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Department of Analytical Chemistry

Studijní program: Farmacie

DIPLOMOVÁ PRÁCE
MASTER'S THESIS

Chemiluminiscenční hodnocení celkové antioxidační kapacity potravin

Chemiluminescent evaluation of total antioxidant capacity in foods

Hradec Králové 2014 Jitka Vlčková

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Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

Jitka Vlčková

CONTENT

SOUHRN

Práce se zabývá měřením a hodnocením celkové antioxidační kapacity (TAC) ve vzorcích červeného vína ošetřených čtyřmi čeřícími činidly (třemi různými typy želatiny a polyvinylpolypyrrolidonem) v koncentracích 10, 20 a 40 g/hL. Hlavním cílem bylo zjistit, jak čeřidla v různých dávkách ovlivňují TAC. Druhá část se zabývá vztahy mezi změnami TAC a obsahů jednotlivých antioxidačních komponent ve vzorcích (celkového množství polyfenolů, antokyanů, kyseliny gallové a nízkomolekulárních fenolů). Celková antioxidační kapacita byla hodnocena metodou TEAC (Trolox equivalent antioxidant capacity assay).

Teoretická část diplomové práce stručně shrnuje základy o luminescenci, zaměřené na chemiluminiscenční reakce. Teorie volných radikálů a antioxidanty, zejména ty, které jsou zahrnuty v červeném víně, jsou také popsány. Proces vinařství, především čiření je také obsažen v této části.

Experimentální část popisuje metody stanovení celkové antioxidační kapacity, princip metody CL (chemiluminescent) assay založené na systému luminol/křenová peroxidáza.

Výsledky ukazují že rozdílné chování polyvinylpolypyrolidonu (PVPP) oproti želatině. Zatímco želatina snižuje TAC, PVPP má tendenci působit opačně. Z hlediska koncentrace se ukázalo, že rozdíly v TAC mezi vzorky obsahujícími různé množství čeřidel jsou minimální. Ve srovnání celkové antioxidační kapacity s obsahem jednotlivých antioxidačních komponent ve vzorcích se ukázala jistá spojitost TAC s celkovým obsahem polyfenolů (PPT). Vztahy mezi obsahem antokyanů, kyseliny gallové, nízkomolekulárních fenolů a TAC nebyly prokázány.

Z hlediska zachování celkové antioxidační kapacity vína, se PVPP ukázal jako dobré čeřidlo; i v koncentraci 40 g/hL byly zachovány hodnoty TAC a PPT.

ABSTRACT

An investigation presented is dealing with the measurement and evaluation of the total antioxidant capacity (TAC) in samples of red wine clarified with four fining agents (polyvinylpolypyrrolidone and three different types of gelatin). All wine samples were treated by concentrations of 10, 20 and 40 g/hL of finings The main aim was to discover how the particular agents in different dosages influence the TAC. The second part describes relations between changes in TAC in content of single antioxidant components (total polyphenols, anthocyanins, gallic acid and simple phenols). Total antioxidant capacity in red wine samples was evaluated using the Trolox equivalent antioxidant capacity assay (TEAC).

A theoretical part of the thesis briefly summarizes basics about luminescence, focused on chemiluminescent reaction. Theory of free radicals and antioxidants, especially those included in red wine are also described. Process of winemaking aimed at fining is also contained in this part.

An experimental part describes techniques of determination of the total antioxidant capacity, principle of chemiluminescent assay based on the luminol/peroxidase system.

The results show the different behaviour of gelatin from the polyvinylpolypyrrolidone (PVPP). In general gelatin tends to reduce TAC, whilst PVPP tends to act vice versa. The differences in TAC between samples containing different amounts of finings were insignificant. The comparison of TAC with amount of various antioxidant components in the samples showed that antioxidant capacity is related to the total polyphenol content. Relations between contents of anthocyanins, gallic acid, low-molecular phenols and TAC weren´t proved.

In terms to save the total antioxidant capacity in wine, PVPP was proved as a good treatment; even the concentration 40 g/hl retains the values of the TAC and the PPT.

1 Introduction

1.1 General aspects of Luminescence

1.1.1 Characteristics of luminescence

Luminescence is emission of light by certain materials when they are relatively cool. Luminescence may be seen in neon and fluorescent lamps; television, radar, and X-ray fluoroscope screens; organic substances such as luminol or the luciferins in fireflies and glowworms; certain pigments used in outdoor advertising; and also natural electrical phenomena such as aurora borealis. In all these phenomena, light emission does not result from the material being above room temperature, and so luminescence is often called cold light. The practical value of luminescent materials lies in their capacity to transform invisible forms of energy into visible light.

Luminescence emission occurs after an appropriate material has absorbed energy from a proper source (such as ultraviolet or X-ray radiation, electron beams, chemical reactions, and so on). The energy lifts the atoms of the material into an excited state, and then, because excited states are unstable, the material undergoes another transition, back to its unexcited ground state, and the absorbed energy is liberated in the form of either light or heat or both (all discrete energy states, including the ground state, of an atom are defined as quantum states). The excitation involves only the outermost electrons orbiting around the nuclei of the atoms. Luminescence efficiency depends on the degree of transformation of excitation energy into light, and there are relatively few materials that have sufficient luminescence efficiency to be of practical value. (1)

Many types of luminescence have been identified, designated by identifying the source of energy responsible for the production and emission of light (**Table 1**). (2)

type	source
chemiluminescence	chemical reaction
bioluminescence	enzymatic reaction
electroluminescence	electric current in ionized gas or semiconductor
triboluminescence	breaking of asymmetrical bonds in a crystal
photoluminescence	absorption of IR, visible or UV light
radioluminescence	radioactive material incorporated in phosphorus
sonoluminescence	sound waves in liquid
thermoluminescence	temperature or radiation

Table 1: Types of luminescence

1.1.2 Chemiluminescence

Chemiluminescence (CL) is the production of electromagnetic radiation (UV, visible, or IR) by a chemical reaction between at least two reagents in which an electronically excited intermediate or product is obtained and subsequently relaxes to the ground state with emission of a photon or by donating its energy to another molecule which then luminescences. CL emission can be characterized by the four parameters of **color**, **intensity**, **rate of production**, and **decay of intensity**, and the reaction conditions have a significant effect on the progress of the chemiluminescence. The intensity of light emission depends on the rate of the chemical reaction, the efficiency of production of the excited state, and the efficiency of light emission from the excited state. In recent years, CL has become a powerful analytical tool for sensitive and selective detection of chemical species. The **advantages** of CL for quantitative analytical applications include superior sensitivity, low detection limits, wide linear dynamic ranges, and speed of response,

but it has some **disadvantages** such as poor reproducibility and long observation times, although not all the reactions are slow. (3)

1.1.2.1 Principle of chemiluminescent reaction

In chemiluminescence the excitated energy level is generated by a chemical schema reported in **Fig. 1**.

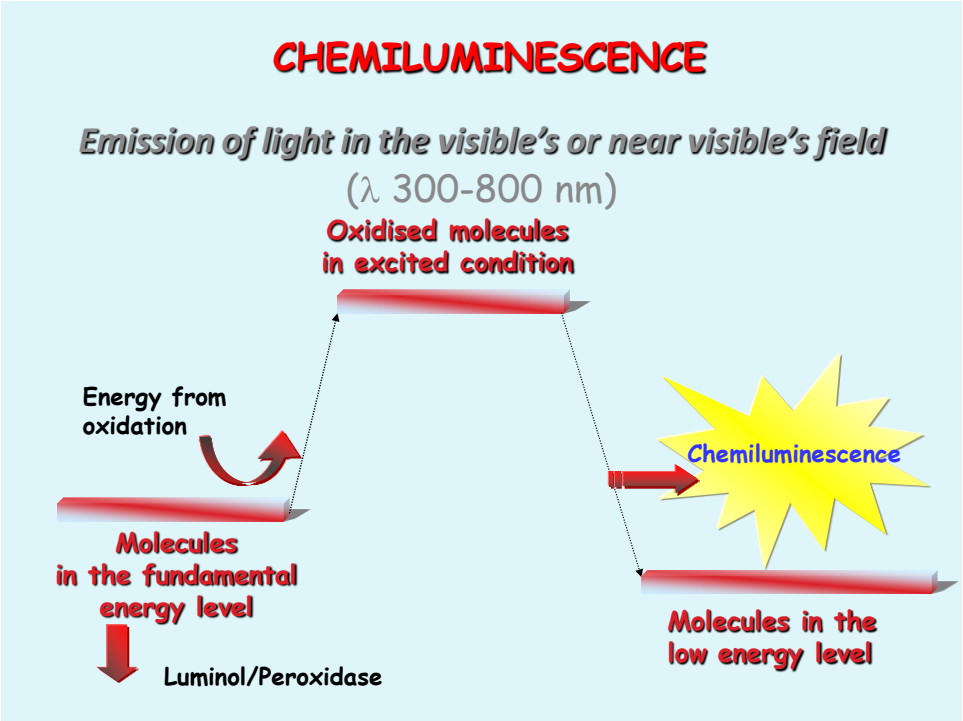

Fig. 1: Diagram of emissions of chemiluminescent system

The term Bioluminescence (BL) is outlining specific forms of luminescence that occurs in living organisms, in which the reactions producing emissions are catalyzed by enzymes. The bioluminescent systems most commonly used in the analysis are luciferase from fire-fly, glow-worm and bacterial luciferase. (4)

In case of bioluminescence the excited state is produced by an enzymatic reaction (**Fig. 2**).

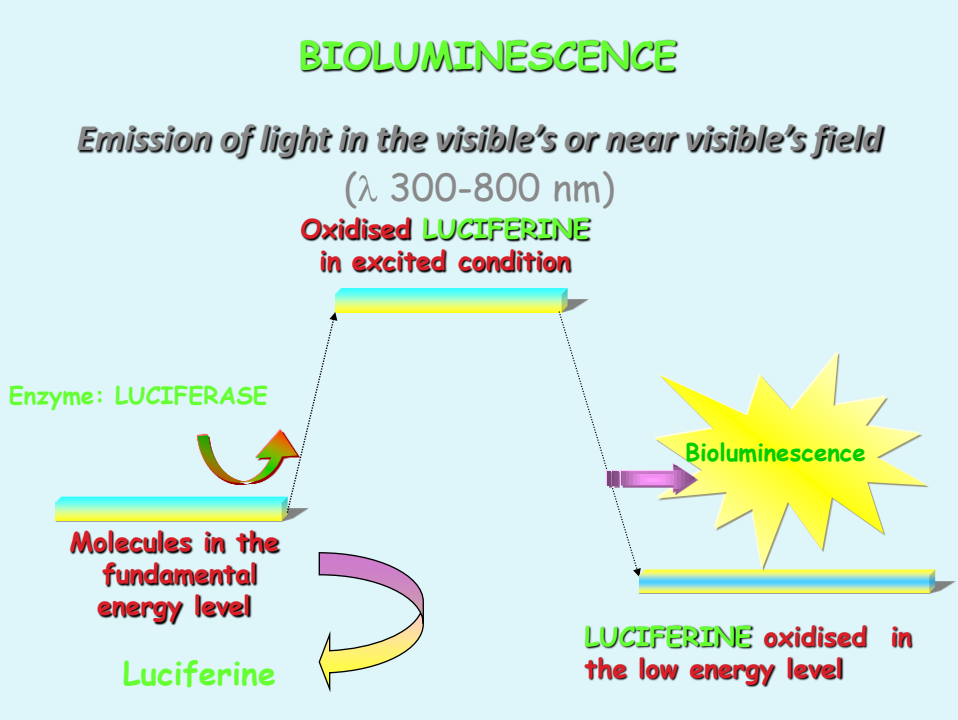

Fig. 2: Diagram of emissions of bioluminescent system

Chemiluminescent and bioluminescent reactions usually involve the cleavage or fragmentation of the O-O bond an organic peroxide compound. Peroxides, especially cyclic peroxides, are prevalent in light emitting reactions because the relatively weak peroxide bond is easily cleaved and the resulting molecular reorganization liberates a large amount of energy (**Fig. 3**). (3)

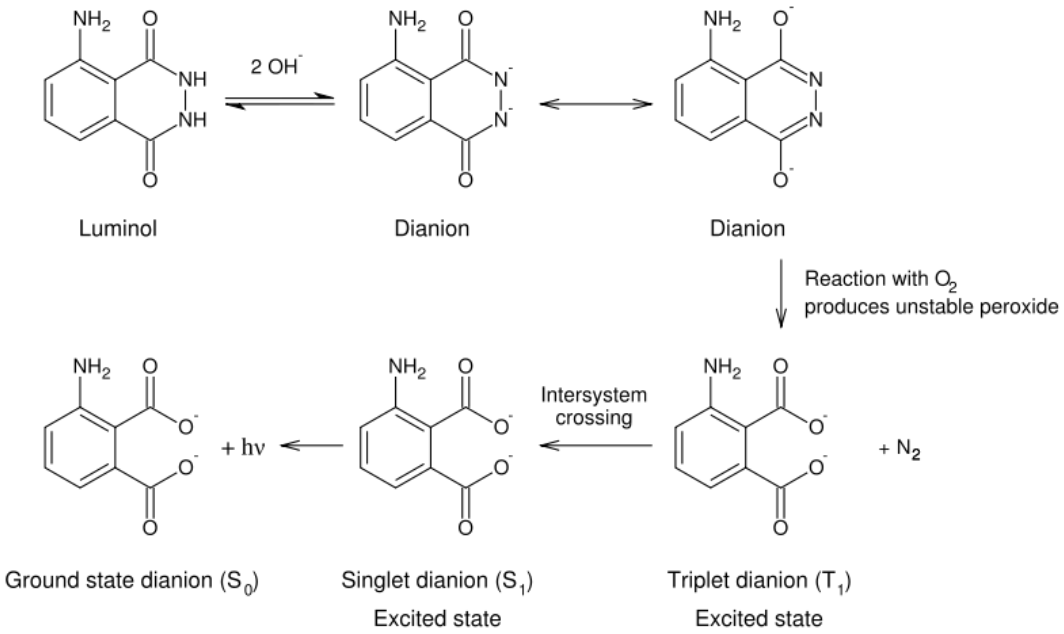

Fig. 3: Principle of chemiluminescent reaction

The production of these metastable compounds can be controlled by enzymatic processes, obtaining a reaction product in a state of excited singlet or triplet. The light is emitted during the passage of this intermediate excited state to the ground state and this process is called **direct chemiluminescence.** (2)

In general only the singlets have been involved. The amount of energy needed to produce a photon is quite high, around about 200 kJ, and varies depending on the wavelength of emission.

