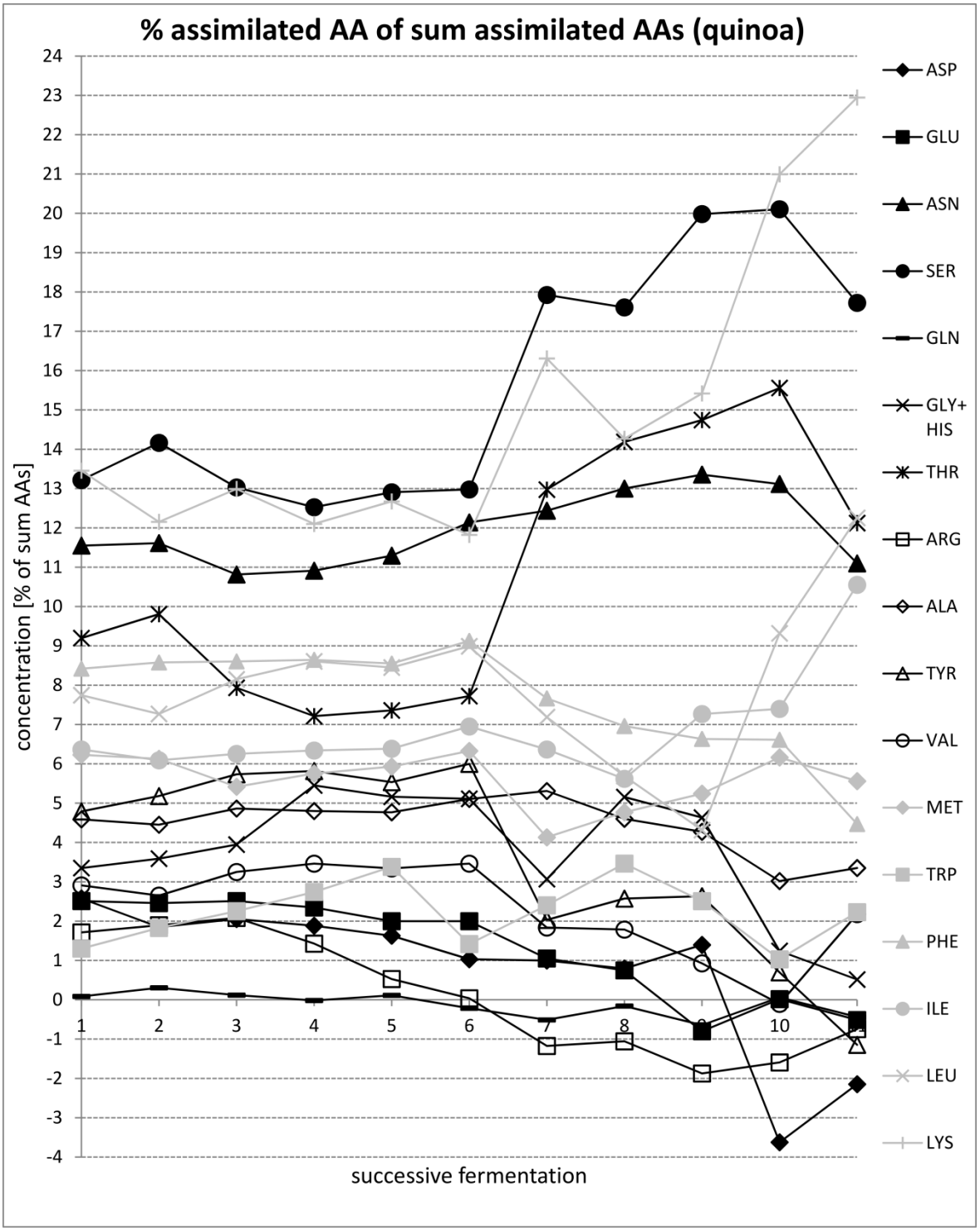

Figure 47: The line chart of the final assimilation of a particular amino acid relative to the sum assimilated amino acids for eleven successive fermentations of the quinoa wort
Slika 47: Črtni diagram končne asimilacije posamezne aminokisline glede na vsoto vseh asimiliranih aminokislin tekom enajst zaporednih fermentacij kvinojine pivine

In addition, the final uptake of particular AA in regard to its initial concentration as a function of serial repitching was also investigated (Figure 48, Figure 49 and Figure 50). In barley, AAs, i.e. Asn, Ser, Leu, Ile, Lys, Phe and Thr, which on average were assimilated the most efficaciously (> 90%), were also assimilated the most consistently (RSD < 5%). But on average, only 40% of initial Trp and 60% of initial Gly+His were assimilated although the exact values varied between successive fermentations considerably (RSD = 58 and 20%, respectively). In general, the serial repitching influenced particular AAs differently. In buckwheat, only the assimilation of Val, Gln, Met, Asp and Gly+His was lower than 90%. As mentioned above, serial repitching had a negligible influence on the AA assimilation which is here supported by the low RSD values (< 5%). In the case of quinoa, AAs were assimilated to very distinct degrees which were manifested by a somehow stratified pattern of Figure 7. After F6, the assimilated share of all AAs fell markedly but for some of them, i.e. Gly+His, Met, Ile and Lys it increased again later on. For both buckwheat and quinoa it seems that the serial repitching influenced particular AAs more similarly compared to barley.

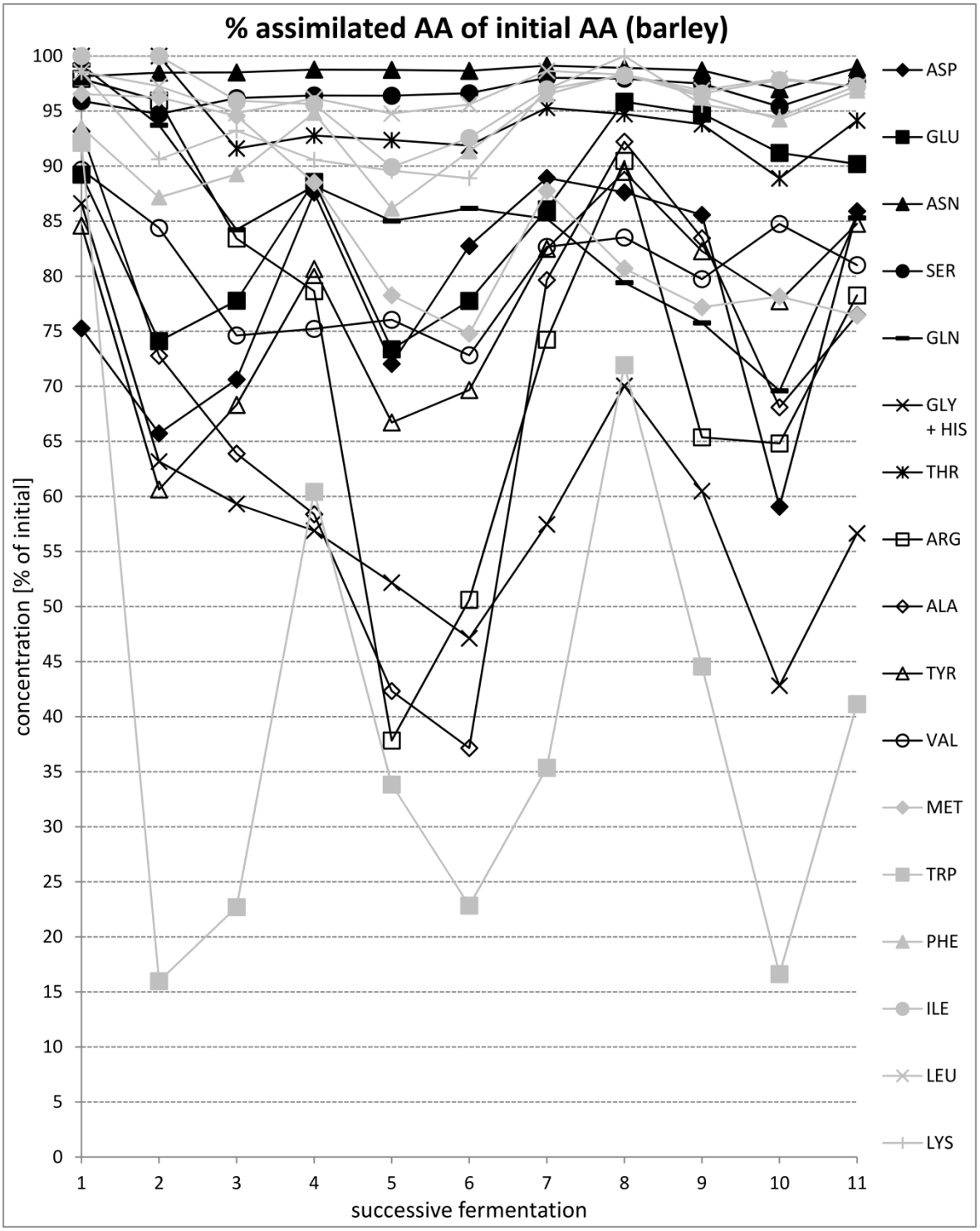

Figure 48: The line chart of the final uptake of a particular amino acid in regard to its initial concentration after eleven successive fermentations of the barley wort
Slika 48: Črtni diagram končne asimilacije posamezne aminokisline glede na njeno začetno koncentracijo tekom enajst zaporednih fermentacij ječmenove pivine

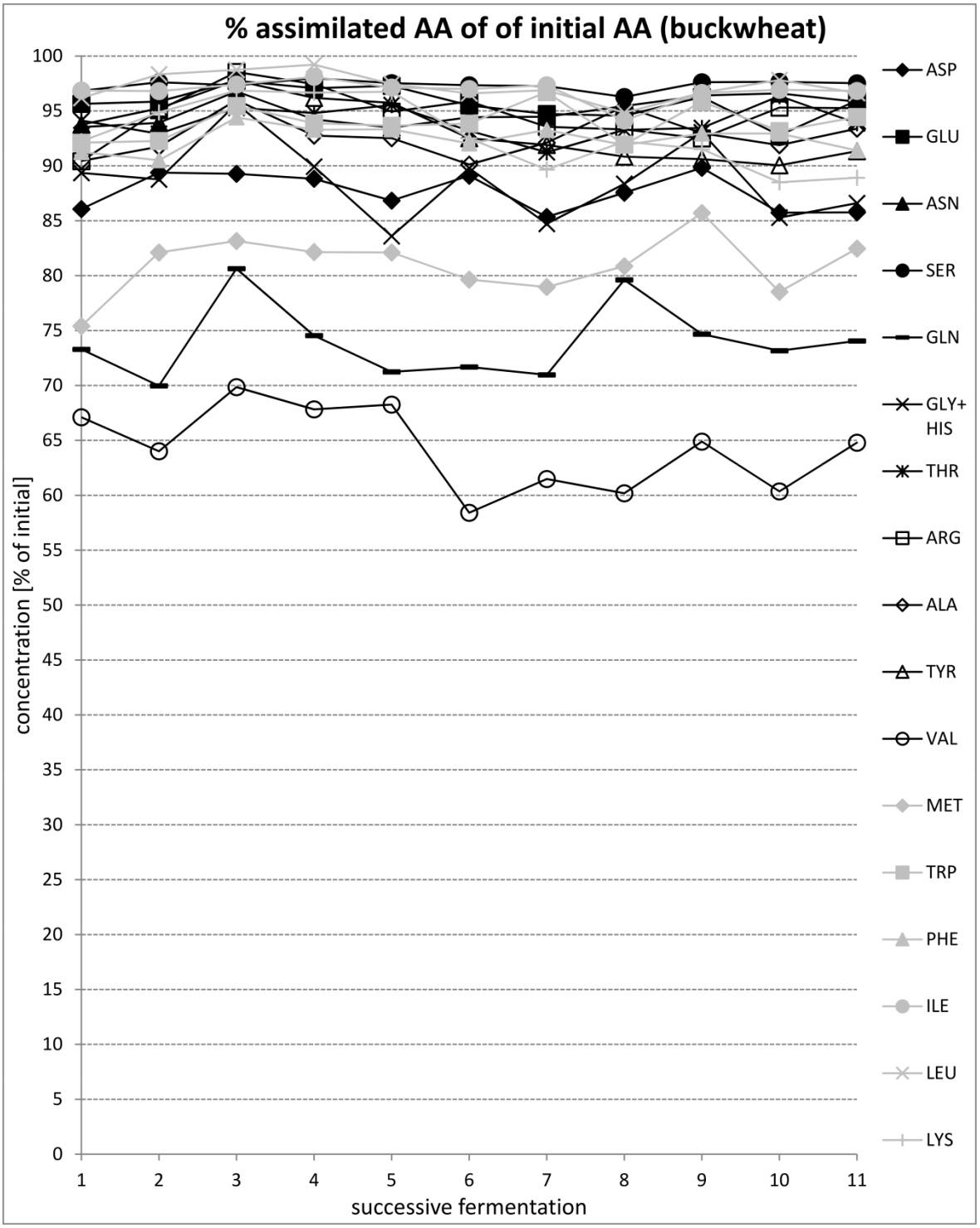

Figure 49: The line chart of the final uptake of a particular amino acid in regard to its initial concentration after eleven successive fermentations of the buckwheat wort
Slika 49: Črtni diagram končne asimilacije posamezne aminokisline glede na njeno začetno koncentracijo tekom enajst zaporednih fermentacij ajdove pivine

