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Determination of Phytosterols in Beer.

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ABSTRACT

Extraction of phytosterols as lipid substances from various plant sources, including corn, is a subject of many foreign publications. However, studies that identify the behavior and influence of phytosterols in the beer making process were not found in the literature. Phytosterols present in cereals as free sterols, fatty acid esters and phenolic acids, glycosides and acylated glycosides. The study showed that phytosterols' content is entirely dependent on the raw material used. The more a malt part in the grain, the higher is phytosterol content. Phytosterol content accumulates in beer due to the extraction of raw materials from unmalted grain products, malt and hops.

Keywords: Phytosterols; raw materials for beer production; beta-sitosterol; campesterol; stigmasterol.

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INTRODUCTION

Phytosterols are plant-derived sterols extracted from the non-saponifiable fraction of plant lipids. Over 200 natural phytosterols have been identified, stigmasterol, brassicasterol and beta-sitosterol being the most common ones. Their melting points are 170 °C for stigmasterol, 148 °C for brassicasterol, and 140 °C for beta-sitosterol (Piironen *et al.*, 2000). Figure 1 shows the chemical structures of these phytosterols. Phytosterols represent a group of plant-derived cyclic secondary monoalcohols.

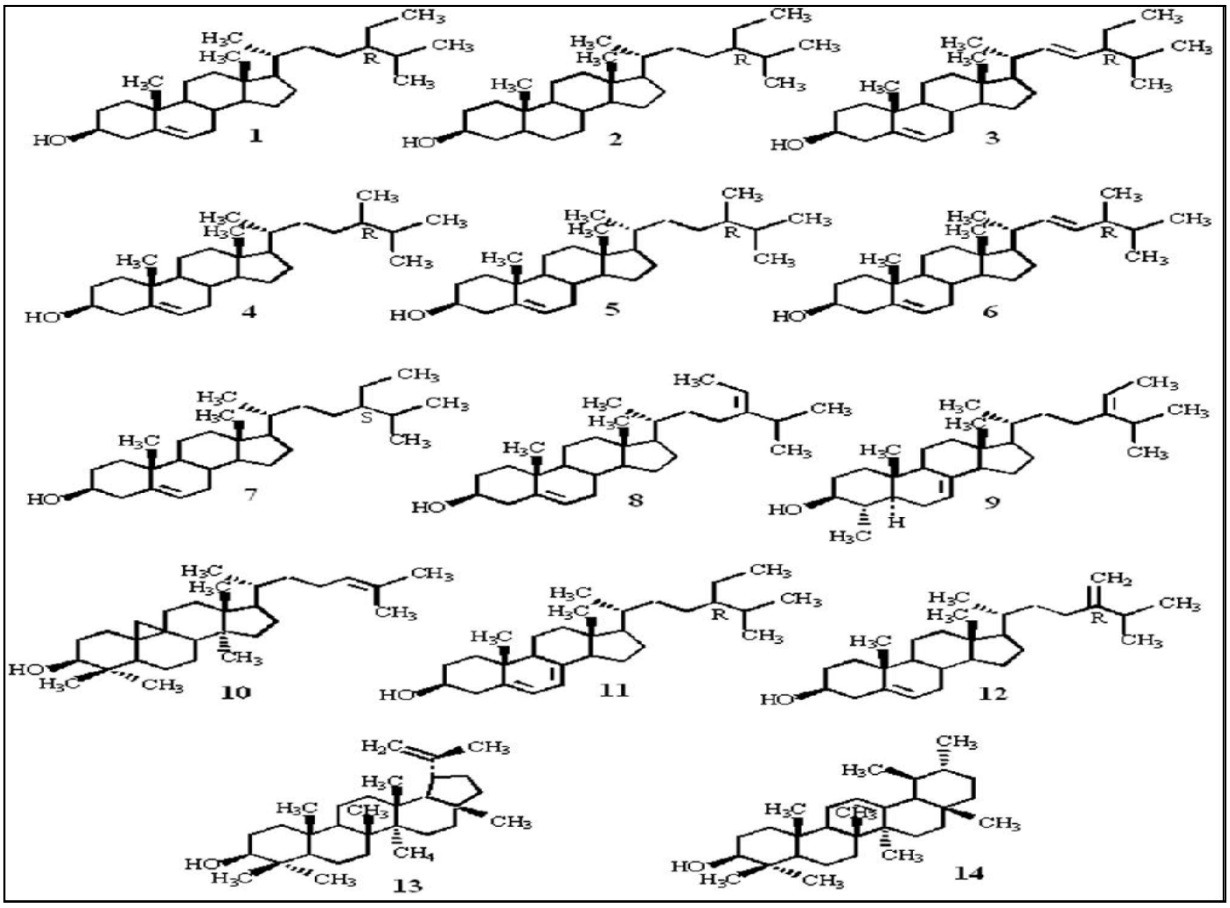

Figure 1. Main phytosterols and related compounds: 1 – sitosterol (b-sitosterol, stigmast-5-en-3b-ol); 2 – sitostanol (stigmastan-3b-ol); 3 – stigmasterol (stigmasta-5,22E-diene-3b-ol);4 – campesterol ((24R)-ergost-5-ene-3b-ol); 5 – campestanol ((24R)-ergostan-3b-ol); 6 – brassicasterol ((24R)-ergosta-5,22E-diene-3b-ol); 7 - g-sitosterol ((24S)-stigmast-5-ene-3b-ol); 8 – fucosterol (stigmasta-5,24(28)-diene-3b-ol); 9 - a1-sitosterol (citrostadienol, 4a-methylstigmasta-7,24(28)Z-diene-3b-ol); 10 – cycloartenol; 11 – 7-dehydrositosterol (stigmasta-5,7-diene-3b-ol); 12 – 24-methylenecholesterol (ergosta-5,24(27)-diene-3b-ol); 13 – lupeol (b-viscol, fagarasterol, lup-20(29)-ene-b-ol); 14 - a-amyrin (a-amyrenol, urs-12-ene-3b-ol).

Phytosterols in raw plant material are associated with different compounds, linked to them through the side chain, such as desmethylsterols – sitosterol, campesterol, stigmasterol, avenasterols and stanols, constituting the major part of plant-derived phytosterols (Moreau *et al.*, 2002; Piironen *et al.*, 2000). Monomethyl- and dimethylsterols are found less frequently. Raw materials contain sterols in form of so-called conjugated sterols – fatty acid esters (SE), phenolic acid esters (SPHE), glycosides (SG) and acylated glycosides (ASG), which can possess various chemical, technological and nutritional properties. Cholesterol usually amounts to 1-2% of all plant sterols, coming up to 5% or more in certain plant families, species, organs, or tissues (Moreau *et al.*, 2002).