In reaction of **indirect chemiluminescence** the excited product of the reaction is not the real emitter of light, but moves the activation energy to an acceptor that emits the light.

The components which become part of a chemiluminescent reaction are:

1. Substrate or chemiluminescent substrates that are processed in the electronically excited state, responsible for the reactions of direct light or transfer energy in those indirect;
2. An electron acceptor such as oxygen, if this is an reaction of oxidation;
3. A catalyst, e.g. an enzyme or a metal ion which reduces the activation energy and therefore increases the speed of reaction;
4. Cofactors necessary to convert one or more substrates in a form capable of reacting, or interact with the catalyst;
5. An acceptor of energy or electrons if it is an indirect reaction. (5)

1.1.2.2 Efficiency of chemiluminescent reaction

In direct reaction efficiency of chemiluminescent reaction is determined by the primary chemical reaction, the character of chemical reagents and the environment in which the excited product is found. That is why it is required that the reaction provides:

• Sufficient energy excitation

• At least one compound capable of moving to excited state

• A high speed of reaction that will produce in time a sufficient number of photons

• A system of reaction that encourages the production of excited states compared to the ground state. (2)

In order to achieve the highest levels of sensitivity, a chemiluminescent reaction must be as efficient as possible in generating photons of light. Each chemiluminescent compound or group can produce no more than one photon of light. A perfectly efficient reaction would have a chemiluminescence quantum yield (Φ CL) of one, i.e. one photon/molecule reacted according to the equation:

$$\Phi CL = \Phi CE * \Phi F * \Phi R$$

The chemiexcitation quantum yield (Φ CE) is the probability of generating an electronic excited state in a reaction and has a value between 0 and 1, with 0 being a completely dark reaction and, when 1, all product molecules are generated in the excited state. The most useful chemiluminescent reactions will have Φ CE of about 10^{-3} or greater. The fluorescence quantum yield (ΦF) is the probability of the excited state emitting a photon by fluorescence rather than decaying by other processes. This property, which can have values between 0 and 1 is frequently at least 0.1. The reaction quantum yield (ΦR) is the fraction of starting molecules which undergo the luminescent reaction rather than a side reaction. This value is usually about 1. (3)

1.2 Oxidative stress

Free radicals such as reactive oxygen species are formed during a variety of biochemical reactions and cellular functions (such as mitochondria metabolism). The steady-state formation of pro-oxidants (free radicals) is normally balanced by a similar rate of consumption by antioxidants. Oxidative stress results from an imbalance between formation and neutralization of pro-oxidants.

Free radical formation and the effect of these toxic molecules on cell function (which can result in cell death) are collectively called "oxidative

stress." These free radicals are highly reactive, unstable molecules that have an unpaired electron in their outer shell. They react with (oxidize) various cellular components including DNA, proteins, lipids / fatty acids and other products. These reactions between cellular components and free radicals lead to DNA damage, mitochondrial malfunction, cell membrane damage and eventually cell death (apoptosis - which is the term for programmed cell death). (6)

1.2.1 Free radicals

Free radicals are atoms, molecules or ions with unpaired electrons on an otherwise open shell configuration. (7) They are represented especially by reactive oxygen radicals (ROS - reactive oxygen species) and nitrogen radicals (RNS - reactive nitrogen species) that have significant physiological and patogenetical importance. (8)

Some free radicals are a normal part of a healthy metabolism, others appear, or the amount of them increases in the process of illness or mental and physical stress. Free radicals cause rapid detritions of tissue cells, especially when their quantity in the body increases trough the poor nutrition and stay in dirty areas. Free radicals occur in vivo and have a number of physiological functions, great attention is currently paid to them and their negative effects on the body are monitored in a number of diseases.

Free radical molecules engender in three ways:

- homolytical cleavage of covalent chemical bonds, each fragment obtained an unpaired electron
- by the addition of one electron to a normal molecule (reduction)
- By loss of one electron (oxidation)

The body is receiving free radicals from the outside; however, there is a large number which is generated during metabolism. According to this, we divide causes of formation of free radicals to exogenous and endogenous.

Exogenous causes:

- Ionizing radiation (g a X-rays)
- UV, blue light (treatment of hyperbilirubinemia by newborn)
- High content of pollutants in the air
- Smoking
- Intoxication (alcohol, chlorophorm,carbon tetrachloride)
- Diet

Endogenous causes:

- The creation of uric acid - by accidents, necroses, post-operative conditions
- Fate phagocyte and microphages (inflammation, burns, septic condition)
- The emergence of methaemoglobin
- Formation of prostaglandins
- Increased metabolism of estrogen
- Auto oxidation of thiols
- Hyperglycemia (9)

1.2.1.1 Reactive oxygen species

The most important ROS are the superoxide anion (O_2-·), hydroxyl radical (OH·), nitric oxide (NO·), and hydrogen peroxide (H_2O_2). The primary ROS formed *in vivo* are superoxide and H_2O_2. H_2O_2 is generated through nonenzymatic or enzymatic dismutation of superoxide. However, the most reactive and harmful ROS is the hydroxyl radical, which can be formed from H_2O_2 and superoxide (**Fig. 4**), but also via the reaction of superoxide with NO to produce peroxynitrite (OONO-), which decomposes to form NO_2 and OH· (10)

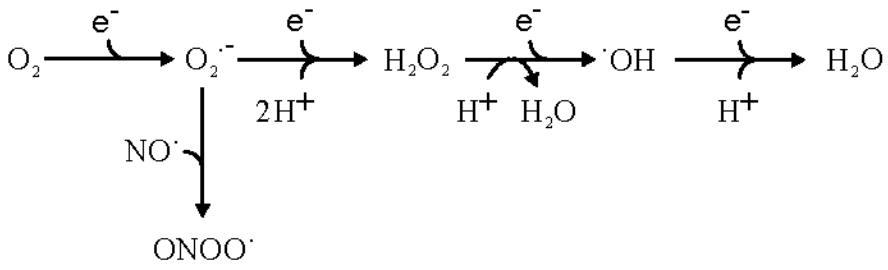

Fig. 4: Formation of the hydroxyl radical

1.2.1.2 Positive effects of free radicals

Free radicals in the human organism does not need to be only harmful, they can play a number of positive roles. In the immune system, they allow to leukocytes and macrophages to defense against infection in the respiratory flash. One of the physiological functions of free radicals is the destruction of pathogens by phagocytes. In the membrane of phagocytes NADPH-oxidase enzyme is present and simplifies one-electron reduction of molecular oxygen to superoxide. Afterwards it is converted to a more efficient ROS, of which the hydrochlorous acid is the most important, which destroys the pathogen. (9) (11)

They also participate on reactions that create some important substances (biosynthesis of cholesterol and bile acids), or apply to the detoxification of certain xenobiotics, and many drugs. Hydrogen peroxide is necessary for the oxidation of iodide to elemental iodine, which is used for iodization of aromatic nuclei of thyronine.

Superoxide and hydrogen peroxide also participate on successful egg fertilization by sperm. While superoxide disrupts the egg-membrane, enabling the sperms to penetrate trough it, hydrogen peroxide with the help of molecules of tyrosine, contained in the eggs, prevents their further penetration.

Nitric oxide, which is also one of the free radicals, has a significant vasodilator effect. Its importance is also in regulating immune processes, during erection, and as neurotransmitter. (11)

1.2.2 Oxidative damage: Protection

Reparative processes of the body cannot fully eliminate the damage important functions in the organism. One way to protect the body against the influence of radicals is the action of antioxidants. (12)

Antioxidant protection of the body is a complex set of mechanisms that work in combination, have to be complementary with each other, moreover, and must be balanced with prooxidative substances, namely the production of free radicals.

Criteria for antioxidants in the role of antioxidant protection:

- Antioxidants must be able to react with ROS in the place of the body, where the ROS is is located.
- During interaction of ROS and antioxidant, more reactive form of ROS cannot be created.
- There has to be enough antioxidant in the body that can afford to react with ROS and to ensure sufficient protection. (13)

1.2.3 Antioxidants

Every substance, which protects target molecule against oxidative damage, can be called antioxidant. (14)

1.2.3.1 Antioxidant enzymes

Superoxide dismutase (SOD)

Superoxide dismutase is a naturally occurring enzyme that protects the body against active oxygen free radicals by scavenging excess superoxide. Low or undetectable levels of superoxide dismutase and catalase allow oxygen radicals to form in anaerobic bacteria and to inactivate other bacterial enzyme systems. As an enzyme, SOD has particular value as an antioxidant that can help to protect against cell destruction. It has the distinct ability to neutralize superoxide, one of the most damaging free radical substances in nature. Like so many other protective compounds which naturally occur in the body, it decreases with age, making cells

much more vulnerable to the oxidants which cause aging and disease. Research in the areas of inflammatory, hepatic, allergic, tumorigenic, metabolic, cardiovascular, vision, and neurological disorders indicate the supportive function of SOD in the prevention and alleviations of such symptoms. SOD is also important in many other aspects of health and longevity. SOD plays an important role in the body's antioxidant system, intervening in the first transformation by dismuting the most reactive forms of oxygen (and therefore the most dangerous for the cells) – the superoxide free radicals – into ions that are less reactive. This transformation is called dismutation, thus its name dismutase (an enzyme that stops mutation). (15)

The SOD-catalyzed dismutation of superoxide may be written with the following half-reactions:

- $M^{(n+1)+} - SOD + O_2^- \rightarrow M^{n+} - SOD + O_2$
- $M^{n+} - SOD + O_2^- + 2H^+ \rightarrow M^{(n+1)+} - SOD + H_2O_2$.

where M = Cu (n=1); Mn (n=2); Fe (n=2); Ni (n=2).

In humans, three forms of superoxide dismutase are present. SOD 1 is located in the cytoplasm, SOD 2 in the mitochondria and SOD 3 is extracellular. SOD 1 and SOD 3 contain copper and zinc, while SOD 2 has manganese in its reactive centre. (16)

Glutathione peroxidase (GSHPx)

Glutathione peroxidase is an enzyme with peroxidase activity whose main biological role is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. During the reaction glutathione is oxidized simultaneously.

$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$

GSH represents reduced monomeric glutathione and GS–SG represents glutathione disulfide. Glutathione reductase then reduces the oxidized glutathione to complete the cycle: (17)

$GS–SG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$

Glutathione peroxidase is a selenium-containing tetrameric glycoprotein, that is, a molecule with four selenocysteine amino acid residues. As the integrity of the cellular and sub cellular membranes depend heavily on glutathione peroxidase, the antioxidative protective system of glutathione peroxidase itself depends heavily on the presence of selenium.

There are several isozymes, which vary in cellular location and substrate specificity. Glutathione peroxidase 1 is the most abundant version, found in the cytoplasm of nearly all mammalian tissues, whose preferred substrate is hydrogen peroxide. Glutathione peroxidase 4 has a high preference for lipid hydroperoxides; it is expressed in nearly every mammalian cell, though at much lower levels. Glutathione peroxidase 2 is an intestinal and extracellular enzyme, while glutathione peroxidase 3 is extracellular, especially abundant in plasma. (18)

Catalase

Catalase is a common enzyme found in nearly all living organisms, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen. Catalase has one of the highest turnover numbers of all enzymes; one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen per second.

Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide. The optimum pH for catalase is approximately 7, while the optimum temperature varies by species.

The reaction of catalase in the decomposition of hydrogen peroxide is:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Catalase can also oxidize different toxins, such as formaldehyde, formic acid, phenols, and alcohols. In doing so, it uses hydrogen peroxide according to the following reaction:

$$H_2O_2 + H_2R \rightarrow 2H_2O + R$$

Hydrogen peroxide is a harmful by-product of many normal metabolic processes: To prevent damage, it must be quickly converted into other, less dangerous substances. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules. Catalase works at an optimum temperature of 37 °C, which is approximately the temperature of the human body and is usually located in a cellular organelle called the peroxisome.

Catalase is used in the food industry for removing hydrogen peroxide from milk prior to .cheese production another use is in food wrappers, where it prevents food from oxidizing. Catalase is also used in the textile industry, removing hydrogen peroxide from fabrics to make sure the material is peroxide-free. A minor use is in contact lens hygiene - a few lens-cleaning products disinfect the lens using a hydrogen peroxide solution; a solution containing catalase is then used to decompose the hydrogen peroxide before the lens is used again. Recently, catalase has also begun to be used in the aesthetics industry.