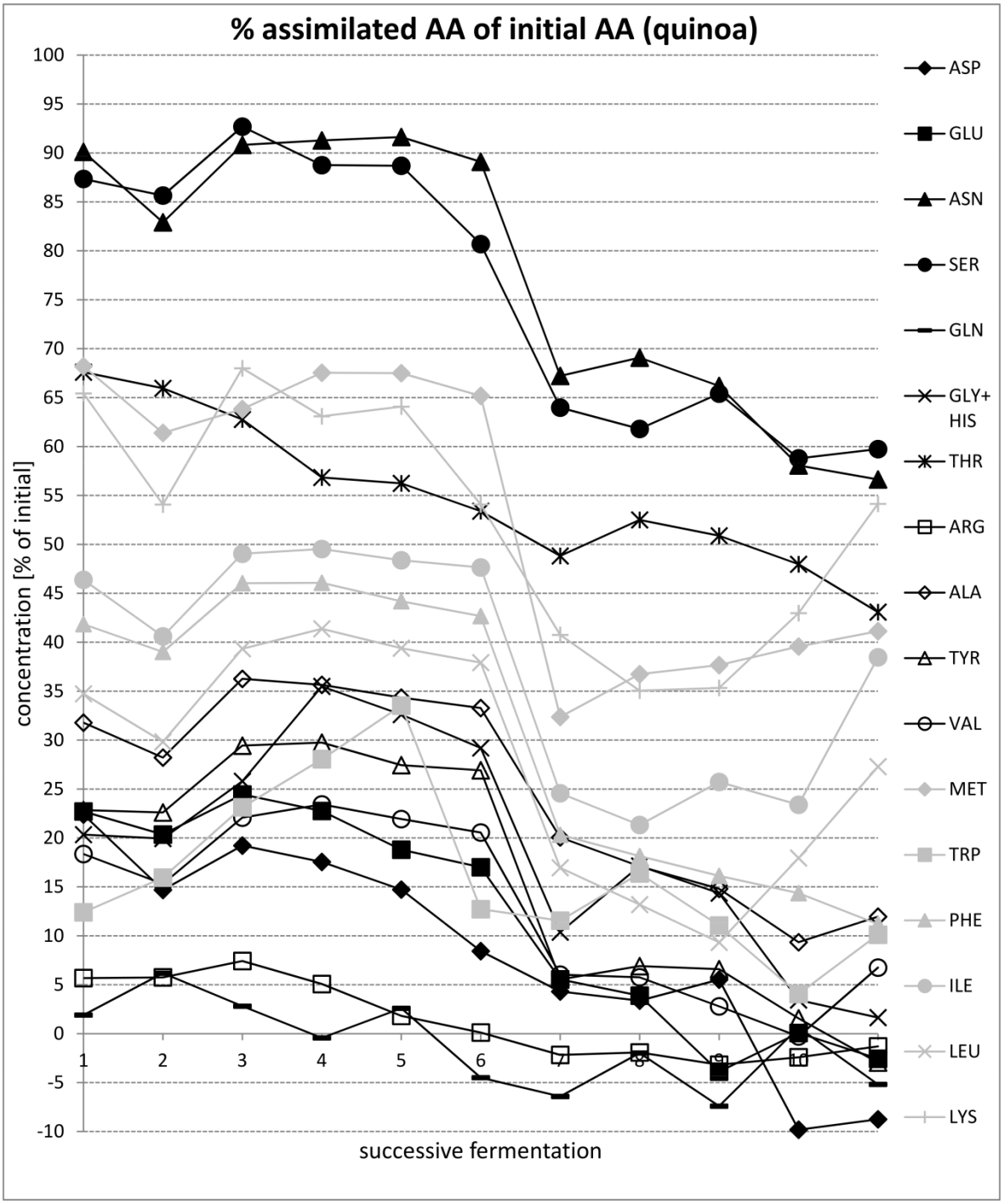

Figure 50: The line chart of the final uptake of a particular amino acid in regard to its initial concentration after eleven successive fermentations of the quinoa wort
Slika 50: Črtni diagram končne asimilacije posamezne aminokisline glede na njeno začetno koncentracijo tekom enajst zaporednih fermentacij kvinojine pivine

As a matter of interest, it is worth mentioning that we found a sort of confirmation for our previous assumption that different relative concentrations of the 30.7 kDa protein band between raw materials could be due to differing expression of Met16p, which is involved in the metabolism of sulphur-containing amino acids (Figure 24 and Figure 25). In particular, there was a negative correlation observed between average relative protein concentration and initial concentration of Met in the barley, buckwheat and quinoa wort.

4.2.2.5 Volatile compounds

The content of important volatile compounds (VCs) in beverages from the barley, buckwheat and quinoa malt and their overall significance were already discussed in Section 4.1.5. Here, the main focus has been given to the production dynamics of the selected VCs as a function of successive fermentation and raw material type, i.e. barley, buckwheat and quinoa. Because the attenuation time, i.e. the last day when a daily change in the extract content was higher than or equal to 0.1%, was raw material- and successive fermentation-dependent (Table 47), the time axis was relativized throughout the entire article. In order to quantitatively support the visual observations, some quantitative statistical concepts were used. Where appropriate, a designation $\Delta_{max\text{-}min}$ was used to express the difference between the maximal and minimal value. The coefficient of variation (CV) was used as a normalized measure of data dispersion around the average value. In addition, the non-linear regression was performed fitting the four-parameter symmetrical sigmoidal curve to the experimental data of a particular VC.

Three statistics, namely AT_{50} (the percentage of attenuation time needed to produce a half of total produced VC), curve slope (describes the steepness of the curve; higher absolute values belong to steeper curves) and R^2 (percentage of the goodness of fit) were considered for further discussion (Table 54 and Table 55). RSD values of all three statistics for eleven successive fermentations of each raw material were calculated. Their product, termed "the serial repitching factor" (SRf), was used to support the visual evaluation of the serial repitching and its influence on the VC production profile (greater influence of the serial repitching gave higher SRf value). However, the production profile of acetaldehyde and methanol differed drastically from the sigmoidal curve. In those cases and in some others, results were described "ambiguous fit" (A.G.), "not converged (N.C.)" or "interrupted fit" (I.R.), similarly as in the case of some AAs (Section 4.2.2.4). Although these cases of the socalled "bad regression" cannot be quantitatively included into the SRf calculation, they were still considered during the data interpretation in a more holistic way.

Table 54: The AT_{50}, curve slope and R^2 values of methanol, 1-propanol, isobutanol, 2- and 3-methylbutanol and 2-phenylethanol for eleven successive fermentations of the barley, buckwheat and quinoa wort
Preglednica 54: Vrednosti AT_{50}, naklona regresijske krivulje in R^2 za 1-propanol, izobutanol, 2- in 3-metilbutanol in 2-feniletanol tekom enajst zaporednih fermentacij ječmenove, ajdove in kvinojine pivine

		methanol			1-propanol			isobutanol			2- & 3-methylbutanol			2-phenylethanol		
wort type	F	AT_{50} [%]	curve slope [x100]	R^2 [%]	AT_{50} [%]	curve slope [x100]	R^2 [%]	AT_{50} [%]	curve slope [x100]	R^2 [%]	AT_{50} [%]	curve slope [x100]	R^2 [%]	AT_{50} [%]	curve slope [x100]	R^2 [%]
barley	1	N.C.	N.C.	N.C.	49.97	2.09	99.49	54.52	4.91	99.58	57.82	3.02	99.90	54.84	4.06	99.80
	2	N.C.	N.C.	N.C.	A.G.	A.G.	A.G.	42.57	3.61	99.32	44.10	2.76	99.83	42.71	3.55	99.44
	3	N.C.	N.C.	N.C.	33.85	2.83	99.20	43.93	4.25	98.75	44.81	2.18	99.90	43.35	2.99	99.14
	4	N.C.	N.C.	N.C.	35.66	4.13	99.57	41.87	4.42	99.32	44.34	3.46	99.86	46.18	3.64	99.33
	5	N.C.	N.C.	N.C.	49.54	1.75	99.79	53.50	2.73	99.72	49.55	1.98	99.61	50.56	3.38	99.87
	6	N.C.	N.C.	N.C.	45.55	3.01	99.37	52.74	4.87	99.30	48.39	2.58	99.66	48.76	5.98	99.09
	7	N.C.	N.C.	N.C.	40.02	2.91	99.73	44.36	3.39	99.70	48.11	2.76	99.88	47.32	4.20	99.10
	8	N.C.	N.C.	N.C.	42.80	3.62	99.89	47.26	5.08	99.87	47.99	3.36	99.83	45.41	4.80	99.75
	9	N.C.	N.C.	N.C.	44.53	3.23	99.49	48.85	3.68	99.60	48.78	2.86	99.64	46.01	4.38	99.31
	10	N.C.	N.C.	N.C.	49.66	2.66	98.84	57.04	3.99	99.55	53.57	2.44	99.39	52.28	3.54	99.06
	11	N.C.	N.C.	N.C.	41.09	4.16	98.83	45.27	4.35	99.73	47.37	3.66	99.88	45.62	4.79	99.72
buckwheat	1	N.C.	N.C.	N.C.	45.64	5.98	99.84	43.77	7.01	99.36	49.71	5.32	99.84	56.65	6.31	99.66
	2	N.C.	N.C.	N.C.	42.10	8.63	99.58	40.94	9.05	99.62	44.86	6.30	99.90	51.28	6.73	99.79
	3	N.C.	N.C.	N.C.	44.41	6.67	98.91	44.60	5.54	97.86	50.40	7.93	98.80	A.G.	A.G.	A.G.
	4	N.C.	N.C.	N.C.	39.94	15.32	99.78	40.32	4.44	99.67	44.78	4.90	99.52	44.67	6.16	98.82
	5	N.C.	N.C.	N.C.	40.50	10.85	99.18	29.12	5.21	99.39	35.33	4.90	99.79	49.22	6.20	99.73
	6	N.C.	N.C.	N.C.	43.55	3.90	98.76	43.26	2.41	97.42	53.08	4.48	98.22	52.68	4.92	98.66
	7	N.C.	N.C.	N.C.	43.98	4.40	99.49	38.21	5.06	97.75	47.34	3.04	99.37	59.58	3.41	99.71
	8	N.C.	N.C.	N.C.	49.22	3.28	98.71	28.83	1.67	99.54	52.66	1.47	99.13	51.09	3.80	98.14
	9	N.C.	N.C.	N.C.	35.86	3.70	99.14	24.92	3.10	98.72	38.26	2.53	99.06	58.05	2.86	99.14
	10	N.C.	N.C.	N.C.	38.25	2.92	99.60	12.63	1.57	99.30	A.G.	A.G.	A.G.	54.59	1.41	98.65
	11	N.C.	N.C.	N.C.	44.09	2.30	98.73	17.93	1.75	97.41	54.92	10.94	98.71	57.19	10.61	99.48
quinoa	1	N.C.	N.C.	N.C.	46.98	5.31	98.72	48.25	5.49	99.57	45.83	6.31	99.39	52.67	4.45	98.90
	2	N.C.	N.C.	N.C.	43.46	4.04	98.84	40.79	3.65	99.55	41.19	3.48	99.38	50.85	3.40	99.24
	3	N.C.	N.C.	N.C.	26.29	3.39	98.17	31.70	3.30	98.62	33.92	3.79	99.61	42.45	3.07	98.98
	4	N.C.	N.C.	N.C.	27.14	3.88	98.76	25.56	3.76	99.63	24.60	4.39	99.78	32.63	4.60	98.63
	5	N.C.	N.C.	N.C.	33.81	6.02	99.82	32.46	4.62	99.60	31.58	5.54	99.86	38.96	5.08	99.37
	6	N.C.	N.C.	N.C.	55.91	4.51	99.80	49.84	5.24	99.29	52.85	4.61	99.80	51.22	7.46	98.44
	7	N.C.	N.C.	N.C.	41.88	1.52	98.42	A.G.	A.G.	A.G.	35.21	1.19	98.68	65.20	1.40	99.31
	8	N.C.	N.C.	N.C.	44.72	4.64	98.70	34.19	5.92	97.40	37.88	4.18	99.15	51.47	3.53	98.29
	9	N.C.	N.C.	N.C.	32.14	2.36	99.61	26.02	2.42	99.84	27.52	3.02	99.91	34.06	2.51	99.23
	10	N.C.	N.C.	N.C.	47.65	1.98	89.30	59.47	3.16	95.78	54.20	4.24	96.99	87.03	0.64	97.75
	11	N.C.	N.C.	N.C.	58.42	10.18	99.55	56.01	5.91	98.27	53.87	5.70	99.04	53.15	3.83	99.24

F – the number of successive fermentation (F1-F11). AT_{50} – the percentage of attenuation time needed to achieve 50% of the total change in the extract or ethanol content. R^2 – the goodness of curve fit. conc. – concentration. A.G. – ambiguous fit. N.C. – fit not converged.

Table 55: The AT_{50}, curve slope and R^2 values of acetaldehyde, ethyl acetate, isoamyl acetate, 2-phenylethyl acetate and the sum of VCs for eleven successive fermentations of barley, buckwheat and quinoa wort
Preglednica 55: Vrednosti AT_{50}, naklona regresijske krivulje in R^2 za acetaldehid, etil acetat, izoamil acetat, 2-feniletil acetat in vsoto vseh obravnavanih hlapnih spojin tekom enajst zaporednih fermentacij ječmenove, ajdove in kvinojine pivine