Sterol biosynthesis in plant sources starts with acetic and mevalonic acids, undergoing the multistage enzymatic process to be transformed to the immediate precursor – squalene, which has different cyclization pathways in living organisms (Mekhtiev, & Misharin, 2007). Sterols are themselves precursors for other

steroids – aglyconic parts of steroid glucosides, which regulate the plant growth. They are also a part of cell membrane, being included in the phytolipid complex, and have impact on the cellular metabolism of plants.

Content and composition of phytosterols are influenced by both hereditary background and growth conditions – temperature, humidity; the amount of phytosterols decreases in case of higher germination temperatures, and, vice versa, increases in case of lower temperatures (Vlahakis, & Hazebroek, 2000).

Phytosterols, by virtue of their lipid nature, can be oxidized in the course of food processing and storage. Sterol reactivity and the rates of destruction, apparently, depend on the sterol structure, mostly the presence of unsaturated bonds within the cycle, temperature and matrix composition (Dutta, & Savage, 2002; Lampi *et al.*, 2002; Lampi *et al.*, 2000; Oehrl *et al.*, 2001; Soupas *et al.*, 2002; Whitaker *et al.*, 1997).

Efficient extraction of phytosterols from the food matrix is the most important part of sample preparation. The losses caused by an unsuitable extraction solution and technology cannot be compensated in the course of further analysis. When comparing extraction techniques, one should pay attention to various conjugated forms of phytosterols, i.e., free alcohols, esters, glycosides, or acylated glycosides. Solubility of different phytosterol compounds varies significantly. Nonpolar lipid solvents are effective for the extraction of free and fatty acids – etherified phytosterols. More polar phytosterolglycosides are poorly soluble in nonpolar solvents; therefore, more polar solvents should be used for the extraction.

Food matrix also has major influence on how easily phytosterols are released. Phytosterols of raw and refined oils can be easily dissolved in a nonpolar solvent with the sample. Most phytosterols of oilseeds, nuts and other tissues with high fat content are also quite easily extracted by solvents, whereas phytosterol extraction through solid cell walls or from complex polysaccharide-protein matrices of moist plant tissues and gramineous plants is much more challenging. Moreover, one should avoid enzymatic treatment to minimize loss, transformation, and formation of artifacts.

