# Karakterizacija i procjena kvalitete rakija "Biska"

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# CHARACTERIZATION AND QUALITY EVALUATION OF MISTLETOE SPIRITS

Work was done in the Department of Food Engineering in the Laboratory for Fermentation and Yeast Technology at Faculty of Food Technology and Biotechnology at University of Zagreb under mentorship of Damir Stanzer, PhD, associate professor at Faculty of Food Technology and Biotechnology at University of Zagreb with help from Jasna Mrvčić, PhD, associate professor at Faculty of Food Technology and Biotechnology at University of Zagreb, Jasenka Gajdoš Kljusurić, PhD, full professor at Faculty of Food Technology and Biotechnology at University of Zagreb and Karla Hanousek Čiča, mag.ing.biotechn, assistant at Faculty of Food Technology and Biotechnology at University of Zagreb.

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# CHARACTERIZATION AND QUALITY EVALUATION OF MISTLETOE SPIRITS

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**Abstract:** Moderate and regular consumption of phenolic-containing alcoholic beverages has been associated with health benefits. The aim of this work was to evaluate mistletoe spirits quality and whether they contain polyphenols and have antioxidant activities. The total polyphenol content (TPC) and antioxidant activity were estimated using spectrophotometric methods (Folin-Ciocalteu, DPPH and FRAP). Some spirits have high TPC, DPPH and FRAP antioxidant activities and stimulation of moderate consumption of mistletoe spirit is justified. As analysis of aroma compounds is one of the most important steps in the evaluation of spirit quality, the aroma composition of mistletoe spirits was determinated for the first time, using GC/MS, solid-phase microextraction (SPME). A total number of 166 aroma compounds in mistletoe spirit samples were determinated. FTIR spectra were registered to create calibration models to predict TPC and antioxidant activities in unknown mistletoe spirits. The determination coefficients in calibration and prediction models were greater than 0.8, suggesting strong correlation of predicted parameters based on the FTIR spectra.

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# KARAKTERIZACIJA I PROCJENA KVALITETE "BISKA" RAKIJA

*Maja Rupert, 713/N*

**Sažetak:** Umjerena konzumacija alkoholnih pića koja sadrže polifenole povezana je s pozitivnim učincima na zdravlje. Cilj ovog rada bio je procijeniti kvalitetu rakija "Biska" i utvrditi sadrže li polifenole i imaju li antioksidativnu aktivnost. Ukupni polifenoli i antioksidativna aktivnost određeni su spektrofotometrijskim metodama (s Folin-Ciocalteu reagensom, DPPH i FRAP metodom). S obzirom da neki uzorci sadrže visok udjel polifenola i imaju visoku anitoksidacijsku aktivnost, umjerena konzumacija "Biske" je opravdana. Određivanje aroma važan je korak u procjeni kvalitete. U ovom radu po prvi put su određeni spojevi rakija "Biske" koji pridonose sveukupnoj aromi. Koristeći GC/MS SPME metodu, detektirano je 166 spojeva aroma u uzorcima rakije "Biske". Snimljeni su spektri pomoću FTIR-a s ciljem kreiranja modela za brzu procjenu udjela polifenola i antioksidacijske aktivnosti u nepoznatim uzorcima "Biske". Determinacijski koficijenti u primijenjenim modelima bili su viši od 0.8, što dokazuje da su modeli kvalitetni i pouzdani.

**Ključne riječi:** Biska, rakija, polifenoli, FTIR, aroma
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# 1. INTRODUCTION

Since ancient times alcoholic beverages has been closely associated with diet, drunk in all societies of the world and based on the ingredients, which are locally available. For many years, moderate and regular consumption of alcoholic beverages has been associated with health benefits, with no scientific basis. Moderate alcohol consumption is defined as up to one drink per day for women and up to two drinks per day for men and only by adults of legal drinking age (De Salvo et al., 2016). However, over the last two decades, several studies around the world have demonstrated that moderate intake of alcoholic beverages produces positive effects on antioxidant activity, lipid profile and the coagulation system (Lindberg and Amesterdam, 2008) that may explain the reduction in the risk of cardiovascular disease (CVD) (Arranz et al., 2012), overall mortality (Gronbaek et al., 1995) and other diseases observed in moderate drinkers. By contrast, alcohol abuse or binge drinking has undoubtedly been related to a large number of medical, social and work related problems (negative effects), including the development of alcohol dependence syndrome, several chronic diseases (liver cirrhosis, cardiomyopathy, encephalopathies, polyneuropathy, dementia) and accidents which eventually lead to death (Arranz et al., 2012). The Guidelines also do not recommend that individuals who do not drink alcohol start drinking for any reason. However, some beverages such as red wine or cognac might reduce mortality more than other alcoholic beverages due to the presence of polyphenol compounds (Mrvcic et al., 2012). Polyphenol compounds have no known nutritional function, but they may be important for human health. Consumption of phenolic-containing alcoholic beverages transiently raises total phenol concentration and enhances the antioxidant activity of plasma. This is compatible with suggestions that moderate alcohol usage and increased antioxidant intake decrease the risk of coronary heart disease (Duthie et al., 1998).

In Croatia, spirit drinks have traditionally been a part of the diet as well. Today, there are numerous types of distilled spirits, which are produced by different technologies. "Biska" (mistletoe spirit) is one of them. It is the traditional spirit drink in Istria, one of Croatia's Adriatic regions, produced by maceration of mistletoe (*Viscum album*) in fermented grape marc brandy. The plant mistletoe is a rich source of numerous pharmacologically active compounds (Luczkiewicz et al., 2001), which are used for the treatment of some non-communicable diseases. During the maceration, those compounds are extracted and pass into the alcohol base enriching it.

In this work, fourteen samples of mistletoe spirit will be studied to determine total polyphenol content (TPC), antioxidant activities (DPPH and FRAP) and aroma that are characteristic for this type of spirit. The aim of this work will be to investigate whether mistletoe spirits contain polyphenol compounds and have antioxidant activities and whether stimulation of moderate consumption of mistletoe spirit can be justified. In addition, FTIR spectra will be registered to check if mistletoe spirit samples contain phenolic molecules and if this this method can serve to identify the molecular structure. Moreover, as analysis of aroma compounds is one of the most important steps in the evaluation of spirit quality, using GC/MS analysis aroma in mistletoe spirit samples will be detected and characteristic aroma compounds for mistletoe spirit will be determinated. What is more, pH of mistletoe spirit samples will be measured in order to check whether pH can serve for quick detection if mistletoe spirit was obtained by the maceration in ethyl alcohol of agricultural origin or in distillate made from various raw materials. To conduct statical analyses the MS Office Excel tool- XLStat will be used. Principal component analysis (PCA) will be used to create biplots, which will visualize total polyphenol content (TPC), DPPH and FRAP measured values vs. samples and sample compound distributions vs. samples. In this work FTIR spectra in combination with chemometrics (Partial least squares (PLS) will be used to create calibration models for fast prediction of total polyphenol content (TPC) and total antioxidant capacities (DPPH and FRAP) in unknown mistletoe spirit samples.

# 2. THEORETICAL PART

## 2.1. STRONG ALCOHOLIC BEVERAGES

Alcoholic beverages are very popular worldwide. They are produced in various countries from various raw materials. According to the Regulation of the European Parliament and of the Council (No 110/2008) alcoholic beverages are intended for human consumption, they possess particular organoleptic qualities and have a minimum alcoholic strength of 15 % vol. They have been produced: (i) either directly by the distillation, with or without added flavorings, of naturally fermented products, or by the maceration or similar processing of plant materials in ethyl alcohol of agricultural origin, (ii) or by the mixture of a spirit drink with one or more other spirit drinks, and/or ethyl alcohol of agricultural origin or distillates of agricultural origin, and/or other alcoholic beverages, and/or drinks. Alcoholic beverages that are produced by the alcoholic fermentation and distillation exclusively obtained from the different types of raw material and shall be classified into categories according to the content of alcohol. Alcoholic drinks are those which contain minimum 2.0 % and maximum 15.0 % vol. alcohol. Strong alcoholic beverages, also called spirit drinks, are those which contain minimum 15.0 % vol. alcohol. There are many categories of spirit drinks, such as Rum, Whiskey, Brandy, Grain and Wine spirits, Grape marc spirit, Fruit and Fruit marc spirits, Vodka, Gin, Liqueur and many others. Alcohol production is based on alcohol fermentation of sugars from grapes, fruits or some other plant sources that are rich in sugars and starch, such as plums, peaches, apricots, cherries, grains or potatoes. Ethyl alcohol of agricultural origin which is used in the production of strong alcoholic beverages should not have detectable taste and smell other than that of the raw material, with minimum alcoholic strength by 96.0 % vol.

Depending on the process of production, amount of alcohol or sugar in drinks and the quality, strong alcoholic beverages can be divided into three groups: naturally strong alcoholic beverages, artificially strong alcoholic beverages and aromatized wines (Grba, 2000).

Naturally strong alcoholic beverages are those which were produced by the alcoholic fermentation and distillation exclusively obtained from the raw material, characterized with special aroma from raw material, have no addition of alcohol, do not contain added flavoring substances. According to the raw material from which they are made, naturally strong alcoholic beverages, can be: Fruit spirit (Grape spirit, etc.), Grain spirits (Whiskey, Vodka, etc.) or Sugar spirits (Rum, etc.)

Artificially strong alcoholic beverages are produced by the maceration of plant materials in ethyl alcohol of agricultural origin and by the distillation of macerate that is later combined with ethyl alcohol and aromatic substances. They contain natural aroma from a plant which was used for maceration and do not contain unwanted compounds which occur during the fermentation.

Aromatized wines are produced by the maceration of aromatic herbs and/or spices and/or flavoring foodstuffs in the wine with or without adding sugar. The wine used in the preparation of an aromatized wine must, before enrichment, be present in the finished product in a proportion of not less than 75 %. The description 'aromatized wine' may be replaced by 'wine-based aperitif (Grba, 2010).

Depending on the raw material, technology of production and the conditions in which alcohol was produced, strong alcoholic beverages can be divided into five groups: I. Spirits, II. Special natural spirits, III. Strong alcoholic beverages made in special procedure, IV. Liqueur, V. Cocktails. Spirits are obtained from distillation of the marc of grapes or fruits without adding ethyl alcohol, aroma, sugar or other carbohydrates. Special natural spirits are spirits obtained from an extracts from aromatic plants and natural spirit. They are rich in bioactive compounds from plants and have healing properties. Either strong alcoholic beverages made in special procedure are produced exclusively by alcoholic fermentation and distillation, from molasses, or a mash made from malted cereals, potatoes, etc. In this group are Whiskey, Rum, Vodka, Gin, etc. Liqueurs are produced by flavoring ethyl alcohol of agricultural origin or a distillate of agricultural origin or one or more spirit drinks or a mixture thereof, sweetened and with the addition of products of agricultural origin or foodstuffs such as cream, milk or other milk products, fruit, wine or aromatized wine. The minimum alcoholic strength by volume of liqueur shall be 15 %. Cocktails are made by mixing two or more strong alcoholic beverages with fruit juices, aroma or wine (Mujic, 2010).

### 2.1.1. Spirits with traditional ingredients

From the history of the production of alcoholic beverages it’s known that the method of the distillation was used for producing distillates from aromatic plants in medical purposes, and later for producing an alcohol, also in medical purposes. Only doctors, monks and alchemists kept secrets of the productions. The preparation of special, aromatic spirits has a long tradition. For their production the most often are used grape spirits and aromatic plants. In the preparation of this kind of spirits the most important is to use a plant which will enrich the spirit with

bioactive compounds. That is why for this kind of spirits only specific plants that have known special properties are used. Large number of bioactive compounds from roots, flowers, stains, leaves and seeds are extracted, stabilized and conserved by alcohol. There is convincing evidence that low to moderate consumption of this kind of spirits have a positive effect on the health (WHO/FAO, 2003). Furthermore, these plants give the color, taste and aroma to the spirit (Mujic, 2010). Raw plant material can be stored and kept short without losing quality and has to be processed as soon as possible after harvest. To preserve bioactive compounds and aroma different methods are used. Some of them are maceration, digestion, percolation, infusion and distillation.

There are different groups of bioactive compounds in different plants, such as essential oils, tannins, polyphenols, saponins, terpenoids, lectins etc. Each group has specific properties with different target tissue. For example, essential oils have antibacterial and antifungal properties, encourage digestion and kidneys, polyphenols have a positive effect on the heart and cardiovascular system and have antiviral, antibacterial and antifungal properties, terpenoids are anticancer constituents. According to the plant or plants and methods that were used in the preparation properties of the beverage are defined.

### 2.1.2. Health benefits of moderate alcohol consumption

In many parts of the world, drinking alcoholic beverages is a common feature of social gatherings. Society generally has a poor connotation associated with drinking alcohol. However, it can also have health benefits, depending on the consumer's physical and physiological characteristics as well as the amount consumed. Several cohort studies have pointed out that light-to-moderate alcohol consumers have an increased survival compared to abstainers. Current evidence also suggests the protective effects of moderate drinking on cardiovascular system including coronary heart disease (CHD), ischemic stroke, peripheral arteriopathy and congestive heart failure. Positive effects have also been reported for moderate alcohol consumption on cellular aging damage, cognitive function and dementia. These effects have been observed in a variety of patients, including diabetics, hypertensive subjects and those with previous CHD. The underlying mechanisms to explain these protective effects against CHD include an increase in high-density lipoprotein (HDL) cholesterol, a decrease in platelet aggregation, a reduction in the levels of fibrinogen and an increase in insulin sensitivity, which have been attributed to the ethanol content (Arranz et al., 2012). Heavy alcohol consumption, however, can negatively affect

neurologic, cardiac, gastrointestinal, hematologic, immune, psychiatric and musculoskeletal organ systems (Standridge et al., 2004). The epidemiological relation between light-to-moderate alcohol consumption (defined as up to about 3-4 standard units per day, 1 unit being defined as 8 g of ethanol) and all-cause mortality reflects the competing risks and benefits of drinking. The risks of heavier drinking, above about 3-4 standard units per day, outweigh the benefits and are not in question (Rodney and Beaglehole, 1995). In addition, moderate alcohol consumption has the psychological benefits. It has been reported that alcohol in moderate amounts is effective in reducing stress and increase overall affective expression, happiness, euphoria, conviviality and pleasant and carefree feelings. Tension, depression and self-consciousness have been reported to decrease with equal doses. Low alcohol doses have been found to improve certain types of cognitive performance. Included here are problem-solving and short-term memories. Heavy drinkers and abstainers have higher rates of clinical depression than do regular moderate drinkers. Alcohol in low and moderate doses has been effective in the treatment of geropsychiatric problems (Baum-Baicker, 1985).

However, according to the recent studies some beverages such as red wine or cognac might impact mortality more than other alcoholic beverages due to the presence of polyphenol compounds (Carusio et al., 2008). Consumption of phenolic-containing alcoholic beverages transiently raises total phenol concentration and enhances the antioxidant activity of plasma. This is compatible with suggestions that moderate alcohol usage and increased antioxidant intake decrease the risk of coronary heart disease (Duthie et al., 1998).

## 2.2. MISTLETOE SPIRIT

Mistletoe spirit is an authentic Istrian brandy made from special type of spirit produced from the grape marc, mistletoe and honey. It is light brown in color with greenish reflections with expressed fragrance from aromatic compounds. It has exquisite taste in which taste of honey and caramel is dominant, with note of coffee mixed with mild taste of dried raisins. In the end tingle of peppermint mint comes to taste. It contains 60 to 64 % water, 36 to 40 % vol. ethyl alcohol, 0.20 to 2 % vol. methyl alcohol, 200 to 1800 mg $L^{-1}$ total acids and minor amounts of ingredients from mistletoe obtained by maceration (Table 1). Name originated from the Latin name for mistletoe (*Viscum album*). The special type of spirit produced from the grape marc is the base for mistletoe spirit with typical smell and taste, content of alcohol 40 to 52 % vol. (Mujic, 2010). The mistletoe spirit can be produced from other types of spirit as well. The base

spirit in the production can be ethyl alcohol, grape brandy, plum brandy and other distillates made from various raw materials.

**Table 1**. Basic technological parameters and composition of mistletoe spirit (Mujic, 2010)

| Base | Mistletoe spirit | |
|---|---|---|
| Type of ingredient | | Amount |
| 1. | Mistletoe - leaves (g/l) | up to 30 |
| | Maceration (days) | 20 to 45 |
| 2. | Honey (g/l) or Sugar (g/l) | up to 50 or up to 30 |
| Content of the finished spirit | | Amount |
| Ethanol % vol. | | 36 to 40 |
| Aldehydes mg/l | | 500 to 1500 |
| Esters mg/l | | 1000 to 1500 |
| Methanol % vol. | | 0,20 to 2,0 |
| Higher alcohols mg/l | | 1400 to 5500 |
| Total acids mg/l | | up to 1800 |
| Extract g/l | | up to 110 |

2.2.1. Komovica- special type of spirit produced in Istria

In the original recipe the base for the mistletoe spirit is distillate produced by distillation of the residual from winemaking, the marc called "komovica". Similar spirits have equivalent appellations, for example as Italian "grappa", French "eau-de-vie de marc", Spanish "aguardente", Portuguese "bagaceira", Cypriote "zivania", Greek "tsipouro" and the Georgian product "tshiatshia" (Apostolopoulou et al., 2005).

"Komovica" is a distillate of the grape marc. The marc is the skins, pulp and seeds left over from winemaking and pressing the grapes. It was originally made to prevent waste by using these leftovers. The quality of the marc is the first and most important factor for obtaining a good product and distillers are well aware of this, which is why they take great care in choosing the raw material. The freshness and good condition of the marc are all-important, since any deterioration in these qualities will inevitably have a bearing on the end product (Anonymous 1, 2003). The raw material for the production of this special type of spirit should not contain any impurities, such as sugared water, sugar, fluid obtained by washing equipment, steams etc

(Mujic, 2010). For the wine-maker the whole grape is important, but for the "komovica"-maker the skin is the most important part of the grape, because it contains huge amounts of aromatic and coloring substances. The skin's aromatic substances constitute the primary aroma of the "komovica", because they are already present first and since the beginning in this part of the grape. The composition of the marc is variable depending on the content and amount of individual parts, depending on freshness of the marc. During the storage, microbiological and chemical processes are happening and the composition of the marc is changing. Before proceeding with the distillation of the marc, it must contain alcohol. This is possible only when the marc has been fermented; when the sugar contained in it has been converted into alcohol. Alcohol fermentation can be done in two ways; spontaneously, with naturally present micro flora or by adding selected yeasts (Nikicevic and Tesevic, 2010). Spontaneous fermentation is complex microbial process, which is the result of activity of various species of yeasts, lactic acid bacteria and molds. Of these, yeasts are the main group responsible for alcoholic fermentation. The most significant yeasts present are *Klockera apiculata*, *Metschnikowia pulcherrima* i *Candida stellata*, which are non-*Saccharomyces* yeasts (Combina et al., 2005). Their activity is expressed in the beginning of the fermentation, because later are inhibited by ethyl alcohol. They are responsible for aroma production, esters, higher alcohols and acids (Swiegers et al., 2005). Controlled fermentation is done with inoculated selected yeast, the most often *S. cerevisiae*. It is dominating during the whole fermentation and it has important role in suppression other non-*Saccharomyces* yeasts (Ciani et al., 2010). Characteristics of selected yeasts define the speed of fermentation and quality of finished product. Marc coming from the production of white wines is not left to soak in the must during the fermentation. It is rich in sugars but not alcohol and therefore must be fermented before being distilled. Marc coming from the production of rosé and red wines has undergone brief soaking in the must during fermentation and already contains alcohol. It must be fermented for shorter time than marc coming from the production of white wines (Anonymous 1, 2003). Fermentation must be carried out under controlled conditions, in the closed containers without oxygen. Immediately after the fermentation, distillation has to be done (Mujic, 2010). Distillation is a process of separating the component or substances from a liquid mixture by selective evaporation and condensation. Distillation may result in essentially complete separation, or it may be a partial separation that increases the concentration of selected components of the mixture. In either case the process exploits differences in the volatility of the mixture's components. The aim of the distillation is to extract ethyl alcohol and aroma in

favorable ratio, so the finished product has high quality and wanted characteristics (Nikicevic and Tesevic, 2010). The principle of alcoholic distillation is based upon the different boiling points of alcohol (78.3 °C) and water (100 °C). If a liquid containing ethyl alcohol is heated to a temperature above 78.3 °C but below 100 °C and the vapor coming off the liquid is condensed, the condensate will have a higher alcohol concentration, or strength. In the marc to be distilled however, other volatile components evaporate during the heating and are transferred in the distilled liquid. Many of these substances are unpleasant and unwanted, and must be eliminated. Fortunately, the various volatile substances in the marc evaporate at different temperatures. By carefully controlling the distilling process the unwanted components can be eliminated while maintaining all the substances of quality. This separation, or elimination, of the unpleasant and unwanted substances is called rectification and is obtained by removing the heads and tails of the distillate. The head is the first part of the distilled liquid to be produced and mainly contains unpleasant substances that would give an unpleasant sour taste, as well as methyl alcohol, which is toxic, and therefore has to be eliminated. These substances have a lower evaporation point than the "noble" substances and therefore are the first to be produced. The skill of the distiller consists of the ability to establish when the head of the distillate ends and when the so-called heart, the best part of the "komovica", rich in ethyl alcohol and aromatic substances starts to come out. The distiller's skill is also judged by his ability to recognize the end of the heart and the start of the tail**,** the last part of the distillate, which will be eliminated, because it contains unpleasant fatty and oily substances. "Komovica" is obtained by selecting the heart, the middle part of the distillation, and discarding the head and tail, the first and last parts of the distillation process. During the distillation a large number of ingredients from the marc passes into the spirit. The most important are: water, alcohols, esters, aldehydes and acids. The amount of those ingredients in finished product depends on the region where grapes grew, type of grapes, time of storage the marc, method of storage, method of distillation, final finishing process and spirit aging (Mujic, 2010). After the distillation, distillate has sharp and inharmonious smell and taste. That is why it has to be stored and aged. During the storage and aging, many chemical processes are happening and concentration of aromatic compounds increases, which increases quality of the spirit. The quality and composition of the resulting spirit will depend, in part, on how the alcoholic fermentation of the raw material has been carried out and on storage conditions. Another very important factor in the production of this type of alcoholic drink is the distillation technique (Cortes et al., 2005).