wort type	F	acetaldehyde			ethyl acetate			isoamyl acetate			2-phenylethyl acetate			sum VCs		
		AT_{50} [%]	curve slope [x100]	R^2 [%]	AT_{50} [%]	curve slope [x100]	R^2 [%]	AT_{50} [%]	curve slope [x100]	R^2 [%]	AT_{50} [%]	curve slope [x100]	R^2 [%]	AT_{50} [%]	curve slope [x100]	R^2 [%]
barley	1	N.C.	N.C.	N.C.	64.26	3.76	99.81	62.99	4.48	98.69	66.26	2.94	98.96	57.96	2.95	99.88
	2	N.C.	N.C.	N.C.	52.17	3.73	99.64	49.26	3.80	98.84	48.06	4.32	99.91	42.55	3.43	98.78
	3	N.C.	N.C.	N.C.	52.52	3.23	99.86	48.15	4.26	99.01	47.95	4.80	99.66	42.19	2.43	99.29
	4	N.C.	N.C.	N.C.	50.59	4.15	99.12	49.94	5.08	99.34	49.29	4.88	99.64	43.25	3.85	98.86
	5	N.C.	N.C.	N.C.	57.42	4.25	99.89	59.30	4.49	99.79	62.51	7.12	98.01	41.32	3.37	99.41
	6	N.C.	N.C.	N.C.	55.20	4.44	99.09	58.98	3.87	98.75	A.G.	A.G.	A.G.	42.70	4.07	98.61
	7	N.C.	N.C.	N.C.	52.89	4.30	99.47	54.22	4.75	98.90	54.62	4.00	99.50	44.08	5.32	98.63
	8	N.C.	N.C.	N.C.	55.52	5.28	99.98	56.28	5.78	99.87	53.99	7.36	99.85	48.90	7.12	96.82
	9	N.C.	N.C.	N.C.	55.30	4.99	99.91	56.01	5.87	99.86	57.03	6.26	99.71	41.03	3.93	99.37
	10	N.C.	N.C.	N.C.	67.73	3.92	99.81	63.38	5.02	99.91	68.71	3.31	99.62	47.99	1.90	98.27
	11	N.C.	N.C.	N.C.	54.00	4.70	99.94	55.63	4.87	99.77	52.51	6.90	99.85	41.37	4.27	99.64
buckwheat	1	N.C.	N.C.	N.C.	55.66	7.63	99.62	53.82	7.10	99.89	57.39	7.17	99.66	53.19	6.27	99.38
	2	N.C.	N.C.	N.C.	49.36	8.63	99.77	48.07	9.68	99.64	52.47	9.10	99.37	47.02	6.58	99.76
	3	N.C.	N.C.	N.C.	52.19	10.01	99.52	53.05	9.57	99.54	54.80	11.97	98.11	50.31	9.62	98.52
	4	N.C.	N.C.	N.C.	47.84	4.86	99.46	54.27	3.45	99.62	49.17	9.43	97.18	43.15	4.33	99.20
	5	N.C.	N.C.	N.C.	50.59	8.43	99.65	47.35	15.30	99.30	53.65	7.11	99.71	41.19	4.48	99.27
	6	N.C.	N.C.	N.C.	54.75	9.20	98.90	50.56	4.77	98.05	56.77	11.48	97.97	49.24	3.62	98.53
	7	N.C.	N.C.	N.C.	57.35	4.24	99.90	55.91	4.90	99.33	59.44	8.08	99.71	52.34	3.24	99.22
	8	N.C.	N.C.	N.C.	50.01	3.95	98.85	57.90	4.29	99.30	55.74	6.33	97.99	49.00	2.12	99.69
	9	N.C.	N.C.	N.C.	54.70	3.38	99.55	54.35	2.45	98.83	57.81	3.23	99.25	42.97	2.35	98.56
	10	N.C.	N.C.	N.C.	52.10	4.18	99.56	66.55	2.05	99.37	50.23	5.36	99.40	37.73	1.27	99.86
	11	N.C.	N.C.	N.C.	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	55.97	11.00	99.71	54.83	8.41	97.27
quinoa	1	N.C.	N.C.	N.C.	56.60	5.13	99.14	56.66	30.60	99.40	A.G.	A.G.	A.G.	47.39	5.19	99.28
	2	N.C.	N.C.	N.C.	51.71	5.15	99.56	50.63	7.74	99.08	53.26	6.04	91.45	43.14	3.41	99.47
	3	N.C.	N.C.	N.C.	42.16	2.27	99.14	41.94	19.94	97.84	65.03	27.22	99.96	26.60	4.19	99.26
	4	N.C.	N.C.	N.C.	34.45	3.11	99.32	39.83	6.10	99.06	A.G.	A.G.	A.G.	16.95	3.38	99.61
	5	N.C.	N.C.	N.C.	40.53	4.29	99.68	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.	26.49	4.35	99.79
	6	N.C.	N.C.	N.C.	48.59	3.23	97.17	53.15	18.43	98.38	70.16	9.47	99.90	46.41	3.95	99.53
	7	N.C.	N.C.	N.C.	58.07	3.13	97.24	63.89	3.77	96.92	53.08	12.90	94.00	23.63	1.73	98.25
	8	N.C.	N.C.	N.C.	55.53	5.34	98.68	51.43	9.53	99.78	50.00	79.60	89.22	36.93	5.06	99.40
	9	N.C.	N.C.	N.C.	47.25	1.92	99.06	43.80	4.81	97.96	47.43	17.18	98.97	17.56	2.56	99.08
	10	N.C.	N.C.	N.C.	62.93	5.08	92.88	60.05	5.14	97.59	62.99	4.00	97.45	A.G.	A.G.	A.G.
	11	N.C.	N.C.	N.C.	61.34	9.26	98.12	62.57	18.59	98.37	A.G.	A.G.	A.G.	52.98	7.82	98.37

F – the number of successive fermentation (F1-F11). AT_{50} – the percentage of attenuation time needed to achieve 50% of the total change in the extract or ethanol content. R^2 – the goodness of curve fit. conc. – concentration. VCs – volatile compounds. A.G. – ambiguous fit. N.C. – fit not converged.

Sum VCs

The sum content of important VCs during eleven successive fermentations of the barley, buckwheat and quinoa wort differed considerably between successive fermentations and these differences were very similar for barley and buckwheat (Figure 51). Besides the similar visual impression of their VC profile, there were other similarities between barley and buckwheat, in particular: (i) an average final production of VCs was ~210 mg/L for barley and ~230 mg/L for buckwheat, (ii) the $\Delta_{max-min}$ values of final VC content for barley and buckwheat were practically the same (106 and 107 mg/L, respectively) and (iii) in both raw materials, there was a lower final content of VCs after F4 or F5 but in F11, the final concentration raised again. In addition, in both barley and buckwheat, the influence of serial repitching was not prominent in early stages of a particular fermentation but it became obvious after ~30% of attenuation time, which is here expressed with similar average AT_{50} values of barley and buckwheat (45 and 47%, respectively) and with their relatively low CV of AT_{50} values (0.11 and 0.12, respectively).

In quinoa, the sum content of important VCs during eleven successive fermentations was exceedingly different from those of barley and buckwheat from practically every point of view. In particular: (i) an average final production of VCs was almost two times lower (~130 mg/L) which correlates well with the lower amino acid uptake (Section 4.2.2.4), (ii) the $\Delta_{max-min}$ value of final VCs content was around four times lower (26 mg/L) which means that the final content of VCs was very little influenced by serial repitching. Instead (and opposed to barley and buckwheat), the most obvious differences between successive fermentations were observed during the first 60% of attenuation time of a particular fermentation. This visual observation is further supported by low average AT_{50} value (34%) and with high CV of AT_{50} values (0.39).

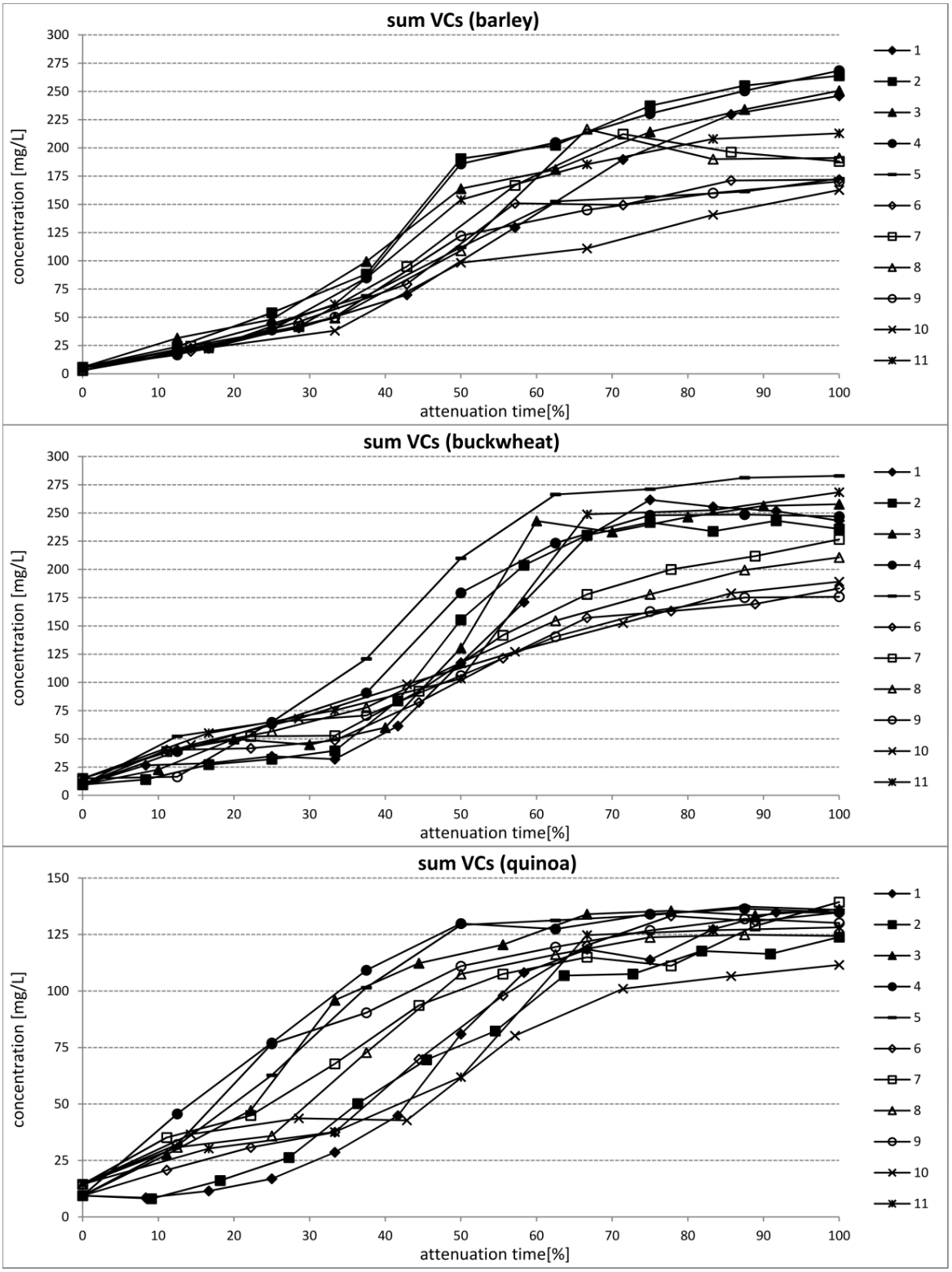

Figure 51: The production profile of sum important volatile compounds for eleven successive fermentations of the barley, buckwheat and quinoa wort (Brečko, 2014)
01-11: the numbering of a particular successive fermentation.
Slika 51: Profil tvorbe vseh obravnavanih hlapnih spojin za enajst zaporednih fermentacij ječmenove, ajdove in kvinojine pivine (Brečko, 2014)
01-11: številčenje posamezne zaporedne fermentacije.

Methanol and acetaldehyde

The methanol and acetaldehyde profiles showed substantially unique properties not observed in the case of esters and higher alcohols (Figure 52). This is expected since methanol is not a fermentation by-product and acetaldehyde is an intermediate in primary metabolism. In particular, methanol and acetaldehyde concentrations did not show any constant trend of increase or decrease during fermentation nor there was any regression curve that would fit their experimental data and that would describe the 'concentration-attenuation time' dependency (Table 54 and Table 55).

Among the VCs under the consideration, methanol was the only compound that was initially present in all three worts (Figure 52). Its initial concentrations were the lowest in barley (2-5 mg/L) followed by quinoa (7-9 mg/L) and buckwheat (8-13 mg/L). During fermentation, in barley there were random fluctuations of methanol content observed and the final concentrations differed greatly ($\Delta_{max-min}$ = 5 mg/L) meaning that in some fermentations the methanol content even increased. In general, the influence of serial repitching in barley was great but without any obvious pattern.

In buckwheat, there was mostly one increase of methanol content right at the beginning of the particular fermentation. Later on, the concentrations fell to the initial levels mostly until 40% but no later than 80% of attenuation time. The final concentrations in buckwheat were in the range of initial concentrations and very similar between each other ($\Delta_{max-min}$ = 2 mg/L), despite the relatively high initial concentrations. In general, serial repitching of buckwheat wort showed a constant and predictable influence without the final increase in methanol concentration.

In the case of quinoa, the methanol content did not change much during particular fermentation and its final concentrations were very similar ($\Delta_{max-min}$ = 2 mg/L) and lower or equal to the initial concentrations.

In general, the dynamics of acetaldehyde content seemed very little influenced by serial repitching in barley, a bit more in buckwheat, and a lot in quinoa (Figure 52). In all cases, acetaldehyde was practically not present in wort but its concentration increased at the very beginning of particular fermentation. In barley, the initial increase resulted in acetaldehyde concentrations of 5-15 mg/L after 10% of attenuation time but only in F1 it did not changed until the end of fermentation. In subsequent fermentations, there was a transient increase of acetaldehyde concentrations (35-60 mg/L) with a peak value at around 50% of attenuation time with the exception of F5-F8 where a peak value was observed a bit later (60-80% of attenuation time). The final concentrations of acetaldehyde in barley were between 10 and 20 mg/L being the lowest in F1 and F7-F9.

In buckwheat, the initial increase of acetaldehyde concentrations was successive fermentation-dependent (0.55 mg/L at 10% of attenuation time). Later on, there were two different types of acetaldehyde concentration profiles observed. In F1-F3 and F7-F9 there was a double increase in acetaldehyde concentration with the first peak value between 20 and 40 and the second between 60 and 90% of attenuation time. On the contrary, only one transient increase of acetaldehyde concentrations with a peak value between 20 and 40% of attenuation time was observed in F4-F6 and F10-F11. The final acetaldehyde concentrations (1-5 mg/L) were lower than in barley.

In quinoa, the first two fermentations differed markedly from subsequent ones by the fact that the acetaldehyde concentrations were very low throughout fermentation with no prominent increase. In F3-F11, the initial increase of acetaldehyde concentrations were similar to barley (5-15 mg/L) as well as was the fact that only one transient increase of acetaldehyde concentrations occurred with a peak value between 30 and 70% of attenuation time. The final concentrations (5-15 mg/L) were slightly lower than in barley.