One of the techniques to enhance the rate of phytosterol extraction from complex food matrices is matrix degradation induced by alkalis, acids or enzymatic hydrolysis. Alkaline solvents effectively decompose lipid and protein matrices, while acidic hydrolysis is necessary to dissolve certain polysaccharides, such as starch. As alkaline hydrolysis is commonly used to separate sterols from saponifiable lipids, one could skip the preliminary lipid extraction before the hydrolysis. One should remember that such treatment also leads to hydrolyzation of phytosterol conjugates, which makes this technique inappropriate, if uninterrupted molecular species of conjugated phytosterols are to be studied. Hydrolysis can also have adverse effects on certain phytosterols, such as Δ7-phytosterols. Although phytosterols are relatively resistant to oxidation, compared to polyunsaturated lipids, certain precautions should be taken during the sample preparation. One should avoid heat, light and air exposure, some researchers add antioxidants to the mixtures used in the extraction process (Ostlund *et al.*, 2002; Stefani *et al.*, 2000).

There are different techniques of phytosterol extraction from food matrix, varying in efficiency, specificity, reliability and affordability. Therefore, phytosterol validation (for instance, detection of the compounds) should be included to improve each sample extraction procedure for each type of food matrix.

In case of direct extraction of phytosterols from food matrix, sample particles should of the same small size to improve the extraction rate. Seeds and dry plant materials can be regrounded with powdered glass (Normén *et al.*, 2001), coffee grinder (Pillow *et al.*, 1999), mortar (Pennington, 2002), crusher (Stefani *et al.*, 2000), or bladed grinder-machine. Some studies include thorough size-based selection of particles with the help of a bolter (Vries *et al.*, 1997). Humid tissues can be homogenized in the solvent.

Phytosterols can be extracted from air-dried plant material or from moist tissues. In case of dry material, water-immiscible solvents, such as hexane, were used, but moist samples require water-miscible solvents and solvent mixtures for efficient lipid extraction (Phillips *et al.*, 1999). In case of nonpolar solvents used for extraction, proper sample drying is very important, as residual moist can isolate phytosterols from the solvent.

Both polar and nonpolar solvents, as well as their mixtures, were used to release the lipids from the sample. Nonpolar solvents extract phytosterols together with lipids and are often used for the extraction of

oilseeds. Polar solvents are more selective, as they release polar phytosterol compounds more effectively than nonpolar solvents. As the majority of phytosterols of oilseeds exists in the form of free alcohols or fatty acid esters, they are easily extracted by nonpolar solvents, while other plant materials, such as gramineous plants, also contain glycosylated phytosterols, soluble in polar solvents.

Hexane is used to extract phytosterols from poppy and sunflower seeds (Schothorst, & Jekel, 1999), must (Nair *et al.*, 1984), maize fibers (Stefani *et al.*, 2000) and oilseeds (Johansson-Kornfeld, 1979), whereas petroleum ether is used in case of pumpkin seeds (Ostlund *et al.*, 2002). Hexane and methylenchloride proved to be equally efficient for phytosterol extraction from dry ginseng seeds, as the results were similar. Raw oil can be obtained after the extraction with the help of centrifugal separation or filtration, for instance, through cellulose acetate or glass-fiber filters (Johansson-Kornfeld, 1979).

Depending on the efficiency of the procedure, the latter should be repeated in order to provide full extraction of lipids. Extraction is usually repeated three times. Solvent extraction is more effective, if the sample is heated and the extraction process is continuous. Dry and homogenized samples are weighed in Soxhlet apparatus in the extraction shells, undergoing constant extraction with hot solvent during several hours. Continuous extraction of lipids with hot hexane have been used in case of sesame seeds (Pennington, 2002), avocado mesocarps (Homberg, & Bielefeld, 1989), roasted coffee (Ferrari *et al.*, 1996), while more polar acetone has been used for cocoa butter (Reina *et al.*, 1999). Lipid extraction can also be enhanced with ultrasonic bath (Ostlund *et al.*, 2002) and by moist sample grinding with sodium sulfate prior to the extraction (Phillips *et al.*, 2002).

There have been numerous foreign studies on the extraction of phytosterins as substances of lipid nature from various plant sources, including grain. However, no research on the behavior and the impact of phytosterols on beer brewing has been found in the literature.