1.2.3.2 Vitamin E

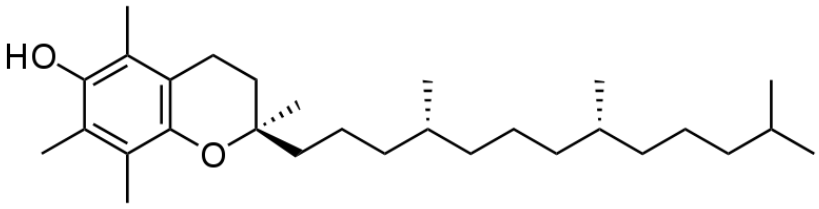

Fig. 5: Vitamin E

Vitamin E (**Fig. 5**) is the collective name for a set of 8 related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties. Of these, α-tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and using this form.

Structure of α-tocopherol

It has been claimed that α-tocopherol is the most important lipid-soluble antioxidant, and that it protects cell membranes from oxidation by reacting with

lipid radicals produced in the lipid peroxidation chain reaction. This would remove the free radical intermediates and prevent the oxidation reaction from continuing. The oxidized α-tocopheroxyl radicals produced in this process may be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol.

Particularly high levels of vitamin E can be found in the following foods: Almonds, Asparagus, Avocado, Nuts, Olives, Red Palm Oil, Seeds, Spinach and other green leafy vegetables. (19)

1.2.3.3 Vitamin A

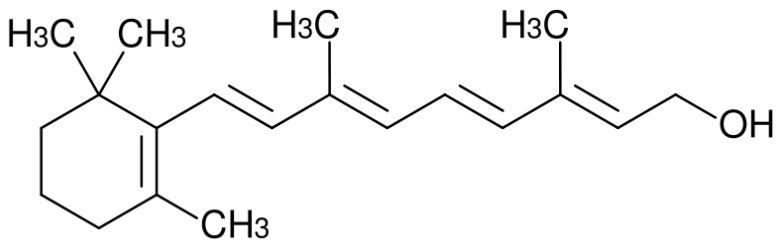

Fig. 6: Vitamin A

Vitamin A (**Fig. 6**) is a general term that refers to fat-soluble compounds that are similar in structure and biologic activity to retinol. Vitamin A also refers to dietary precursors of vitamin A. The precursors of vitamin A (retinol) are the carotenoids (most commonly beta-carotene). The retinoids are the most active form of vitamin A.

Structure of retinol

Retinol, the active form of vitamin A, is rarely found in foods. Instead, precursors to retinol, fatty acid retinyl esters, are found in the human diet. The esters are commonly found in foods of animal origin, such as egg yolks, liver, fish oil, whole milk and butter.

Retinoids are most commonly used in the treatment of skin diseases. The role the retinoids play in epithelial cell formation is very important in the treatment of skin cancer, acne, and acne-related diseases. Vitamin A also has antioxidant properties.

However, beta-carotene has been noted as having pro-oxidant properties. Despite these discrepancies vitamin A is known to help repair damaged tissue and therefore may be beneficial in counter-acting free radical damage. (20)

1.2.3.4 Vitamin C

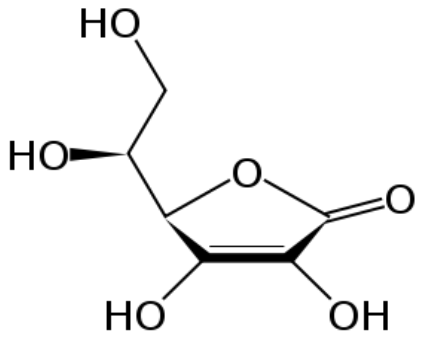

Fig. 7: Vitamin C

Vitamin C (ascorbic acid, AA, **Fig. 7**) is the major water-soluble antioxidant within the body. The vitamin readily donates electrons to break the chain reaction of lipid peroxidation. The water-soluble properties of vitamin C allow for the quenching of free radicals before they reach the cellular membrane. Tocopherol and glutathione also rely on AA for regeneration back to their active isoforms. The relationship between AA and glutathione is unique. Vitamin C reduces glutathione back to the active form. Once reduced, glutathione will regenerate vitamin C from its DHAA or oxidized state.

Vitamin C has the ability to sequester the singlet oxygen radical, stabilize the hydroxyl radical, and regenerate reduced vitamin E back to the active state. These functions work to halt peroxidation of cellular lipid membranes. (21)

1.2.3.5 Flavonoids and polyphenols

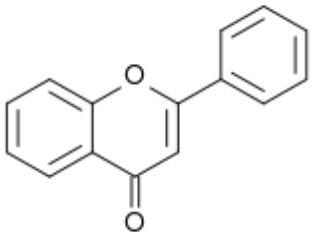

Fig. 9: Flavonoids

Flavonoids (**Fig. 9**) are polyphenols naturally occurring in fruits, vegetables, grains, tea, and wine. Epidemiological studies have indicated that flavonoids are preventive in coronary heart disease, stroke, and certain cancers through their antioxidant, anti-inflammatory, antiallergic, and antiviral activities. As such, flavonoids are generally considered to be beneficial to consumers' health and present one of the most important bioactive food components. On the other hand, there is considerable evidence that flavonoids may have prooxidant and DNA-damaging activity. (22)

1.2.4 Importance of diet in protection against damage from free radicals

The diet has only marginally affect on the activity of enzymes with antioxidant activity by providing an adequate supply of cofactors required for the activities of these enzymes (selenium, glutathione), but dietary intake of macromolecular antioxidants both water- and fat-soluble (vit. E, vit. C, vit. A, retinol, anthocyanins, and catechins), can heavily influence size of oxidative damage.

Main dietary sources of antioxidants:

- Soybeans
- The green and black tea
- coffee
- red wine

- spices (salvia)
- olive oil
- onions
- fruits and vegetables

The diet may also influence the level and quality of molecules susceptible to oxidation, such as fatty acids, which may present a different degree of unsaturation, which makes them more or less exposed to the radical attack.

As the fat ingested by diet is the main source of spectrum of lipids and lipoproteins, healthy eating is an important factor in prevention and treatment of cardiovascular disease.

The atherosclerosis and coronary diseases are multifactorial diseases associated with various causes such as dyslipidemia, hypertension, diabetes and obesity. Among these factors, the concentration of cholesterol carried by low-density lipoprotein (LDL) is one of the most important. (23) In fact, low cholesterol levels highly decrease the probability of occurrence of diseases like atherosclerosis (24).

Decades of research has clearly shown that the diet has a strong influence on levels of lipids and lipoproteins in serum, being lipids in diet factors most directly involved. (25) (26)

In the diet of western countries, three saturated fatty acids lauric, palmitic myristic are the main contained in food and represent 60-70% of all saturated fatty acids received by diet and it has been shown that these lipids are responsible for increase of total cholesterol (23)

The polyunsaturated fatty acids found most in diet is linoleic acid, dominant in plants (such as sunflower).Other polyunsaturated fatty acids (PUFA) physiologically important are the alpha-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid. Despite the ability of diet rich on PUFA to reduce the level of the plasma cholesterol, level of unsaturation of fatty acids affect the sensitivity of LDL to oxidative modifications, making these particles more susceptible to peroxidation.

The main monounsaturated fatty acid (MUFA) in diet is oleic acid, the main fat of olive oil.

Both mono and polyunsaturated fatty acids significantly reduce the LDL when substituted for saturated fatty acids. When MUFA replace saturated fatty acids, it can reduce the levels of total cholesterol and LDL, without altering the HDL, which is effectively reduced by PUFA. Moreover, the MUFA lead to a positive change in lipid profile lipoprotein, generating LDL more resistant to oxidative modification. (27) A high intake of monounsaturated combines the advantages of both lowering cholesterol and reducing the susceptibility of LDL to oxidation. This may be crucial because the oxidation of lipoproteins is widely recognized as a factor that contributes to the etiology of arthrosclerosis. (28) The consumption of olive oil also ensures an appropriate intake of essential polyunsaturated fatty acids. Diet has some influence on the composition of fatty acids in cell membranes: a diet rich in monounsaturated fatty acids promotes a high content of these substances in cell membranes, inducing the cells themselves a higher resistance to oxidative damage. (29)

1.2.5 Antioxidants in red wine

The phenol compounds in wine include a large group of several hundred chemical compounds, known as polyphenolics that affect the taste, color and mouth feel of wine. This large group can be broadly separated into two categories - flavonoids and non-flavonoids. Flavonoids include anthocyanins and tannins which contribute to the color and mouth feel of the wine. Non-flavonoids include stilbenes such as resveratrol and compounds derived from acids in wine like benzoic, caffeic and cinnamic acid. In wine grapes, phenolics are found widely in the skin, stems and seeds. (30)

1.2.5.1 Flavonoids

In red wine, up to 90% of the wine's phenolic content fall under the classification of flavonoids. These phenols, mainly derived from the stems, seeds and skins are often leeched out of the grape during the maceration period of winemaking. The amount of phenols leeched is known as extraction. They contribute to

the astringency, color and mouthfeel of the wine. Within the flavonoid category is a subcategory known as flavonols, which includes the yellow pigment inducing phenol-quercetin. Like other flavonoids, the concentration of flavonols in the grape berries increases as they exposed to sunlight. (30)

1.2.5.1.1 Tannins

Tannins are astringent, bitter plant polyphenols that either bind and precipitate or shrink proteins.

They are usually divided into **hydrolyzable tannins** (base unit is gallic acid – **Fig. 10**) and **condensed tannins** (proanthocyanidins, base unit is flavones – **Fig. 11**).

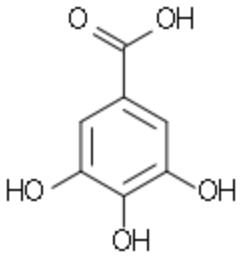

Fig. 10: Gallic acid

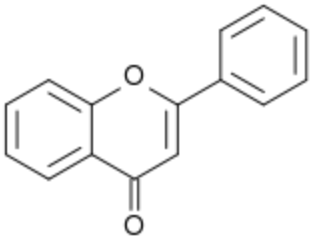

Fig. 11: Flavone

Tannins refer to the diverse group o chemical compounds in wine that can affect the color, aging ability and texture of the wine. While tannins cannot be smelt or tasted, they can be perceived during wine tasting by the tactile drying sensation and sense of bitterness that they can leave in the mouth. This is due to the tendency of tannins to react with proteins, such as the ones found in saliva. In food and wine pairing, foods that are high in proteins (such as red meat) are often paired with tannic wines to minimize the astringency of tannins.

Tannins in wine can come from many sources and the tactile properties differ depending on the source. Tannins in grape skins and seeds (the latter being especially harsh) tend to be more noticeable in red wines. The stems of th grape bunches also contain tannins. Tannins extracted from grapes are condensed tannins, which are polymers of proanthocyanidin monomers. Hydrolysable tannins are

extracted from the oak wood the wine is aged in. Hydrolysable tannins are more easily oxidised than condensed tannins.

Tannins play an important role in preventing oxidation in aging wine and appear to polymerize and make up a major portion of the sediment in wine. (31)

Gallic acid

Gallic acid is an organic acid, also known as 3,4,5-trihydroxybenzoic acid. (32) It is a plant phenolic compound, which form moieties on flavonoid rings. Though existing in a number of astringent plants, is usually prepared from tannic acid.

Gallic acid is much inferior to tannic acid as a topical astringent; but administered internally, it is more powerful as a remote astringent. Indeed, tannic acid, in its passage through the system, becomes changed into gallic acid. As a remote astringent, gallic acid has been found very beneficial in *uterine, pulmonary,* and *nephritic hemorrhages*, and all *hemorrhages* of a passive character. (33)

Gallic acid is also commonly used in the pharmaceutical industry. It is used as a standard for determining the phenol content of various analytes by the Folin-Ciocalteau assay; results are reported in *gallic acid equivalents*. Gallic acid can also be used to synthesize the hallucinogenic alkaloid mescaline. (32)

1.2.5.1.2 Anthocyanins

Anthocyanins are water-soluble vacuolar pigments that may appear red, purple, or blue according to pH. They are responsible for the blue to red colors found in flowers, fruits and leaves. In wine grapes, they develop during the stage of veraison when the skin of red wine grapes change color from green to shading from red to black. As the sugars in the grape increase during ripening, so does the concentration of anthocyanins.

The anthocyanins are subdivided into the sugar-free anthocyanidin aglycones and the anthocyanin glycosides

Anthocyanins are glucosides of anthocyanidins, the basic chemical structure of which is shown in **Fig. 12**. (34)

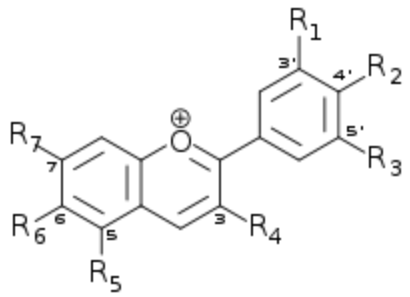

Fig. 12: Anthocyanidin

1.2.5.1.3 Other flavonoids

- **Catechins** are flavonoids that contribute to the construction of various tannins and contribute to the perception of bitterness in wine. They are found in highest concentrations in grape seeds but are also in the skin and stems. Catechins play a role in the microbial defense of the grape berry, being produced in higher concentrations by the grape vines when it is being attacked by grape diseases such as downy mildew.
- **Vanillin** is a phenolic aldehyde most commonly associated with the vanilla notes in wines that have been aged in oak. Some trace amounts of vanillin are found naturally in the grapes themselves but they are most prominent in the lignin structure of oak barrels. (31)

1.2.5.2 Non-Flavonoids

1.2.5.2.1 Resveratrol

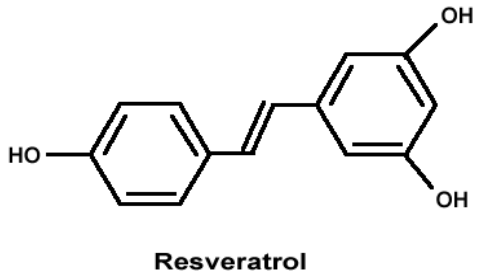

Fig. 13: Resveratrol

Resveratrol (**Fig. 13**) is a phytoalexin, a class of antibiotic compounds produced as a part of a plant's defense system against disease.