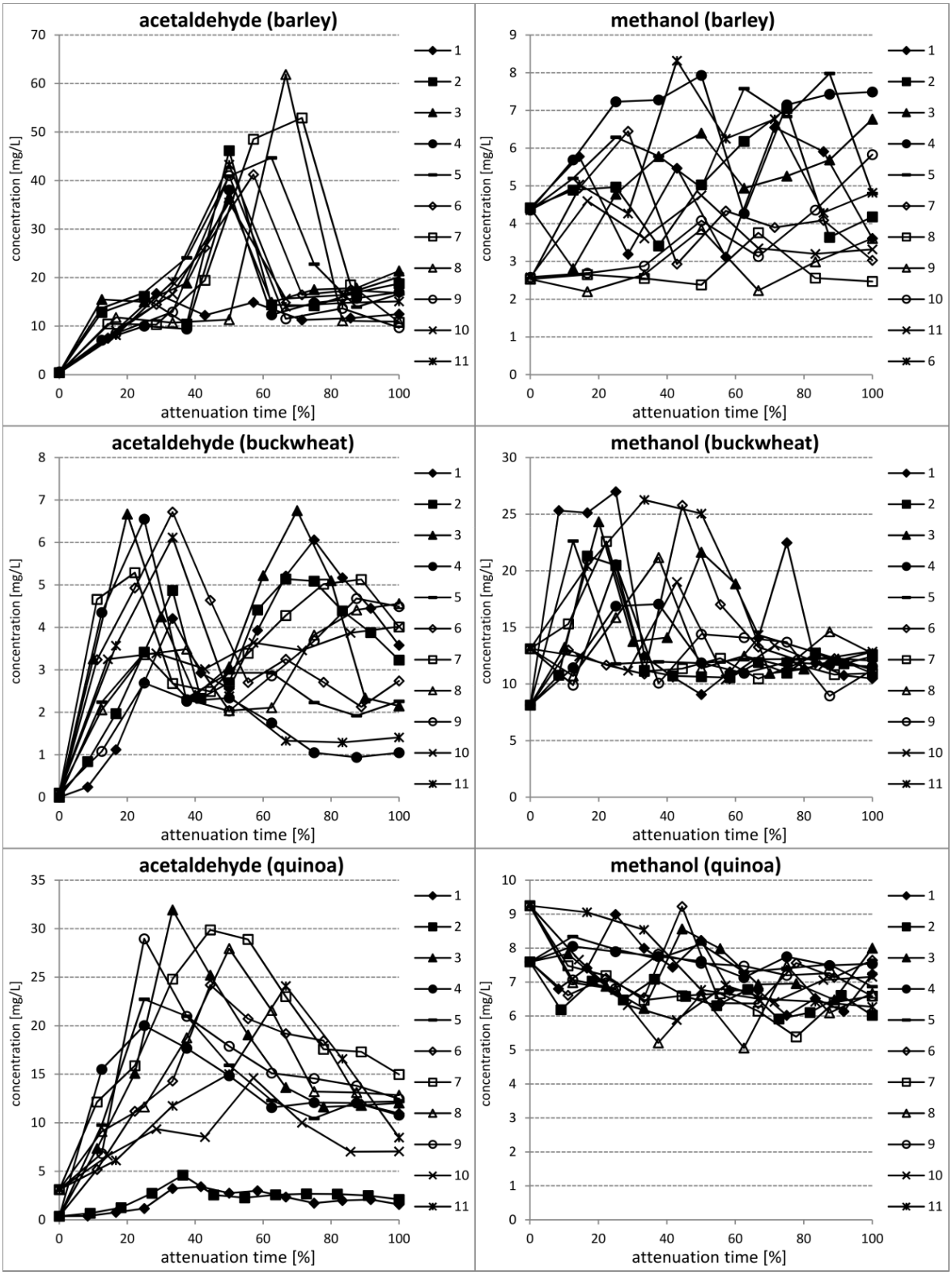

Figure 52: The production profile of methanol and acetaldehyde during eleven successive fermentations of the barley, buckwheat and quinoa wort (Brečko, 2014)
01-11: the numbering of a particular successive fermentation.
Slika 52: Profil tvorbe metanola in acetaldehida za enajst zaporednih fermentacij ječmenove, ajdove in kvinojine pivine (Brečko, 2014)
01-11: številčenje posamezne zaporedne fermentacije.

Higher alcohols

They are all fermentation products of yeast thus normally not present in wort; however, 1-propanol and 2-phenylethanol were also found in the buckwheat and quinoa wort although their concentrations were very low, which is in agreement with the findings in final beverages (Table 20 and Table 21). We have examined five different higher alcohols but only four distinct chromatographic peaks were differentiated because 2-methylbutanol and 3-methylbutanol coeluted. In all cases, their synthesis began at the very beginning of fermentation. In barley, their overall synthesis was the least influenced by serial repitching whereas in buckwheat, this influence was the most prominent in regard to the final concentration ($\Delta_{max-min}$ = ~100 mg/L). In quinoa, the most prominent influence of serial repitching was observed during the first two-thirds of fermentation time which resulted in great differences in AT_{50} ($\Delta_{max-min}$ = ~30%). An important observation here was that regardless the particular higher alcohol and the raw material used, the production during the first half of fermentation was the fastest in F4 and somehow also in F3 and F5, a characteristics which can thus be ascribed solely to yeast. In those fermentations, also the final concentrations were often the highest.

In barley, 2- and 3-methylbutanol were the main higher alcohols followed by 2-phenylethanol (Figure 53). The influence of serial repitching was significant although relatively low (SRf < 130) and without obvious pattern.

In buckwheat, average final concentrations of 2- and 3-methylbutanol and iso-butanol were much higher than in barley whereas the contents of 1-propanol and 2-phenylethanol were comparable (Figure 54). The influence of serial repitching was greater than in barley (SRf > 250) and with two distinct types of VC production profile. In F15 and F11, the fitted sigmoidal curve was stepper and the sum of final concentrations was higher (225-275 mg/L) whereas in F6-F10, the fitted curve was closer to the straight line which resulted in lower sum of final concentrations (175-225 mg/L).

In quinoa, average concentrations of 2- and 3-methylbutanol and isobutanol were similar to barley whereas the contents of 1-propanol and 2-phenylethanol were by half lower than in barley (Figure 55). It is also evident that the production of all higher alcohols was greatly affected by serial repitching (SRf > 800).

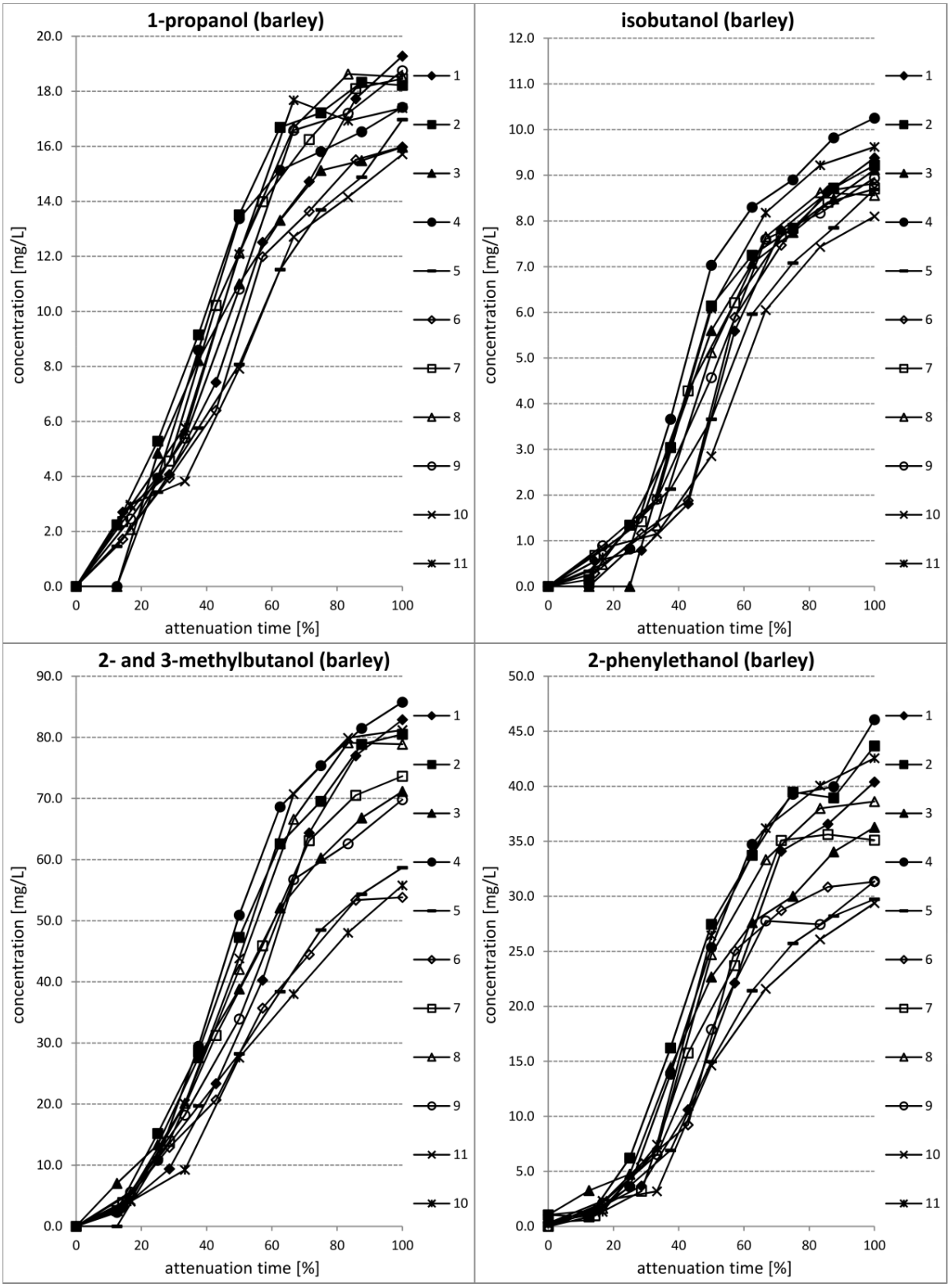

Figure 53: The production profile of important aromaactive higher alcohols during eleven successive fermentations of the barley wort (Brečko, 2014)
01-11: the numbering of a particular successive fermentation.
Slika 53: Profil tvorbe pomembnih aromatičnih višjih alkoholov za enajst zaporednih fermentacij ječmenove pivine (Brečko, 2014)
01-11: številčenje posamezne zaporedne fermentacije.

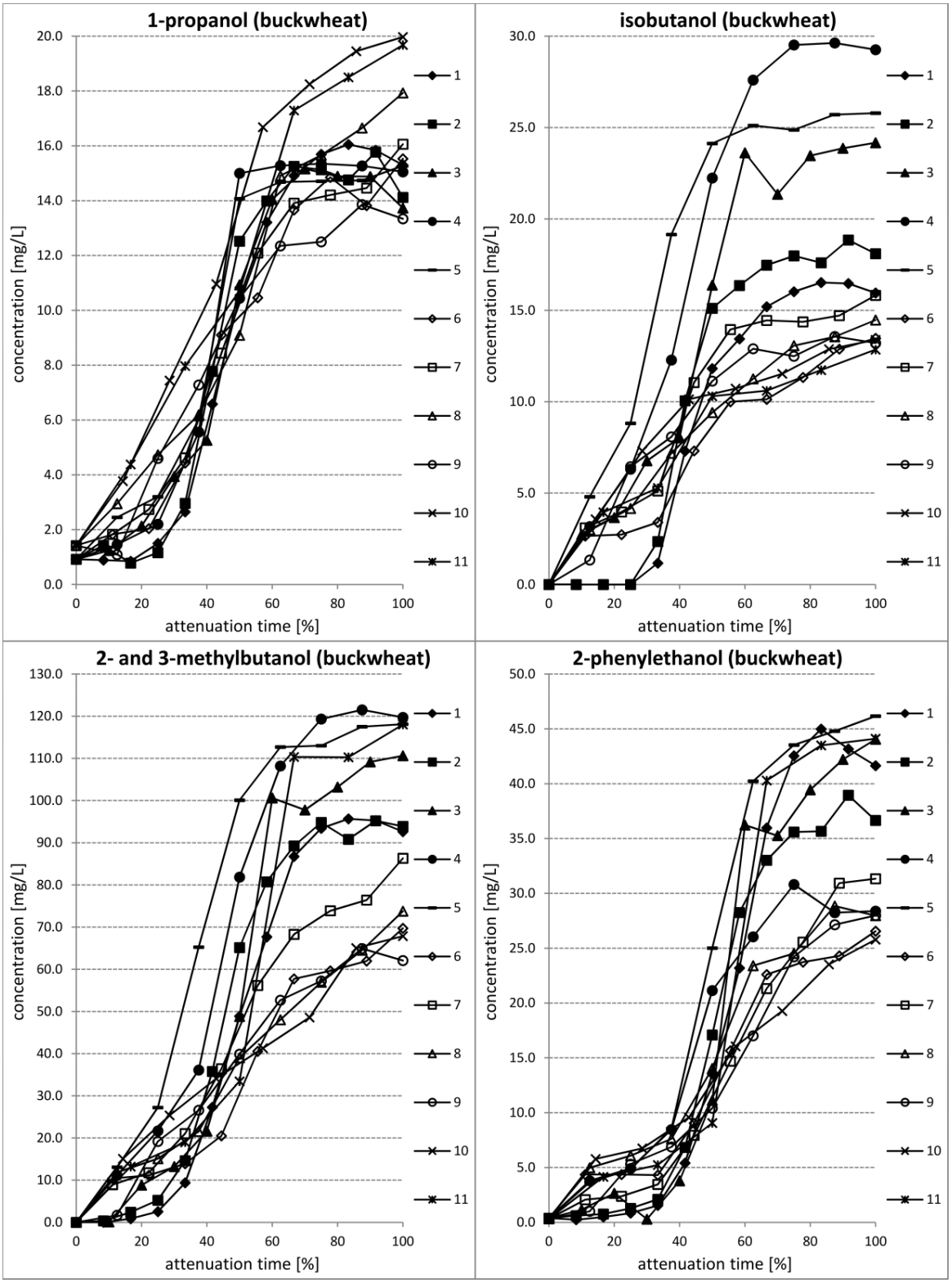

Figure 54: The production profile of important aromaactive higher alcohols during eleven successive fermentations of the buckwheat wort (Brečko, 2014)
01-11: the numbering of a particular successive fermentation.
Slika 54: Profil tvorbe pomembnih aromatičnih višjih alkoholov za enajst zaporednih fermentacij ajdove pivine (Brečko, 2014)
01-11: številčenje posamezne zaporedne fermentacije.