It is known that raw materials for beer production, according to GOST 31711-2012, include brewer's barley and wheat malt, unmalted grains – barley, wheat, rice, corn, etc. Raw materials listed above contain phytosterols.

Phytosterol ability to form complex compounds with certain organic substances – alcohols, phenols, acids and their derivatives, amines, proteins, carbohydrates, inorganic salts and acids – has been studied long ago (Chung, 2000).

This is the reason for their accumulation in different parts of a grain. For instance, germ and bran fractions are known to be the best sources of sterol among all the grinding products. Generally, phytosterol content in different grinding products of grain crops (rye, wheat, oat, barley, etc.) is, apparently, correlated with ash content (Lu *et al.*, 2007; Meledina *et al.*, 2013).

Phytosterols of barley, the main grain crop used in brewing, are localized throughout a kernel, like in other gramineous plants, and can be classified as starchy or non-starchy lipids (Li *et al.*, 2007). Barley contains 0.8 mg/g of sterols – stigmasterol, β-sitosterol, camposterol and brassicasterol, where β-sitosterol makes up to 60% of all barley sterols, prevailing over all phytosterols (Lu *et al.*, 2007).

In gramineous plants, phytosterols can be found as free sterols, fatty acid and phenolic acid esters, glycosides and acylated glycosides. The presence of these groups varies in different gramineous plants and in different parts of a kernel (Dzhafarov *et al.*, 2010).

One should note that phytosterols change their structure type during storage (Jilian, 2011). For instance, total content of sterols in wheat flour over long-term storage (5 years) has not changed, the content of free sterols has decreased, while the content of esters has increased.

In the course of malting, part of lipids is utilized for metabolic respiration, part of them is oxidized due to more acidic conditions within the endosperm caused by dissolving high-molecular compounds. During the technological process of grain doughing, both raw and malted, most lipids pass into spent grains.

Therefore, considering the set of literature on the role of phytosterols in brewing process, many questions still do not have answers – whether beer contains phytosterols, the sources of phytosterols, the influence of phytosterols on the end product.

METHODOLOGY

Instrumental procedure of phytosterol detection has been developed in order to answer the question of the presence of phytosterols in beer (Rapota, & Tyrsin, 2014).

Preparation of standard solutions for chromatographic analysis of malt, wort and hop samples. A portion of cholesterol (100 mg ± 0.001 g) was weighed using analytical balance, dissolved in the mixture of 50 cm^3 of ethanol and 15 cm^3 of dichloromethane, diluted to the mark with distilled water, filtered if necessary. β-sitosterol stock solution was prepared in the same way. 1 cm^3 of these stock solutions of cholesterol and β-sitosterol (1 mg/cm^3) was transferred to the 100 cm^3 measuring flasks, diluted in the mixture of 50 cm^3 of ethanol and 15 cm^3 of dichloromethane, then 35 cm^3 of distilled water were added to the solutions, which were then mixed until complete dissolution. The solutions were stored for 1 month at the temperature of 4-8 °C.

Preparation of standard solutions for chromatographic analysis of beer samples. A portion of cholesterol (100 mg ± 0.001 g) was weighed using analytical balance, dissolved in the mixture of 50 cm^3 of ethanol and 15 cm^3 of dichloromethane, diluted to the mark with distilled water, filtered if necessary. β-sitosterol and campesterol stock solutions were prepared in the same way. 1 cm^3 of these stock solutions of cholesterol and β-sitosterol (1 mg/cm^3) was transferred to the 100 cm^3 measuring flasks, diluted in the mixture of 50 cm^3 of ethanol and 15 cm^3 of dichloromethane, then 35 cm^3 of distilled water were added to the solutions, which were then mixed until complete dissolution. The solutions were stored for 1 month at the temperature of 4-8 °C.

Preparation of the phytosterol complex of the raw material. Phytosterol extraction from brewer's barley malt, wort and granulated hops was conducted according to MacMurrey and Morrison (1970), using dichloromethane. Phytosterols of the raw material were separated by column liquid chromatography in two ways:

- Using Agilent Hypersil ODS 2.0 x 125 mm column, Agilent Technologies 1200 Series detector, Alltech 3300 ELSD light scattering detector, and polyether ether ketone adsorbent;
- Using LS/MSD Trap SL mass detector, Agylent 1100 Series LS/MS detector, APCI ion source with the velocity of nebulizer gas (nitrogen) of 10 L/minute and nebulizer pressure of 50 psi, curtain gas temperature of 350 °C and separation energy of 1.2.