### 2.2.2. Mistletoe

Mistletoe is the common name for evergreen semi parasite plants in the order *Santalales*. Mistletoe attaches to and penetrates the branches of a tree or bush by a structure called the haustorium, a specialized organ that penetrates the living tissue of its host and absorbs water and nutrients, transferring them to the parasite through the xylems of the host and parasite (Figure 1). Most mistletoe parasitizes a variety of hosts, including apple, hawthorn, lime, ash, cedar of lebanon and many others. As semi parasite, mistletoe has green leaves and stems that contain chlorophyll, which means that although it depends on its host for water and mineral nutrients, it is able to photosynthesize and create its own carbohydrates using sunlight. Mistletoes are slow growing but persistent. Their natural death is determined by the death of the hosts or by complete removal from the host. Although there are many varieties of mistletoe, most studies have been done on *Viscum album* L., European mistletoe. It is widely appreciated that the chemical composition of mistletoe is not stable and depends not only on the biosynthesis but also on species of the host tree and growing conditions, such as ambient temperature, carbon dioxide, concentration and the time of harvest, and the manufacturing process (Luczkiewicz et al., 2001). The influence on the chemical composition, biological and antioxidant activity can also have a solvent used for extraction in investigation (Vicas et al., 2011). Furthermore, for many compounds that have been found in the European mistletoe, were shown that are not produced by the mistletoe, but from the host tree and absorbed by mistletoe. In the last years, many studies have been done on different medicinal plants, especially on those, which were used as therapeutically herbs for a long time. Modern phytochemical research showed that mistletoe twigs and leaves are a rich source of numerous pharmacologically active compounds (Luczkiewicz et al., 2001), which are used for the treatment of some non-communicable diseases (Choudhary et al., 2010). New interests appeared when its potential in cancer treatment was determined (Zänker and Kaveri, 2015).

**Figure 1.** Mistletoe on the host tree (own picture)

A number of phytochemicals including lectins, polysaccharides, alkaloids, terpenoids, proteins, amines, peptides, polyphenols, flavonoids, phytosterols, and amino acids characterizes European mistletoe. Thanks to numerous bioactive phytochemicals, European *Viscum album* L. possesses wide-reaching biological activities, including immunomodulatory, antioxidant, cytotoxicity, anti-tumor, anti-hypertensive, sedative, anti-diabetic, and hepato-protective. Meanwhile, it has also displayed significant inhibitory bioactivity against human cancer cell lines. In addition, extract and phytochemicals can inhibit inflammation and prevent the development of cancer (Singh et al., 2016).

Special attention has always been given to its potential as a medicinal plant. From the time of Hippocrates, physicians have reported mistletoe to be beneficial for many diseases, especially epilepsy, infertility and diseases of the spleen. Juice or powder gained from leaves, berries, or stems were given as a drink or applied as a plaster or ointment. Since the 1920s, extracts of *Viscum album* have been used in cancer treatment. The German Commission has approved mistletoe extracts and preparations as a curative in degenerative and inflamed joints and as a palliative therapy for malignant tumors (Nazaruk and Orlikowski, 2015).

The following sections describe the most important components of *Viscum album* identified until now.

Viscotoxins are plant thionins synthesized in the leaves and stems. They are a mixture of low-molecular weight cysteine rich and basic proteins consisting of 46 amino acids. The polypeptide chains are attached through few disulphide bonds, which are giving them a compact

structure and high stability against denaturing conditions such as heat and proteases. Their amphipathic nature helps in the inducing cytotoxicity in eukaryotic cells by interfering with the cell membrane and altering its integrity. Seven different isomers have been characterized and their biological effect can vary. The viscotoxins reveal a high structural and pharmacological association with snake (cobra) cardio toxins. Impact of the viscotoxins on human granulocytes was studied by flow cytometry and it was found that at concentrations 25 and 250 μg $mL^{-1}$ enhanced phagocytosis and burst activity against *E. coli* infection in respiratory track (Singh et al., 2016.).

The main group of chemicals of *Viscum album* is lectins. They are classified as type II ribosome-inactivating proteins; consist of two peptide chains, an A-chain comprising three distinct individual domains and a B-chain containing two domains with similar configurations (Nazaruk and Orlikowski, 2015). The chain A inhibits protein synthesis by degrading the 28S rRNA in ribosomes of eukaryotic cells and accelerates apoptosis. While, the chain B is capable of binding to glycoconjugates of cell surface and thereby permitting into the cell of the toxic subunit. The content of lectins is highest in the winter. Sprouts and shoots contain the highest concentrations (Singh et al., 2016). Mistletoe lectins are widely used in histochemical applications as markers for macrophage- derived cells and in histrochemistry with human breast cancer (Van Damme et al., 1998.). Though a large number of the anti-tumoral properties of mistletoe preparations have been attributed to the lectins, it is possible that new researches on other components, especially polyphenols, would show and open new perspectives.

Another group of chemicals found in mistletoe is polyphenols. The influence of the host tree may have a key role in a range of flavonoids, phenolic acids and phenypropanoids of mistletoe leaves or stems. It was also shown that the mistletoe stem extracts contained lower levels of phenolics, as compared to leaves (Vicas et al., 2011). Examined plants had a rich but diversified qualitative and quantitative content of these compounds. The flavonoids and phenolic compounds are natural antioxidants, acting as electron donors and hence protect living cells and tissues from free radical mediated oxidative stress such as aging and human degenerative diseases (Finkel and Holbrook, 2000). The most investigated chemical property of the phenolic compounds is their antioxidant, antiviral and antibacterial activity. In the study was shown that mistletoe has radical scavenging activity and protective effect against hydroperoxide generation and that the antioxidant activity could differ depending on the harvesting time of the plant as

well as nature of the host tree (Shahaboddin, 2011). The mistletoe grown on lime tree in summer showed the highest activity (Onay-Ucar et al., 2006). Phenolic compounds, phenylpropanoids and flavonoids, isolated from this plant, caused vascular relaxation in dose-dependent manner, which can also contribute to the lowering of blood pressure (Deliorman et al., 2000).

Phenylpropanoids are the important bioactive molecules of the European mistletoe, with extremely diverse structures and wide-spectrum medicinal effects. The leaves and stem were found to contain several phenylpropanoids. They play an important bioactive role, with extremely diverse wide-spectrum medicinal effects (Singh et al., 2016).

Terpenoids, liposoluble compounds, are the next group of compounds in *Viscum album*. Isolated from the herb were triterpenes $\beta$-amyrin acetate, oleanolic acid, betulinic acid and a mixture of phytosterols (stigmasterol, $\beta$-sitosterol) and their glucosides. Triterpenes are important anticancer constituents of mistletoe, but they dissolve in water poorly (Nazaruk and Orlikowski, 2015).

Cyclic peptides, amino acids, proteins, alkaloids, amines (histamine and cetylcholine), jasmonic acid, cysteine, glutathione, vitamin C, and xanthophyl and different types of polysaccharides have also been identified. Some of them were found in the berries and leaves. The content of them is varied depending on the host plants (Singh et al., 2016).

Contemporarily, most of the research on *Viscum album* deals with its anti-tumoral activity. In numerous clinical trials, scientist and physicians have demonstrated the benefits of mistletoe extracts and isolated compounds were found to be effective in the treatment of cancer and led to cancer regression (Nazaruk and Orlikowski, 2015). What is more, it has been observed that mistletoe therapy improved the quality of life and extended the survival time in patient with cancer, depending on the reaction of the patient and the stage of the disease, doses and routes of application, which were used in the therapy. Many animal studies, as well as non-randomized, randomized and cohort studies showed that mistletoe extracts exhibit potential therapeutic effects in breast and gynecological cancers (Kienle et al. 2009).

*Viscum album* has been documented as a traditional treatment of diabetes. A tea prepared from leaves of mistletoe is used traditionally to treat diabetes in the West Indies. This treatment has been shown also to relieve the diabetic symptoms of severely hyperglycemic streptozotocin-diabetic mice, including polydipsia, hyperphagia and body weight loss. Despite being long

advocated as an effective traditional treatment for diabetes, few scientific studies have attempted to evaluate the efficacy and possible mode of action of mistletoe. Aqueous extracts produced a dose-dependent insulin-releasing effect in clonal B-cells. It has also been demonstrated that active constituents are heat resistant (Gray and Flatt, 1999). A significant decrease in serum glucose level accompanied by an increase of the serum insulin level was observed in alloxan-hyperglycaemic rabbits and rats after administration of mistletoe water extract. The extract enhanced the serum's antioxidant activity that is very important in the prevention of diabetic complications (Shahaboddin et al., 2011).

2.2.3. Honey

Honey is composed primarily of the sugars glucose and fructose; its third greatest component is water. Honey also contains numerous other types of sugars, as well as acids, proteins and minerals (White et al., 1962; White, 1980). It contains other disaccharides that make up over 7 percent of its composition. Some of the disaccharides in honey are maltose, sucrose, kojibiose, turanose, isomaltose, and maltulose. In addition, honey also contains carbohydrates known as oligosaccharides that may function as prebiotics. These are medium-sized carbohydrates, containing more than three simple sugar sub-units, often made of mono- and disaccharides. Honey is a natural source of readily available carbohydrates providing 64 calories per tablespoon. Furthermore, honey contains a variety of phytochemicals (as well as other substances such as organic acids, vitamins, and enzymes) that may serve as sources of dietary antioxidants. The amount and type of these antioxidant compounds depends largely upon the floral source variety of the honey. In general, darker honeys have been shown to be higher in antioxidant content than lighter honeys (Gheldof et al., 2002). Honey has the capacity to serve as a natural food preservative. Research has demonstrated the potential for honey to reduce enzymatic browning in fruits and vegetables and prevent lipid oxidation in meats. Other researchers have identified the flavonoids in honey, particularly caffeic acid and ferulic acid, as the most likely contributors. (Mundo et al., 2004). Honey naturally contains small amounts of enzymes that are introduced into honey by the bees during various phases of the honey manufacturing process. The predominant enzymes in honey are diastase (amylase), invertase ($\alpha$-glucosidase) and glucose oxidase. Enzymes play an important role in honey and contribute to its functional properties (White, 1980). Honey contains a number of acids, which include amino acids (0.05- 0.1 %) and organic acids (0.57 %, range: 0.17- 1.17 %). The average pH of honey is 3.9 (with a typical range of 3.4 to 6.1). The carbohydrates found in honey have the ability to

improve the intensity of desirable aroma compounds and reduce the intensity of others (Mujic, 2010).

Honey is added to the spirit because it enhances sweetness intensity, decreases sourness, decreases the bitterness intensity and increases the acceptability of the spirit. Honey gives typical scent and flavor to the spirit and has benefits for human health (Mujic, 2010).

### 2.2.4. Technological process of production mistletoe spirit

The first phase in the production of mistletoe spirit is the production of the base spirit. As mentioned before, base spirit can be produced from different kind of raw materials. Produced base spirit should contain about 40 % vol. alcohol. If the base spirit is stronger than 40 % vol. alcohol, it can be diluted with distilled water. Next step in the production is maceration. For the maceration, small amount of prepared base spirit should be taken and dry or fresh leaves of mistletoe have to be left in it. For one liter of mistletoe spirit approximately 30 grams of leaves should be used. The maceration lasts 20 to 45 days, dependent on the amount of leaves left in the base spirit. Bigger amount of leaves means shorter time of maceration. The amount of leaves and the duration of the maceration affect the amount of ingredients that come into the mistletoe spirit, especially, pigments. Longer maceration gives darker color of the mistletoe spirit. Macerate should be filtered and added to the rest of prepared base spirit. After adding the macerate, honey should be added as well, about 50 grams of honey per liter of spirit. It should be mixed all together and left in order for compounds to connect with each other. Connecting compounds should last at least 30 days. After that, spirit should be filtered and left in bottles of various shapes (Mujic, 2010).

# 3. EXPERIMENTAL PART

## 3. 1. MATERIALS

### 3.1.1. Mistletoe spirit samples

In order to perform this study, fourteen samples of mistletoe spirits were analyzed, five from an industrial origin and nine from homemade sources. The spirit samples were obtained from Istria (Croatia). They were collected directly from the producers, but the available equipment and the processes followed are unknown. Spirits were named with numbers from one to fourteen (Figure 2). Samples, which were produced in industrial way are 8, 9, 10, 13, 14 and they had the declaration of manufacturer. Samples 1-7, 11 and 12 were homemade ones. Samples 9 and 14 were obtained by extract of mistletoe in ethyl alcohol of agricultural origin. Samples 8 and 13 were obtained by leaf maceration of mistletoe in the grape marc spirit. Sample 10 was obtained by leaf maceration of mistletoe in the grape distillate.

**Figure 2.** Mistletoe spirit samples (own picture)

### 3.1.2. Chemicals

Chemicals used in experimental part of work:

- distilled water
- 96 % vol. ethanol obtained from Kefo (Ljubljana, Slovenia)
- Folin-Ciocalteu reagent obtained from Kemika (Zagreb, Croatia)
- Gallic acid obtained from Sigma-Aldrich (Steinheim, Germany)
- Sodium carbonate anhydrous ($Na_2CO_3$) obtained from Gram-mol (Zagreb, Croatia)
- 2,2-diphenyl-1-picrylhydrazyl (DPPH) obtained from Fluka (Buchs, Switzerland)
- 2,4,6-Tris(2-pyridyl)-s-tirazine (TPTZ) obtained from Fluka (Buchs, Switzerland)

- Iron (III) chloride ($FeCl_3$) obtained from Kemika (Zagreb, Croatia)
- Trolox obtained from Sigma-Aldrich (Steinheim, Germany).
- Sodium acetate anhydrous ($C_2H_3NaO_2$) obtained from Gram-mol (Zagreb, Croatia)
- Chloride acid (HCl) obtained from Fisher scientific (Loughborough, UK)

## 3.2. METHODS

### 3.2.1. Determination of total polyphenol content (TPC)

Principle of method

Determination of the total polyphenol content (TPC) in samples has been conducted by the Folin-Ciocalteu method (Singleton and Rossi, 1965). The Folin-Ciocalteu reagent (FCR), also called the gallic acid equivalence method (GAE), is a mixture of phosphomolybdic and phosphotungstic acid complexes. The method relies on the transfer of electrons in alkaline medium from phenolic compounds to form a blue chromophore constituted by a phosphotungstic/ phosphomolybdenum complex where the maximum absorption depends on the concentration of phenolic compounds. The reduced Folin-Ciocalteu reagent is detectable with a spectrophotometer at 760 nm, the color intensity is proportional to the proportion of polyphenols in the sample. Generally, gallic acid is used as the reference standard compound and results are expressed as gallic acid equivalents (mg $L^{-1}$).

Procedure of work

500 µl of Folin-Ciocalteu's solution was added to the 300 µl sample (samples 1-14 were diluted 10 times with distilled water) and 6 mL distilled water, agitated to homogenize and left to stand in the dark for 5 min in order to perform reaction. 1.5 mL of $Na_2CO_3$ solution (200 g $L^{-1}$) and distilled water were added to make up the total volume of 10 mL. The samples were left for 2 h in the dark and then absorbance was measured at 760 nm. All spectrophotometric measurements were performed by UV-VIS spectrophotometer UV-Vis Unicam. The calibration curve was prepared with gallic acid solution (GA) ranging from 0 to 300 mg $L^{-1}$, and the results were expressed as gallic acid equivalents (mg GAE $L^{-1}$) (Figure 3). These solutions were prepared in the way that 0.5 g of gallic acid was dissolved in distilled water in Erlenmeyer flask of total volume of 50 mL and from this solution 100, 150, 200, 250 and 300 mg $L^{-1}$ (GA) dilutions were prepared. All the measurements were performed in duplicate.

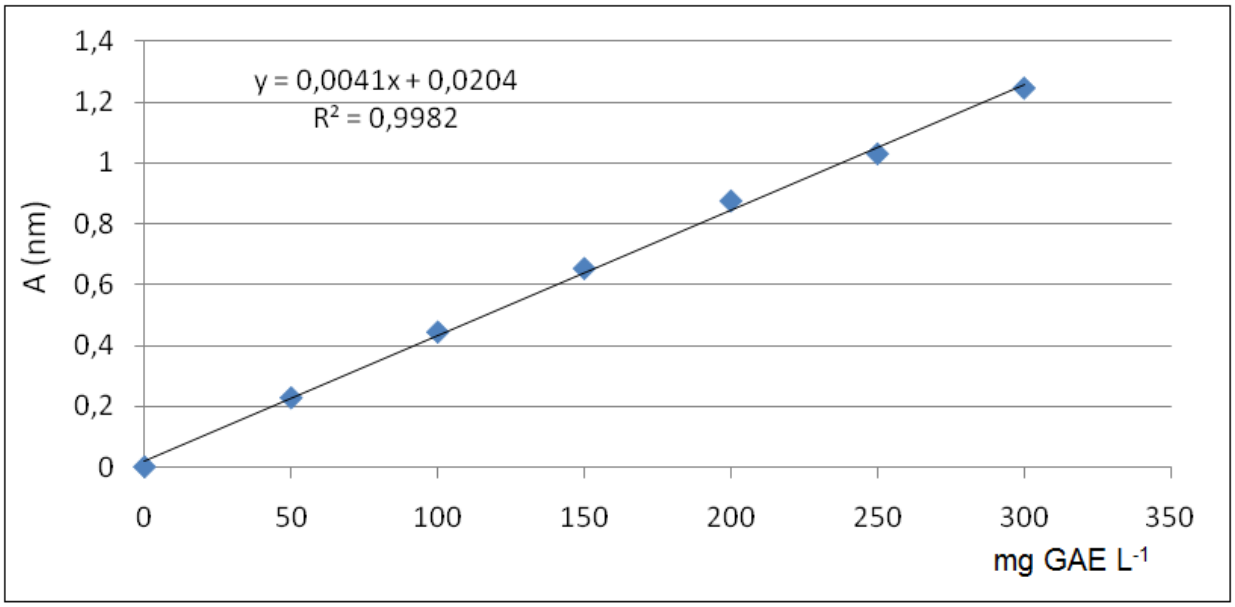


**Figure 3.** The calibration curve for determination of total polyphenol content (TPC) by Folin-Ciocalteu method

3.2.2. Determination of antioxidant activity

Methods used for determining antioxidant activity are based on the study of a reaction in which a free radical are generated and inhibited by the addition of the sample whose antioxidant power is being measured. In this work, for the determination of antioxidant activity characteristics of spirit drinks, the DPPH radical scavenging activity and ferric ion reducing antioxidant power (FRAP) were used.

*3.2.2.1. DPPH assay*

Principle of method

The DPPH assay is popular in natural product antioxidant studies. One of the reasons is that this method is simple and sensitive. This assay is based on the theory that a hydrogen donor is an antioxidant measures compounds that are radical scavengers. 1,1-Diphenyl-2-picrylhydrazyl (DPPH•) is a stable free radical. Figure 4 below, shows the mechanism by which DPPH• accepts hydrogen from an antioxidant. DPPH• is one of the few stable and commercially available organic nitrogen radicals. The antioxidant effect is proportional to the disappearance of DPPH• in test samples. DPPH• shows a strong absorption maximum at 525 nm (purple). The color turns from purple to yellow followed by the formation of 1,1-diphenyl-2-picrylhydrazine

(DPPH) upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 525 nm (MacDonald-Wicks et al., 2006; Moon and Shibamoto, 2009).