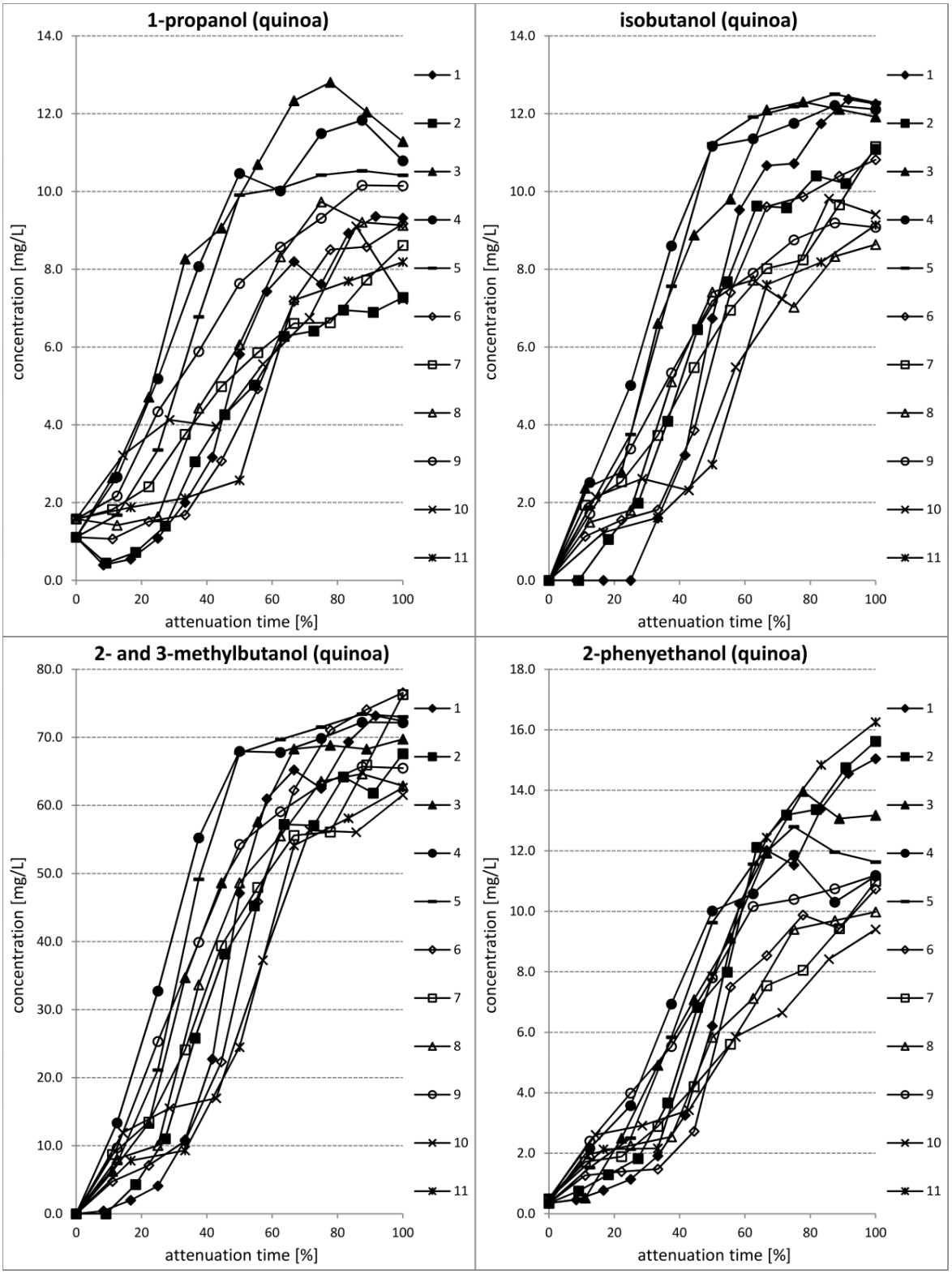

Figure 55: The production profile of important aromaactive higher alcohols during eleven successive fermentations of the quinoa wort (Brečko, 2014)
01-11: the numbering of a particular successive fermentation.
Slika 55: Profil tvorbe pomembnih aromatičnih višjih alkoholov za enajst zaporednih fermentacij kvinojine pivine (Brečko, 2014)
01-11: številčenje posamezne zaporedne fermentacije.

Esters

The three most important esters in beer were examined (Table 55, Figure 56, Figure 57 and Figure 58) and on average, their sum values in barley and buckwheat were comparable, whereas their sum content in the quinoa beverage was 45 times lower. In all cases, the synthesis of particular ester began between 30 and 40% of attenuation time which is not uncommon since esters are derivates of higher alcohols.

In all three raw materials, two distinct types of production profiles could be distinguished although the grouping of particular fermentations was different among them. Fermentations with the fast production rate of esters and with their high final concentrations were F1-F4 in barley, F1, F2, F5, F7, F8 and F11 in buckwheat, and F1, F2, F8, F9 and F11 in quinoa. In all other fermentations, the overall rate of ester production was significantly lower whereas differences in absolute concentrations between these two groups were the most prominent in barley.

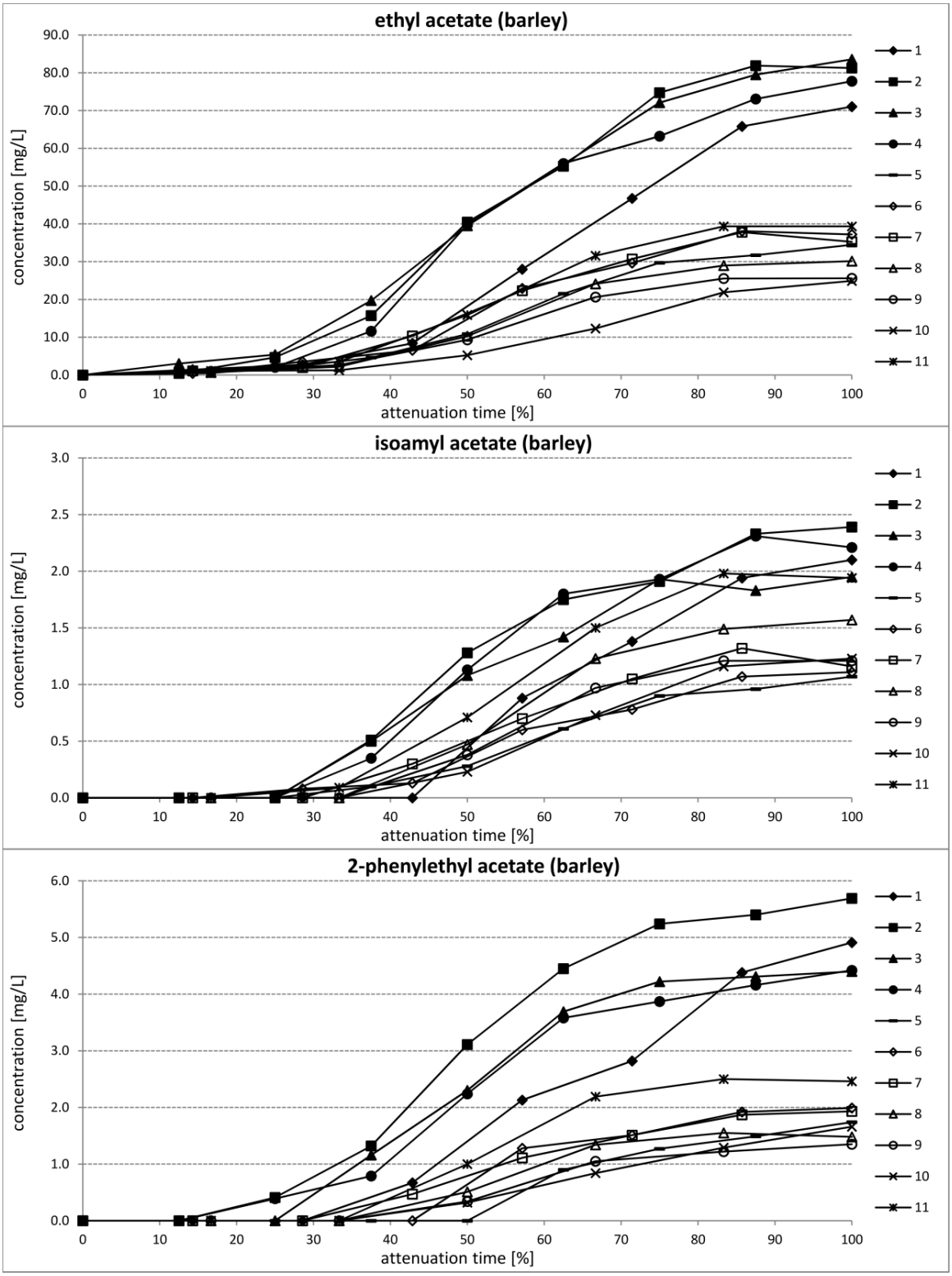

Figure 56: The production profile of important aromaactive esters during eleven successive fermentations of the barley wort (Brečko, 2014)
01-11: the numbering of a particular successive fermentation.
Slika 56: Profil tvorbe pomembnih aromatičnih estrov za enajst zaporednih fermentacij ječmenove pivine (Brečko, 2014)
01-11: številčenje posamezne zaporedne fermentacije.

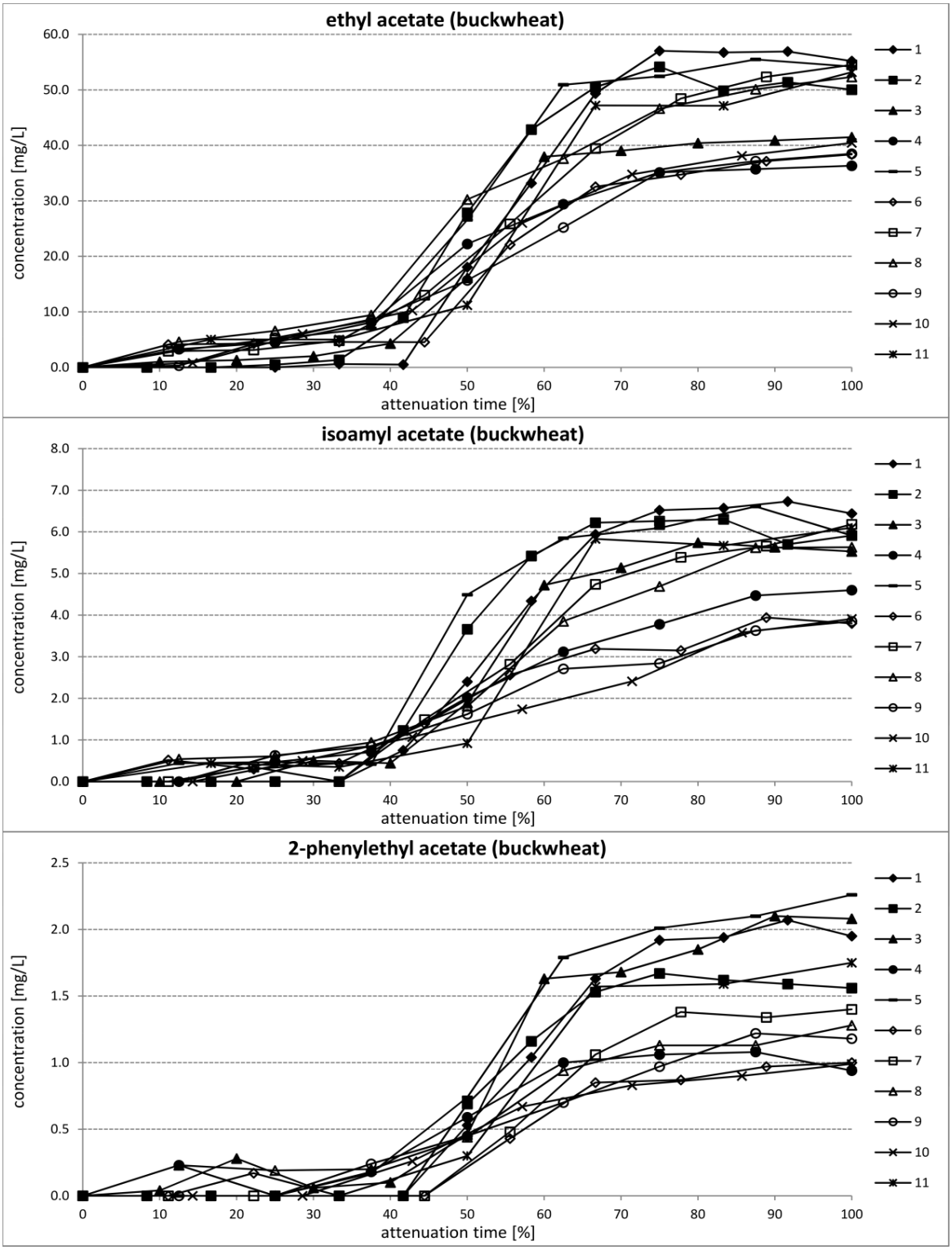

Figure 57: The production profile of important aromaactive esters during eleven successive fermentations of the buckwheat wort (Brečko, 2014)
01-11: the numbering of a particular successive fermentation.
Slika 57: Profil tvorbe pomembnih aromatičnih estrov za enajst zaporednih fermentacij ajdove pivine (Brečko, 2014)
01-11: številčenje posamezne zaporedne fermentacije.

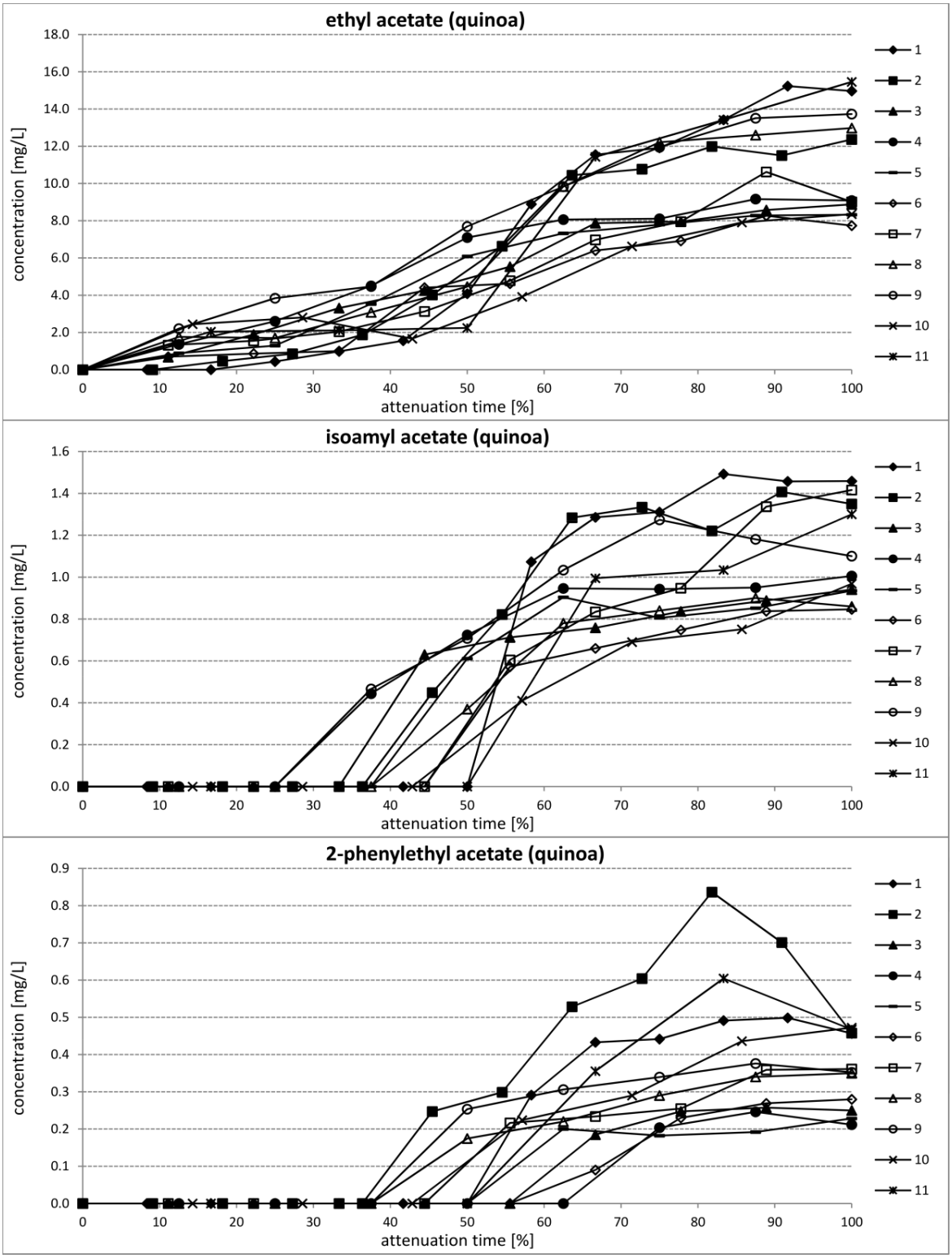

Figure 58: The production profile of important aromaactive esters during eleven successive fermentations of the quinoa wort (Brečko, 2014)
01-11: the numbering of a particular successive fermentation.
Slika 58: Profil tvorbe pomembnih aromatičnih estrov za enajst zaporednih fermentacij kvinojine pivine (Brečko, 2014)
01-11: številčenje posamezne zaporedne fermentacije.