Separation and quantitative analysis of beer phytosterol complex were conducted by column chromatography in three ways:

- Using LS/MSD Trap SL mass detector, Agylent 1100 Series LS/MS detector, APCI ion source with the velocity of nebulizer gas (nitrogen) of 10 L/minute and nebulizer pressure of 50 psi, curtain gas temperature of 350 °C, separation energy of 1.2, and Agilent Hypersil ODS 2.0 x 125 mm column;
- Using LS/MSD Trap SL mass detector, Agylent 1100 Series LS/MS detector, APCI ion source with the velocity of nebulizer gas (nitrogen) of 10 L/minute and nebulizer pressure of 50 psi, curtain gas temperature of 350 °C, separation energy of 1.2, and Symmetry C8 2.1 x 150 mm column;
- By gas chromatography using Agylent 7890A GC/MC with mass detector.

The refined technique was used to investigate phytosterol composition of raw malt (light malt of Pilsen type, provided by a Belgian company) and resulting wort, produced by congress method. Figures 2-3 show the results.

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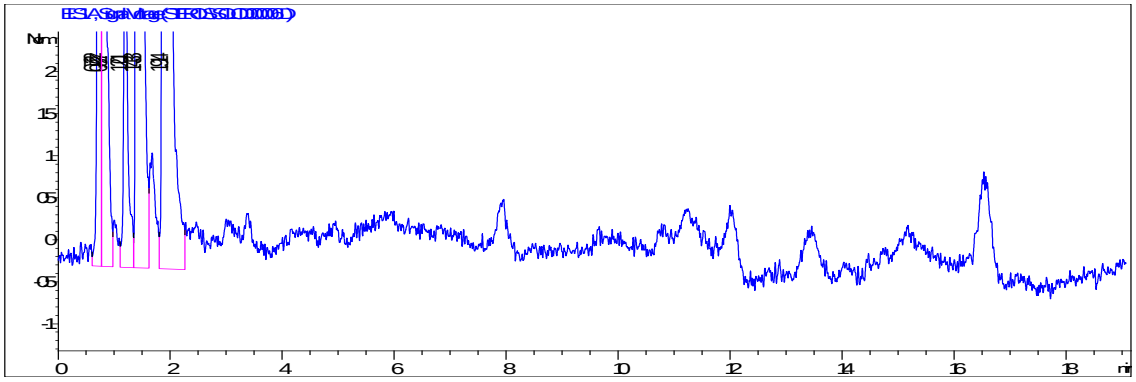

Figure 2. Malt phytosterol chromatogram.

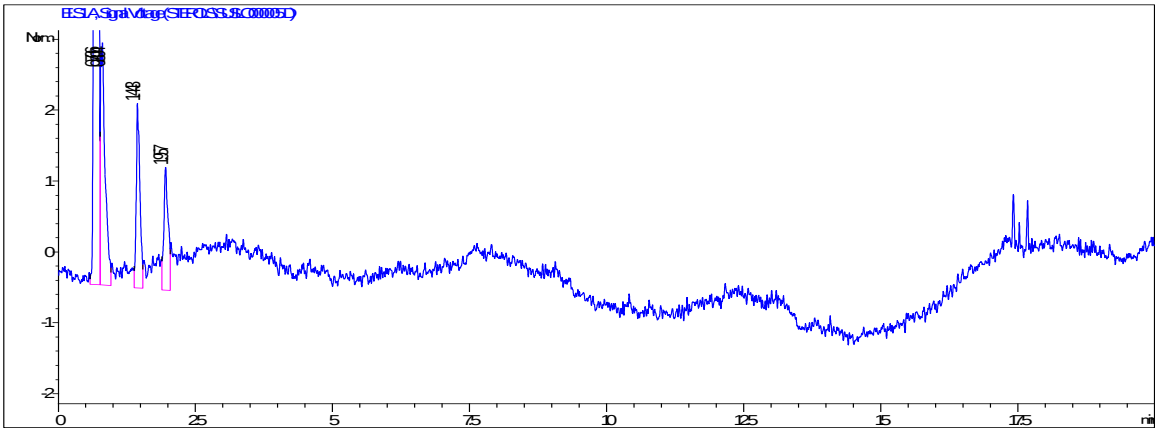

Figure 3. Wort sample chromatogram.