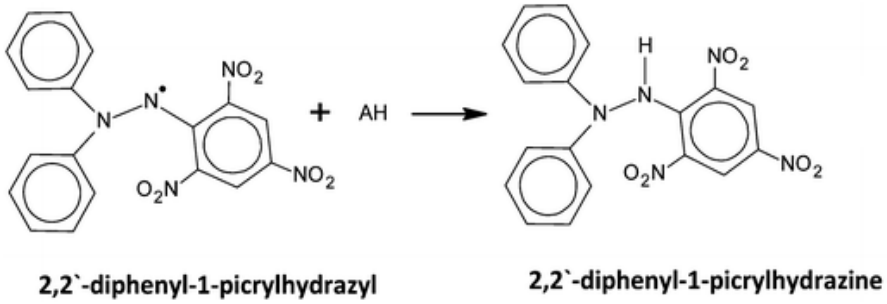


**Figure 4.** DPPH• free radical conversion to DPPH by antioxidant compound (Pyrzynska and Pękal, 2013)

Preparation of reagents

0.03943 g of DPPH was dissolved in 96 % vol. ethanol in Erlenmeyer flask of total volume of 10 mL. In order to prepare 0.1 mM working solution, 0.5 mL of original solution of DPPH and 96 % vol. ethanol were added in Erlenmeyer flask to make up the total volume of 50 mL.

0.01575 g of Trolox was dissolved in 96 % vol. ethanol in Erlenmeyer flask of total volume of 5 mL. In order to dilute this solution 50 times and make 0.05 mM solution of Trolox, 200 µl of original solution and 96 % vol. ethanol were added in Erlenmeyer flask to make up the total volume of 10 mL.

The working DPPH reagent and Trolox were prepared fresh on the day of the analysis.

Procedure of work

Samples 1-14 were diluted 5 times with distilled water. 2 mL of 96 % vol. ethanol, 200 µl of diluted sample and 2 mL of 0.1 mM solution of DPPH reagent were mixed. The absorbance at 525 nm was measured after 30 min of incubation in the dark. DPPH reagent and ethanol were used as a blank reference. Samples percentage of DPPH radical inhibition was compared with 0.05 mM Trolox. The percentage of DPPH inhibition was calculated by the following equation: % of DPPH radical scavenging activity = 100 x [Ao - As]/ Ao, where Ao is the absorbance of

DPPH solution with ethanol, while As is the absorbance of a DPPH solution with the sample. All experiments were performed in duplicate.

*3.2.2.2. FRAP assay*

Principle of method

The FRAP assay was presented as a novel method for assessing "antioxidant power". The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method employing an easily reduced yellow colored oxidant, Fe (III). Ferric to ferrous ion reduction at low pH (3.6) causes an intense blue colored ferrous-tripyridyltriazine complex $[Fe(II)(TPTZ)_2]^{2+}$ to form (Figure 5). The reaction is nonspecific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore directly related to the combined or total reducing power of the electron donating antioxidants present in the reaction mixture. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration (Benzie and Strain, 1999).

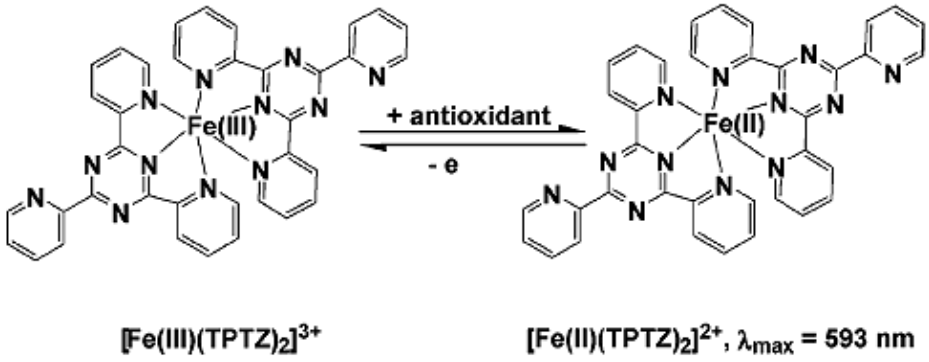


**Figure 5.** Ferric to ferrous ion reduction (Benzie and Strain, 1999)

Preparation of reagents

In order to prepare 10 mM solution TPTZ in 40 mM HCl, 0.0312 g of TPTZ was dissolved in 40 mM HCl in Erlenmeyer flask of total volume of 10 mL.

In order to prepare 20 mM iron (III) chloride aqueous solution, 0.0541 g of iron (III) chloride was dissolved in distilled water in Erlenmeyer flask of total volume of 10 mL.

In order to prepare 300 mM acetate buffer (pH = 3.6), 0.372 g of sodium acetate anhydrous, 3.2 mL of acetic acid and distilled water were added to make up the total volume of 10 mL.

FRAP reagent solution was made of the mixture of acetate buffering agent, prepared TPTZ solution and iron (III) chloride aqueous solution in volume ratio 10:1:1, respectively. The working FRAP reagent was prepared fresh on the day of the analysis.

Procedure of work

The FRAP assay was performed as previously described by Benzie and Strain (1999) with some modifications. Samples 1-14 were diluted 5 times with distilled water. 80 μL of the examined sample was mixed with 2080 μL of FRAP reagent and 240 μL distilled water. After the reaction at 37°C for 5 min the absorbance at 595 nm was measured. The standard curve was constructed by using serial dilution (0.1-2.0 mM) of Trolox stock solution (Figure 6). The final results were expressed as mM Trolox equivalents.

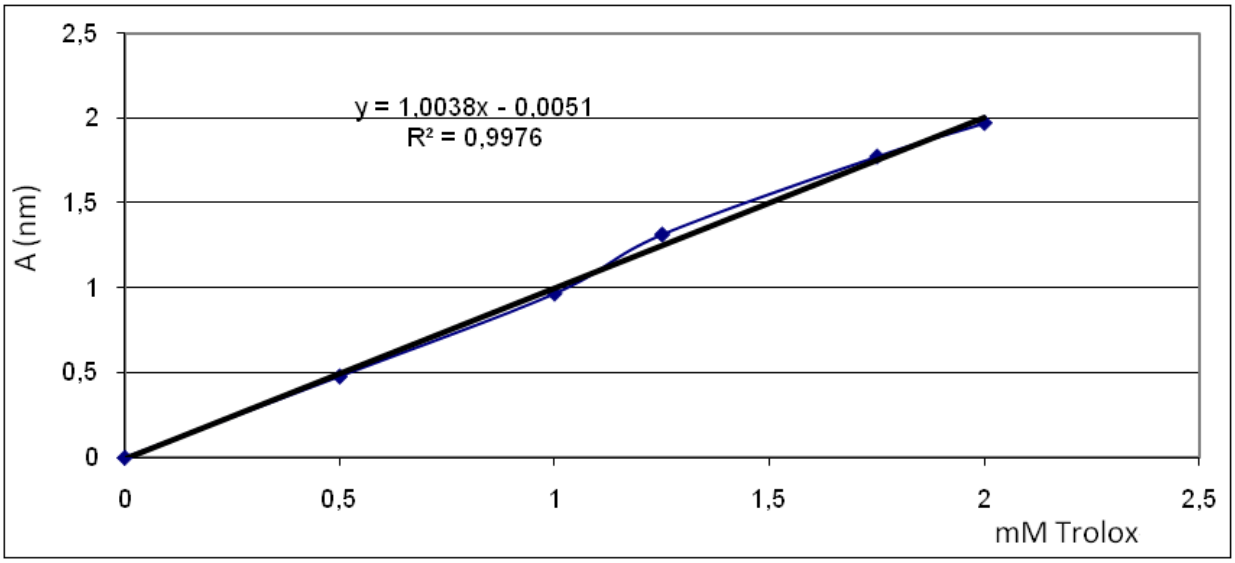


**Figure 6.** The calibration curve for determination of antioxidant activity by FRAP assay

3.2.3. Fourier Transform Infrared Spectroscopy (FTIR)

Principle of method

Infrared spectroscopy (IR spectroscopy or Vibration Spectroscopy) involves the interaction of infrared radiation with matter. This technique is completely complementary

providing characteristic fundamental vibrations that are extensively used for the determination and identification of molecular structure (Larkin, 2011). Infrared light is fundamentally weak, but may induce vibration excitation of covalently bonded atoms and groups. When a molecule absorbs a photon, the molecule gains energy and moves from a lower vibration energy state to a higher vibration energy state. The light is reflected and recombined at the beam splitter before passing through the sample and to the detector (Figure 7). As the intensity of the recombined light is recorded at the detector, the moveable mirror travels towards the beam splitter, producing an interferogram that tells the intensity and frequency of each wave.

The FTIR (Fourier Transform Infrared Spectroscopy) technique, in combination with chemometrics is a fast and reproducible way to identify different parameters of different food and beverage products. The FTIR is increasingly used for the determination of methanol in alcoholic beverages or to verify the authenticity of different fruit juices. The Near-Infrared (NIR) spectroscopy was also used to estimate the ethanol content in alcoholic beverages or to verify the adulteration of different beverages (Coldea et al., 2013). In this study the FTIR spectra were registered for 14 mistletoe samples in order to determine and identify characteristics groups in molecular structers and in order to create calibration models to predict total polyphenol content (TPC) and total antioxidant activities (DPPH and FRAP) in unknown mistletoe spirit samples. The resulting spectra were evaluated using a Partial least squares (PLS) model. The main requirements for these approaches are speed, high degree of automation and cost effectiveness. Direct spectroscopic analyses are well suited in this context, especially because measurements can be done very quickly, without sample preparation and reagent consumption. Application of mid-IR spectroscopy is of special interest due to the presence of sharp and specific absorption bands (Schindler et al., 1998).

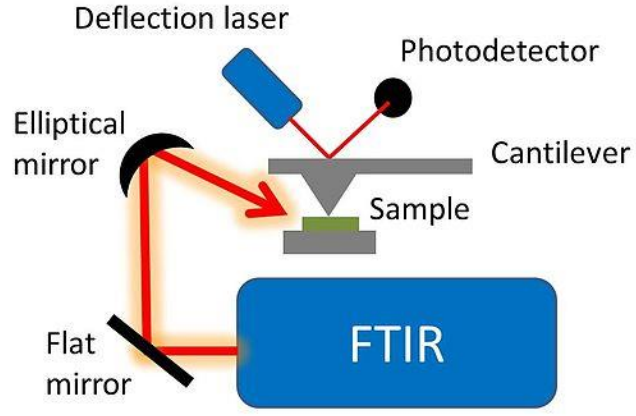


**Figure 7.** FTIR spectrometer scheme (Hammiche et al., 1999)

Procedure of work

The 2000 System Perkin Elmer instrument operated by PERGRAMS software running on Windows 95 platform was used to register FTIR spectra (Figure 8). As reference, the background spectrum of air was collected before the acquisition of the sample spectrum. After each sample, the crystal was rinsed with water and then dried with a soft tissue. To record spectra, sample was placed in measuring holder-dedicated accessory of System 2000 spectrometer and placed in measuring chamber. The transmission technique was applied to conduct 10 scans for each of the studied spirit in spectral range 4000-370 $cm^{-1}$. For each spirit 8 spectra were registered, 122 spectra all together.

**Figure 8.** The 2000 System Perkin Elmer instrument (own picture)

3.2.4. GC/MS analysis of aroma compounds

Principle of method

Gas chromatography (GC) is a powerful tool in the analysis of alcoholic beverage products. The aroma compounds tend to be volatile in nature, which fulfills one of the main requirements of GC. Solid-phase microextraction (SPME) is a solid phase extraction sampling technique that involves the use of a fiber coated with an extracting phase, which extracts different kinds of analytes from different kinds of media, that can be in liquid or gas phase. SPME is ideally suited for routine analysis, and for detection of compounds present at higher concentration (Demyttenaere et al., 2003). This technique, based on absorption and/or adsorption mechanism, depending on the fibre coating, can be successfully applied for polar and non-polar compounds in gaseous, liquid and solid samples and can be easily coupled with various

analytical instruments such as GC, GC/MS and HPLC. In SPME, analytes establish equilibrium among the sample matrix, the headspace above the sample, and a polymer-coated fused fiber. After extraction, the SPME fiber is transferred to the injection port of separating instruments, where desorption of the analytes takes place and analysis is carried out. Because analytes are concentrated on the fiber, and are rapidly delivered to the column, minimum detection limits are improved and resolution is maintained (Anonymous 2, 2017).

The GC/MS instrument is made of two parts. The gas chromatography, known as GC portion, separates the chemical mixtures into pulses of pure chemicals. And the mass spectrometry, known as MS, identifies and quantifies the chemicals (Figure 9). The GC separates chemicals based on their volatility or ease with which are they evaporate into gas . In general, the small molecules travel more quickly than larger molecules. The sample, the mixture of molecules, is injected into the GC and it is carried by inert (non-reactive) gas through the instrument, usually helium. The inject port is heated to 300°C to cause the chemicals become gases. The column is heated to move the molecules through the column. Inside the oven is the column which is a proximately 30 meter long thin tube with a special polymer coating on the inside. Chemicals with high volatility travel through the column more quickly than chemicals with low volatility. After passing through the GC, the chemical pulses continue to the MS. The MS is used to identified chemicals based on their structure. The molecules are blasted with electrons, which cause them to break into pieces and turn into positively charged paricles called ions. This is important because the particles must be chared to pass through the filter. As the ions continue through the MS, they travel through an electromagnetic field that filters the ions based on mass. A detector counts the number of ions with a specific mass. The data from MS is sent to a computer and plotted on a graph called a mass spectrum (Bull, 2008; OSU, 2017).

Compared to conventional techniques this technique offers many advantages such as high sensitivity and reproducibility, does not require solvent and combines extraction and pre-concentration in a single step without pre-treatment of samples, it is fast and versatile. SPME is a technique that requires a previous optimization of the extraction parameters that can affect extraction efficiencies, in order to obtain high recoveries of volatiles. Some of these sampling conditions are fibre sorbent (absorvent/ adsorvent) phase, extraction temperature and extraction time.

Although the use of solid-phase microextraction (SPME) for the aroma compounds analysis of orange juice, beer, fruit flavoured malt beverages, vodka, whisky and especially wine has been reported some years ago, the literature on SPME of spirits is much more recent and less extensive (Demyttenaere et al., 2003). The aim of this method was to analyse the composition of volatiles of mistletoe spirit and to find out which compounds pass from mistletoe plant to the spirit.

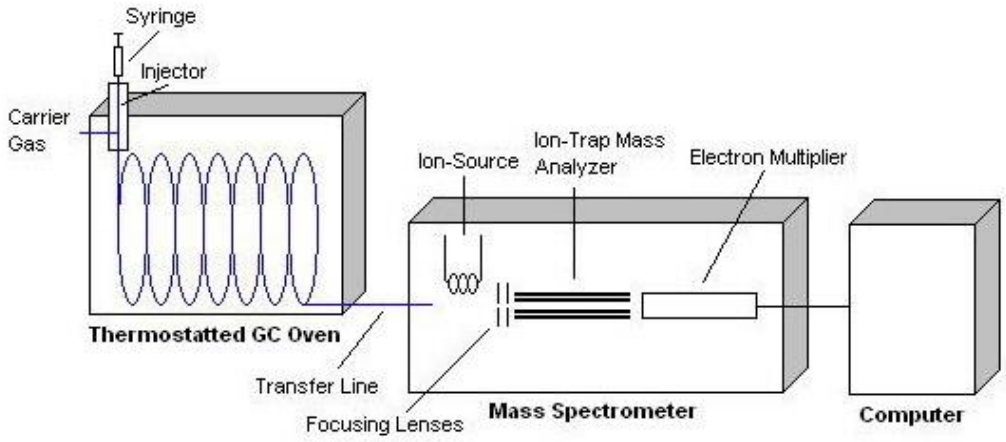


**Figure 9.** Schematic diagram of a GC/MS (Bayemi, 2012)

Procedure of work

Aliquots of 7 ml of spirit were pipetted into glass vials, which were closed with silicone septa (Figure 10). For manual sampling 50/ 30 μm divinylbenzene/ Carboxen on poly (dimethylsiloxane) (DVB/ CAR/ PDMS) was used on a 1 cm StableFlex fibre, which is recommended for aroma compounds (volatiles and semivolatiles) (Demyttenaere et al., 2003), (Figure 11). Samples were analyzed after 20 min of SPME sampling at 25°C using DVB/ CAR/ PDMS fibre (Figure 12). In sampling, the needle was passed through the septum that seals the vial, then the plunger was depressed and the fiber is exposed to headspace above the sample. Organic analytes were adsorbed to the coating on the fiber. After adsorption equilibrium was attained, the fiber was drawn back into the needle and was withdrawn from the sample vial. The fibre was introduced into the GC/MS injector, where adsorbed analytes were thermally desorbed and delivered to the instruments column.

**Figure 10.** Glass vial with aliquot (own picture)

**Figure 11.** SPME fibre (own picture)

**Figure 12.** SPME sampling (own picture)

Analyses were performed with Shimadzu QP2010S gas chromatograph interfaced with a mass spectrometer (Figure 13). Separations were performed using a 20 m x 0.18 mm (i.d.) capillary column coated with a 0.18 μm film of ZB-5ms stationary phase (5 %-Phenyl-Arylene-95 %-Dimethylpolysiloxane), which is equivalent to DB-5m. Helium was used as a carrier gas with a 0.78 ml $min^{-1}$ column flow rate and 3.8 ml $min^{-1}$ total flow rate. The analyses of the

SPME samples were performed with GC/MS under following conditions: the oven program temperature used was 35-220°C at a rate of 5°C $min^{-1}$ and 220-240°C at a rate of 10°C $min^{-1}$, with a final temperature hold for 5 min, resulting in total run of 44 min. The SPME fiber was thermally desorbed in the programmed temperature vaporizer injector at 250°C during 3 min without using split mode. Determinations were made in duplicate.

**Figure 13.** GC/MS QP2010S instrument (own picture)

3.2.5. pH assay

The measurements of pH of all samples were done with Oakton pH plus Meter Kit pH meter. The system was calibrated by placing the electrodes in buffer pH 4.

3.2.6. Statistical analysis

*3.2.6.1. XLStat*

XLStat is a tool of statistical add-ins for Microsoft Excel. In this work, XLStat was used to conduct statistical analyses. It was used to do Principal component analysis (PCA) and Partial least squares (PLS) regression.

*3.2.6.2. Principal component analysis*

Principal component analysis (PCA) is a multivariate technique that analyzes a data table in which observations are described by several inter-correlated quantitative dependent variables. It is probably the most popular multivariate statistical technique and it is used by almost all scientific disciplines. It is also likely to be the oldest multivariate technique. The goals of PCA are to extract the most important information from the data table, to compress the size of the data set by keeping only this important information, to simplify the description of the data set, and to analyze the structure of the observations and the variables. In order to achieve these goals, PCA computes new variables called principal components which are obtained as linear combinations of the original variables. The first principal component is required to have the largest possible variance (i.e., inertia and therefore this component will "explain" or "extract" the largest part of the inertia of the data table). The second component is computed under the constraint of being orthogonal to the first component and to have the largest possible inertia. The other components are computed likewise. The values of these new variables for the observations are called factor scores, and these factors scores can be interpreted geometrically as the projections of the observations onto the principal components (Abdi and Williams, 2010).

In this work PCA was used to visualize total polyphenol content (TPC), DPPH and FRAP measured values vs. samples, as well as, to visualize sample compound distributions vs. samples. PCA analyses are presented as biplots that are an enhanced scatter plots that uses both points and vectors to represent structure.