While present in other plants, such as eucalyptus, spruce, and lily, and in other foods such as mulberries and peanuts, resveratrol's most abundant natural sources are Vitis vinifera, labrusca, and muscadine grapes. The highest concentration is in the skin, which contains 50-100 micrograms (μg) per gram.

The resveratrol content of wine is related to the length of time the grape skins are present during the fermentation process.

The health-food industry is claiming that resveratrol is the wine component responsible for the "French Paradox"- the low incidence of heart disease among the French people, who eat a relatively high-fat diet.

Resveratrol inhibits lipid peroxidation of low-density lipoprotein (LDL), prevents the cytotoxicity of oxidized LDL, and protects cells against lipid peroxidation. It is thought that because it contains highly hydrophilic and lipophilic properties, it can provide more effective protection than other well-known antioxidants, such as vitamins C and E. Reduced platelet aggregation has also been demonstrated in studies on resveratrol, further contributing to its prevention of atherosclerosis.

Resveratrol is also being studied to see how it affects the initiation, promotion, and progression of cancer.Resveratrol appears to decrease tumor promotion activity by inhibiting cyclooxygenase-1 (COX-1), an enzyme that converts arachidonic acid to pro-inflammatory substances that stimulate tumor-cell growth. (35)

1.3 Winemaking

Winemaking, or vinification, is the production of wine, starting with selection of the grapes and ending with bottling the finished wine.

1.3.1 Primary fermentation

During the primary fermentation, the yeast cells feed on the sugars in the must and multiply, producing carbon dioxide gas and alcohol. The temperature during the fermentation affects both the taste of the end product, as well as the speed of the fermentation. For red wines, the temperature is typically 22 to 25 °C.

1.3.2 Cold and heat stabilization

Cold stabilization is a process used in winemaking to reduce tartrate crystals (generally potassium bitartrate) in wine. During the cold stabilizing process, the temperature of the wine, after fermentation, is dropped to close to freezing for 1-2 weeks. This will cause the crystals to separate from the wine.

During "heat stabilization", unstable proteins are removed by adsorption onto bentonite, preventing them from precipitating in the bottled wine.

1.3.3 Secondary fermentation and bulk aging

The secondary fermentation and aging process, take three to six months. The wine is kept under an airlock to protect the wine from oxidation. The result of these processes is that the originally cloudy wine becomes clear.

1.3.4 Malo lactic fermentation

Malolactic fermentation is carried out by bacteria which metabolize malic acid and produce lactic acid and carbon dioxide. The resultant wine is softer in taste and has greater complexity. The process is used in most red wines.

1.3.5 Laboratory Tests

Whether the wine is aging in tanks or barrels, tests are run periodically in a laboratory to check the status of the wine. Common tests include °Brix (measurement of the dissolved sugar-to-water mass ratio of a liquid), pH, residual sugar, free or available sulfur, total sulfur, volatile acidity and percent alcohol.

1.3.6 Blending and fining

Fining agents are used during winemaking to remove tannins, reduce astringency and remove microscopic particles that could cloud the wines. Gelatin has been used in winemaking for centuries and is recognized as a traditional method for wine fining, or clarifying.

Besides gelatin, other fining agents for wine are often derived from animal and fish products, such as casein, egg whites, egg albumin, bone char, bull's blood, isinglass (Sturgeon bladder), PVPP (a dairy derivative protein), lysozyme, and skim milk powder.

Non-animal-based filtering agents are also often used, such as Bentonite (a volcanic clay-based filter), Diatomaceous Earth, cellulose pads, paper filters and membrane filters (thin films of plastic polymer material having uniformly sized holes). (36)

Polyvinylpyrrolidone (PVPP), gelatin, or bentonite have been shown to reduce phenolic levels and alter the color and sensory characteristics of wines. Data found in the literature show that gelatin has little influence on young red wines because it affects only the colloidal compounds, whereas PVPP typically binds and removes smaller molecular weight phenolic compounds, and bentonite, which is volcanic

aluminum silicate clay with exchangeable cationic components, is used to reduce the protein content of wines. Bentonite also absorbs polyphenol oxidase, phenols, and other positively charged molecules. (37)

1.3.6.1 Gelatin

Gelatin is a protein, that is, it is a polymer of amino acids joined together by peptide bonds. Hence, proteins can be depicted as long molecules with many different side chains, which accounts for varying properties. Aminoacid Proline is very important in that it imparts a twist to the chain and affects the shape of the protein molecule and its rigidity.

Gelatin has been used for the clarification or fining of wine since the Roman civilization.

Fining Reactions

- The primary reaction occurring with gelatin is a complex formation between polyphenols in the wine and the protein of gelatin to give the desired floccular precipitate.
- The second reaction, less well understood, but equally important, is the complex formation between the natural proteins of the wine and the added protein, gelatin.
- The third reaction is between bentonite or silica sol (which should be added after the gelatin) which absorbs or complexes with any residual dissolved protein, be it gelatin or natural protein in the beverage.

A disadvantage of gelatin fining is the difficulty of gelatin dissolution. It requires both heat and time, and in addition, gelatin solutions gel on cooling, and furthermore, they should not be stored for more than a few hours at a time because gelatin is an excellent nutrient for most forms of microbiological life. (38)

1.3.6.2 Polyvinyl polypyrrolidone (PVPP)

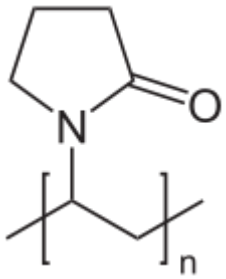

Fig. 14: Polyvinylpolypyrrolidone

PVPP (**Fig. 14**) is a high molecular weight fining agent made of cross linked monomer of polyvinlypyrrolidone. It complexes with phenolic and polyphenolic components in wine by adsorption and attracts low molecular weight catechins. It removes bitter compounds and browning precursors in both red and white wines. PVPP is quick acting with no preparation required. Wines must be filtered to remove the PVPP and wines may seem more astringent when the bitter compounds are removed. PVPP is sold as Polyclar® V and VT. (39)

1.3.7 Preservatives

The most common preservative used in winemaking is sulfur dioxide, another one is potassium sorbate. Sulfur dioxide has two primary actions, firstly it is an anti microbial agent and secondly an anti oxidant. Without the use of it, wines can readily suffer bacterial spoilage no matter how hygienic the winemaking practice. Potassium sorbate is effective for the control of fungal growth, including yeast, especially for sweet wines in bottle.

1.3.8 Filtration

Filtration in winemaking is used to accomplish two objectives, clarification and microbial stabilization. In clarification, large particles that affect the visual appearance of the wine are removed. In microbial stabilization, organisms that affect the stability of the wine are removed therefore reducing the likelihood of re-fermentation or spoilage.

1.3.9 Bottling

A final dose of sulfite is added to help preserve the wine and prevent unwanted fermentation in the bottle. (36)

2 Aim of the thesis

The first aim of this thesis was to measure and evaluate the total antioxidant capacity (TAC) in samples of red wine and figure out if and to what degree every single fining agent influence TAC.

Second aim was to evaluate how the particular dosages influence TAC, that means if TAC of red wine is reduced by adding larger amount of the fining agent or not.

For this purpose it has been made use by evaluation of:

- ➔ influence of different finings on particular wines
- ➔ effect of particular dosages of finings on particular wines
- ➔ interaction between different finings and wines independently on dosages

The last aim of this work was endeavor to discover relations between changes in TAC in content of single antioxidant components like total polyphenols (TPP), anthocyanins, gallic acid and simple phenols.

3 Materials and methods

3.1 Reagents

- **Luminol** (5-amino-2,3-dihydro-1,4-phtalazinedione), analytical grade, Merck (Darmstadt, Germany)

- **Horseradish peroxidase** (HRP) , E.C. 1.11.1.7 , Grade II , Boehringer Mannheim (Germany)

- **Hydrogen peroxide 30%** , Merck (Germany)

- **Trolox** (6-hydroxy-2,5,7,8-teramethylchroman-2-carboxylic acid) 98% pure (Fluka, Milan, Italy). Trolox is a vitamin E analogue, is water soluble and was used as reference antioxidant to determine antioxidant capacity.

- **Phosphate buffer** 0,1 M ; pH 7,4

- **Water** pyrogen-free, reagent-grade prepared with a Mill-Q system (Millipore, Milan, Italy)

- **Wine samples** Measurement was accomplished on samples of red wine obtained from the Faculty of agriculture, university of Bologna, Italy. Samples of three different wines (A,B,C) treated by three different fining agents in concentrations of 10, 20 and 40 g/hL. Each sample was a triplicate. The set contained 117 samples, 107 put through fining treatment and 9 untreated samples.

Table 2: Characteristics of wine samples before adding the finings

		A	B	C
PPT	mg/L	3035	1856	948
anthocyanins	mg/L	341	581	58,8
gallic acid	mg/L	23,96	12,61	7,62
simple phenols	mg/L	1,77	2,68	1,63
points of color		20	12	6

PPT = total amount of polyphenols

As you can see from **table 2**, wine A is rich for total polyphenols, also has biggest amount of gallic acid, on the contrary wine B contains more anthocyanins and simple phenols. Wine C is very poor with low levels of all compounds.

- **Fining agents**

  - **PVPP** (polyvinylpolypyrrolidone): fining able to prevent oxidative processes, it modifies the content of low molecular weight compounds.
  - **GOP** (powder gelatin) : pure protein fining, gelatin of animal origin certified BSA free, food grade
  - **GL** (liquid gelatin) : pure protein-based gelatin (food grade) in solution; composition: gelatin of animal origin certified BSE free 45%, sulfur dioxide 0,5 %, citric acid 0,5%, aqua destillata ad 100%
  - **GA** (atomized gelatin) : pure animal gelatin, product of bovine origin (food grade), odorless and tasteless

3.2 Instruments

Luminometer LKB-Wallac 1250

Producer: Turku, Finland

Luminometer was used to measure light emission. It is using a photomultiplier tube as a detector. It converts the light, which is produced by the sample, into an electrical signal. The signal was amplified and recorded on paper by means of an LKB 2210 recorder, which transformed it into mV.

3.3 Methods

3.3.1 Chemiluminescent analysis

3.3.1.1 Preparation of reagents

- **Preparation of Luminol solution**

The 2 mM solution of luminol is prepared by dissolving 0.0177 g of luminol in 50 mL of 0.1M solution of phosphate buffer pH 7,4. Because luminol is poorly soluble in water, it is necessary, before addition of phosphate buffer, to dissolve it in few drops of dimethylsulfoxide (DMSO). The luminol solution is stored in refrigerator under temperature of 4°C in the dark for months.

- **Solution of peroxidase (POD)**

The stock solution of peroxidase is prepared by solubilizing 10 mg of Horseradish peroxidase in 10 mL of phosphate buffer. Peroxidase is soluble in water. The work solutions were prepared by diluting of stock solution in 0,1 M phosphate buffer pH 7,4 in different rates (1:10, 1:15, 1:20 and 1:50). The diluted solutions are preserved 7 days in refrigerator under temperature of 4°C protected from the light before using. After that they are stored in the same way.

- **Solution of Hydrogen peroxide**

The 10 mM solution is prepared by adding 100 μL of Hydrogen peroxide 30% to 10 mL of 0,1 M phosphate buffer pH 7,4. The solution is prepared fresh every day before using.

- **Solution of Trolox**

The stock solution of trolox (concentration 1mM) is prepared by solubilizing 2,5 mg of trolox in 10mL of 0,1 M phosphate buffer pH 7,4. This solution is divided into volumes of 200 μL and stored in the freezer till the moment of preparation of calibration curve.

- **Chemiluminescent mixture (CLM)**

Chemiluminescent mixture is prepared just before use by mixing 9 mL of 10 mL hydrogen peroxide solution and 1 mL of luminol solution. This solution is protected from light by storing in vessel packed in tinfoil under room temperature.

3.3.1.2 Preparation of calibration curve:

In the time of preparation calibration curve, the stock solution of trolox has been diluted in properly with 0,1 M phosphate buffer pH 7,4. The suitable dilutions are shown in **Table 3**.

Table 3: Preparation of calibration curve

concentration of trolox	volume of stock solution	volume of phosphate buffer
20 μM	100 μL	5 mL
10 μM	100 μL	10 mL
5 μM	100 μL	20 mL
3 μM *	83,3 μL	25 mL
2 μM	20 μL	10 mL
1,75 μM *	17,5 μL	10 mL
1,5 μM	15 μL	10 mL
1,25 μM *	12,5 μL	10 mL
1 μM	10 μL	10 mL
700 nM	7 μL	10 mL
350 nM *	3,5 μL	10 mL
175 nM *	3,5 μL	20 mL
87,5 nM *	3,5 μL	40 mL

* concentrations were prepared by dilution of stronger concentrations of trolox solutions

All the concentrations of trolox solution were subsequently measured by way of LKB and times of inhibition of oxidative process were calculated from record on paper of LKB 2210. Time of inhibition was evaluated like time from injecting of solution of trolox to reaching 30 % light emission with regard to the original value and noted as a calibration curve (dependence of time of inhibition of light emission on concentration of trolox).