5 CONCLUSIONS

The investigation of buckwheat and quinoa for brewing purposes gave a plethora of new basic knowledge as well as many useful information a habitual or commercial brewer can use. Since the exploration of a commercial potential of buckwheat and quinoa for brewing purposes was our main focus, evaluations of buckwheat and quinoa have been made from two distinct points of view, i.e. (i) whether pseudocereal is convenient to be commercially used for the preparation of gluten-free fermented beverages *per se* and (ii) whether this convenience also include a substitutional potential for barley as a brewing raw material.

Our first hypothesis "*Amino and fatty acid content of the buckwheat and quinoa beer-like beverages is higher as compared to common, standard barley beer, and aldehyde and ketone content does not differ significantly*" was only partly confirmed. In comparison to barley, the amino and fatty acid content was higher only in quinoa beverages. In addition, aldehyde and ketone contents differed significantly, being both a function of a raw material used and yeast species implemented for fermentation.

Our second hypothesis "*Non-conventional malt sources have no impact on cell-wall protein profile, chromosomes length, and flocculation capacity but they vary significantly between individual yeast batches*" was rejected in the first part and confirmed in the second part. Biochemical changes of yeast were both raw material- and batch number-dependent.

Our hypothesis "*Functional yeast cell age results in changed metabolism in a sense that it becomes less suitable for the fermentation of the buckwheat and quinoa wort*" was rejected in the case of buckwheat and only partly confirmed in the case of quinoa. In particular, the serial repitching of quinoa wort seems limited to six successive fermentations at most.

Considering the conclusions regarding the analysis of fermentation medium, our hypothesis "*The fermentation profile of organic compounds and metal cations vary significantly between individual yeast batches and these variations are specific for malted barley, buckwheat, and quinoa products, respectively*" was, generally speaking, confirmed.

In general, both raw materials showed a good potential for brewing purposes when different yeast species were used. Buckwheat handling was easy, the process operation was similar to barley and the resultant beverage more or less resembled typical beer in many ways, which makes buckwheat suitable for habitual beer consumers who seek a traditional and well-established beer character. On the other hand, quinoa was slightly more difficult to deal with, making the necessity to adapt some brewing procedures. Its beverage had a very distinct overall appearance, which was hardly suggestive of beer; its near to black colour, nutty aroma, greyish foam, and an astringent taste makes it a beverage of choice for special occasions. However, the low alcohol content and exceptional nutritive properties, such as minerals, amino acids, and other, suggest quinoa beverage could be suitable for a more frequent use.

6 SUMMARY (POVZETEK)

6.1 SUMMARY

The realization that a healthy lifestyle, including nutrition, reduces the risk of disease and increases health and well-being has received a huge amount of publicity. Historically, beer is one of the world's oldest prepared beverages, possibly dating back to the early Neolithic, when cereals were first farmed. Since the introduction of the beer purity law (*ger*. Reinheitsgebot) in 1516, barley has been traditionally used as the main ingredient of beer. To facilitate consumer requirements, other cereals (rice and maize) and pseudocereals (buckwheat, quinoa, and amaranth) have been investigated as brewing ingredients because of the absence of gluten and the presence of compounds that are claimed to have positive effects on health. Since customers are looking for innovative products and additional health benefits while consuming food or drinks, the nutritive properties of buckwheat and quinoa make attempts to investigate these raw materials for malting and brewing purposes worthwhile. But when predictions about the suitability of a raw material for brewing purposes are being made, it is useful to compare its characteristics with others, already well-established in brewing, in particular barley malt. Barley (*Hordeum vulgare* L.) and barley malt have historically been shown to be exceptionally suited for beer brewing purposes and malted grain is thus the single most important raw material for beer production nowadays. In the case of buckwheat, results collected so far strongly suggest that with the aid of commercial enzymes, buckwheat malt has the potential for replacing barley malt as a gluten-free material. Still more extensive work is required to optimize fermentation performance and beer characteristics (e.g. flavor, aroma, and foam development). Regarding quinoa, extensive research has been carried out worldwide on the agricultural aspects of quinoa, but little has been done on a physiological level or its malting and brewing potential.

One part of our research was aimed to prepare bottom- and top-fermented and low-alcohol beverages from the buckwheat and quinoa and explore their physical, chemical, and sensory properties. Therefore, brewing with 100% malted buckwheat (*Fagopyrum esculentum*, Moench) and 100% malted quinoa (*Chenopodium quinoa*, Wild.) was performed and different species of the brewing yeast were used for fermentation. In particular, besides the bottom-fermenting strain TUM 34/70 (*Saccharomyces pastorianus*, E. C. Hansen) that is commonly used for the industrial lager beer brewing, the bottom fermenting yeast for the production of low-alcohol beers (*Saccharomycodes ludwigii*, E.C. Hansen) and the classical top cropping strain used for the production of Kölsch beer TUM 177 (*Saccharomyces cerevisiae*, Meyen) were used. Existing malting, mashing and fermentation methods known from the literature, with some modification, were followed. To assure a reference for a useful evaluation of brewing potential of buckwheat and quinoa malt, the same procedures were used for brewing with 100% malted barley malt.

Compared to barley, the analysis of brewing attributes of buckwheat and quinoa showed lower malt extract, longer saccharification time, higher total protein and fermentable amino nitrogen content, and higher values of iodine test and colour. Fermentability values, the wort pH and the soluble protein content were similar for barley and buckwheat but different for quinoa whereas only values of viscosity and beverage pH were similar between barley and quinoa. All buckwheat beverages and especially quinoa beverages contained a superior level of metal cations. The fermentable carbohydrate content in the buckwheat wort was comparable to barley but lower in quinoa; however, worts derived from both pseudocereals predominately contained glucose. This situation was the main reason that the yeast strain TUM SL17 did not produced low-alcohol beverages, as it was the case in barley. The amino acid content of the buckwheat wort was similar to barley whereas their content in the quinoa beverage was almost twice as high. Regarding the amino acid assimilation, this process was primarily raw material-dependent. The content of volatile compounds commonly associated with beer aroma was comparable between the barley and buckwheat beverage fermented with TUM 34/70 and TUM 177 but significantly lower in quinoa. The situation was different in the case of TUM SL17 which, in comparison to other two yeasts, produced less, similar and more of the selected volatile compounds in the case of barley, buckwheat and quinoa, respectively. However, the quinoa wort and beverages contained some distinctive volatile substances not found in other beverages which were obviously formed during malting. The organoleptic perception of the buckwheat beverage was better than that of the quinoa, although both showed a good general acceptance. An important practical observation was that the beverages fermented with TUM SL17 were rated the best. This situation was the most obvious for quinoa thus this yeast should be preferred when considering quinoa for brewing purposes. In sum, buckwheat seems pretty similar to barley whereas quinoa shows many unique properties.

Gluten-free beer-like beverages from malted buckwheat and quinoa are somehow close to their commercial production, but rather high expenses are expected due to the relatively high price of grain, some technological adaptations of process and the need for external enzyme supplementation during mashing. One of the common and efficient cost reduction measures in the industrial scale is serial repitching of the yeast biomass, which has not been studied for the buckwheat and quinoa wort fermentation before. In that manner we have monitored possible changes in yeast's proteins and chromosomal DNA during eleven serial repitchings of the yeast *Saccharomyces pastorianus* strain TUM 34/70 for fermentation of the barley, buckwheat and quinoa wort. Samples were taken every twenty-four hours after pitching, analyzed for the particular amino acid content and thoroughly statistically evaluated. Karyotypes showed changes in regard to the raw materials used and many responsible candidate proteins are suggested which could cause these differences. Different relative expression of some protein bands was also linked to the proteins involved in yeast stress response and proteins involved in fermentation performance.

Results suggest that serial repitching of the strain TUM 34/70 seems suitable for the production of gluten-free beer-like beverages from buckwheat and quinoa.

Besides the analysis of serial repitching on the karyotype and protein profile of *Saccharomyces pastorianus* TUM 34/70, our research also explored the influence of serial repitching on the composition of the barley, buckwheat and quinoa fermentation medium. Regarding the fermentation performance and the uptake dynamics of metals and fermentable carbohydrates, in general, both pseudocereals show higher variations of all examined attributes during the successive fermentation. However, in buckwheat these differences are still closer to those of barley and they rather fluctuate with no obvious tendency, whereas differences in quinoa were pretty prominent. They often show a directional trend, which indicated a general and integral weakening of the yeast from the sixth successive fermentation on. The assimilation of fermentable carbohydrates lessens and the metal uptake seems poorly controlled.

Considering the uptake dynamics of amino acids during eleven successive fermentations, the term "completion time" (t95), here defined as the percent attenuation time necessary for ~95% of the total assimilation, has been introduced for the first time. In addition, "the serial repitching factor" (SRf) was also used for the first time to support the visual evaluation of the influence of serial repitching. Amino acids that were essentially affected by serial repitching were glutamine, arginine, alanine and tryptophan in barley, aspartate, glutamate and tryptophan in buckwheat, and all in the quinoa wort fermentation. As opposed to buckwheat and quinoa, in barley amino acids behaved more or less independently from each other, which for buckwheat and quinoa indicates a more general, systemic changes of yeast. From the amino acids point of view, buckwheat can be fully regarded as a suitable gluten-free substitute for barley beer since the amino acid assimilation was very consistent and hardly influenced by the serial repitching, especially regarding the final amino acid assimilation. In the case of quinoa, the assimilation of all amino acids became importantly affected after the sixth fermentation and probably unsuitable for the production of beer-like beverages. Results suggest no substitutional potential of quinoa for barley beer but if a nutrient-rich beverages of choice from quinoa malt are meant to be prepared, it seems that the serial repitching is limited to six fermentations at most.

From the customer point-of-view, the production dynamics of important volatile compounds typically associated with beer aroma is the most important. The term "serial repitching factor" (SRf) was used to support the visual evaluation of the influence of serial repitching. Results showed that levels of methanol in the quinoa wort fermentation were only a little higher than in barley and practically successive fermentation-independent. In addition, the behaviour of acetaldehyde in quinoa was similar to barley. However, there was a two-fold lower final production of important aroma compounds compared to barley

and buckwheat and for this reason quinoa could not be apprehended as a gluten-free substitute of bottom-fermented barley beer. Regarding the buckwheat wort fermentation, two- to three-times lower final acetaldehyde content than in barley is desirable, whereas relatively high methanol content could represent some inconvenience. Barley and buckwheat showed comparable sum concentrations and similar overall profiles of important aroma compounds. From this perspective buckwheat seems promising substitute for barley as a brewing raw material.

In very short, overall conclusions of our comprehensive study are that buckwheat shows adequate brewing properties to substitute barley for the commercial preparation of bottom-fermented gluten-free beer-like beverages when yeast is repitched for at least eleven times. On the contrary, quinoa practically shows no substitutional potential for barley beer; however, it has many nutritious advantages thus the commercial preparation of unique, bottom-fermented gluten-free "non-beer-like" beverages − when yeast is repitched for six times at most − seems reasonable.

6.2 POVZETEK

V zadnjem času je ogromno publicitete deležno zavedanje, da zdrav način življenja, predvsem zdrav način prehranjevanja, zmanjša tveganje za pojav bolezni in izboljša splošno počutje. Koncept "funkcionalne hrane" ima izvor na Japonskem v 80. letih 20. stoletja. Novembra 1995 je Evropska komisija predstavila program "Znanost o funkcionalni hrani v Evropi" (FUFOSE), ki je postavil koncepte za znanstven pristop pri obravnavi funkcionalne hrane. Sestavni del tega programa je tudi nova definicija funkcionalne hrane:

> *"Hrano lahko označimo kot funkcionalno, če se dovolj jasno dokaže , da pozitivno vpliva na eno ali več telesnih funkcij ne glede na hranilno vrednost, in to na način, ki je relevanten za dobro počutje in zmanjšanje tveganja za pojav bolezni."*

Funkcionalna pijača mora vsebovati biološko aktivne snovi. Trenutno se le-te delijo v šest različnih skupin, in sicer: sekundarni metaboliti rastlin, prebiotski ogljikovi hidrati, omega-3 maščobne kisline, konjugirana linolna kislina, peptidi mlečnih beljakovin in Maillardovi produkti. Skladno s tem in z zgornjo definicijo lahko pijače na osnovi psevdožit štejemo med funkcionalne pijače.