*3.2.6.3. Partial least squares regression*

In FTIR analysis, a large number of data are produced and different treatments have been developed to analyze these data used Partial least squares (PLS) regression to analyze data from FTIR spectra in order to predict red wine total antioxidant activity (Versari et al., 2010). PLS statistical analysis is a robust chemometric method for data treatment of complex mixtures if a suitable number of calibration samples with the appropriate variation in composition across the range for the unknown samples is available (Silva et al., 2014). PLS regression is a recent technique that generalizes and combines features from principal component analysis and multiple regression. The technique is based on the so-called bilinear projection, meaning that two sets of variables (x, y) are linked to each other by means of linear projection models. Instead of

multiple linear regression (MLR) being applied to the full set of regressors, the information carried by the original x variables is projected onto a smaller set of latent, uncorrelated variables called PLS components (Bauer et al., 2008). Its goal is to predict or analyze a set of dependent variables Y from a set of independent variables or predictors X. It is a method for constructing predictive models when the factors are many and highly collinear. When Y is a vector and X is full rank, this goal could be accomplished using ordinary multiple regression. The emphasis is on predicting the responses and not necessarily on trying to understand the underlying relationship between the variables. For example, PLS is not usually appropriate for screening out factors that have a negligible effect on the response. However, when prediction is the goal and there is no practical need to limit the number of measured factors, PLS can be a useful tool. PLS can be used to construct a linear predictive model for the observed parameter (alcohols, phenols, etc.), based on the FTIR spectrum (Abdi, 2003; Geladi and Kowalski, 1986). The aim of using PLS in this work was to develop a FTIR methodology for screening total polyphenol content and total antioxidant activities in mistletoe spirit samples using infrared spectral data, without sample pre-treatment. PLS models were developed for the prediction of these parameters: TPC, DPPH and FRAP. This method could be important for the rapid screening of spirit phenolic composition and evaluation of antioxidant activity.

# 4. RESULTS AND DISCUSSION

## 4.1. TOTAL POLYPHENOL CONTENT AND ANTIOXIDANT ACTIVITY

### 4.1.1. Total polyphenol content (TPC)

Determination of the total polyphenol content (TPC) in samples has been conducted by the Folin-Ciocalteu method. Even with the undefined chemical nature of Folin-Ciocalteu reagent, the total polyphenol content assay by Folin-Ciocalteu reagent is convenient, simple and reproducible and it is commonly accepted assay and routinely practiced in dietary antioxidant research laboratories throughout the world (Huang et al., 2005). Average and standard deviation values obtained for total polyphenol contents of 14 samples are presented in Figure 14. Results for TPC show there are important differences among the samples studied, probably due to differences in the spirit making techniques and composition of the raw materials: the total polyphenol content in the samples ranged from 49 to 545 mg GAE $L^{-1}$, with an average value of 196 ± 15 mg GAE $L^{-1}$. It is widely appreciated that the chemical composition of mistletoe is not stable and depends not only on the biosynthesis, but also on the type of host plant and growing conditions, such as ambient temperature, carbon dioxide concentration and season of the year (Luczkiewicz et al., 2001). Therefore a number of difficulties occur in comparison of mistletoe spirit samples. The highest polyphenol content were measured in samples 6 and 1. These samples are similar in chemical composition, so they might be obtained from the same base spirit. Even though, both samples might be produced from the same base spirit, there are some differences in total polyphenol content between them. Sample 6 has higher total polyphenol content than sample 1. The reason why these two samples have higher polyphenol content compared to other samples could be the maceration technique. During the maceration process polyphenols are extracted and pass into the alcohol base enriching it (Mrvcic et al., 2012). It might be that maceration was carried out for longer time or under better conditions, such as temperature, in a spirit base with higher ethanol content, etc. Because of lack of information about equipment and the processes of production, it is difficult to say the main reason for higher polyphenol content. Sample 8 has the lowest polyphenol content. This sample was obtained by leaf maceration of mistletoe in the grape marc spirit and it was commercially produced (bottled), the same as sample 13. Although, samples 8 and 13 were produced from the same base spirit, parameters show there are important differences between them. The same case appears between

samples 9 and 14, which were obtained by maceration of mistletoe in the refined ethanol. Even though, in samples 12 and 2 the largest number of compounds were detected, their polyphenol content is not as high as in samples 6 and 1. Compared to other spirit drinks obtained by maceration of other herbs also rich in polyphenols, for example *Artemisia absinthium*, *Salvia divinorum*, *Melisae folium*, *Pimpinella anisum* and *Lavandula officinalis*, mistletoe spirit samples have higher polyphenol content (Mrvcic et al., 2012). Therefore, mistletoe spirit should have the precedence when choosing this kind of alcoholic beverage in order to have greater benefits for health. Based on obtained results that some samples have high polyphenol content, the results can be used to recommend the moderate consumption of mistletoe spirit.

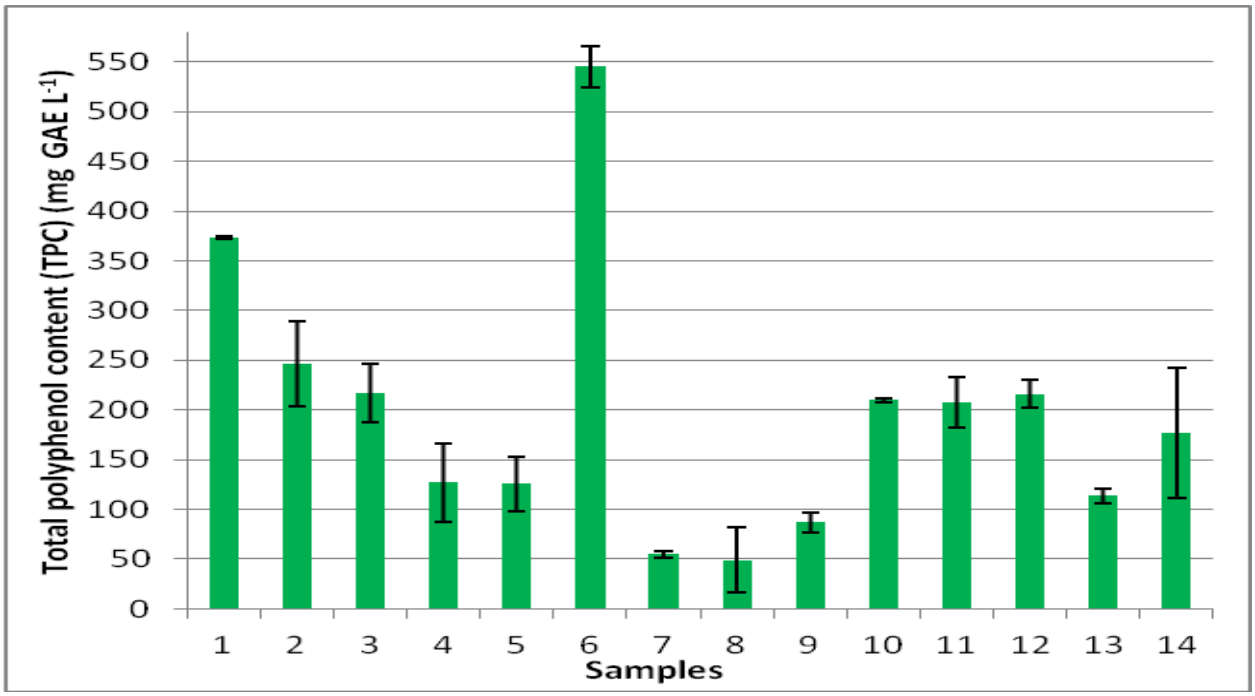


**Figure 14**. Total polyphenol content (TPC) of mistletoe spirit samples (1-14)

For the determination of antioxidative activity characteristics of spirit drinks, the DPPH radical scavenging activity and ferric ion reducing antioxidant power (FRAP) were used.

4.1.2. DPPH assay

In the DPPH assay, a higher percentage inhibition corresponds to a higher antioxidant capacity for this radical. In the present study, DPPH inhibition ranged from 2 % to 63 %. Wide range of DPPH inhibition is suggesting that there are differences between samples in the antioxidant activity. These differences can be the result of different technique of the production, time of maceration and raw materials. One study showed when the activities of the same type of mistletoe extracts, collected from the same host tree, but in different seasons, were compared using the DPPH assay, it was found that the antioxidant activity was, in general, higher in spring (Vicas et al., 2011). Average and standard deviation values obtained for DPPH inhibition of 14 samples are presented in Figure 15. Samples that have the highest DPPH inhibition are sample 6 and 2, followed by 1 and 3. It can be noticed that the order at column height for TPC does not correspond to column height for DPPH inhibition for all samples. For example, sample 1 had higher TPC than sample 2, but has lower DPPH than sample 2. Only for samples 6 and 3 fit, that are at the same position in order for TPC and DPPH inhibition. In addition, in the MS Office Excel with tool- XLStat, linear regression model was created in order to show the correlation between total polyphenol content (TPC) and antioxidant activity measured by DPPH method. DPPH assay of analyzed samples has a moderate correlation with total polyphenol (TPC), with correlation coefficient ($R^2$= 0.6463) (Figure 16). The correlation coefficient is suggesting that the antioxidant potential is reflected by a more complex synergy of active molecules, not only polyphenols. The predicted percentage of DPPH inhibition can be calculated by the following equation of the model: % inhibition of DPPH= 4.02462 + 0.13614 x TPC.

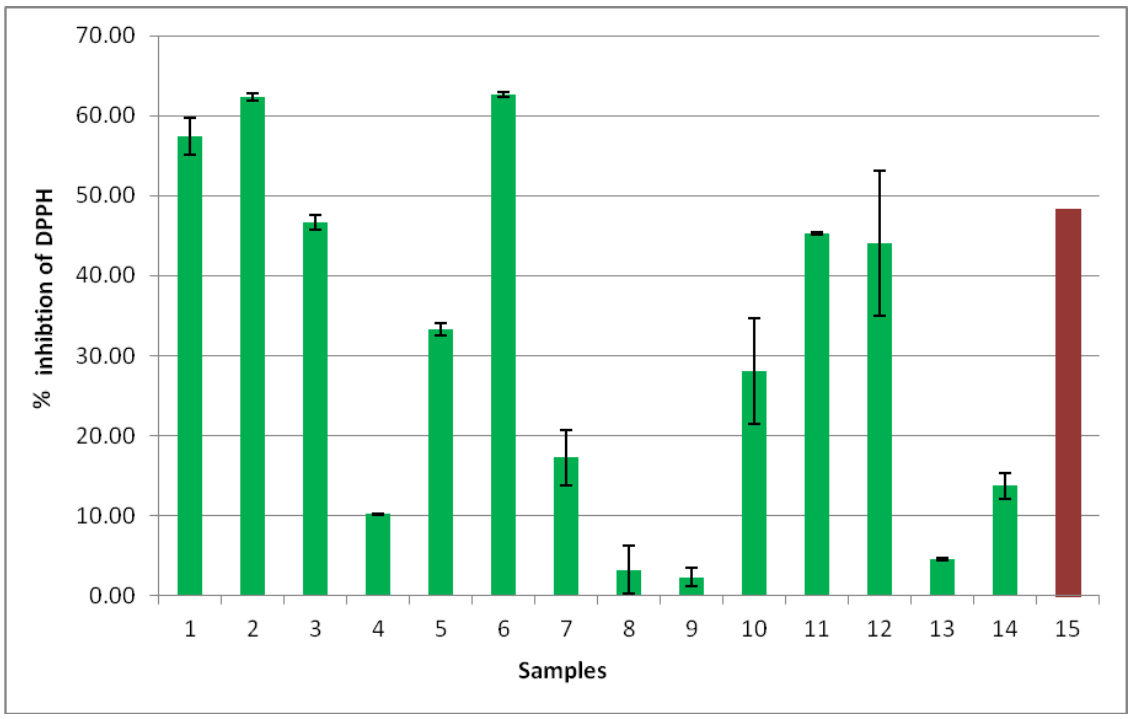


**Figure 15**. DPPH assay of mistletoe spirit samples (1-14) and 0.05 mM Trolox (15)

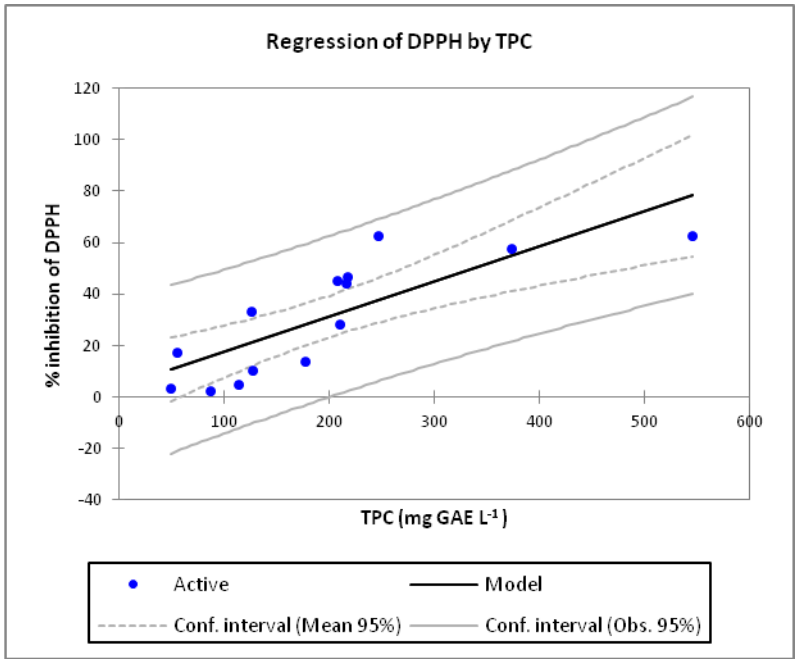


**Figure 16**. Relation between total polyphenol content (TPC) and DPPH assay

4.1.3. FRAP assay

Considering the antioxidant potential of mistletoe spirit due to its content of polyphenols, antioxidant activity was measured by FRAP method. FRAP assay measures the capacity of reducing metals, namely, ferric to ferrous iron. FRAP values in mistletoe spirit samples varied between 0.7900 and 3.3677 mM Trolox. Average and standard deviation values obtained for FRAP assay of 14 samples are presented in Figure 17. First three samples in order that have the highest TPC also have the highest FRAP values. Other samples varied in the order, but the differences were not as large as in DPPH. In addition, in the MS Office Excel with tool- XLStat, linear regression model was created in order to show the correlation between total polyphenol content (TPC) and antioxidant activity measured by FRAP method. FRAP assay of analyzed samples has a strong correlation with total polyphenol content, with correlation coefficient ($R^2$= 0.8782) (Figure 18). The correlation between total polyphenol content (TPC) and FRAP assay is suggesting that the antioxidant activity of mistletoe spirit may be correlated with the total polyphenol content. The predicted mM Trolox equivalents for FRAP assay can be calculated by the following equation of the model: FRAP= 0.56955 + 0.00576 x TPC

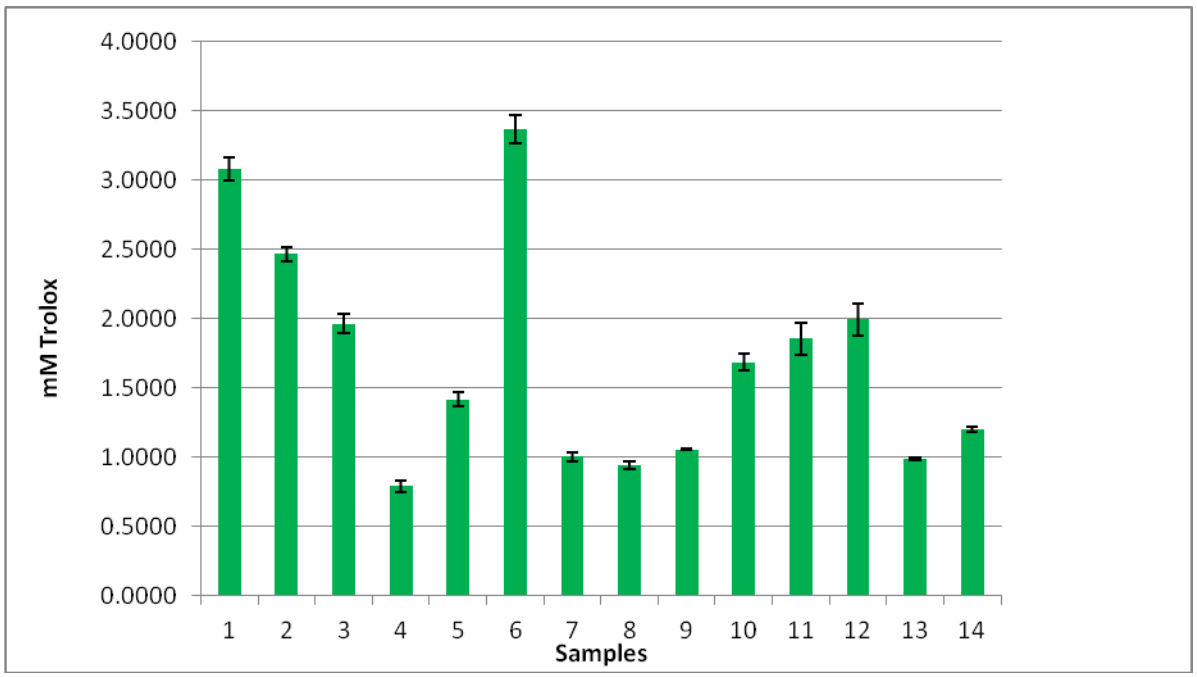


**Figure 17.** FRAP assay of mistletoe spirit samples (1-14)

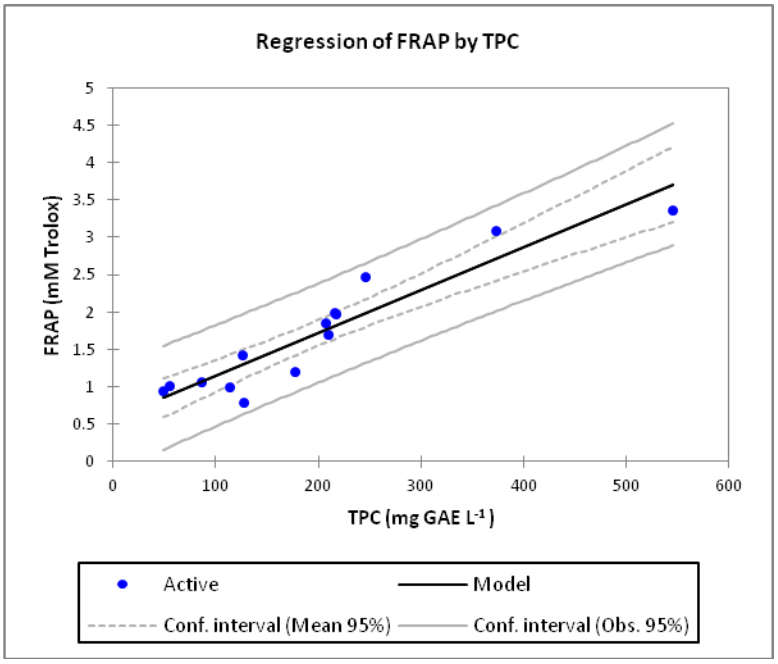


**Figure 18**. Relation between total polyphenol content (TPC) and FRAP assay

Results show that total polyphenol content (TPC) of mistletoe spirit samples has a stronger correlation with FRAP assay than with DPPH assay. As far as it is known, consumption of phenolic-containing alcoholic beverages transiently raises total phenol concentration and enhances the antioxidant activity of blood plasma in healthy volunteers. This is compatible with suggestions that moderate alcohol usage and increased antioxidant intake decrease the risk of coronary heart disease. For the reason that some samples have high polyphenol content, DPPH and FRAP antioxidant activities, the results can be used to stimulate the moderate consumption of mistletoe spirit.

## 4.2. FTIR ANALYSIS

FTIR spectra were registered for 14 mistletoe samples in order to determine and identify characteristics groups in molecular structers. FTIR spectra were analyzed and a typical spectrum for the whole spectral range (4000-370 $cm^{-1}$) is presented in Figure 19. Spectra showed that all samples had characteristic bands. The intense band detected in the 3627-2971 $cm^{-1}$ region originated from compounds with -OH groups such as water and ethanol, which are major compounds in these samples, was not useful in this work. Region 1800–900 $cm^{-1}$ was selected for working range that is shown in Figure 19, where an overlay of all samples analyzed is presented. These wavelengths are part of the fingerprint region and include infrared typical absorption of phenolic molecules such as the stretching band of carbonyl (C=O) groups (1712-

1704 $cm^{-1}$) and C=C stretching bands (1609-1608 and 1519-1516 $cm^{-1}$), which are typical of aromatic molecules. Moreover, signal from the phenols can be found in the region 1680-900 $cm^{-1}$. The absorption at 1448-1444 $cm^{-1}$ corresponds to antisymmetric in-plane bending of -CH3. Furthermore, in the same spectral region corresponding to the phenyl nuclei (C=C bonds), there are also bands of deformation of -CH2- groups. The peak at 1376-1373 $cm^{-1}$ is associated with symmetric in-plane bending of -CH3. The absorption at 1340–1339 $cm^{-1}$ has been assigned to CH bending and CH2 wagging. The peak at 1281-1278 $cm^{-1}$ corresponds to in-plane bending of O-H. The bands at 1207, 1110-1107, 1067-1062 $cm^{-1}$ correspond to the stretching vibration of C-O. These assignments are based on previous work on phenolic compounds in wine (Tarantilis et al., 2008). FTIR spectra analysis confirmed that mistletoe spirit samples contain phenolic molecules and that this method can serve to identify the molecular structure.