As can be seen from the chromatograms 2-3, numerous peaks corresponding to various substances have been observed, presumably nonpolar lipids (mono-, di- and triglycerides, free fatty acids). Malt and wort samples are characterized by similar peaks, reflecting specifically β-sitosterol, stigmasterol, campesterol and brassicasterol.

RESULTS

Hereby, one could note that malting leads to the release of phytosterols from the conjugates with proteins, starch-like carbohydrates and others (β-glucans, etc.) due to barley endosperm fermentation caused by hydrolytic enzymes of different types – those are the phytosterols found as solutes in wort, as the rest of them, a significant proportion conjugated with polyphenols and inorganic salts of the envelopes, stay in the spent grains, which can cause excessive content of phytosterols in beer due to the extraction of the spent grains constituents, coming in contact with wort (Bedner *et al.*, 2008; Borisenko, 2006).

Our studies prove that β-sitosterol, stigmasterol and campesterol of hops transfer to wort during the hopping process.

Further to our research (Farrington *et al.*, 1981; Hakala *et al.*, 2002), we performed quantitative and qualitative analysis of foreign beer samples, as, according to the European regulations, the information stated on the label must be in accordance with the raw materials actually used.

HPLC determination of phytosterols in beer samples.

Several techniques of phytosterol extraction from beer have been investigated, as our subject has not been studied yet:

- Beer sample was exposed to dichloromethane extraction;

- Beer sample was mixed with hydrochloric acid with consequent dichloromethane extraction upon the application of heat;
- Beer sample was mixed with 20% methanol solution, stirred, then ammonium sulfate, acetone solution and hexane solution were added to the flasks in that order, after the incubation the upper layer was dried with sodium sulfate, saponified with alkaline methanol solution, then ammonium sulfate was used to precipitate proteins, while the residual phytosterols were extracted with dichloromethane.

Sample preparation according to three methods did not allow us to extract phytosterols from beer using high performance liquid chromatography (HPLC) with mass detection; therefore, we decided to use gas chromatography with mass detection, which required an altered beer sample preparation: the internal standard was introduced to the beer sample (in order to control the efficiency of sample preparation), 1N solution of hydrochloric acid was added; the flasks were heated using water bath during 30 minutes, cooled down, then saturated solution of ammonium sulfate was added to each flask, the flasks were shaken for 15 minutes; then dichloromethane solution was added to the flasks, which were then shaken for 15 minutes until phase separation, the lower phase was filtered, the solvent was removed, the constituents were extracted with dichloromethane and used for further analysis.

The table summarizes physical and chemical properties of studied beer samples.

Table 1: Phytosterol content in different types of beer

Beer brand, country of origin	Beer type	Raw materials	Content			
			Initial wort extract, % of weight	Alcohol, % of vol.	phytosterols, µg/L	
					campesterol	stigmosterol
"Krusovice Cerne", the Czech Republic	dark filt. pasteur.	Light malt Caramel malt Dark malt	10.0	3.8	29.3	25.2
"Birra Moretti", Italy	light filt. pasteur.	Light malt barley	10.6	4.6	10.4	9.7
"Cēsu Nefiltrētais", Latvia	light unfilt. unpasteur.	Light malt buckwheat	11.0	5.4	16.8	12.7
"Spaten", Germany	light filt. pasteur.	Light malt	12.0	5.2	21.0	11.7
"Tsingtao", China	light filt. pasteur.	Light malt rice	11.0	4.7	7.9	6.5

According to the data shown in the table, the content of initial extract and the volume fraction of alcohol in beer samples from different countries are similar, but the phytosterol content varies significantly.

DISCUSSION

It has been hypothesized that there is a certain correlation between higher phytosterol content and beer type, particularly concerning unfiltered beer, as brewer's yeasts also contain phytosterols, playing an important part in regulating the life of the cells (Iofe, 1986). It would be reasonable to assume that unfiltered beer would contain considerable amounts of phytosterols, but this assumption was not confirmed: unfiltered beer Cesu Nefiltrates had relatively low phytosterol content compared to filtered beer.

Hops also contain phytosterols, but hopping rate is approximately the same in all the samples, which are mostly the samples of light beer, except for one sample of Czech dark beer. However, dark beer is marked by low hopping rate, as the original technology is aimed to emphasize caramel flavours, not dampen them with intense hop taste.