Svetovna pojavnost celiakije je ocenjena na enega izmed 100 prebivalcev. Zaradi tako velike pogostosti je ena izmed najobičajnejših znanih prehranskih preobčutljivosti. Zanjo je značilen imunski odziv na zaužiti gluten pri genetsko podvrženih posameznikih. Edino uspešno zdravljenje je strogo doživljensko upoštevanje diete brez najmanjšega sledu

glutena, ki ga naravno najdemo v žitih (pšenica, pira, rž, ječmen) in žitnih izdelkih, ni pa prisoten v psevdožitih (ajda, kvinoja, amarant)

Zgodovinsko gledano je pivo ena izmed svetovno najstarejših proizvajanih pijač, saj začetki njene proizvodnje segajo v zgodnji neolitik, ko se je pričelo načrtno gojenje žitaric. Sprva se je pivo proizvajalo iz ječmena, ki je vse od uvedbe Zakona o čistosti (*nem*. "Reinheitsgebot") leta 1516 tudi tradicionalno glavna sestavina. Da bi zadovoljili različne želje in potrebe potrošnikov, se je pričelo raziskovanje drugih žit (riž, koruza) in psevdo-žit (ajda, kvinoja, amarant) kot pivovarskih sestavin, predvsem zaradi odsotnosti glutena in prisotnosti različnih snovi, katerim se pripisuje pozitiven učinek na zdravje.

V primeru ajde rezultati, zbrani do sedaj, kažejo, da ima ajdin slad z dodatkom komercialnih encimov visok potencial za nadomestitev ječmenovega sladu pri proizvodnji brez-glutenskih pijač. Kljub temu so potrebne še nadaljnje raziskave za optimiziranje fermentacije in nekaterih lastnosti piva (npr. okus, vonj, razvoj in stabilnost pene). Kar se tiče kvinoje je bilo narejenih mnogo študij, a predvsem z agronomskega vidika, zelo malo pa na področju fiziologije ter slajenja in potencialni uporabnosti v pivovarstvu. V posebni študiji s kvinojo kot pivovarsko sestavino so naprimer ugotovili, da ima optimalno slajena kvinoja rahlo višji ekstrakt kot ječmenov slad. Potrošniki pri prehranjevanju in pitju nenehno iščejo inovativne produkte in koristi za zdravje. Zaradi ugodnih hranilnih lastnosti ajde in kvinoje se zdi raziskovanje njunega slajenja in pivovarske uporabnosti smiselno. Vendar je pri ugotavljanju primernosti (psevdo)žit za pivovarske namene pomembno, da opravimo ustrezno primerjavo z že uveljavljenim, tj. ječmenom. Ječmen (*Hordeum vulgare* L.) in ječmenov slad sta se izkazala kot izjemno primerna za uporabo v pivovarske namene in slajen ječmen tudi danes ostaja daleč najpomembnejša surovina v pivovarstvu.

Praktično vsi standardi, analizni postopki in tehnološko znanje o slajenju, drozganju in fermentaciji temeljijo na raziskavah in proizvodnih izkušnjah ječmena in ječmenovega slada. Za nobeno drugo žito oz. psevdo-žito niso postopki tako optimizirani kot za ječmen, saj je njegova agronomska pridelava usmerjena v čim večji delež ogljikovih hidratov in nizko encimsko aktivnost. Vendar je splošne načine vrednotenja (psevdo)-žita in postopke pridelave pijač, ki se uporabljajo za ječmen, mogoče uspešno uporabiti pri katerem koli (psevdo)-žitu. Standardni postopki se ne spremenijo, ni pa še popolnega soglasja, katera žita (poleg brezglutenskih psevdo-žit), bi lahko služila kot učinkovita surovina za pijače.

Ajda izvira iz osrednje Azije, v vzhodno in srednjo Evropo pa so jo prinesla nomadska ljudstva. Proteini ajdovih semen vsebujejo enako ali večjo količino vseh aminokislin v primerjavi s proteini pšenice, z izjemo glutamina in prolina, ki ju je na splošno manj. Med lipidi predstavljajo linolna, oleinska in palmitinska kislina 88% vseh maščobnih kislin. S tipično 80% nenasičenih maščobnih kislin in več kot 40% esencialne polinenasičene linolne kisline je ajda prehransko bogatejša kot ostala žita. Optimalno encimsko aktivnost

pri ajdinem sladu dosežemo s 96-urno kalitvijo pri 15°C. V tem času poteče zadovoljiva sprememba zrn, hranila pa se ne porabijo pretirano. Poleg tega se med slajenjem občutno poveča delež rutina, biofunkcionalnega polifenola. Za sladico, pridobljeno iz takšnega slada, je značilna nizka fermentabilnost in visoka viskoznost v primerjavi z ječmenovo sladic). Poleg tega so študije pokazale, je vsebnost encimov tako v zrnju kot tudi v semenih znatno nižja kot pri ječmen). Z naraščajočimi količinami dodane α-amilaze pri drozganju se povečajo obarvanost, celokupni ekstrakt, učinkovitost filtracije sladice, fermentabilnost in skupni fermentabilni ekstrakt, viskoznost pa se zmanjša. V primeru postopnega večanja količin dodane amiloglukozidaze pa ima za posledico večjo fermentabilnost, večji skupni fermentabilni ekstrakt ter povečanje vrednosti celokupnega topnega dušika, prostega aminskega dušika in Kolbachovega indeksa.

Kvinoja je bila glavna poljščina pred-Kolumbovskih kultur v Latinski Ameriki. Zanimanje zanjo v zadnjem času ponovno narašča, vse od kar je bilo pokazano, da ima zelo dobre hranilne lastnosti. Aminokislinski profil posamezne proteinske frakcije kaže na uravnoteženo vsebnost esencialnih aminokislin, s še posebej visokim deležem lizina (4,5-7.0%). Razmerja esencialnih aminokislin so blizu priporočilom Organizacije združenih narodov za prehrano in kmetijstvo. Stopnja nenasičenosti maščob je več kot 75%, po nekaterih raziskavah pa celo 87% in več. Količina mineralov v semenih je približno dvakrat večja kot pri žitih, količina vitaminov pa je primerljiva tistim v žitih. Do sedaj je bilo opravljenih zelo malo raziskav glede uporabe kvinoje kot pivovarske surovine. Več je znanega o značilnostih škroba v semenih. Le-ta je mnogo bolj viskozen kot škrob pšenice in amaranta. V *in vitro* pogojih semena kvinoje hitro kalijo, a zelo slabo v prsti. Po 36-urnem slajenju α-amilazna aktivnost naraste za 4-krat, kar sicer ni ugodno za postopek slajenja, je pa koristno pri drozganju in varjenju. Z uporabo 'metode odzivnih ploskev' so optimizirali pogoje slajenja kvinoje. Slajenje kvinoje kot tako ima tudi širšo uporabnost za živilske namene, saj izboljša dostopnost hranil in njihovo biološko vrednost.

Pivovarska kvasovka ima omejeno število razmnoževanj. Vsaka celica v populaciji ima končno število delitev preden umre. Študije starostnega fenotipa tako haploidnih laboratorijskih kot poliploidnih pivovarskih sevov so pokazale, da se posledice staranja odražajo kot morfološke, metabolne in genetske spremembe. Takšne so npr. povečanje velikosti celice, spremembe njene oblike in površine, spremenjen generacijski čas, metabolizem oslabi, spremenjena pa sta tudi genska ekspresija in sinteza proteinov. Na kvaliteto piva močno vpliva biokemijsko dogajanje v kvasni celici tekom fermentacije. Osnovnega pomena so zmožnost ločitve kvasovk od piva, ko je to potrebno, hitra in učinkovita poraba sladkorjev ter proizvodnja relativno velikih količin etanola in ustreznih razmerij aromatičnih spojin. Mnogo notranjih in zunanjih dejavnikov vpliva na učinkovitost in kvaliteto fermentacije in kakovost končnega produkta, vendar razmnoževalno staranje do sedaj ni bilo smatrano kot pomemben dejavnik. V industriji alkoholnih pijač je fermentacija pri proizvodnji piva edinstvena, saj del kvasovk po

končani fermentaciji uporabimo v naslednji. Postopek zaporednih uporab kvasovke večkrat ponovimo, konkretno število ponovitev pa je odvisno predvsem od pričakovanj glede kvalitete proizvoda ter strategije in načel pivovarskega podjetja.

Večina v industriji uporabljanih kvasovk je poliploidnih ali anevploidnih. Čeprav fizikalne in fermentativne karakteristike kvasne kulture lahko izgledajo konsistentne, je bilo opaženo, da pri ponovno uporabi kvasovke prihaja do genetskih mutacij. Vendar takšne spremembe genoma niso nujno takoj vidne, se pa lahko prenašajo v naslednje generacije. Takšna dogajanja lahko vodijo do genetskega zdrsa znotraj populacije, kar potrjuje opažena nestabilnost kvasnega genoma. V mnogih komercialnih sevih pivovarske kvasovke tipa "lager" so opazili polimorfizme dolžine kromosomov, ki pa so najpogostejši pri kromosomih z geni, neposredno udeleženimi pri fermentaciji, npr. *FLO1, FLO5, FLO9, FLO10, HXK1* and *MAL4*. Poleg tega "telomerna hipoteza" predlaga, da je število celičnih delitev zabeleženo s postopnim izgubljanjem telomernih sekvenc in lahko služi kot biološka ura, ki določa število celičnih delitev preden nastopi staranje celic. Analiza spontanih mutant, ki izkazujejo povečano stopnjo flokulacije, je pokazala, da te spremembe nastopijo po približno 6-10 generacijah pri pivovarski kvasovki tipa "lager". Spremembe flokulacijskih karakteristik so sicer najpogostejše posledice genetskih mutacij, vendar lahko genetski zdrs povzroči tudi druge, npr. spremenjena dinamika koriščenja sladkorjev pivine, zmanjšanje količine diacetila, kopičenje "petite" mutant in ostale.

Izražanje proteinov pri kvasovki *S. cerevisiae* je tako kot pri vseh evkariontih, natančno uravnavan proces, v katerega je posredno ali neposredno vključenih na tisoče genov. Tako spekter in absolutne koncentracije celičnih proteinov kot tudi njihovo razmerje so posledica razvojne stopnje celičnega cikla, starosti celice, dostopnosti hranil ter okoljskih pogojev, npr. pH, temperature in koncentracija kisika. Nadzorovana in ustrezna flokulacija na koncu fermentacije je v pivovarski industriji zelo pomembna, saj predstavlja poceni, enostaven in okolju prijazen način ločitve kvasovk od piva. Običajno pivovarska kvasovka izraža štiri glavne proteine, udeležene pri flokulaciji, za katere nosijo zapis štirje *FLO* geni. Vsi se, podobno kot nekateri drugi za pivovarstvo pomembni geni, nahajajo blizu kromosomskih telomer, zaradi česar so pogosteje podvrženi genetskim mutacijam. Konkretno, ti geni nosijo zapis za lektinu podobne protein, znane kot adhezini, zimolektini oz. flokulini. Poleg prej omenjenih sprememb, povezanih s flokulini je malo znanega o diferencialnem profilu celičnih proteinov v odvisnosti od zaporedne uporabe pivovarske kvasovke. Neposredni in takojšnji učinki generacijske starosti kvasovk na njihovo fermentacijsko sposobnost in biokemijske posledice še praktično niso bili raziskovani. Tako v komercialnih kot v laboratorijskih fermentacijah je sicer bilo pokazano, da zaporedna uporaba kvasovk postopno vpliva na fiziologijo, flokulacijo, površinski naboj in viabilnost kvasovk, vendar je zelo malo znanega o metabolnih posledicah.

Različne vrste piva so si podobne v mnogih pogledih, vendar lahko že majhne razlike v sestavi močno vplivajo tako na izgled kot tudi na aromo. Pivo je kemijsko zelo kompleksna pijača, saj vsebuje okrog 800 različnih organskih spojin. Večina jih je v zelo majhnih količinah, vendar imajo nekatere kljub temu znaten vpliv na dojemanje vonja in okusa. Največ kemijskih spojin v pivu izvira iz surovin (slad, hmelj in voda), nekatere pa so stranski produkti kvasnega metabolizma med fermentacijo in ravno te so odgovorne za edinstven aromatski značaj piva. V pivovarstvu sta železo in baker nezaželena, saj lahko imata negativne posledice, ker delujeta kot pro-oksidanta ter tako pospešujeta nezaželeno staranje piva. Prisotnost cinka in mangana v sledovih je nujno potrebna za rast in preživetje kvasovke, v prevelikih koncentracijah pa je toksičen. Proteini in peptidi v sladu se tekom drozganja razgradijo do aminokislin, tj. nadaljuje se encimska razgradnja, ki se je začela že med slajenjem. Reakcije deaminacije in transaminacije v kvasni celici so odgovorne za nastanek več različnih organskih kislin, aldehidov, alkoholov in estrov v pivu.

Z vidika kvalitete in arome je prisotnost maščobnih kislin v splošnem nezaželena, še posebej nenasičenih, a so nasprotno zelo cenjene z vidika hranilne vrednosti. Nekatere so pomembni dejavniki arome, druge so pomembne s prehranskega vidika, spet druge (npr. linolna in linolenska) pa zaradi oksidativne degradacije. Pokazali so, da so dolgoverižne maščobne kisline (C_{12}-C_{18}) povezane z obstojnostjo pene. Nekateri aldehidi so produkt kvasnega metabolizma, drugi so rezultat Streckerjeve degradacije aminokislin, tretji pa očitno nastanejo z naključno dekarboksilacijo organskih kislin. Ketoni spadajo, tako kot aldehidi, v skupino karbonilnih spojin. V pivu sta najpomembnejša diacetil (2,3-butandion) in njemu kemijsko soroden 2,3-pentandion.

V prvem delu raziskav smo se osredotočili na varjenje pivu podobnih pijač z uporabo 100% slajene ajde (*Fagopyrum esculentum*, Moench) in 100% slajene kvinoje (*Chenopodium quinoa*, Wild.). Za fermentacijo smo uporabili tri različne pivovarske kvasovke. Konkretno, poleg kvasovke spodnjega vrenja (*Saccharomyces pastorianus*, E. C. Hansen), sev TUM 34/70, ki je običajna izbira pri industrijskem varjenju "lager" piva, smo uporabili še kvasovko spodnjega vrenja (*Saccharomycodes ludwigii*, E.C. Hansen), sev TUM SL 17, ki se uporablja za varjenje piva z nizko vsebnostjo alkohola ter kvasovko zgornjega vrenja (*Saccharomyces cerevisiae*, Meyen), sev TUM 177, ki se uporablja za pripravo klasičnega "Kölsch" piva. Vse tri omenjene seve smo naročili pri "Weihenstephan Research Center for Brewing and Food Quality" Za postopke slajenja, drozganja in fermentacije smo uporabili obstoječe metode iz literature. Za uspešno interpretacijo rezultatov smo vse omenjene postopke ponovili z uporabo ječmenovega slada.