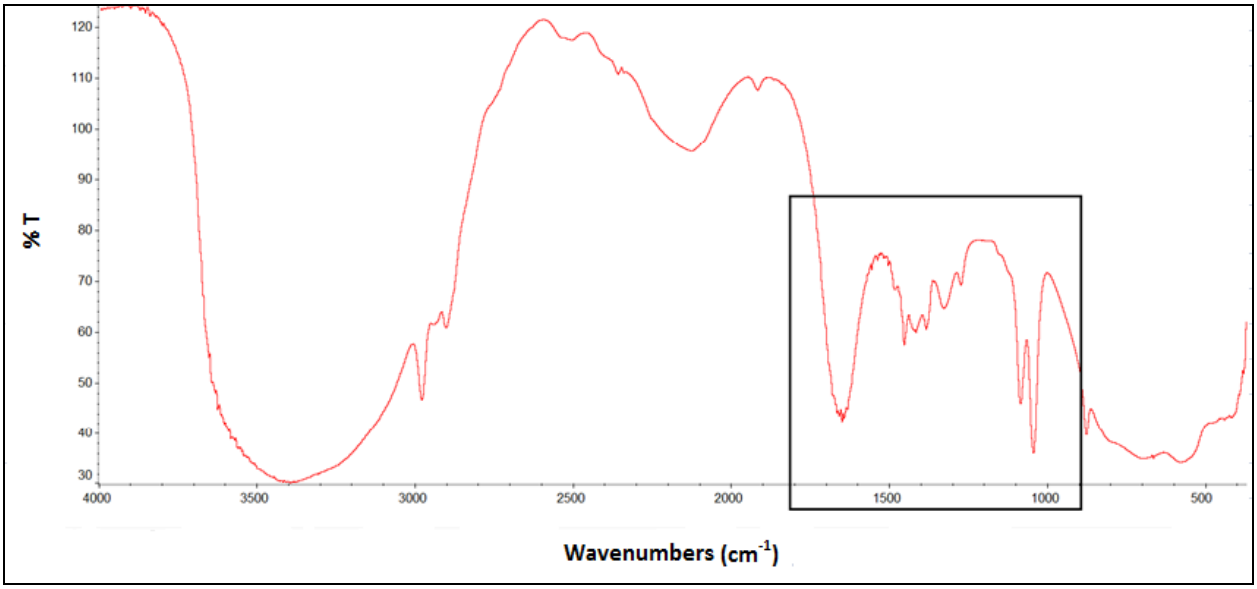


**Figure 19**. FTIR spectrum of a sample of mistletoe spirit: zoom of the selected wavenumber range with an overlay of all samples analyzed in that range

### 4.3. AROMA COMPOUNDS ANALYSIS

The flavor of alcoholic beverages has been considered since early years as an image of their quality. With alcoholic beverages, a human being perceives an overall sensation, chemical analysis measures single aroma compounds qualitatively and quantitatively. Thus perceived sensations must be broken down into clearly defined elements, the chemical compounds (Demyttenaere et al., 2003). These compounds and their influence on the overall aroma are characterized.

A total number of 166 aroma compounds have been identified in the aroma of mistletoe spirit, from which 53 were esters, 32 alcohols, 25 terpenes, 18 carbonyl compounds, such as aldehydes and ketones, 12 alkanes, 9 acetals, 8 acids and 9 other compounds (Annex 1). The esters generally have a pleasant and some of them also a very intense odor and thus it may be assumed that they appear as important aroma components, even if they are present in relatively small amounts. Esters compose the majority of volatiles, such as low molecular weight ethyl esters (C6-C11), high molecular weight ethyl esters (C13-C20), acetate esters and isoamyl esters. Among all aroma compounds, four aroma compounds were detected in each sample, and accounted for an average of 52.6 % of the total aroma compounds (ethyl octanoate, ethyl decanoate, isopropyl myristate and ethyl hexadecanoate). In addition, three aroma compounds were detected in each sample, except sample 9, accounted for an average of 58.8 % of the total aroma compounds (decanal, ethyl dodecanoate, ethyl pentadecanoate) and one aroma was detected in each sample, except sample 5 (ethyl hexanoate). Their presences in samples are shown in Figures 20-27. Differences of volatile constitutes among samples were also discovered (Table 2). Some aroma compounds were detected only in one sample and some samples had lack of some aroma compounds, even though, they were detected in all other samples. Differences may occur because samples were produced from different base spirits and from different mistletoe plants, which grown on different host trees. Interesting is that some of the compounds found in mistletoe plant are not produced by the plant, but obtained from the host tree (Nazaruk and Orlikowski, 2015) and thus it can be also expected to find different compounds in mistletoe spirits.

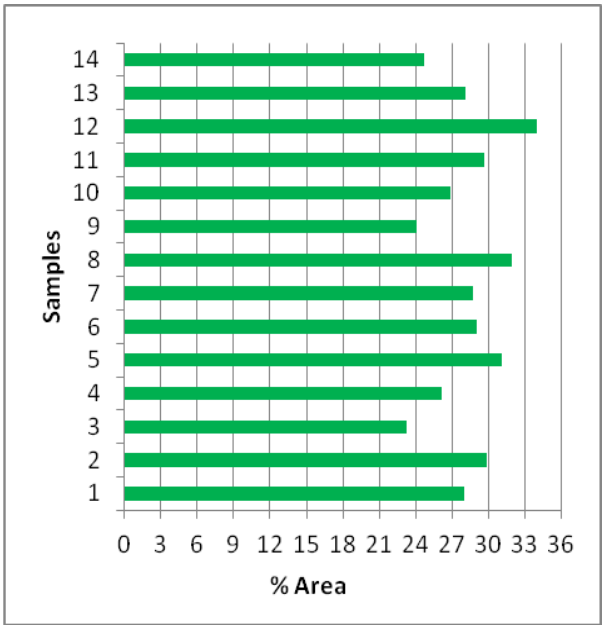


**Figure 20.** Presence of ethyl octanoate in samples (1-14)

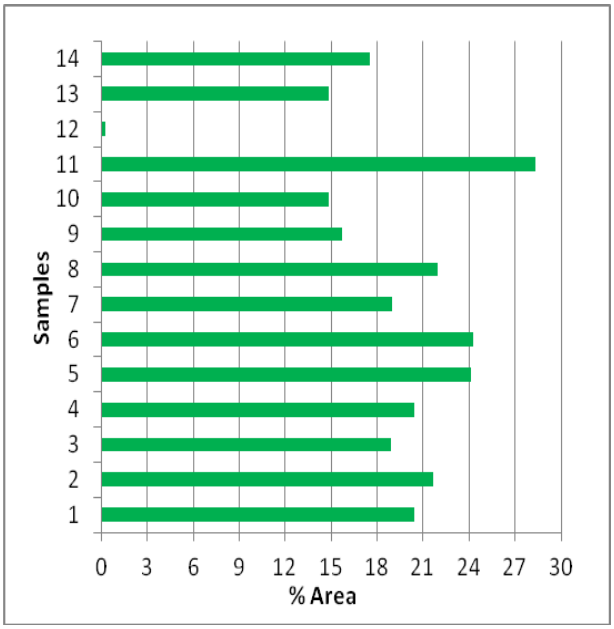


**Figure 21.** Presence of ethyl decanoate in samples (1-14)

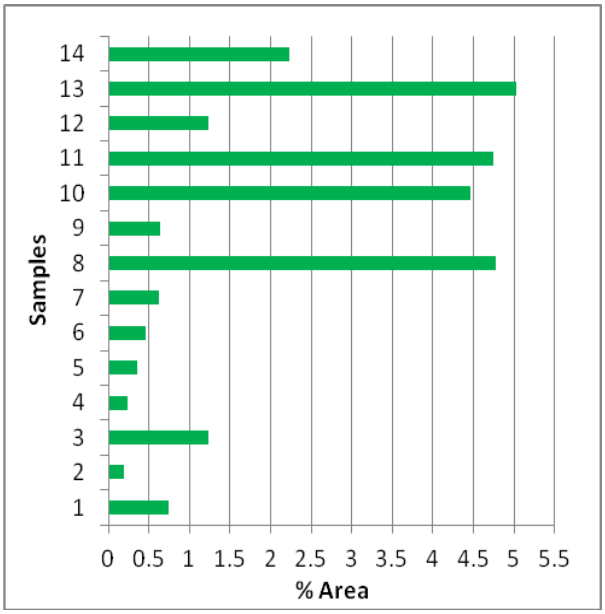


**Figure 22.** Presence of isopropyl myristate in samples (1-14)

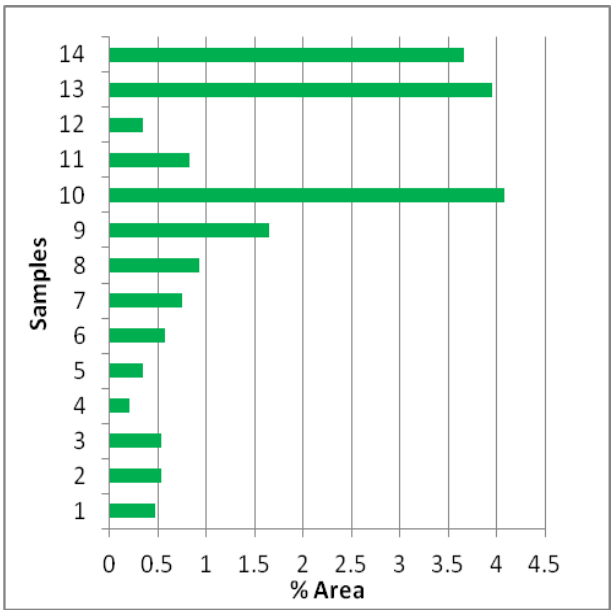


**Figure 23.** Presence of ethyl hexadecanoate in samples (1-14)

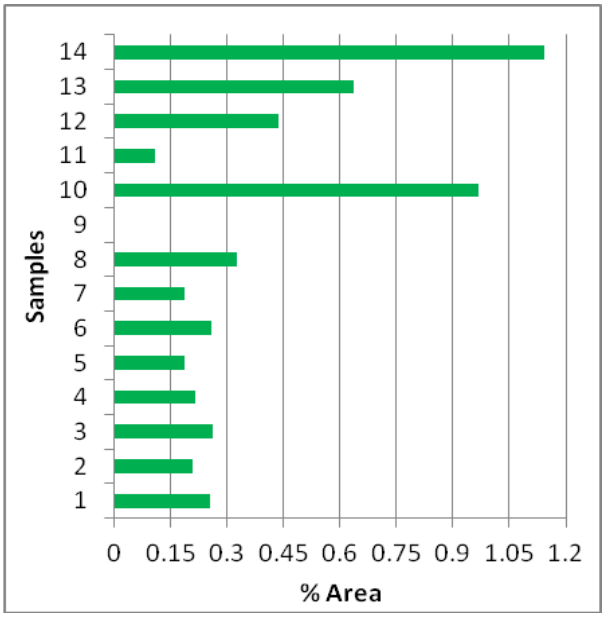


**Figure 24.** Presence of decanal in samples (1-14)

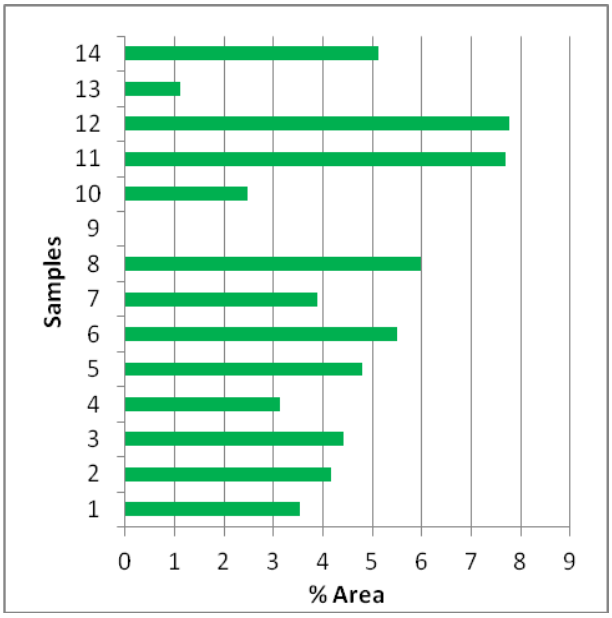


**Figure 25.** Presence of ethyl dodecanoate in samples (1-14)

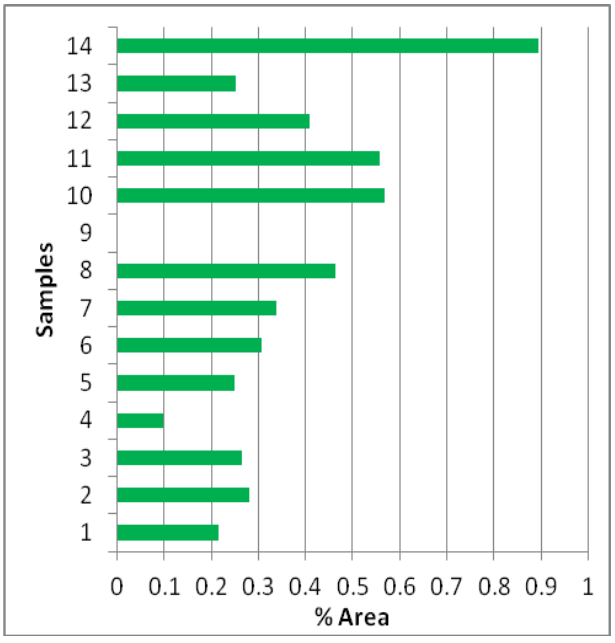


**Figure 26.** Presence of ethyl pentadecanoate in samples (1-14)

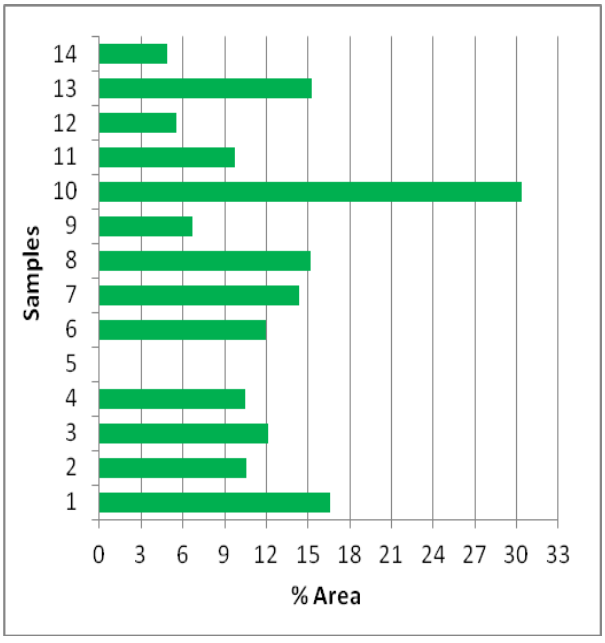


**Figure 27.** Presence of ethyl hexanoate in samples (1-14)

**Table 2.** Differences between samples (1-14) in number of detected compounds

| Sample | Number of detected compounds | Number of compounds detected only in this sample | Number of compounds detected in one extra sample |
|---|---|---|---|
| **1** | 52 | 3 | 3 |
| **2** | 58 | 4 | 3 |
| **3** | 42 | 8 | 2 |
| **4** | 49 | 3 | 2 |
| **5** | 48 | 6 | 2 |
| **6** | 37 | 0 | 0 |
| **7** | 46 | 6 | 3 |
| **8** | 20 | 3 | 0 |
| **9** | 24 | 2 | 5 |
| **10** | 31 | 8 | 3 |
| **11** | 34 | 2 | 1 |
| **12** | 73 | 27 | 4 |
| **13** | 44 | 8 | 4 |
| **14** | 19 | 0 | 2 |

Quantitatively noticeable components are ethyl esters of hexanoic, octanoic, decanoic, dodecanoic and hexadecanoic acids (Figure 28). For these compounds it was reported that they can be produced during fermentation by yeast (Camara et al., 2007), but as they were detected in samples 9 and 14, which were obtained by maceration of mistletoe in ethyl ethanol, it can be concluded that these compounds were created during extraction of acids from plant mistletoe to the alcohol. These compounds make a positive contribution to the general quality of spirit being responsible for their "fruity" and "floral" sensory properties. The concentration of ethyl esters decreases over time due to spontaneous hydrolysis. Those are the main ester component, although isobutyl and isoamyl esters of short- chain fatty acids, called "fruit esters", also appear. Acetates are the result of the reaction of acetyl-CoA with higher alcohols that are formed from degradation of amino acids or carbohydrates (Câmara et al., 2007). Isoamyl acetate, which possesses sensory impact described as "banana and apple" was detected in ten samples. It was produced by yeast during fermentation and had been identified with fruity aroma (Arrieta-Garay et al., 2014). Isoamyl butyrate possesses sensory impact described as "apple and melon-like with tutti frutti notes" (Pino and Queris, 2011), it was detected in two samples. Furthermore, apparently differences were found for isoamyl hexanoate between samples. It was detected in six samples. Hydroxy esters were formed from the esterification of the corresponding hydroxyl fatty acids and are responsible for fruity-floral fragrance, which could be produced from the reduction

of keto acids. Additionally, it was widely considered that hexanoic acid was a saturated fatty acid that had six carbons and one carboxylic group and could be converted into other useful materials such as ethyl hexanoate and hexanol via esterification and hydrogenation (Pino and Queris, 2011). Ethyl hexanoate, the most important aroma contributor with apple peel and fruit odors (Pino and Queris, 2011), was detected in all samples and hexanol in two samples. Previous study had reported that *Clostridium kluyveri* produced hexanoic acid from ethanol upon growing with methane-producing bacterium (Barker and Beck, 1942). Genthner, Davis, and Bryant (1981) revealed that the methanol-utilizing bacteria, *Eubacterium* produced hexanoic acid from methanol. Actually, family *Clostridiaceae*, as an important group in the fermentation, converted organic substances into organic acids, such as hexanoic acids, alcohols, $CO_2/H_2$ and minerals, forming ethyl hexanoate. 1-hexanol is only partially a non-alcoholic fermentation product. It derived from the enzymatic degradation of C18 unsaturated acids of the marc by lipoxygenase, mostly obtaining C6-aldehydes which are reduced by yeasts to alcohols including the reduction of the double bond in 2-3 position. It is responsible for sweet herbal odor with a green top note (Ding et al., 2016). Of the higher esters, ethyl hexadecanoate is interesting because significant amounts of this compound have been detected in all samples. The yeast used a great influence on the production of esters in the fermentation process. The ester content of distilled beverages also depends on whether or not the yeast is present at the time of distillation. If distillation occurs in the presence of yeast, the ethyl ester concentrations of at least decanoic, dodecanoic and hexadecanoic acids increase (Demyttenaere et al., 2003). Numerous carbonyl compounds have been reported as being part of the volatile composition of freshly distilled Calvados, whisky and various brandies or spirits (Ledauphin et al., 2004). The most predominant aldehyde in mistletoe spirit samples was decanal, which was detected in thirteen samples and brings sweet, orange, citrus aroma. Except decanal, the main aldehydes identified were: benzaldehyde, nonanal, hexadecanal and dodecanal. For bezaldehyde is known that has sensory impact described as "almond, fruity, powdery and nutty" and for dodecanal "citrus, orange rindy with floral nuances" (Luebke, 1997). Nonanal is described as "waxy, aldehydic, citrus, with a fresh slightly green lemon peel like nuance, and a cucumber fattiness" (Mosciano, 1995). With the exception of this compound, aldehydes and ketones in general show very low concentrations in distilled spirits. Nevertheless, because of their very low detection threshold, they can produce an important olfactive impact. As carbonyl function is moreover very reactive, an evaluation of these compounds can be strongly depreciated by reaction with the matrix. Conversion of aldehydes