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CONCLUSION

Our study has led us to the conclusion that phytosterol content fully depends on the raw grain materials used for doughing. Higher proportion of malt leads to a higher phytosterol content (Krusovice Cerne and Spaten beer samples, marked by phytosterol levels of 21.0-29.3 μg/dm^3), while the utilization of unmalted grains (other beer samples) provides decrease in phytosterol content down to 7.9-16.8 μg/dm^3.

Chinese beer stands out among the beer samples produced from unmalted grains for the lowest concentration of phytosterols, probably due to higher proportion of rice among the utilized raw grains compared to the proportion of malt, which is also confirmed by organoleptic evaluation, revealing slightly thin taste of the beer.

It should be noted that campesterol content is 7-16% higher than stigmasterol content in all the beer samples, not correlating with the malt charge composition, but rather linked to the specific features of malts and unmalted materials used in brewing process.

To conclude, phytosterols accumulate in beer due to their extraction from raw materials – unmalted grains, malt and pots – Figure 4.

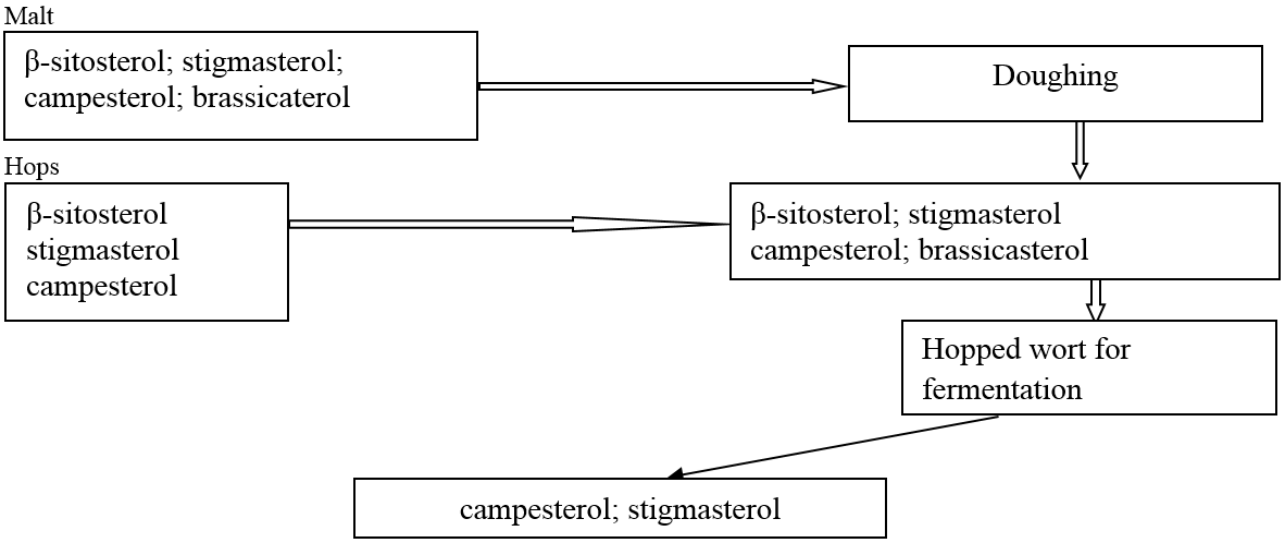

Figure 4. Beer phytosterols.

It is well known that beer is a flexible colloid system formed in a multistage technological process. This colloid system consists of various substances, linked by various interactions, and the part each of these substances plays is crucial for the balanced taste of beer.

In the course of manufacturing process, unsustainable quality of raw materials and technological errors can lead to adverse effects, concerning the issue of unstable beer, which should be stored and sold. These include microbiological issues due to contamination of raw materials, intermediate products, and technological equipment; colloid haze of beer due to insufficient starch and protein dissolution of raw grains (unmalted grains and malt), inappropriate temperature breaks during grain doughing, short duration of wort and hops boiling and poor separation of trub, insufficient removal of suspended matter from cooling wort during the separation of hopped wort.

The reasons, the forecasting methods and the elimination techniques for various types of colloid and microbiological haze have been successfully resolved and properly studied by brewers.

Due to evolving methodological framework, today brewing industry focuses on the issues of investigation, identification and elimination of the problems concerning the substances responsible for the

sensory stability of beer. The studies on the impact of phytosterols on the beer quality and sensory stability are continued (Rapota, & Tyrsin, 2016).

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