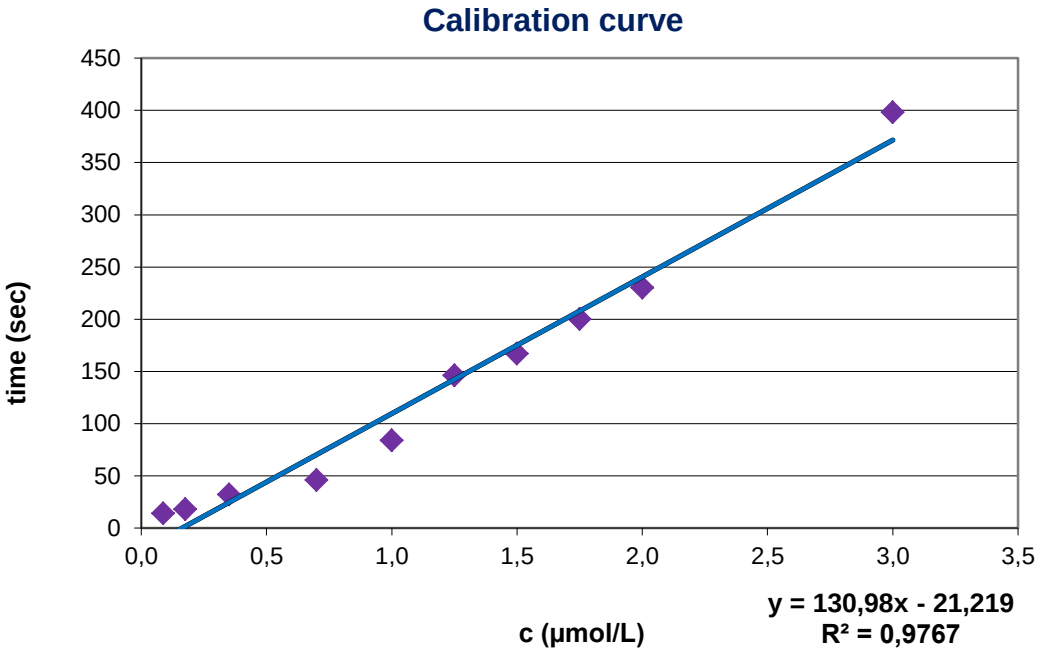

Fig. 15: Calibration curve

Calibration

Because the system peroxidase / luminol as every enzymatic system embodies variance in duration of days, for acquirement of most exact results it is necessary to measure both – calibration curve and samples together each day.

3.3.2 Chemiluminescent assay

Principe of the method used to evaluation total antioxidant capacity

The Trolox equivalent antioxidant capacity assay known as TEAC was used to evaluate the total antioxidant capacity of wine. This method is based on outspread total antioxidant capacity of sample to equivalent concentration of trolox (water soluble analog of vitamin E).

Measuring of chemiluminescence

200 µL of chemiluminecsence mixture and 50 µL of Peroxidase solution were injected in luminometer cuvette and put into the device. The reaction of oxidation of luminol catalyzed by peroxidase solution determines the emission of light. This mixture was the reference system representing 100% light emission in the absence

of inhibition by the sample or standard antioxidant solutions (**Fig. 16**). Paper recording followed the kinetics of emission to pinpoint the peak reaction time, maintained almost constant with a stationary emission for a time interval allowing measurement. To determine the total antioxidant capacity of the samples, 10 mL of the sample or standard solutions were injected into the cuvette when emission had reached the stationary emission. The light signal was immediately inhibited, reaching values close to zero which were maintained for a certain time then gradually increased to return to values close to those observed prior to sample injection (**Fig. 17**). The time between sample injection and the return to an emission 30% of the initial light was measured. The value obtained (in seconds) was a function of the antioxidant capacity of the sample examined. This antioxidant capacity was expressed, fitting the times obtained on the relative calibration curve, in concentration of Trolox (mM).

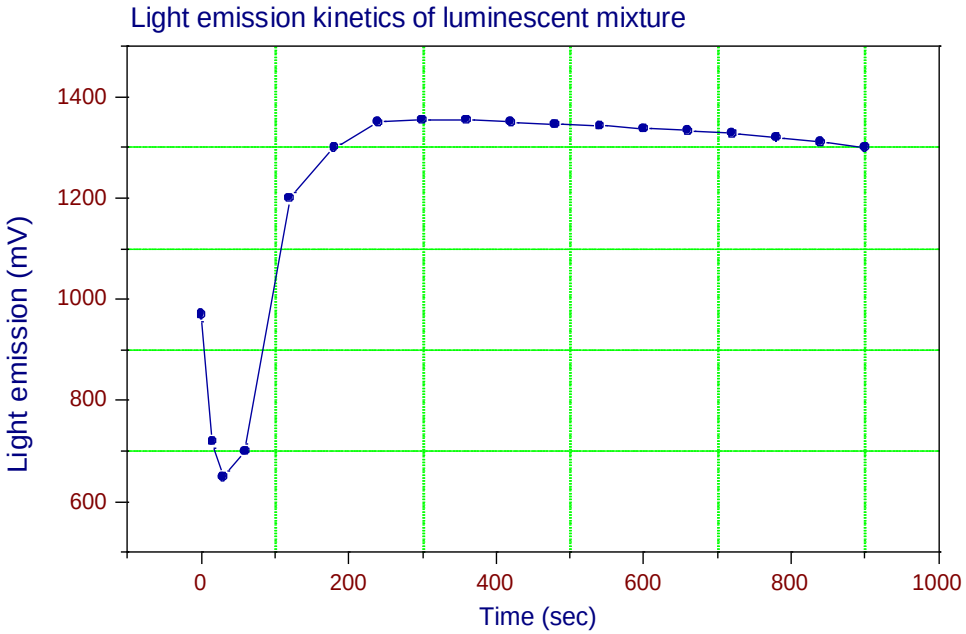

Fig. 16: Light emission kinetics of luminescent mixture

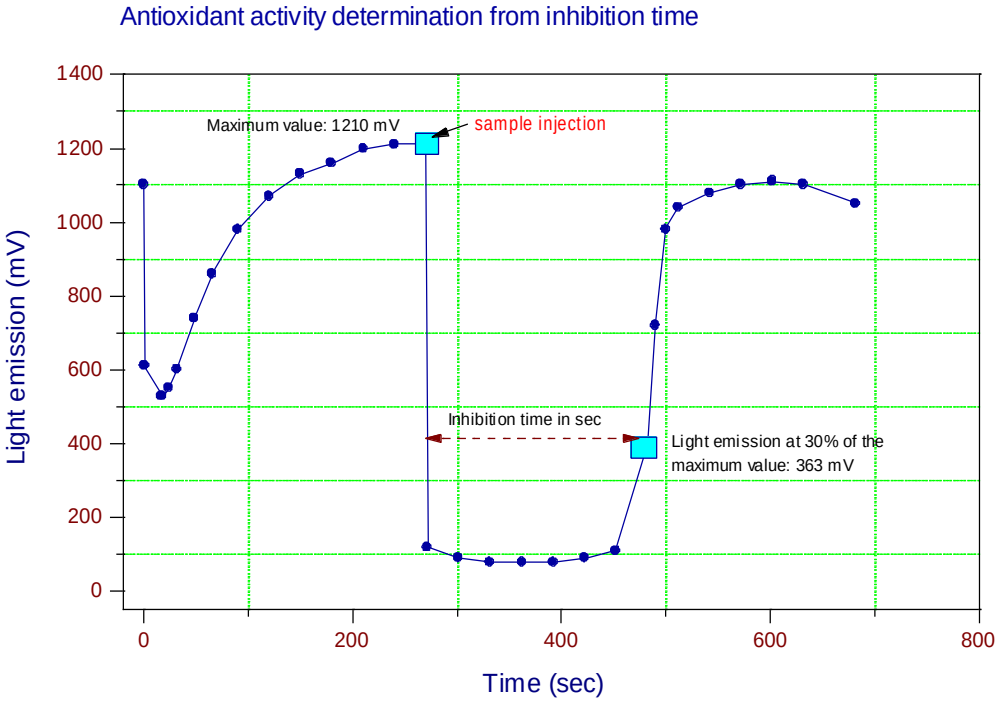

Fig. 17: Antioxidant activity determination from inhibition time

4 Discussion and results

Figure 18 value of TAC of A wine samples for different fining agents independently on their dosage. According to the graph TAC of wine samples containing PVPP and powder gelatin didn't show big changes in contrary of 2 other samples (with liquid gelatin and atomized gelatin) where TAC has decreased. In case of liquid gelatin, measured value was about 50 % lower than origin value.

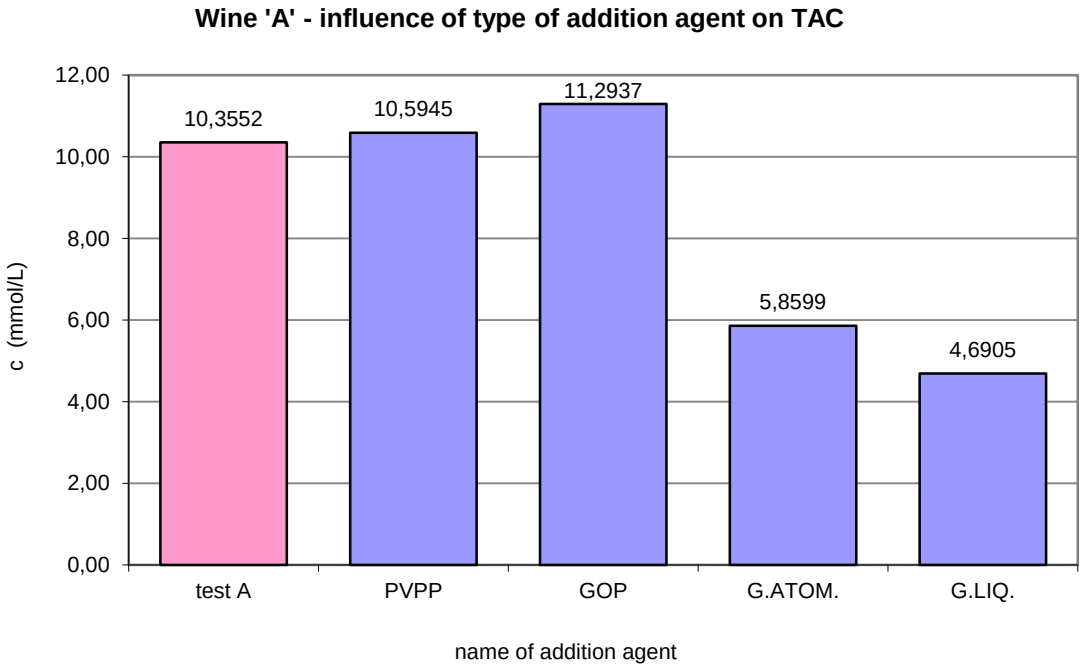

Fig. 18

Figure 19 demonstrates same results, but in case of wine B, that means for wine with lower content of total polyphenols, but higher amount of simple phenols were the original TAC about half value than for A samples. After evaluating TAC it has been shown that adding of fining agents have just small influence on TAC by samples clarified via PVPP and liquid gelatin, the values remained almost the same as for the test sample. TAC by samples containing powder and atomized gelatin has decreased at about one half of origin value.

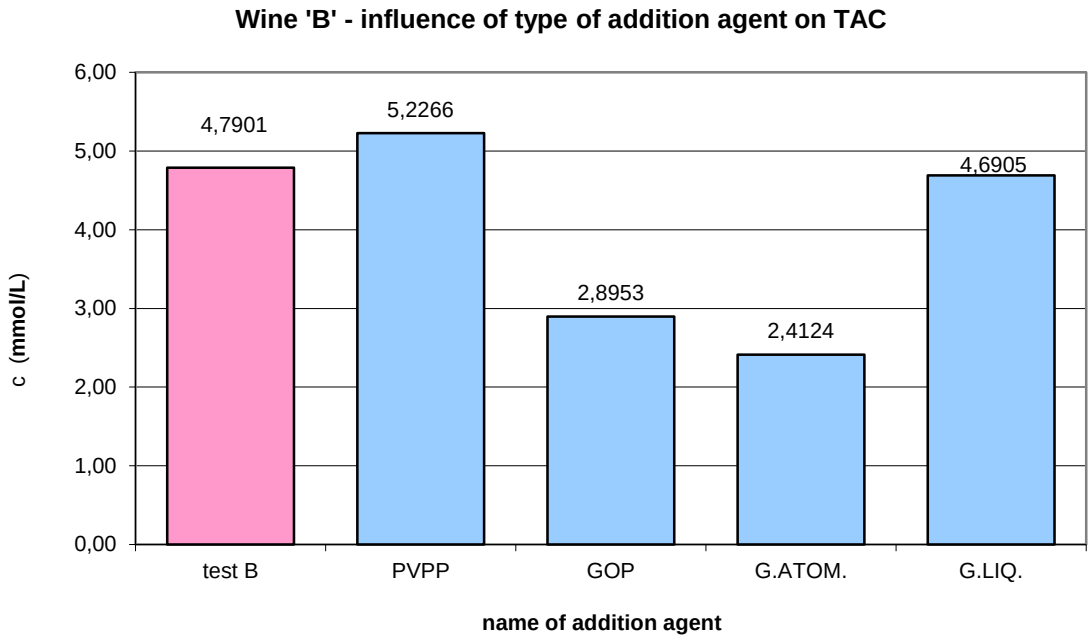

Fig. 19

Figure 20 for sample of C wine (pure wine with low level of TPP and also simple phenols) shows that the prime value of TAC is about 30 % of TAC values of samples A. Results of measurement exposed differences. In case of PVPP the TAC remained on the same level, while by other 3 fining agents has rapidly decreased, especially by samples with powder gelatin.