into acetals was not ably reported by using liquid–liquid extraction with polar solvents in highly ethanolic media. As a consequence, because of their relative instability in complex media, carbonyl compounds usually undergo a derivatization step prior to analysis (Ledauphin et al., 2004). Therefore, some acetals were detected. Such as, isovaleraldehyde diethyl acetal, acetaldehyde ethyl amyl acetal, hexanal diethyl acetal, and octanal diethyl acetal. The most significant acetals among samples were hexanal diethyl acetal, with sensory impact described as "cognac, pear, floral, apple, fruity" and octanal diethyl acetal, with sensory impact described as "green, citrus, oily and fatty with a woody, spicy and fruity nuance" (Mosciano, 1995). A special group of compounds make up the alcohols. Some of them, have a significant impact to the aroma. For example, farnesol with sensory impact described as "mild, fresh, sweet, linden, floral, angelica" (Luebke, 1997), carbitol with slightly ethereal odor (Luebke, 1997), thujanol with sensory impact described as "cooling, minty, eucalyptol, green and terpy with a spicy nuance" and nerol with sensory impact described as "fresh, citrus, floral, green, sweet, lemon/lime and waxy with a spicy depth" (Mosciano, 1995). In addition, medium chain fatty alcohols are detected (C8-C14). They also bring specific aroma and enrich the spirit. For example, nonanol and decanol have sensory imapct described as "fresh, clean, fatty, floral, sweet, orange", dodecanol and tetradecanol are described as "waxy, fatty, honey, coconut" (Luebke, 1997). Higher alcohols fraction is composed mainly by *n*-alcohols of C6 chain length and aromatic compounds such as 2-phenyl ethanol. The presence of these compounds may cause a "flowery" and "sweet" notes which could be considered as a positive characteristic for spirit (Camara et al., 2007). 2-phenyl ethanol, quite exclusive of fermentative origin, was detected in eleven samples. The dominant yeast type may affect its content during the mash fermentation, but mostly by the intensity of the tail cut at the distillation (Ding et al., 2016). It is described as a "rose-like, sweet and perfume-like" and has a positive influence on the aroma (Apostolopoulou et al., 2005). 2-phenyl ethanol is a marked tail component, so it should be present in the distillate in quite low concentrations (Da Porto, 2002). The distiller has the opportunity to produce "hearts" that contain the least possible amount of the above alcohol, following the best selection of the cut points (Apostolopoulou et al., 2005). For instance, 2-phenyl ethanol concentration of one of the homemade sample (4) was very low (0.25 % of whole area in chromatogram) indicating a good fraction separation. On the contrary, increased 2-phenyl ethanol concentration (3.52 % of whole area in chromatogram) was found in sample (8), possibly indicating that the "tails" were not removed in time during the production of this distillate. The next group of compounds is the fatty

acids. The most important fatty acids present in the samples were palmitic, oleic, myristic acids. Oleic acid, known as monounsaturated omega-9 fatty acid, has many health benefits. For example, it reduces blood pressure, increases fat burning to help with weight loss, protects cells from free radical damage, may prevent type 2 diabetes, prevents ulcerative colitis and generates brain myelin (Pravst, 2014). Terpenes, which may have an important contribution on the "floral" and "fruity" aromas of the spirit, make a special group of compounds found in all samples. The most significant terpens are L-limonene, D-limonene, linalool, $\alpha$-terpinolene, $\beta$-bourbonene and camphene. These compounds have already been identified in fresh plums and were reported as volatile constituents of fruits responsible for a wide spectrum of very pleasant aromas (Pino and Quijano, 2012). D-limonene is described as "sweet, citrus and peely" (Mosciano, 1995), L-limonene as " terpene pine herbal peppery", linalool as "citrus, floral, sweet, bois de rose, woody, blueberry", $\alpha$-terpinolene as "fresh, woody, sweet, pine, citrus" (Luebke, 1997), $\beta$-bourbonene as "herbal woody" and camphene as "camphoreous, cooling, pine, woody, terpenic, citrus, green, minty and spicy" (Luebke, 1997).

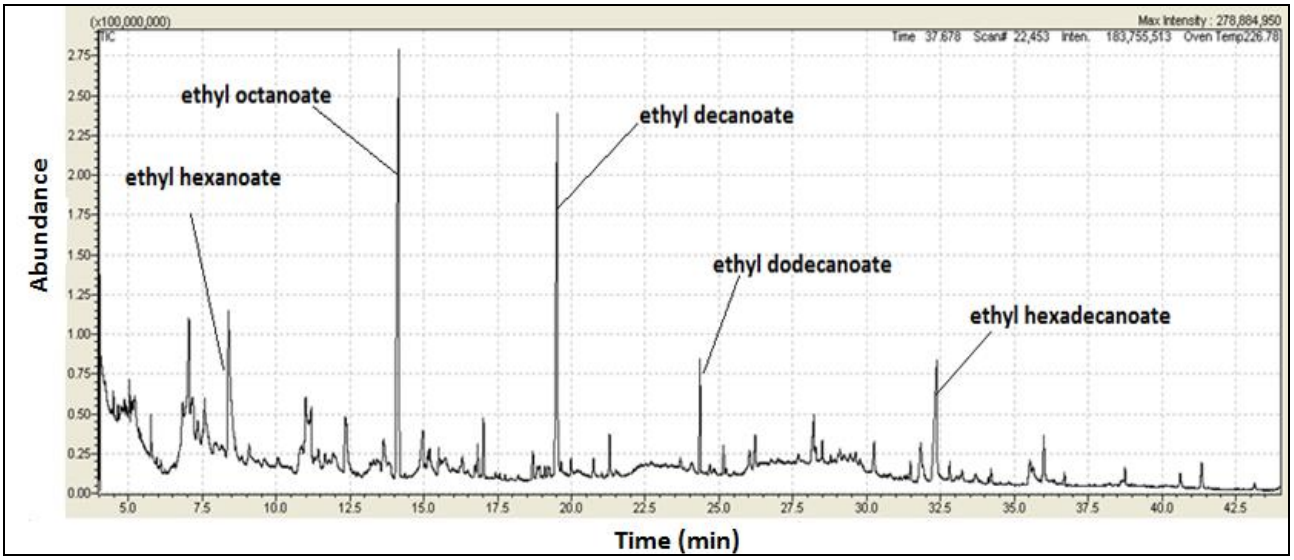


**Figure 28.** Chromatogram obtained after liquid SPME of mistletoe spirit (sample 6) using DVB/CAR/PDMS fibre (20 min, 25°C)

As far as it is known, two samples (9 and 14) were obtained by maceration of mistletoe leaves in the refined ethanol. It can be concluded that all compounds, which were determined in that samples are from the plant mistletoe. Figures 29 and 30 show compounds that were detected in these two samples and their presence in samples. Not all compounds that were detected in these samples were found in other samples. Some of them were detected only in few samples,

such as *p*-cymene, L-limonene, D-limonene, ethyl benzoate, methyl oleate and nonanal. *P*-cymene was also detected in oil obtained from mistletoe leaves and stems and it is described as "fresh, citrus, terpene, woody, spice" (Luebke, 1997). Not only that it has pleasant aroma, but also it is biocidal against spoilage yeasts and *E. coli* in apple juices (Kisko and Roller, 2005). Limonene is a colorless liquid found in essential oils of pine and citrus tree. It exists in two isomeric forms, called L-limonene, D-limonene. In manufacturing, L-limonene is used as a fragrance (Fahlbusch et al., 2003*).* D-limonene is one of the most common terpenes in nature. Being an excellent solvent of cholesterol, D-limonene has been used clinically to dissolve cholesterol-containing gallstones. Because of its gastric acid neutralizing effect and its support of normal peristalsis, it has also been used for relief of heartburn. D-limonene has well-established chemo-preventive activity against many types of cancers. Evidence from a phase I clinical trial shows a partial response in a patient with breast cancer and stable disease for more than six months in three patients with colorectal cancer (Sun, 2007). Ethyl benzoate is an ester that brings sensory impact described as "sweet, wintergreen, fruity, cherry, grape" (Mosciano, 1995). Furthermore, two compounds, ethyl 2-hydroxyisovalerate and L-menthyl acetate, were determined only in those samples. Ethyl 2-hydroxyisovalerate is described as a "pineapple, strawberry, tea, honey" and L-menthyl acetate is described as an "herbal mint rose". On top of that, in sample 9 were determined two more compounds. Those are cyclopentyl 4-ethyl benzoate and *β*-thujone. Only for *β*-thujone are described organoleptic properties "cedar, thujonic, spicy, woody" (Luebke, 1997).

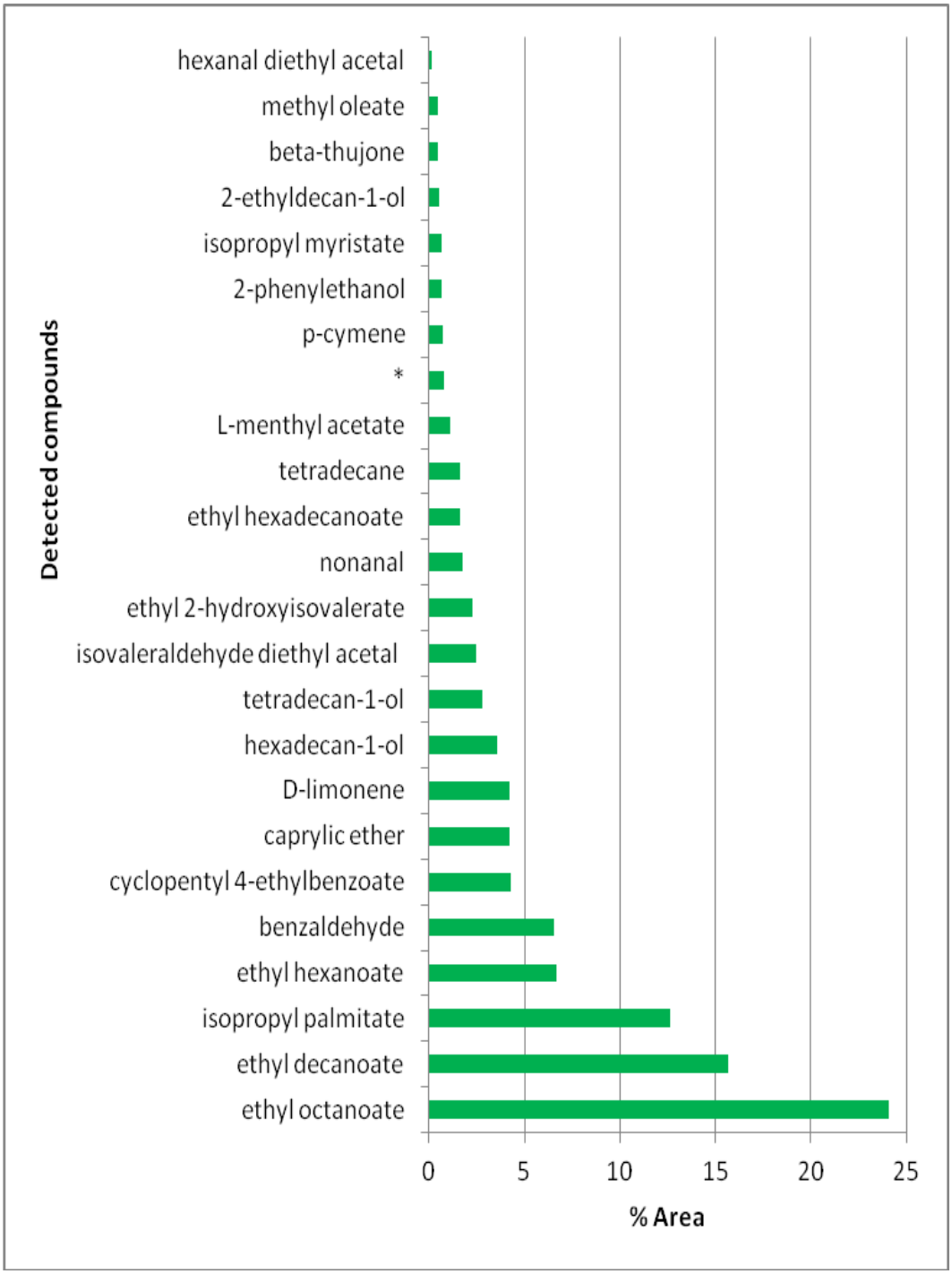


**Figure 29.** Detected compounds in sample 9 (*1-(3,5-ditert-butyl-4-hydroxyphenyl) propan-1-one)

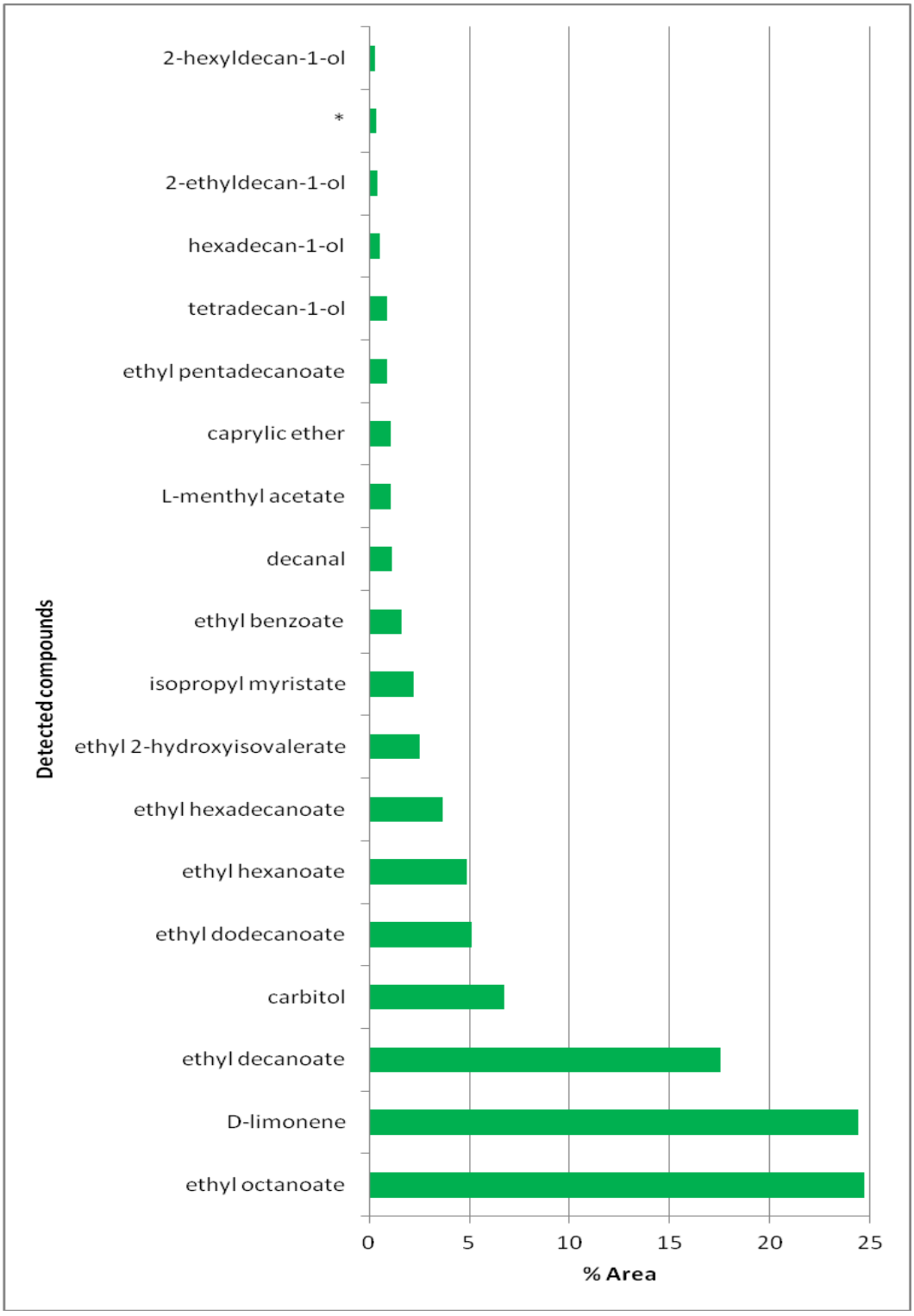


**Figure 30.** Detected compounds in sample 14 (*methyl 14-(2-octylcyclopropyl) tetradecanoate)

To compare samples, 42 target compounds were selected. Each sample that has certain compound is marked with "x" (Table 3). In the table are shown compounds that were detected in most samples.

**Table 3.** List of mistletoe spirit aroma compounds and their presence in samples (1-14)

| Compounds | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Esters** | | | | | | | | | | | | | | |
| ethyl octanoate | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| ethyl decanoate | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| isopropyl myristate | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| ethyl hexadecanoate | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| ethyl dodecanoate | x | x | x | x | x | x | x | x | | x | x | x | x | x |
| ethyl pentadecanoate | x | x | x | x | x | x | x | x | | x | x | x | x | x |
| ethyl hexanoate | x | x | x | x | | x | x | x | x | x | x | x | x | x |
| 3-methylbutyl octanoate | x | x | x | x | x | x | x | x | | x | x | x | | |
| isoamyl acetate | x | x | x | x | | x | x | x | | | x | x | x | |
| diethyl butanedioate | x | x | x | x | | x | x | x | | | x | x | x | |
| ethyl nonanoate | x | x | x | x | x | x | | x | | | x | x | | |
| ethyl heptanoate | x | x | x | x | x | x | | | | | x | x | | |
| methyl hexanoate | x | x | x | x | x | | x | | | | x | x | | |
| methyl octanoate | x | x | x | x | x | | | | | | x | x | | |
| isobutyl caprylate | x | | x | x | x | x | x | | | | | x | | |
| ethyl dec-9-enoate | x | x | x | x | | x | x | | | | | x | | |
| isobutyl caprate | x | x | | x | x | | x | | | | x | x | | |
| 3-methylbutyl decanoate | x | x | | x | x | x | | | | | x | | | |
| methyl oleate | x | x | | x | x | x | | | x | | | | | |
| isopentyl hexanoate | x | | | x | x | x | x | | | | | x | | |
| **Alcohols** | | | | | | | | | | | | | | |
| 2-phenylethanol | x | | x | x | x | x | x | x | x | | x | x | x | |
| tetradecan-1-ol | x | x | | x | x | x | x | | x | x | x | | x | x |
| 2-ethyldecan-1-ol | x | | | x | | x | | x | x | x | | | x | x |
| hexadecan-1-ol | x | x | | x | x | | | | x | | x | | x | x |
| 2-hexyldecan-1-ol | x | x | | x | x | | | | | | x | | x | x |
| nonan-1-ol | x | x | | | | x | | | | x | | x | x | |
| lauryl alcohol | x | x | | | x | | | | | x | | | x | |
| carbitol | | | x | x | | x | x | | | | | | | x |
| **Aldehydes** | | | | | | | | | | | | | | |
| decanal | x | x | x | x | x | x | x | x | | x | x | x | x | x |
| dodecanal | x | x | x | x | x | x | x | x | | | | | x | |
| benzaldehyde | x | x | | | | x | x | | x | x | | | | |
| nonanal | | | | | | x | x | | x | | | x | x | |
| **Terpenes** | | | | | | | | | | | | | | |
| linalool | x | x | x | x | x | | x | | | x | x | x | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| limonene | | x | x | | x | | | | x | | | x | x | x |
| camphene | x | x | x | | x | x | | | | | x | | x | |
| *α*-terpinolene | x | x | | | x | x | x | | | | | | | |
| **Acetals** | | | | | | | | | | | | | | |
| acetaldehyde ethyl amyl acetal | x | x | x | x | x | x | x | x | | | x | x | x | |
| isovaleraldehyde diethyl acetal | x | x | x | x | | x | x | | x | | x | x | | |
| hexanal diethyl acetal | x | x | x | x | | | | | x | x | | x | x | |
| **Acids** | | | | | | | | | | | | | | |
| myristic acid | x | x | | x | x | | x | | | | | | x | |
| oleic acid | x | x | | x | x | | | | | | | | x | |
| **Others** | | | | | | | | | | | | | | |
| caprylic ether | x | x | | x | x | x | x | x | x | x | x | x | x | x |

According to Hayashi et al. (1996), more than 200 compounds from oils of three different mistletoes were identified. Their study has shown that even though they belong to the *Loranthaceae* family their volatile concentrates are different. They have also shown that mistletoes contained some compounds possibly characteristic of their host tree. Although their research was done with oils and different methods were used and column on GC/MS, some compounds which were detected are the same as compounds in this work. The major compounds in their research were hexanoic acid, octanoic acid and decanoic acid, but also found in the spirt. In addition, some terpenoids, such as eucalyptol, linalool, *p*-cymene, *α*-humulene, camphor, nerol and farnesol were also detected. The biological potential of the identified compounds is worth being taken into consideration for detailed studies.

Based on the similarity of the identified compounds with previous studies, it can be concluded that the mistletoe spirit obtained by maceration of stems and leaves of mistletoe is enriched by many value compounds that give healing properties to the mistletoe spirit.