V drugem delu smo preučili vpliv zaporedne uporabe pivovarske kvasovke (*Saccharomyces pastorianus,* E.C. Hansen), sev TUM 34/70, na njene fiziološke in presnovne lastnosti v odvisnosti od sladice iz različnih slajenih (psevdo)žit. Posamezna celica pivovarske kvasovke ima končno razmnoževalno sposobnost, ki se odraža v

omejenem številu celičnih delitev. Poleg tega so kvasovke tekom fermentacije podvržene mnogim stresnim dejavnikom, kot so oksidativni in osmotski stres, spremembe pH, toksičnost etanola, manjšanje količine hranilnih snovi, nizke temperature, itd. Posledično se kvasne celice s časom fiziološko, morfološko in genetsko spreminjajo, kar vpliva na zmogljivost in kvaliteto kvasovke pri proizvodnji alkoholnih pijač. Najizrazitejše so spremembe v privzemu in metabolizmu sladkorjev, proizvodnji etanola in aromatičnih snovi ter pri flokulaciji (reverzibilno tvorjenje skupkov kvasnih celic na koncu fermentacije). Dejstvo je, da so vrsta in stanje uporabljene kvasne kulture kot tudi način njene uporabe pomembne za končni izdelek, vendar šele od nedavno se več pozornosti daje tudi starosti. Število zaporednih uporab kvasovke se je določalo izkustveno, brez utemeljitve in poznavanja osnov. Zato smo pri našem delu ugotavljali, kakšen vpliv ima enajstkratna zaporedna uporaba na fermentacijske sposobnosti. Dnevno smo merili vsebnost železa, bakra, cinka in mangana štirih fermentabilnih sladkorjev, osemnajstih aminokislin in devetih hlapnih snovi, pomembnih za aromo. S tem smo na osnovi podatkov skupaj z že znanimi dejstvi določili najvišje število zaporednih uporab, ki še ustreza kakovostnim zahtevam končnega uporabnika, in sicer ločeno za ječmen, ajdo in kvinojo.

Postavili smo naslednje hipoteze:

- *Vsebnost aminokislin in maščobnih kislin v ajdovi in kvinojini pivu podobni pijači je višja v primerjavi z običajnim, standardnim ječmenovim pivom, vsebnost aldehidov in ketonov pa ni značilno različna.*

- *Funkcionalna starost kvasne celice se kaže v spremenjenem metabolizmu, in sicer v smislu, da postaja manj primerna za fermentacijo.*

- *Fermentacijski profil organskih snovi in kovinskih kationov je značilno različen med posameznimi zaporednimi uporabami pivovarske kvasovke, te razlike pa so specifične za fermentacijske produkte na osnovi slajenega ječmena, slajene ajde in slajene kvinoje.*

- *Nekonvencionalni viri slada nimajo vpliva na profil proteinov celične stene, dolžino kvasnih kromosomov in sposobnost flokulacije, se pa značilno razlikujejo med posameznimi zaporednimi uporabami pivovarske kvasovke.*

Vzorcem slada, pivin in pijač smo določili vrednosti najpomembnejših pivovarskih atributov. Poleg tega je analizni del vključeval določanje aminokislin, nasičenih in nenasičenih maščobnih kislin, aldehidov, ketonov, estrov, alkoholov in kovinskih kationov, in sicer v vzorcih sladice, vzorcih, dobljenih tekom fermentacije in vzorcih končnega izdelka. Rezultate analiz smo interpretirali z vidika kvalitete končnega proizvoda, tj. prisotnosti spojin, ki vplivajo na aromo in rok trajanja ter prisotnost spojin, ki imajo

hranilno vlogo in/ali so biološko aktivne. Poleg tega smo ajdo in kvinojo ovrednotili z vidika primernosti kot brezglutenskega nadomestka za ječmenovo pivo. Kolikor nam je bilo znano, tako podrobna analiza fermentiranih alkoholnih pijač na osnovi 100% slajene ajde in 100% slajene kvinoje še ni bila narejena. Pri eksperimentu "zaporedne uporabe kvasovke" smo poleg zgoraj navedenih analiz kvalitativno in kvantitativno ovrednotili tudi proteinski profil kvasovke in spremembe v velikosti njenih kromosomov. Splošno sprejeto je namreč, da zaporedna uporaba povzroča pri kvasovki različne poškodbe, npr. postopna izguba telomernih sekvenc in diferencialno izražanje proteinov. Na ta način smo lahko bolj jasno podali povezavo med genetiko in biokemijo kvasovke ter kemijskimi karakteristikami produkta fermentacije.

V primerjavi z ječmenom je analiza pivovarskih atributov ajde in kvinoje pokazala nižji ekstrakt v sladu, daljši čas saharifikacije, višje celokupne vsebnosti beljakovin, višje vrednosti fermentabilnega amino dušika ter višje vrednosti jodnega testa in barve. Fermentabilnost, pH sladice in vsebnost topnih beljakovin so bili podobni med ječmenom in ajdo, a drugačni za kvinojo, po drugi strani pa so bile vrednosti viskoznosti in pH pijače podobne med ječmenom in kvinojo. Pijače iz obeh psevdožit, zlasti kvinojine, so vsebovale izjemno visoke koncentracije kovinskih kationov. Vsebnost fermentabilnih ogljikovih hidratov v ajdovi sladici je bila primerljiva z ječmenovo in nižja v kvinojini. Pivini obeh psevdožit sta pretežno vsebovali glukozo, kar je bil glavni razlog, da kvasovka TUM SL17 v tem primeru ni proizvedla pijače z nizko vsebnostjo alkohola, kot se je to zgodilo pri ječmenu. Vsebnost aminokislin v ajdovi pivini je bila podobna tisti v ječmenovi, njihova vsebnost v kvinojinih pijačah pa je skoraj dvakrat večja. Vsebnosti aminokislin so pretežno odvisne od tipa pivine kot od uporabljene kvasovke. Vsebnost izbranih hlapnih spojin, pogosto povezanih z aromo piva je bila v primeru kvasovk TUM 34/70 in TUM 177 primerljiva med ječmenovimi in ajdovimi pijačami, a precej nižja v kvinojih. V primeru kvasovke TUM SL17 pa so bile razlike očitnejše, in sicer je bila vsebnost izbranih hlapnih spojin pri ječmenu manjša, pri ajdi primerljiva in pri kvinoji večja v primerjavi z ostalima dvema kvasovkama. Poleg tega je kvinoja vsebovala nekatere druge hlapne snovi iz skupine pirazinov, pirimidinov in furanov, ki jih ni bilo moč najti v drugih pijač in so najverjetneje nastali pri slajenju.

Vsebnost maščobnih kislin je bila največja v kvinojini pivini in pijačah, še posebej nenasičenih (predvsem linolejske). Ječmenove in ajdove pijače so si bile glede vsebnosti maščobnih kislin zopet zelo podobne. V vseh primerih se je količina kratkoverižnih nasičenih maščobnih kislin povečala in količina dolgoverižnih nenasičenih zmanjšala po fermentaciji. Značilnost kvinojine pivine in pijač je bila znatna količina pentilfurana, vsebnost aldehidov in ketonov pa je bila nižja kot pri ječmenu in ajdi. Če primerjamo različne kvasovke, je bila količina aldehidov in ketonov največja v pijačah, fermentiranih s kvasovko TUM SL17. V splošnem se je vsebnost večine aldehidov in ketonov tekom staranja povečala. Organoleptično dojemanje ajdovih pijač je bilo boljše od kvinojinih,

čeprav so tudi slednje bile v splošnem relativno dobro sprejete. Na tem mestu je potrebno poudariti, da sta bili obe pijači, fermentirani s kvasovko TUM SL17, najbolje ocenjeni. To je bilo še posebej očitno pri kvinoji, zaradi česar se ta kvasovka priporoča kot prva izbira pri uporabi kvinoje v pivovarske namene, navkljub višji produkciji aldehidov in ketonov. Na splošno je veljalo, da so bile ajdove pijače precej podobne ječmenovem pivu, kvinojine pa so kazale številne edinstvene lastnosti.

Brezglutenske pivu podobne pijače iz slada ajde in kvinoje so nekako blizu svoji komercialni proizvodnji, vendar so pričakovani visoki proizvodni stroški zaradi relativno visoke cene zrnja, nekaterih tehnoloških prilagoditev procesa in potrebe po dodatku komercialnem encimskih preparatov. Eden od pogostih in učinkovitih ukrepov za zmanjšanje stroškov v industrijskem merilu je zaporedna uporaba kvasovke, ki še ni bila raziskovana za fermentacijo ajdove in kvinojine sladice. Zato smo spremljali morebitne spremembe profila proteinov in kromosomske DNA pri kvasovki v enajstih zaporednih fermentacijah z uporabo kvasovke *Saccharomyces pastorianus* sev TUM 34/70. Vzorci so bili vzeti vsakih štiriindvajset ur po inokulaciji, analizirani za vsebnost kovin, fermentabilnih ogljikovih hidratov, aminokislin in hlapnih snovi s sledečim temeljitim statističnim ovrednotenjem. Pokazali smo spremembe kariotipa v zvezi z uporabljenimi surovinami in veliko odgovornih kandidatnih proteinov, ki bi lahko povzročili te razlike. Druge razlike v relativnem izražanju proteinov kažejo tudi na spremenjeno izražanje nekaterih beljakovin vpletenih v stresni odziv kvasovke in proteinov, ki sodelujejo pri procesu fermentacije. Rezultati kažejo, da se zaporedna uporaba seva TUM 34/70 zdi primerna za proizvodnjo brezglutenske pivu podobne pijače iz ajde in kvinoje.

Poleg analize vpliva zaporedne fermentacije na kariotip in beljakovinski profil *Saccharomyces pastorianus* TUM 34/70, je naša raziskava preučila tudi vpliv zaporedne fermentacije na sestavo fermentacijskega medija iz ječmena, ajde in kvinoje. Kar se tiče fermentacijskega profila (poraba ekstrakta in tvorba etanola) ter privzema kovinskih kationov in fermentabilnih ogljikovih hidratov, je pri obeh psevdožitih bila v primerjavi z ječmenom opažena večja variabilnost tekom enajst zaporednih fermentacij. Kakorkoli, razlike so v primeru ajde znatno manjše od tistih pri kvinoji. Pri ajdi tudi ni opaziti posebnega vzorca sprememb medtem ko se pri kvinoji kaže usmerjen trend, pogosto v smeri splošnega in celostnega slabljenja kvasne zmogljivosti, zlasti od šeste fermentacije naprej. Najbolj očitne posledice so zmanjšan privzem fermentabilnih sladkorjev in slabo kontrolirana asimilacija kovinskih kationov.

Pri karakterizaciji dinamike privzema aminokislin tekom enajst zaporednih fermentacij smo uvedli nov atribut, imenovan "dovršilni čas" (t95). Le-ta predstavlja delež časa atenuacije v odstotkih, ki je potreben za asimilacijo ~95% skupne asimilacije posamezne aminokisline ali vsote vseh aminokislin. Poleg tega smo uvedli tudi "faktor zaporedne fermentacije" (SRf) pri čemer je njegova večja vrednost pomenila večji vpliv zaporedne

fermentacije na aminokislinsko asimilacijo. Aminokisline, katerih asimilacija je bila najbolj pod vplivom zaporednih fermentacij so glutamin, arginin, alanin in triptofan pri ječmenu, aspartate glutamat in triptofan pri ajdi in vseh osemnajst pri kvinoji. Za razliko od ajde in kvinoje so bili pri ječmenu vplivi zaporednih fermentacij na privzem posameznih aminokislin neodvisni drug od drugega, kar kaže na bolj splošne in sistemske spremembe kvasovke. Z vidika asimilacije aminokislin lahko ajdo jemljemo kot ustrezen brezglutenski nadomestek ječmenovega piva. Asimilacija aminokislin je bila tekom enajst zaporednih fermentacij zelo konsistentna, kar še posebej velja za končno količino asimiliranih aminokislin. V primeru kvinoje je bila asimilacija vseh aminokislin drastično prizadeta od šeste fermentacije naprej. Transport aminokislin v in iz kvasovke izgleda slabo kontroliran in dinamika bistveno odstopa od običajne sigmoidne krivulje. Skladno s tem rezultati kvinojo izključujejo kot pivovarski nadomestek za ječmenovo pivo, vendar to ne pomeni, da ni primerna za pripravo hranilnih pijač s specifičnimi karakteristikami. Kakorkoli, izgleda da je tudi v tem primeru število zaporednih fermentacij omejeno na šest.

Z vidika potrošnika je dinamika nastanka izbranih hlapnih spojin, ki so bistvene za karakter piva, seveda najpomembnejša. Tudi tukaj smo uporabili "faktor zaporedne fermentacije" (SRf), ki nam je kvantitativno pomagal pri vizualni interpretaciji rezultatov. Le-ti so pokazali, da je bila količina metanola tekom fermentacije kvinojine pivine le malo višja od tiste pri ječmenu in poleg tega neodvisna od zaporedne fermentacije, obnašanje acetaldehida pa je bilo podobno. Poleg tega je bila v primeru ajde končna količina vseh obravnavanih hlapnih spojin približno dvakrat nižja, zaradi česar je kvinoja praktično neprimerna kot nadomestek ječmena v pivovarske namene. Kar se tiče ajde je dva- do trikrat nižja vsebnost acetaldehida kot pri ječmenu zelo dobrodošla, a relativno visoke vsebnosti metanola bi lahko predstavljale določene nevšečnosti. Končna količina vseh obravnavanih hlapnih spojin je bila pri ječmenu in ajdi primerljiva, kot so bili tudi profili posameznih fermentacij. Končna ugotovitev je, da je ajda obetajoč nadomestek ječmena pri pripravi brezglutenskih fermentiranih pijač spodnjega vrenja.

Splošni zaključki naše celovite študije so, da je ajda s pivovarskega stališča povsem primerna za nadomestitev ječmena za komercialno pripravo brezglutenskih pivu podobnih pijač, ta primernost pa vključuje tudi zaporedno fermentacijo, in sicer vsaj enajstkrat. Ravno nasprotno pa kvinoja praktično ne kaže nadomestnih možnosti za ječmenovo pivo, vendar pa ima veliko hranilnih prednosti, zato se komercialna priprava edinstvenih, brezglutenskih "pivu ne-podobnih pijač" – ko se kvasovka uporabi največ šestkrat zapored − zdi razumna.

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