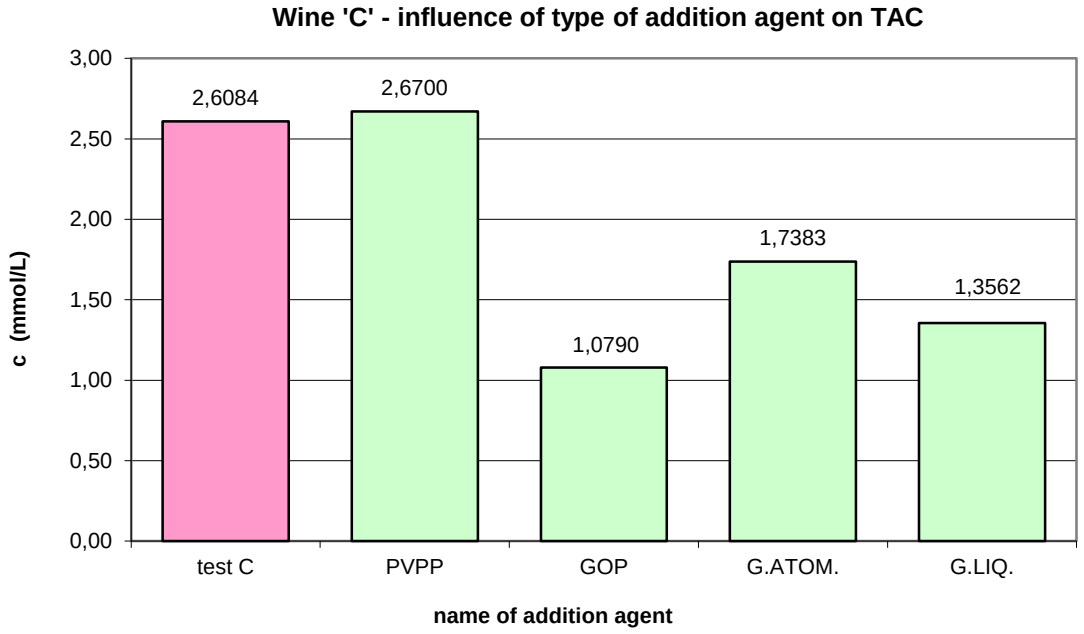

Fig. 20

From previous three graphs is obvious that dependence of total antioxidant capacity on fining agents independently on its amount in the sample didn't deport any special periodicity, even results for different wines are variable. As an example for liquid gelatin the TAC of samples from A and C wines rapidly decreased compared to test sample but in samples of B wines remained on the same level. Parallel differences also showed powder gelatin and less extensive, but noticeable atomized gelatin. Its worth to notice that TAC in each wine sample clarified with PVPP didn't change so rapidly and is comparable with the origin values by not clarified wines.

Figure 21 was prepared to display how the single clarifying agents influence TAC regardless to sort of wine or its amount in it. The TAC in wines clarified in general by PVPP didn't lower at all; the resulting level was even a little bit higher. In cases of all types of gelatin there can be noted some decline. By powder gelatin not that dramatic, the final level is about 85 % of origin value, on the other side fall of the TAC by atomized gelatin and liquid gelatin was more than 40 % in both cases.

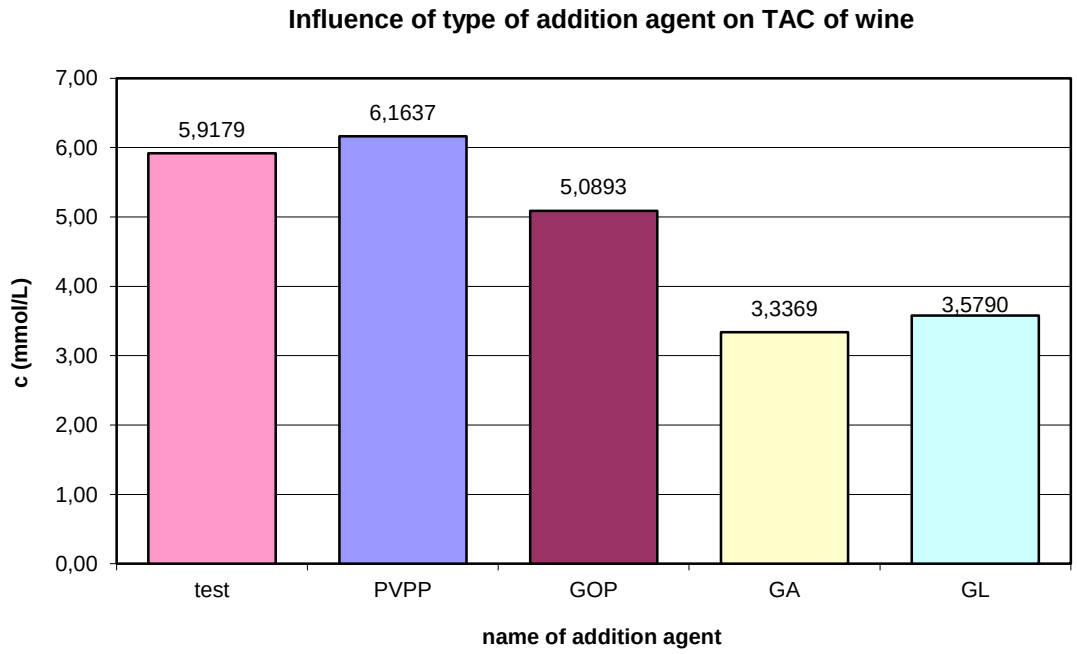

Fig. 21

Following three graphs serve for classification the total antioxidant capacity depending upon amount of fining agents, but independently of its type.

From **Figure 22** for A wine samples is possible to see that amount 10 and 20 g/hL didn't have big influence on TAC and measured values remained on the same level. In case of 40 g/hL amount the TAC has reduced of about 30%.

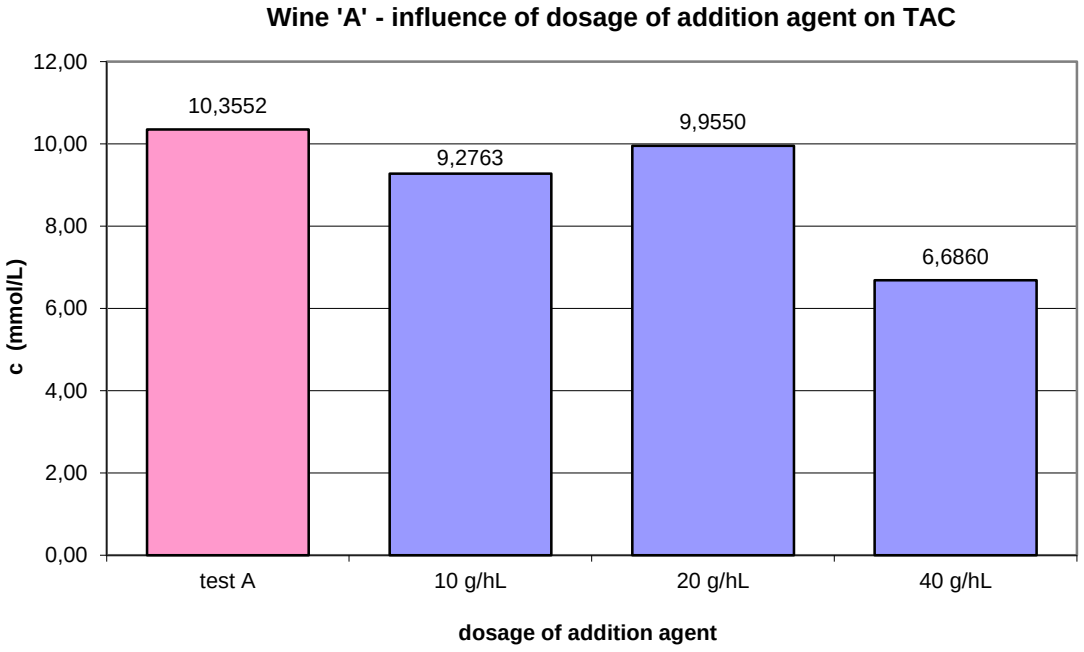

Fig. 22

A little bit different situation was in case of B samples (**Figure 23**). According to results it turns out that the fining agents in these amounts have no bit effect on final TAC, as you can see from the graph in case of concentration 40 g/hL the measured TAC was the highest from all and compared to test sample TAC attained to more than 90 % of primary value. An analogical value was measured for samples containing 10 g/hL of fining agent, in case 20 g/hL concentration TAC decreased to about 65% of origin value.

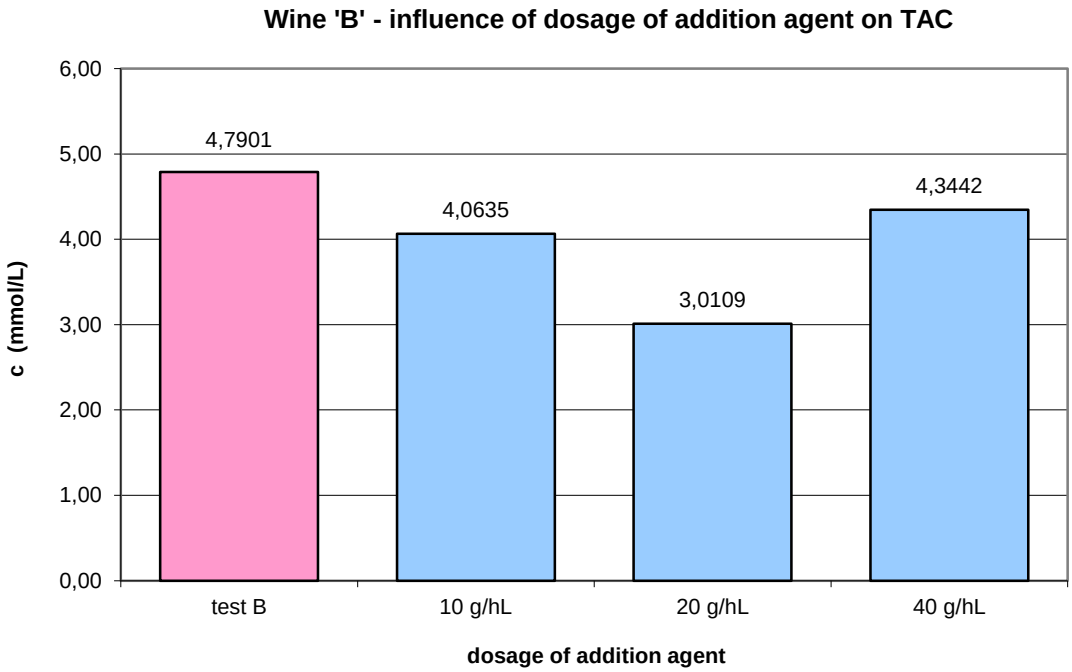

Fig. 23

In case of C samples (**Figure 24**) the opinion that fining agents added to wines in these concentrations (10, 20 and 40 g/hL) has not such a dramatic influence on TAC has been confirmed. As you can see measured values of TAC were very similar in all three groups of samples. The value moved around 65 % of origin value.

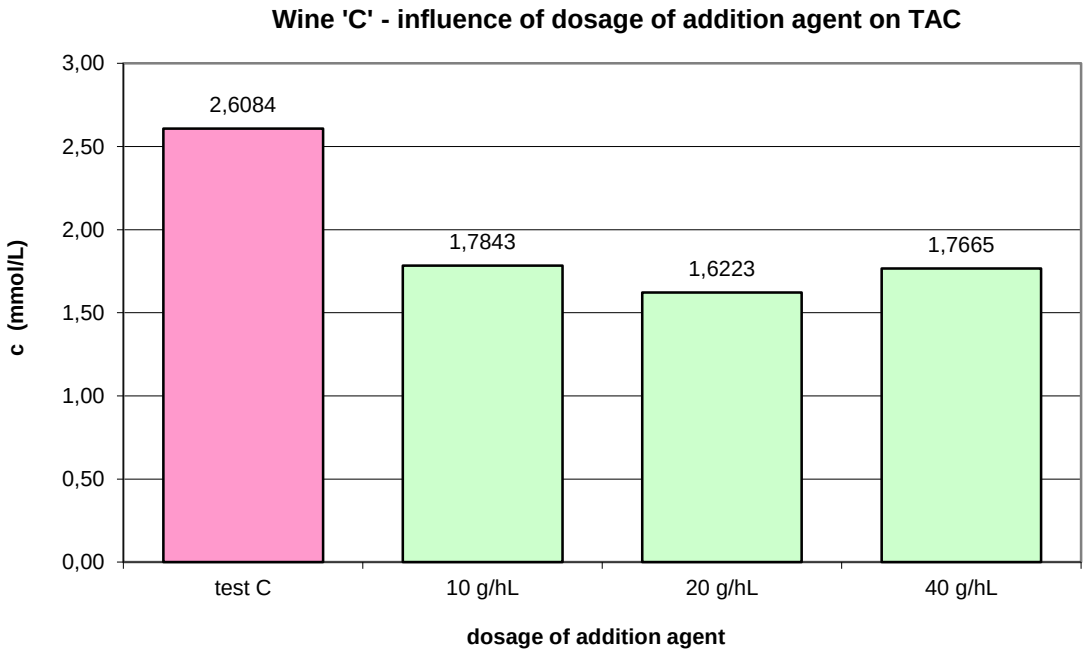

Fig. 24

Previous three graphs have shown size of influence of concentrations on value of total antioxidant capacity. In case of wines B and C the TAC has decreased even by adding small amount of fining agent (10 g/hL), while adding bigger amount of fining didn't show any further rapid decrease of TAC value. On the other hand the TAC of A samples remained more or less equal after adding 10 and 20 g/hL of clarifying agent. In samples with 40 g/hL decrease of about 30 - 40% was noted.