This work reports, for the first time, the aroma profile of mistletoe spirit and also describes compounds that are extracted and pass into the alcohol base during the maceration. Such identification can potentially be used to guarantee beverage quality. The principal aroma compounds identified in this work that are responsible for the characteristic aroma of mistletoe spirit, are ethyl esters of hexanoic, octanoic, decanoic, dodecanoic and hexadecanoic acids, limonene and 2-phenylethanol. It was shown that mistletoe spirit samples can have varied aromatic profile because of many factors, such as, the species of the host tree and growing conditions, the time of harvest of the mistletoe, the manufacturing proceses etc. It was also

shown that spirits could differ in number of detected compounds. Moreover, in samples were also detected compounds, which even at low concentrations, could have sensory impact. Some of those compounds are linalool, $\alpha$-terpinolene, $\beta$-bourbonene, camphene, L-menthyl acetate, farnesol, carbitol, nerol etc. The present work will be useful for further research on the mistletoe spirit. Further investigations are required to reveal the other volatile compounds that contribute to the aroma in mistletoe spirit.

### 4.4. pH ASSAY

The results show that in mistletoes spirit samples the pH ranged from 4.4 to 6.5, averaging 5.2. The lowest pH was measured in samples 2 and 7 and the highest pH was measured in samples 9 and 14 (Figure 31). When an acid is dissolved in water, the pH will be less than 7. When a base, or alkali, is dissolved in water, the pH will be greater than 7. All samples have pH lower than 7. Samples 2 and 7 have the most acids compared to other samples. Samples 9 and 14 have the highest measured pH, which is almost neutral. Those samples were produced by mistletoe maceration in ethyl alcohol of agricultural origin. However, the technical definitions and requirements in the regulation of European parliament and of the council of the European Union, demand that total acidity in ethyl alcohol of agricultural origin, expressed in grams of acetic acid per hectoliter of 100 % vol. alcohol is 1.5. Other samples were produced from the grape marc brandy, or other type of brandies, which contain more acids. On top of that, honey, which is added in the mistletoe spirit, also could have an impact on the pH of the spirit. The average pH of honey is 3.9, because it contains a number of acids. Those mistletoe spirits that have pH 6.5 were obtained by the maceration in ethyl alcohol of agricultural origin, and those that have lower pH than 6.5 were obtained by the maceration in distillates made from various raw materials. Based on this, pH can serve for quick detection whether mistletoe spirit was obtained by the maceration in ethyl alcohol of agricultural origin or in distillate made from various raw materials.

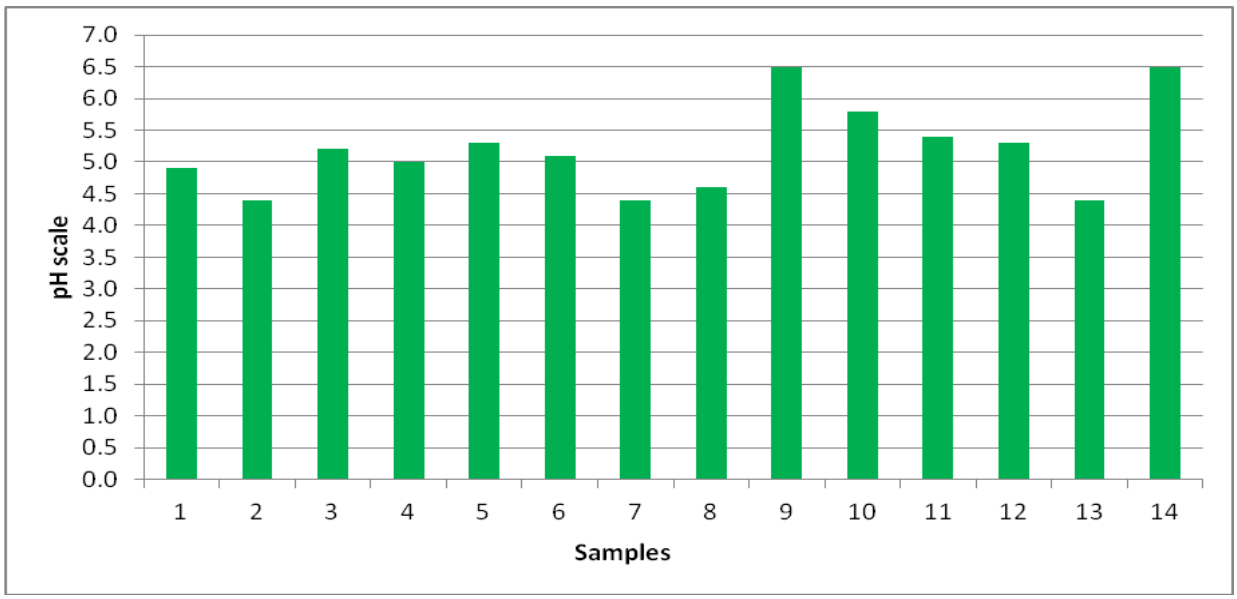


**Figure 31.** pH assay of mistletoe spirit samples (1-14)

### 4.5. Statistical analysis

4.5.1. PCA of TPC, DPPH and FRAP

Principle Component Analysis (PCA) was conducted by use of the the MS Office Excel tool- XLStat, to visualize total polyphenol content (TPC), DPPH and FRAP measured values vs. samples. PCA analysis is presented as a biplot that is an enhanced scatter plot also presenting the cases and variables in one. Red are the vectors presenting following variables: TPC, DPPH and FRAP values and the cases are presented as blue dots representing mistletoe spirit samples (Figure 32).

The observed data set presented the input data matrix consisted of 14 spirits and 3 different methods (observed variables). For those data set was the, sum of all observed variations in the data set 99.10 %, where the first principal component contributes with 92.55 % and the second one with 6.55 %, respectively (Figure 32). All three variables are far from the center. The variable, which represents FRAP is closer to the variable, which represents TPC than to the variable, which represents DPPH. This means that TPC and FRAP are significantly more correlated. In the shown biplot observed cases have larger squared cosines in the first factor (F1) than in the second factor (F2) and they are spread along the PC1. Sample 6 (34.51 %) and sample 1 (16.56 %) are the closest to the vector of variable that represents FRAP (35.38 %), which means that those samples have higher values for FRAP compared to other samples. Generally, samples could be divided in two groups. In the group one are samples 1, 2, 3, 6, 11 and 12. In the

group two are samples 5, 7, 8, 9, 10, 13, 14. Samples from the group one are closer to vectors. This suggests that measured values of TPC, DPPH and FRAP of samples from the group one are higher than values measured in samples from the group two. It is interesting that all samples that are produced in industrial way are in the group two and have lower values for TPC, DPPH and FRAP compared to homemade samples from the group one. Samples 3, 11 and 12 are close to each other, which means that they have similar measured values for TPC, DPPH and FRAP. Sample 2 is further from samples 3, 11 and 12 and further from the center, suggesting that has higher measured values than those three samples.

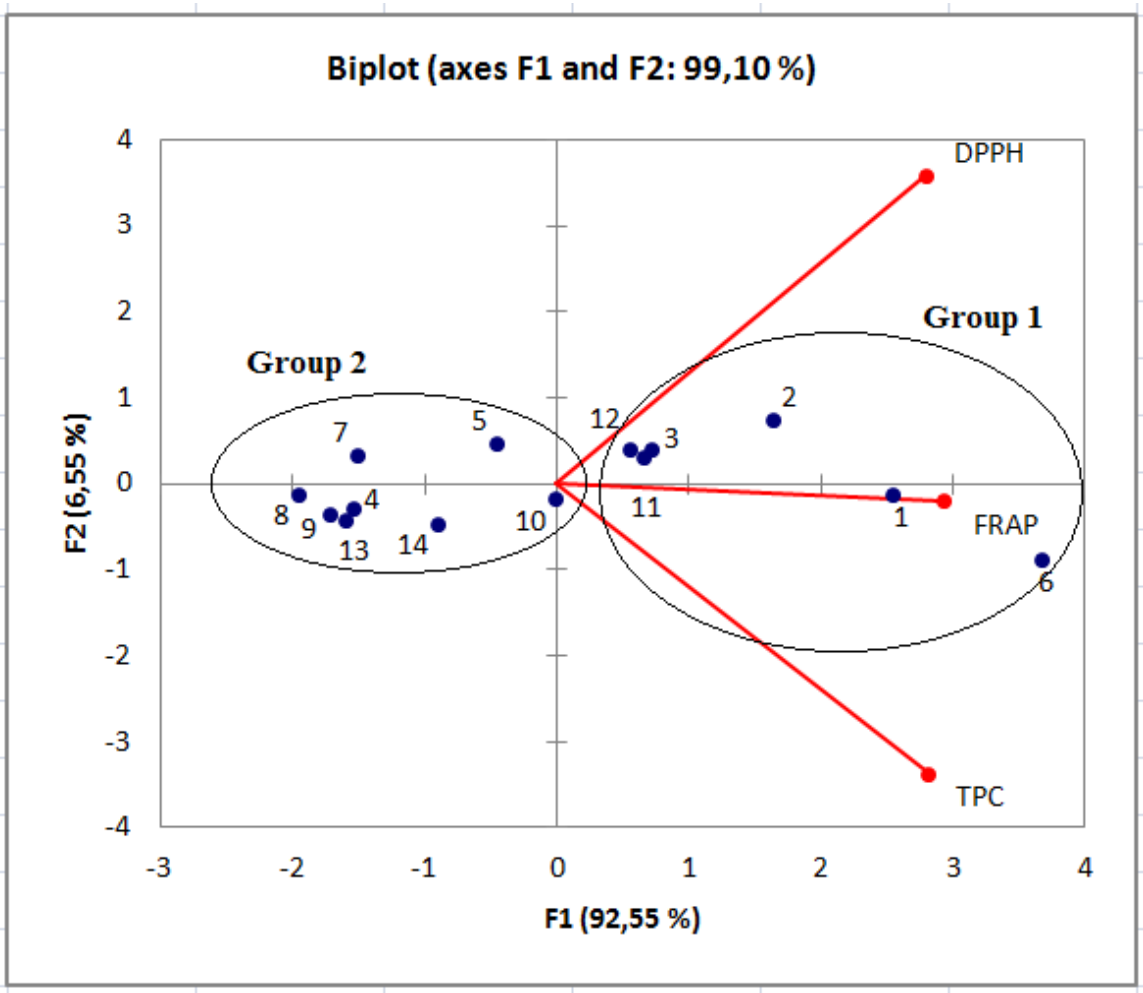


**Figure 32.** A biplot representation of total polyphenol content (TPC), DPPH and FRAP measured values vs. mistletoe spirit samples (1-14) according to the Principal component analysis (PCA)

4.5.2. PCA of aroma compounds

Statistical analysis was conducted by use of the the MS Office Excel tool- XLStat, with aroma compounds that were detected in most samples. Principal component analysis (PCA) was used to visualize sample compound distributions vs. samples. PCA analysis is presented as a biplot that is an enhanced scatter plot that uses both points and vectors to represent structure. A biplot uses points to represent the scores of the observations on the principal components, and it uses vectors to represent the coefficients of the variables on the principal components (Young, 1999). Red vectors of variables represent aroma compounds and the observations are blue dots representing mistletoe spirit samples (Figure 33).

The direction and placement shows, higher squared multiple correlation with the principal components. The length of the vector is proportional to the squared multiple correlation between the fitted values for the variable and the variable itself (Young, 1999). For the observed data set consisted of 14 spirits and 16 different aroma compounds, the sum of all observed variations in the data set is 53.38 %, where the first principal component contributes with 33.67 % and the second one with 19.7 %, respectively (Figure 24). In the shown biplot compounds hexadecanol, ethyl pentadecanoate, tetradecanol, ethyl dodecanoate, decanal and 1-octoxyoctane have the largest squared cosines in the first factor (F1) and presented as the first principal component, PC1. On the other hand, compounds ethyl hexadecanoate, isopropyl myristate and isoamyl acetate have higher values of squared cosines in the second factor (F2) and are presented as the second principal component, PC2. The biplot of the principal component analysis shows that vectors of variables, which are not close to each other, are not correlated. Those vectors that point in the same direction correspond to variables that have similar response profiles, and can be interpreted as those having similar meaning in the context set by the data (Young, 1999). That means that hexadecanol and tetradecanol have similar response profile, but they are not correlated with ethyl dodecanoate and ethyl pentadecanoate. Also, ethyl hexadecanoate and isopropyl myristate have similar response profile and are not correlated with isovaleraldehyde diethyl acetal and isoamyl acetate.

Those parameters that are closer to an observed vector have higher values of the observed parameter. The contribution of the values for observed parameters as well as for samples shows the percentage of their contribution in the first or second factor of the biplot. That means that for sample 9 (73.42 %), which is the furthest from the center and the closest to compounds 2-ethyldecan-1-ol (8,93 %), hexadecanol (13.23 %) and tetradecanol (15.1 %), this compounds are

more characteristic than for other samples. Sample 12 (19.4 %) is the furthest from the center in the first quadrant, being close to decanal, which means that this compound is the most characteristic for this sample. Moreover, the same sample (12) compared to other samples is in the same quadrant to compounds ethyl pentadecanoate, isopropyl myristate and ethyl hexadecanoate, which means that this compounds are significantly represented in sample 12 and more typical for this sample than for other samples. Those samples that are suited far from a vector that represents an observed compound, have the lower contents of that compound. This means that sample 9 compared to other samples has the lowest content of the ethyl dodecanoate, ethyl pentadecanoate and decanal. This claim is supported by the fact that those compounds were not even detected in sample 9. Also, sample 12 is the furthest from compounds 2-ethyldecan-1-ol, hexadecanol and tetradecanol, which means that those compounds are very low or even not present. This is exactly the case, these compounds were not detected in sample 12. Those projecting in the middle have an average amount of those compounds that are in the same quadrant as they take.

Other samples are not so far from each other and not so far from the center. This may suggest that any remaining compound is characteristic only for that sample. Comparing two samples that are close to a vector, that one, which is closer has that certain compound in larger amount compared to other sample. If some sample is far from a vector, it indicates that that certain compound is not present in a significant amount in that sample, or even not present (equal to 0).

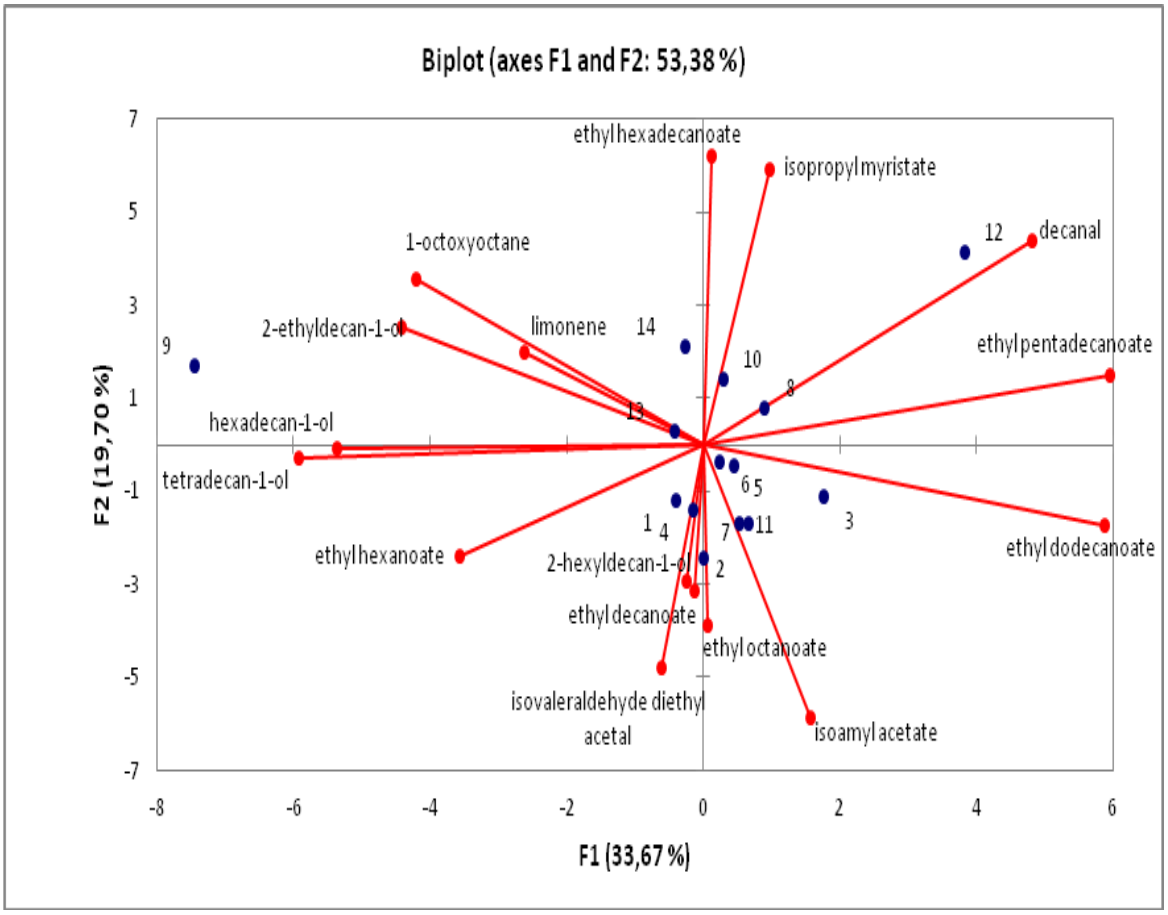


**Figure 33.** A biplot representation of the aroma compounds of mistletoe spirit samples (1-14) according to the principal component analysis

A PCA analysis, which was used to visualize sample compound distributions vs. samples, presented as a biplot can serve as a quick overview of typical aroma compounds for individual samples.

4.5.3. PLS predicting models

In this study the FTIR spectra were registered for 14 mistletoe samples in order to create calibration models to predict total polyphenol content (TPC) and total antioxidant capacities (DPPH and FRAP) of mistletoe spirits in unknown samples. The results obtained from the reference methods and those calculated from infrared spectra (FTIR) of the samples were used to establish the calibration ranges. PLS was used as a statistical tool to build calibration models. The statistical PLS approach used in this work relies on the linear combination of FTIR spectral variables, so-called factors. A factor is a set of components that contains spectral and concentration information and is used to describe the variation in a PLS method model. The

software used automatically selects the number of components/factors for the quantitative analysis. Values of $R^2$ closer to 1 mean a higher probability that the FTIR predicted value (y-axis) is related to the measured parameters (x-axis). Models with values of the determination coefficients higher than 0.8 were considered as acceptable (Silva et al., 2014) because they present good relation between the input and output parameters. Table 4 summarizes the results obtained for the referred parameters.

**Table 4.** Calibration results for implemented FTIR models

| Parameter | Calibration range | Determination coefficient ($R^2$) |
|---|---|---|
| TPC (GAE mg $L^{-1}$) | 1712-1704, 1609-1608, 1519-1516, 1207, 1110-1107, 1067-1062 | 0.803 |
| DPPH inhibition (%) | 1712-1704, 1609-1608, 1519-1516, 1207, 1110-1107, 1067-1062 | 0.934 |
| FRAP (mM Trolox) | 1712-1704, 1609-1608, 1519-1516, 1207, 1110-1107, 1067-1062 | 0.944 |

Obtained coefficients of determination were greater than 0.8 for total polyphenol content (TPC), DPPH and FRAP, suggesting strong correlation of these parameters with the calibration models implemented. Figures 34- 36 show constructed linear predictive models for the component amounts for TPC, DPPH and FRAP based on the FTIR spectrum.