The next graph (**Figure 25**) was prepared to see how the dosage of clarifying agent influence TAC displayed just influence of dosage of fining independently on its type or type of wine also.

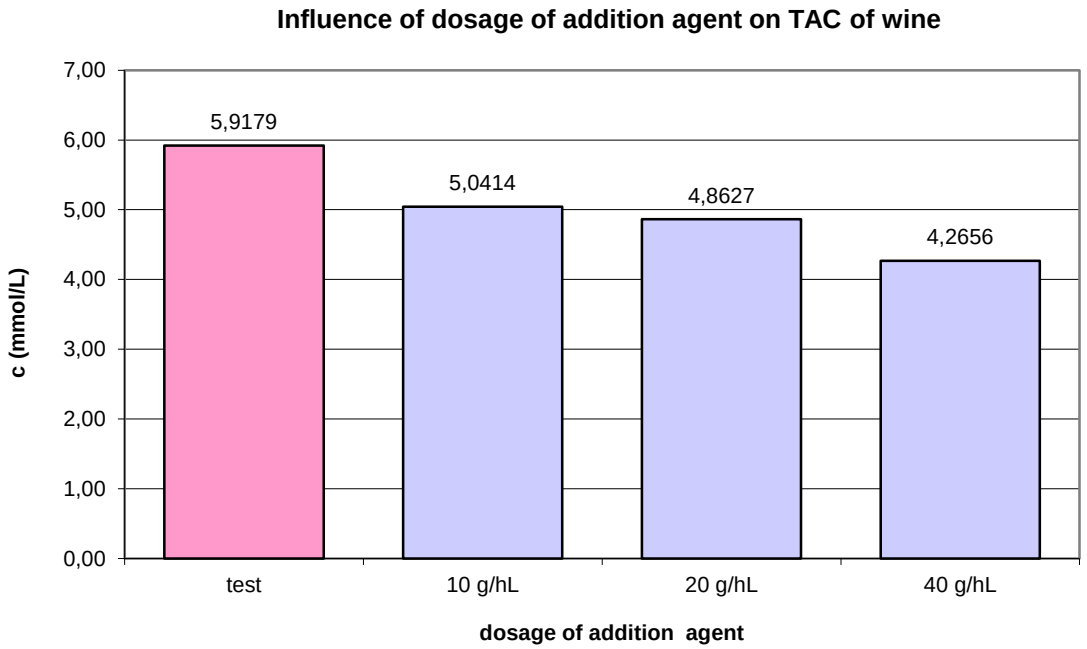

Fig. 25

The results have shown that TAC has let down to 85 % of origin value by adding 10 g/hL of fining agent. In case of amount twice bigger TAC stayed more or less equal (the variation was about 4%), in case of amount 4 times bigger it let down only to 70 %. So it was proved that amounts in order tens grams added to hectoliter of wine have approximately same effect on TAC no matter what it is 10 or 40.

Following three graphs represents interactions between dosages and types of fining agents and its influence on total antioxidant capacity for each series of wine. It is necessary to say that although each sample was a triplicate, measured results differ a lot, the standard deviation is quite big in few cases on the other side it's important to mention that all measurements done on test samples with no fining agent disposed of very little standard deviation, even measured multiple times.

For A samples (**Figure 26**) wines clarified by polyvinylpolypyrrolidone and liquid gelatin, it can't be said that bigger amount of agent increases antioxidant capacity. By samples containing GA and GOP TAC had been decreasing with rising amount of agent, but values of TAC by samples with GOP were different according to value of the test sample. Again TAC took the higher values by samples with PVPP and GOP, by GA and GL it was about from 50 to 70 % of origin value.

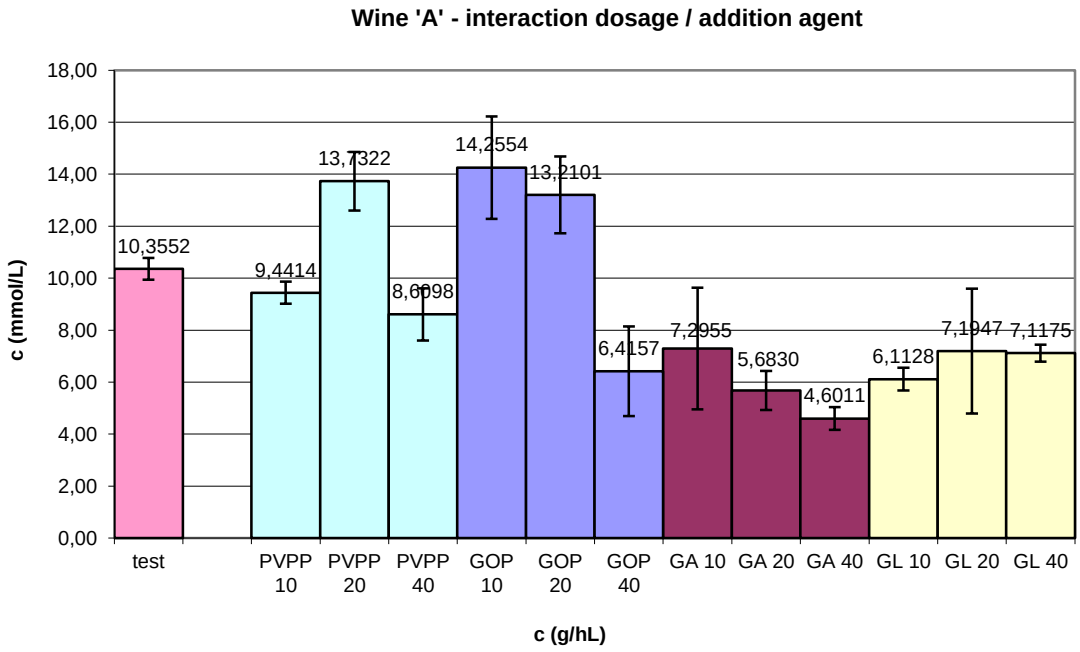

Fig. 26

Samples B (**Figure 27**) also didn't show any correlation between TAC and rising amount of single agents. By PVPP samples the TAC remained almost on same level even for samples containing 40 g/hL of fining, high values of TAC were also measured by GL samples. TAC by samples containing GOP and GA decreased of about 60 %, but independently on amount included in the samples.

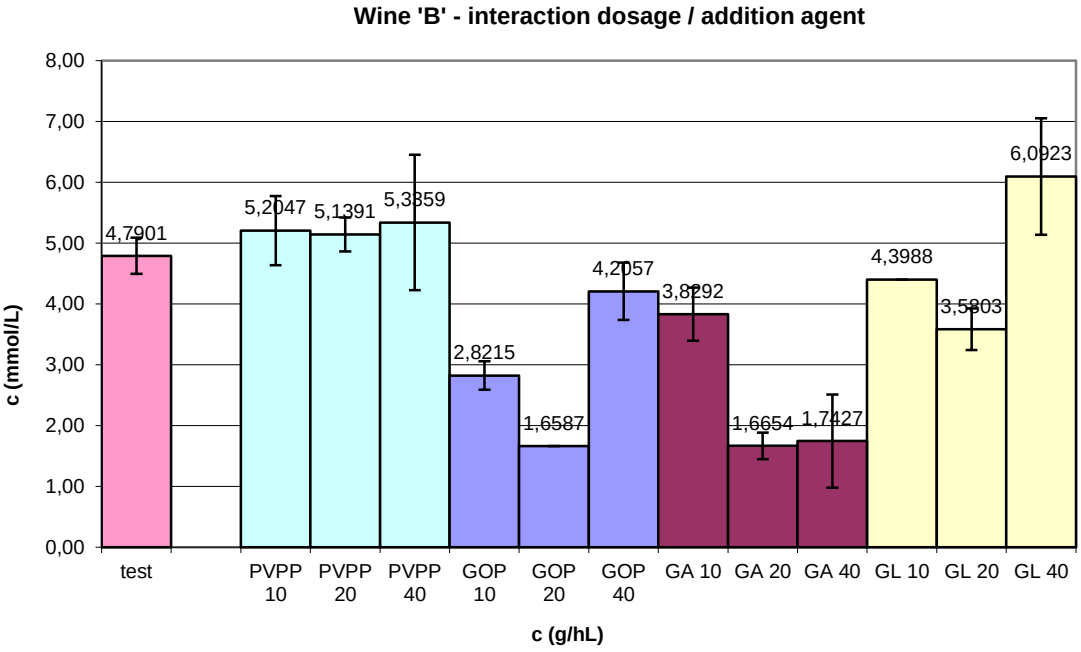

Fig. 27

As for C samples (**Figure 28**), again in case of PVPP the TAC didn't change, in other three groups it has been reached to expressive drop-off, in case of GA sample of about 40 %, by GL of 50 %, by GOP even of 60 %. The TAC decrease dependency on the increasing amount of clarifying agent was not proved again.

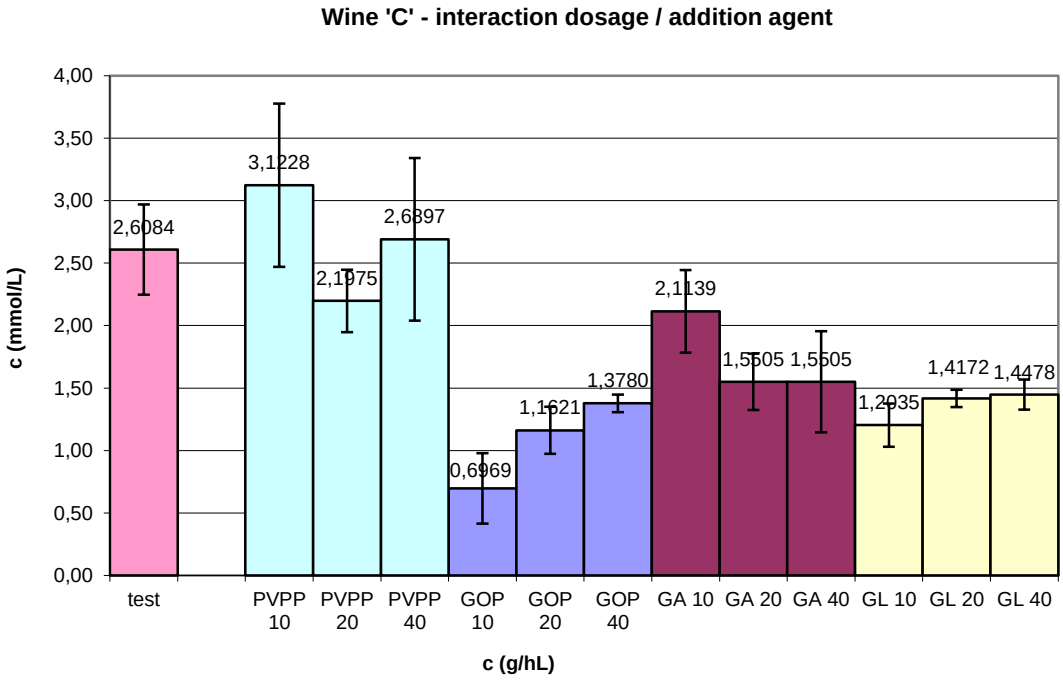

Fig. 28

In the following part of work, I was concentrated on relations between changes in TAC in content of single antioxidant components like total amount of polyphenols (TPP), anthocyanins, gallic acid and simple phenols.

First four graphs (**Figures 29 – 32**) are showing the link between antioxidant capacity and antioxidant components (for each fining regardless of their quantity in the samples) in samples of A.

As it can be seen from the graphs, the TAC doesn´t correlate with quantity of individual components by polyphenols (**Figure 29**), gallic acid (**Figure 30**) and simple phenols (**Figure 32**). The **Figure 31** may be seen a dependence of TAC on the quantity of anthocyanins, although the TAC after treatment by PVPP remains, despite the decrease in the quantity of anthocyanins, high.

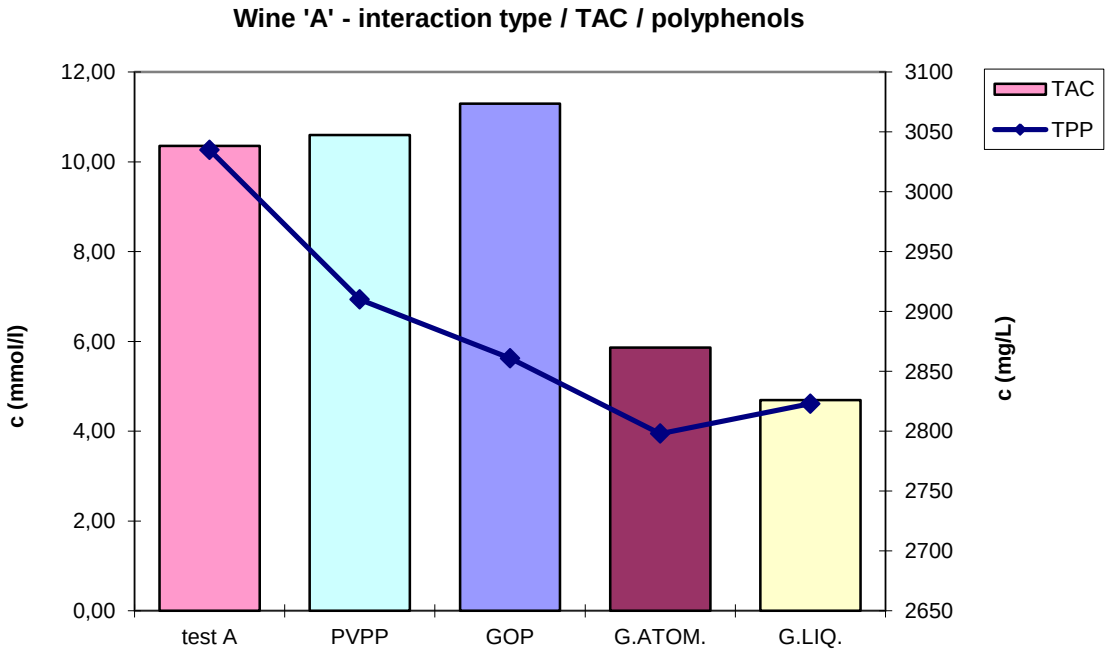
Wine 'A' - interaction type / TAC / polyphenols
12,00
10,00
8,00
6,00
4,00
2,00
0,00
c (mmol/l)
3100
3050
3000
2950
2900
2850
2800
2750
2700
2650
c (mg/L)
TAC
TPP
test A
PVPP
GOP
G.ATOM.
G.LIQ.