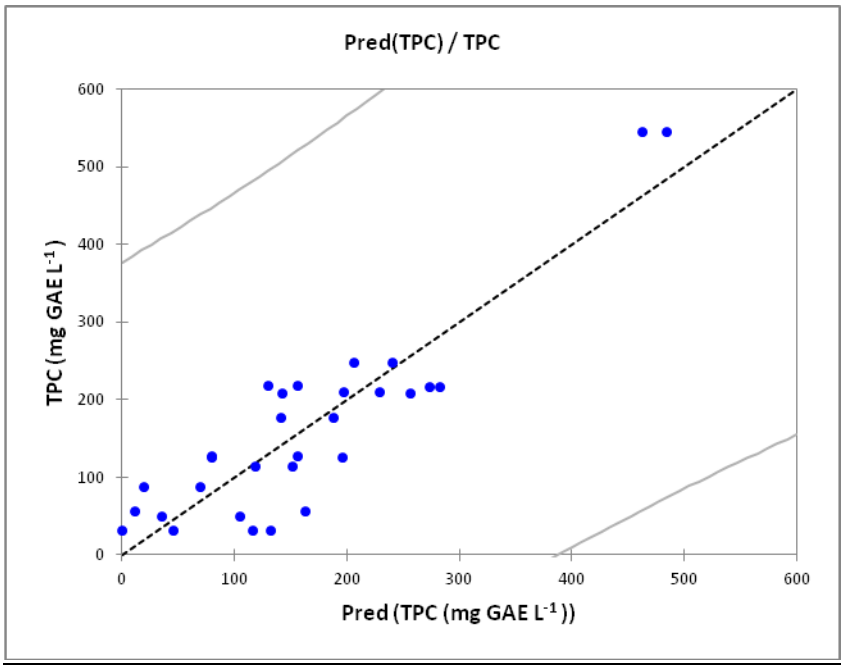


**Figure 34**. PLS predicting model for TPC

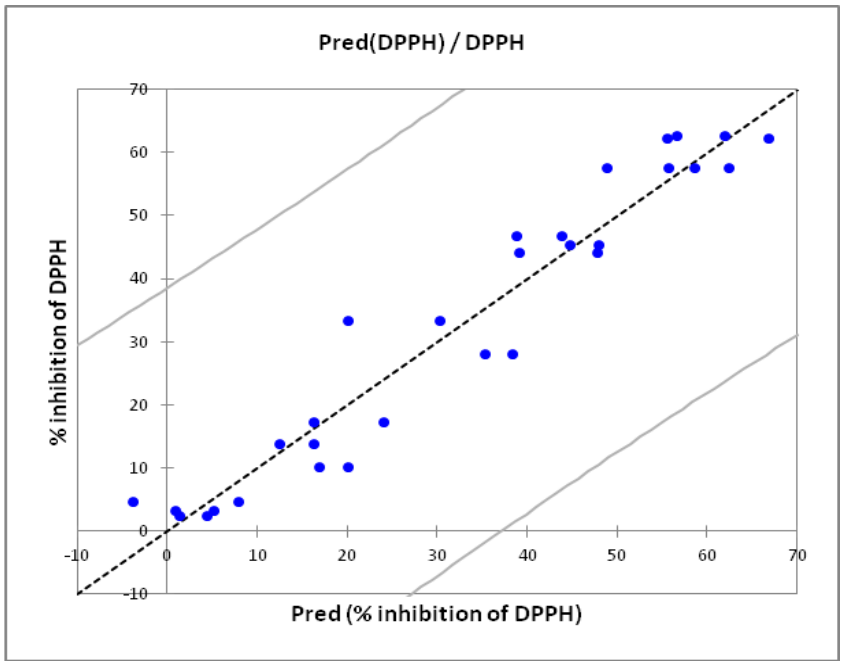


**Figure 35**. PLS predicting model for DPPH

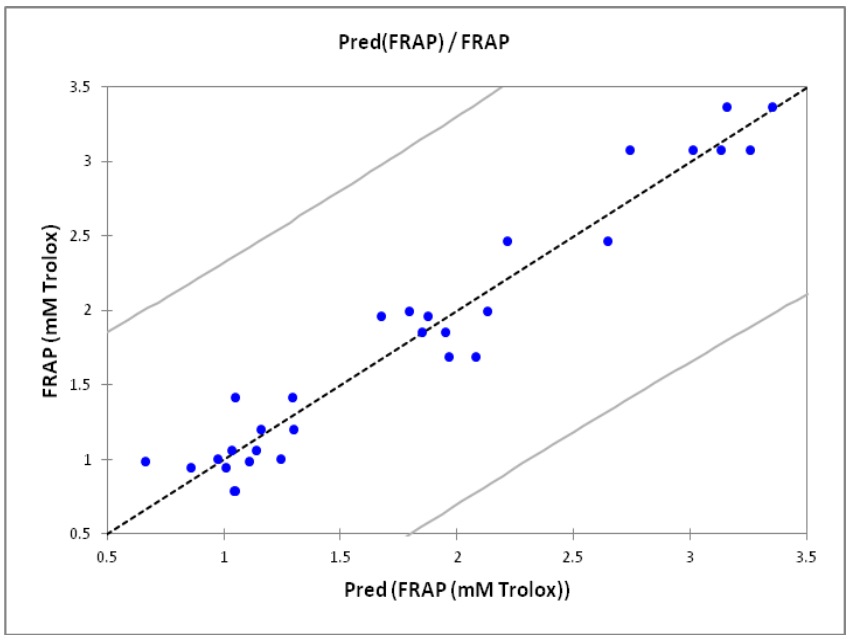


**Figure 36**. PLS predicting model for FRAP

These preliminary results show that FTIR can be a useful tool for rapid screening of total polyphenol content and antioxidant activities in mistletoe spirits. The implemented methodologies may also be used to get rough estimates for total polyphenol content (TPC), DPPH and FRAP antioxidant activities. Further validation of the implemented models should include a larger number of mistletoe spirit samples from different producers and known origin. FTIR-spectroscopy in combination with chemometric data evaluation provides valuable information even for highly complex problems such as spirit analysis. PLS models were successfully developed for the prediction of these parameters: TPC, DPPH and FRAP. Therefore, the proposed method can already be seen as interesting possibility for screening and quality control purposes.

# 5. CONCLUSIONS

1. There are differences in total polyphenol content (TPC), DPPH and FRAP antioxidant activities among the examined mistletoe spirit samples. Among the samples examined, the best results for TPC were obtained for sample 6 (545 mg GAE $L^{-1}$) and sample 1 (373 mg GAE $L^{-1}$). The best results for DPPH were obtained for sample 6 (63 % inhibition of DPPH) and sample 2 (62 % inhibition of DPPH). The best results for FRAP were obtained for sample 6 (3.4 mM Trolox) and sample 1 (3.1 mM Trolox). Because of high polyphenol content and antioxidant activity, mistletoe spirit should have the precedence when choosing strong alcoholic beverage in order to have benefits for health.

2. Results show moderate correlation between DPPH and TPC and strong correlation between FRAP and TPC.

3. FTIR spectra analysis confirmed that mistletoe spirit samples contain phenolic molecules and that this method can serve to identify the molecular structure.

4. A total number of 166 aroma compounds in mistletoe spirit samples were detected, from which 53 were esters, 32 alcohols, 25 terpenes, 18 carbonyl compounds, such as aldehydes and ketones, 12 alkanes, 9 acetals, 8 acids and 9 other compounds. The principal aroma compounds that are responsible for the characteristic aroma of mistletoe spirits that were detected are: ethyl esters of hexanoic, octanoic, decanoic, dodecanoic and hexadecanoic acids, D-limonene and 2-phenylethanol. In samples were also detected compounds, which even at low concentrations could have sensory impact. Some of those compounds are linalool, $\alpha$-terpinolene, $\beta$-bourbonene, camphene, L-menthyl acetate, farnesol, carbitol and nerol.

5. pH can serve for quick detection whether mistletoe spirit was obtained by the maceration in ethyl alcohol of agricultural origin or in distillate made from various raw materials.

6. The goals of PCA have been achieved and the most important information from the data table was extracted and compressed. Biplots were created to visualize total polyphenol content (TPC), DPPH and FRAP measured values vs. samples and sample compound distributions vs. samples. PCA analysis also showed that TPC and FRAP are significantly more correlated than TPC and

DPPH. A PCA analysis, which was used to visualize sample compound distributions vs. samples, presented as a biplot can serve as a quick overview of typical aroma compounds for individual samples.

7. FTIR-spectroscopy in combination with chemometric data evaluation provides valuable information even for highly complex problems such as spirit analysis. PLS models were successfully developed. These preliminary results show that FTIR can be a useful tool for rapid screening of total polyphenol content (TPC), DPPH and FRAP antioxidant activities in mistletoe spirits. Therefore, the proposed method can already be seen as interesting possibility for screening and quality control purposes. Further validation of the implemented models should include a larger number of mistletoe spirit samples from different producers and with known origin.

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# 7. ANNEX

**Annex 1.** All aroma compounds that have been identified in mistletoe spirit samples (1-14) by GC/MS

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ESTERS** | | | | | | | | | | | | | | |
| isoamyl acetate | x | x | x | x | | x | x | x | | | x | x | x | |
| hexyl formate | | | | | | | | | | | | x | | |
| ethyl hexanoate | x | x | x | x | | x | x | x | x | x | x | x | x | x |
| hexyl acetate | | | | | | | | | | x | | x | x | |
| ethyl (E)-hex-2-enoate | | | | | | | | | | | | x | | |
| isoamyl butyrate | | | | | | | | | | | | x | x | |
| ethyl heptanoate | x | x | x | x | x | x | | | | | x | x | | |
| amyl valerate | | | | | | | | | | | | x | | |
| methyl octanoate | x | x | x | x | x | | | | | | x | x | | |
| isobutyl caproate | x | | x | | | | | | | | | x | | |
| ethyl benzoate | | | | | | | | | | x | | x | | x |
| diethyl butanedioate | x | x | x | x | | x | x | x | | | x | x | x | |
| ethyl oct-4-enoate | | | | | | | | | | | | x | x | |
| ethyl octanoate | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| isopentyl hexanoate | x | | | x | x | x | x | | | | | x | | |
| 2-methylbutyl hexanoate | | | | | | | | | | | | x | | |
| propyl octanoate | | | | | | | | | | | | x | | |
| ethyl nonanoate | x | x | x | x | x | x | | x | | | x | x | | |
| methyl hexanoate | x | x | x | x | x | | x | | | | x | x | | |
| isobutyl caprylate | x | | x | x | x | x | x | | | | | x | | |
| ethyl dec-9-enoate | x | x | x | x | | x | x | | | | | x | | |
| ethyl decanoate | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| 3-methylbutyl octanoate | x | x | x | x | x | x | x | x | | x | x | x | | |
| isobutyl caprate | x | x | | x | x | | x | | | | x | x | | |
| ethyl dodecanoate | x | x | x | x | x | x | x | x | | x | x | x | x | x |
| 3-methylbutyl pentadecanoate | | | x | | | | x | | | | | x | | |
| ethyl pentadecanoate | x | x | x | x | x | x | x | x | | x | x | x | x | x |
| isopropyl myristate | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| ethyl palmitate | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| isopropyl palmitate | | | | | | | | | x | | | x | | |
| octadecyl 2-ethylhexanoate | | | | | | | | | | | | x | | |
| linalyl acetate | | x | | | | | | | | | | | | |
| caprylyl acetate | | x | | | | | | | | | | | | |
| ethyl undecanoate | | x | | | x | | x | | | | | | | |

**Extension of annex 1.** All aroma compounds that have been identified in mistletoe spirit samples (1-14) by GC/MS

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3-methylbutyl decanoate | x | x | | x | x | x | | | | | x | | | |
| methyl linoleate | | x | | x | | x | | | | | | | | |
| methyl oleate | x | x | | x | x | x | | | x | | | | | |
| methyl hexadecanoate | | x | | x | x | x | | | | | | | | |
| methyl 14-(2-octylcyclopropyl)tetradecanoate | | | | | | | | | | | | | x | |
| ethyl isopentyl succinate | x | | | | | | | | | | | | | |
| methyl palmitate | x | | | | | | | | | | | | | |
| ethyl (Z)-4-octenoate | | | | x | | | | | | | | | | |
| tetradecan-2-yl acetate | | | | x | | | | | | | | | | |
| ethyl 4-methylpentanoate | | | | | x | | | | | | | | | |
| fenchyl acetate | | | | | x | | | | | | | | | |
| cyclopentyl 4-ethylbenzoate | | | | | | | | | x | | | | | |
| ethyl 2-hydroxyisovalerate | | | | | | | | | x | | | | | x |
| L-menthyl acetate | | | | | | | | | x | | | | | x |
| melon nonenoate | | | | | | | | | | x | | | | |
| ethyl (2E,4E)-deca-2,4-dienoate | | | | | | | | | | x | | | | |
| ethyl nonadecanoate | | | | | | | | | | x | | | x | |
| methyl linolenate | | | | | | | | | | x | | | x | |
| *α*-glyceryl linolenate | | | | | | | | | | x | | | x | |
| **ALCOHOLS** | | | | | | | | | | | | | | |
| octan-1-ol | x | x | | | | x | x | | | | | x | | |
| 2-phenyl ethanol | x | | x | x | x | x | x | x | x | | x | x | x | |
| nonan-1-ol | x | x | | | | x | | | | x | | x | x | |
| trans-*p*-mentha-2,8-dienol | | | | | | | | | | | | x | | |
| 4-methylnonan-4-ol | | | | | | | | | | | | x | | |
| heptatriacontan-1-ol | | | | | | | | | | | | x | | |
| eucalyptol | | x | | | | | | | | | | | | |
| 4-thujanol | | x | | | | | x | | | | | | | |
| decan-1-ol | | x | | | x | | x | | | | | | | |
| nerol | | x | | | x | | | | | x | | | | |
| estragole | x | x | | | x | | | | | | | | | |
| lauryl alcohol | x | x | | | x | | | | | x | | | x | |
| tetradecan-1-ol | x | x | | x | x | x | x | | x | x | x | | x | x |
| methyldiethyl carbinol | | x | | x | x | | | | | | | | | |
| hexadecan-1-ol | x | x | | x | x | | | | x | | x | | x | x |
| 2-hexyldecan-1-ol | x | x | | x | x | | | | | | x | | x | x |

**Extension of annex 1.** All aroma compounds that have been identified in mistletoe spirit samples (1-14) by GC/MS

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1,2,3,4,5-tetradecanepentol | | | | | | | | | | | | | x | |
| tridecan-1-ol | | | | | | | | | | | | | x | |
| hexan-1-ol | x | | | | | x | | | | | | | | |
| 2-ethyldecan-1-ol | x | | | x | | x | | x | x | x | | | x | x |
| dec-3-yn-2-ol | | | | x | | | x | | | | | | | |
| seudenol | | | | | x | | | | | | | | | |
| farnesol | | | | | x | | | | | | | | | |
| sulcatol | | | | | | | x | | | | | | | |
| nopol | | | x | | | | | | | | | | | |
| carbitol | | | x | x | | x | x | | | | | | | x |
| norborneol | | | x | | | | | | | | | | | |
| (E)-prop-1-ene-1-thiol | | | | | | | | x | | | | | | |
| 2-ethylhexan-1-ol | | | | | | | | | | x | | | | |
| 3- thujanol | | | | | | | | | | x | | | | |
| 2-ethyl-2-propylhexan-1-ol | | | | | | | | | | x | | | | |
| **ACIDS** | | | | | | | | | | | | | | |
| myristic acid | x | x | | x | x | | x | | | | | | x | |
| octadecanoic acid | | x | | | | | | | | | x | | x | |
| oleic acid | x | x | | x | x | | | | | | | | x | |
| myrmicacin | | x | | x | | | | | | | | | x | |
| hexadecanoic acid | x | x | | x | | | | | | | | | | |
| linolelaidic acid | x | | | x | | | | | | | | | | |
| docosa-10,12-diynedioic acid | | | | | | | | | | | | | | |
| 2-nonenoic acid | | | | | | | | | | x | | | | |
| **TERPENES** | | | | | | | | | | | | | | |
| *p*-cymene | | | | x | x | | | | x | | | x | | |
| L-limonene | | | | | | | | | x | | | x | | |
| linalool | x | x | x | x | x | | x | | | x | x | x | | |
| linalool oxide | | | x | | | | | | | | | | | |
| 1,2-dihydro-1,5,8-trimethylnaphthalene | | | | | | | | | | | | x | | |
| $\alpha$-terpinolene | x | x | | | x | x | x | | | | | | | |
| ylangene | | | x | | | | | | | | | x | | |
| $\alpha$-copaene | | | | | | | | | | | x | x | | |
| $\beta$-bourbonene | | | x | | | | x | | | | x | x | | |
| trans-caryophyllene | | | x | | | | | | | | | x | | |
| $\alpha$-guaiene | | | | | | | | | | | | x | | |

**Extension of annex 1.** All aroma compounds that have been identified in mistletoe spirit samples (1-14) by GC/MS

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *α*-muurolene | | | x | | | | | | | | x | x | | |
| alloaromadendrene | | | | | | | | | | | | x | | |
| *α* -humulene | | | | | | | | | | | | x | | |
| cedrene | | | | | | | | | | | | x | | |
| *β*-selinene | | | | | | | | | | | | x | | |
| ɗ-cadinene | | | | | | | | | | | | x | | |
| D-germacerene | | | | | | | | | | | | x | | |
| α-panasinsen | | | | | | | | | | | | x | | |
| *α*-calacorene | | | | | | | | | | | | x | | |
| D-limonene | | x | x | | x | | | | | | | | x | x |
| camphor | | x | | | | | | | | | | | | |
| camphene | x | x | x | | x | x | | | | | x | | x | |
| *β*-elemene | | | x | | | | | | | | | | | |
| D-germacrene | | | x | | | | | | | | | | | |
| **ACETALS** | | | | | | | | | | | | | | |
| isobutyraldehyde diethyl acetal | | | | | | | | | | | | x | | |
| isovaleraldehyde diethyl acetal | x | x | x | x | | x | x | | x | | x | x | | |
| acetaldehyde ethyl amyl acetal | x | x | x | x | x | x | x | x | | | x | x | x | |
| pentanal diethyl acetal | | | | | | | | | | | | x | | |
| hexanal diethyl acetal | x | x | x | x | | | | | x | x | | x | x | |
| octanal diethyl acetal | x | | | | | | x | | | | | x | | |
| phenacetaldehyde dimethyl acetal | x | | | | | x | | | | | | | | |
| heptaldehyde diethyl acetal | | | x | | | | | | | | | | | |
| hexadecanal cyclic ethylene acetal | | | | | | | | x | | | | | | |
| **ALDEHYDES** | | | | | | | | | | | | | | |
| nonanal | | | | | | x | x | | x | | | x | x | |
| decanal | x | x | x | x | x | x | x | | x | x | x | x | x | x |
| benzaldehyde | x | x | | | | x | x | | x | x | | | | |
| (E)-non-2-enal | | x | | | | | | | | | | | | |
| tetradecanal | | | | | | | | | | | | | x | |
| methoxycitronellal | | x | | | x | | | | | | | | x | |
| hexadecanal | x | | | x | x | | | | | | | | x | |
| octadecanal | | | | | x | | | | | | | | | |
| hexadecanal | x | | | x | x | | | | | | | | x | |
| cocoa pentenal | | | | | | | x | | | | | | | |
| dodecanal | x | x | x | x | x | x | x | x | | | | | x | |

**Extension of annex 1.** All aroma compounds that have been identified in mistletoe spirit samples (1-14) by GC/MS

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| marine decadienal | | | | | | | | | | x | | | | |
| **KETONES** | | | | | | | | | | | | | | |
| 1-(3,5-ditert-butyl-4-hydroxyphenyl)propan-1-one | | | | | | | | | x | | | x | | |
| 3-thujanone | | x | | | | | | | | | | | | |
| 6-ethoxy-6-methylcyclohex-2-en-1-one | | | | x | | | | | | | | | | |
| 4-Methyl-4-phenyl-2,3:5,6-diepoxycyclohexanone | | | | | | | x | | | | | | | |
| (+)-carvotan acetone | | | | | | | | x | | | | | | |
| *β*-thujone | | | | | | | | | x | | | | | |
| **ALKANES** | | | | | | | | | | | | | | |
| vitispirane | | | | | | | | | | | | x | | |
| tetradecane | | | | | | | | | | | x | x | | |
| 2-cyclopropylidene-1,7,7-trimethylbicyclo[2.2.1]heptane | | | | | | | | | | | | x | | |
| 2-methyl-5-propylnonane | | | | | | | | | | | | x | | |
| 1-chlorotetradecane | | | | | | | | | | | | x | | |
| 2,3-dimethoxy-2-methylbutane | | | | | | | | | | | | | x | |
| hexadecane | x | | | | x | | | | | | | | | |
| octadecane | x | | | | | | | | | | | | | |
| spiro[4.5]decane | | | | x | | | x | | | | | | | |
| patchoulane | | | | | | | x | | | | | | | |
| 4,6-dimethyldodecane | | | x | | | | | | | | | | | |
| 2-methoxy-2,3,3-trimethylbutane | | | | | | | | | | x | | | | |
| **OTHERS** | | | | | | | | | | | | | | |
| caprylic ether | x | x | | x | x | x | x | x | x | x | x | x | x | x |
| Naphthalene, 1,6-dimethyl-4-(1-methylethyl)- | | | | | | | | | | | | x | | |
| azidomethylbenzene | | | | | | | | | | | x | | | |
| 1,3-dimethyl-8-(1-methyl ethyl) tricyclo(4.4.0.0.02,7-)dec-3-ene | | | | | | | | | | | x | | | |
| 1-butanamine | | | | | | | | | | | | | x | |
| methyl vinyl sulfide | | | | | | | | | | | | | x | |
| (1Z)-4-methylen-6-(1-propen-1-yliden)cycloocten | | | | | | | x | | | | | | | |
| 7,7-dimethyl-2-propan-2-yl-5-prop-1-en-2-ylbicyclo[4.1.0]hept-3-ene | | | | | | | x | | | | | | | |
| styrene | | | x | | | | | | | | | | | |