Fig. 29

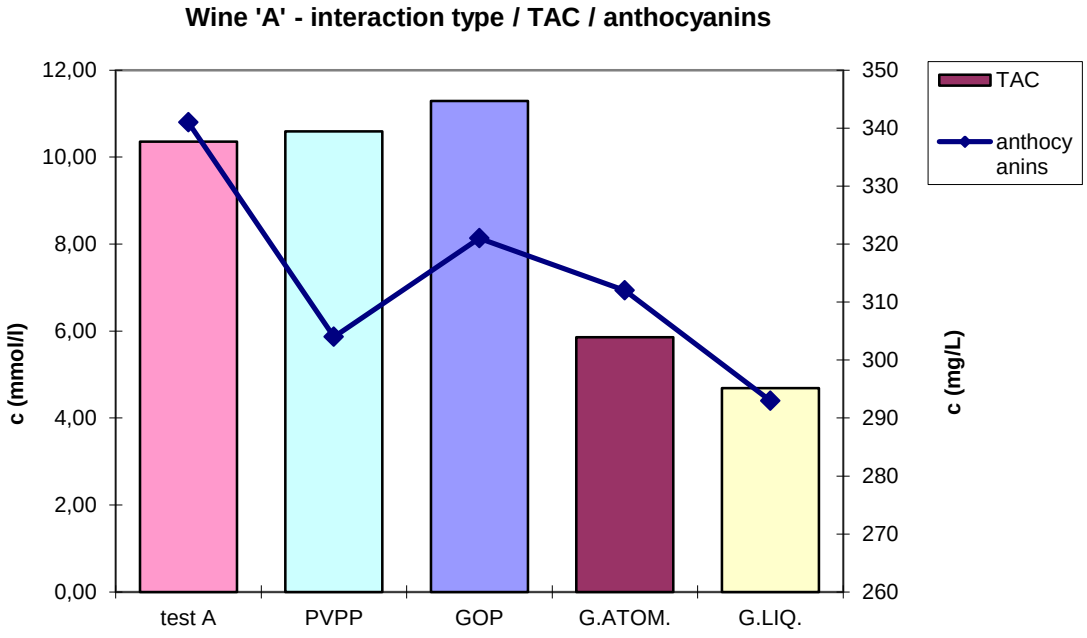
Wine 'A' - interaction type / TAC / anthocyanins
12,00
10,00
8,00
6,00
4,00
2,00
0,00
c (mmol/l)
350
340
330
320
310
300
290
280
270
260
c (mg/L)
TAC
anthocy anins
test A
PVPP
GOP
G.ATOM.
G.LIQ.

Fig. 30

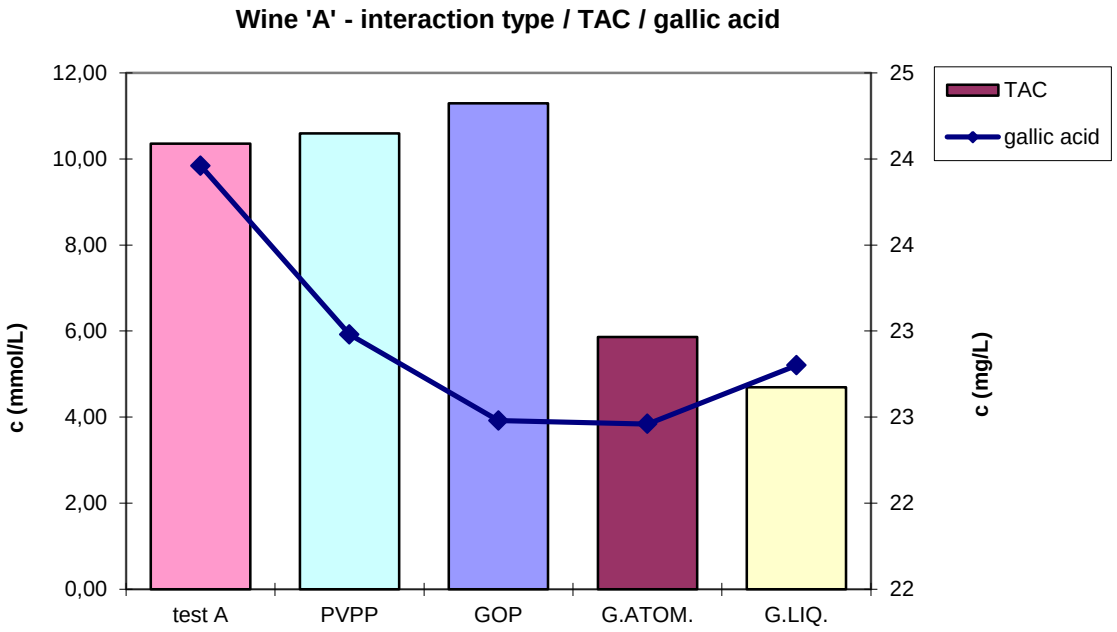

Fig. 31

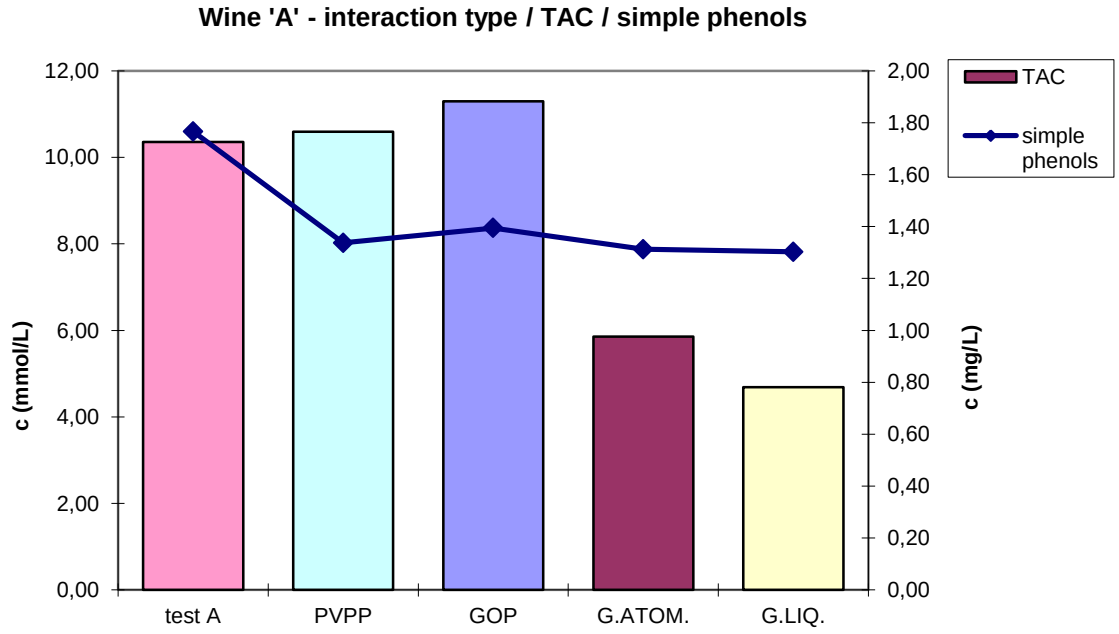

Fig. 32

The same assessment, but for the B samples is displayed in Figures 33 – 36. For comparison of samples with polyphenol compounds (**Figure 33**), we can see that the greatest amount of polyphenols remained in the samples after treatment with PVPP and GL, namely those where the highest TAC was also obtained. For graphs with

anthocyanins (**Figure 34**) and gallic acid (**Figure 35**) for treatment by PVPP there has been a significant decline of both components, while by liquid gelatin the decrease was the smallest, that means it has not been demonstrated that the TAC is dependent on the content of these components in wine. Reduce of the level of simple phenols (**Figure 36**) has not been dramatic and did not correlate with the values of the TAC.

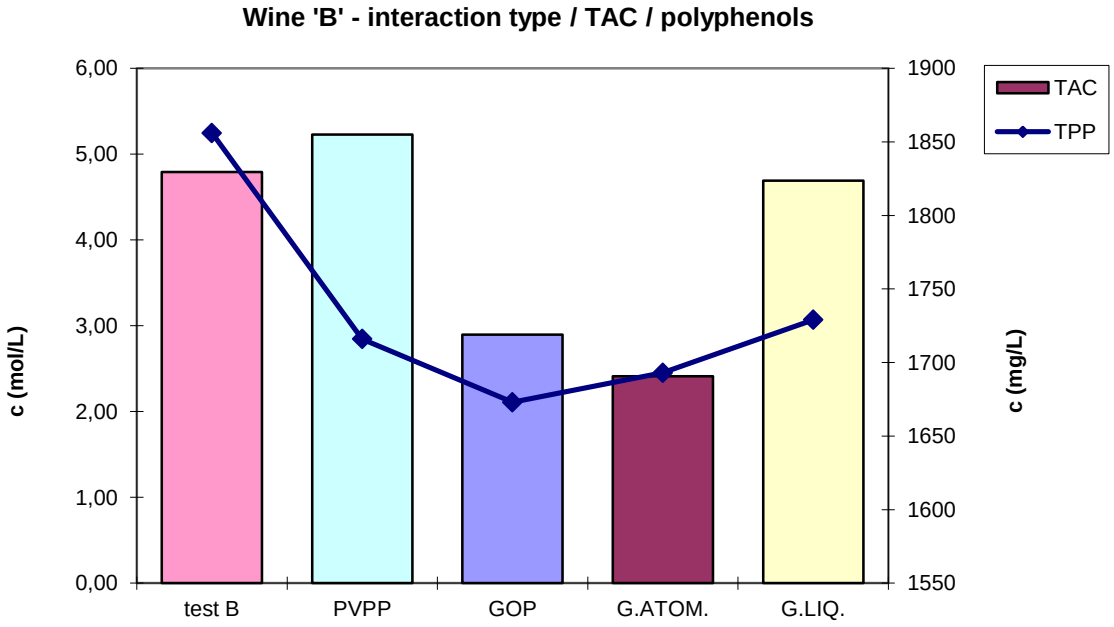

Fig. 33

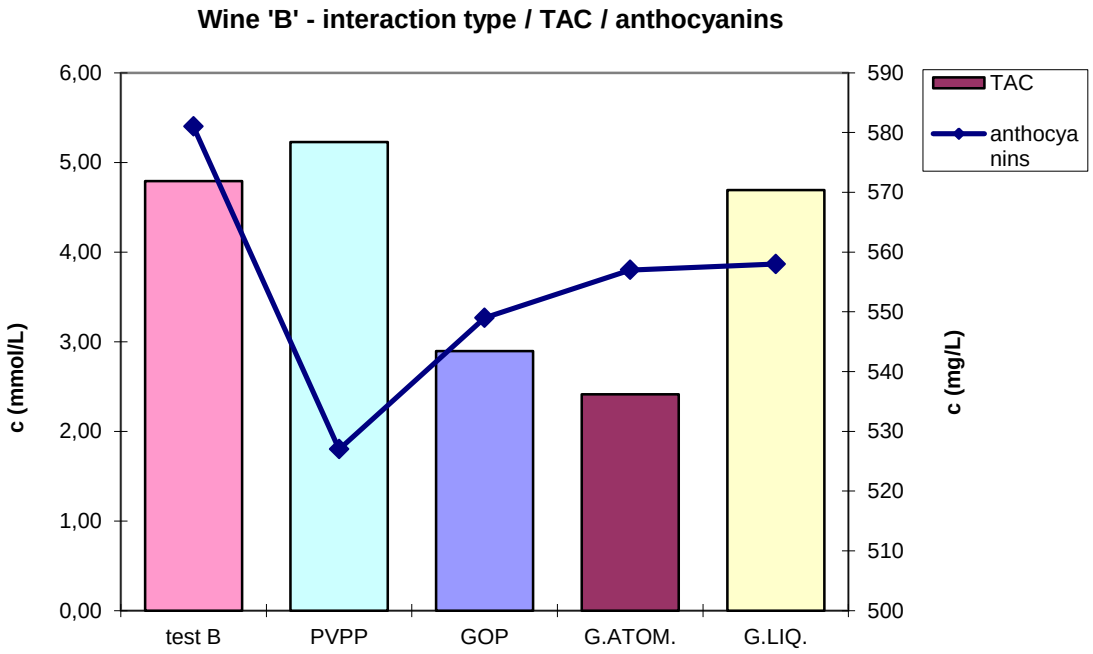
Wine 'B' - interaction type / TAC / anthocyanins
6,00
5,00
4,00
3,00
2,00
1,00
0,00
c (mmol/L)
590
580
570
560
550
540
530
520
510
500
c (mg/L)
TAC
anthocya nins
test B
PVPP
GOP
G.ATOM.
G.LIQ.

Fig. 34

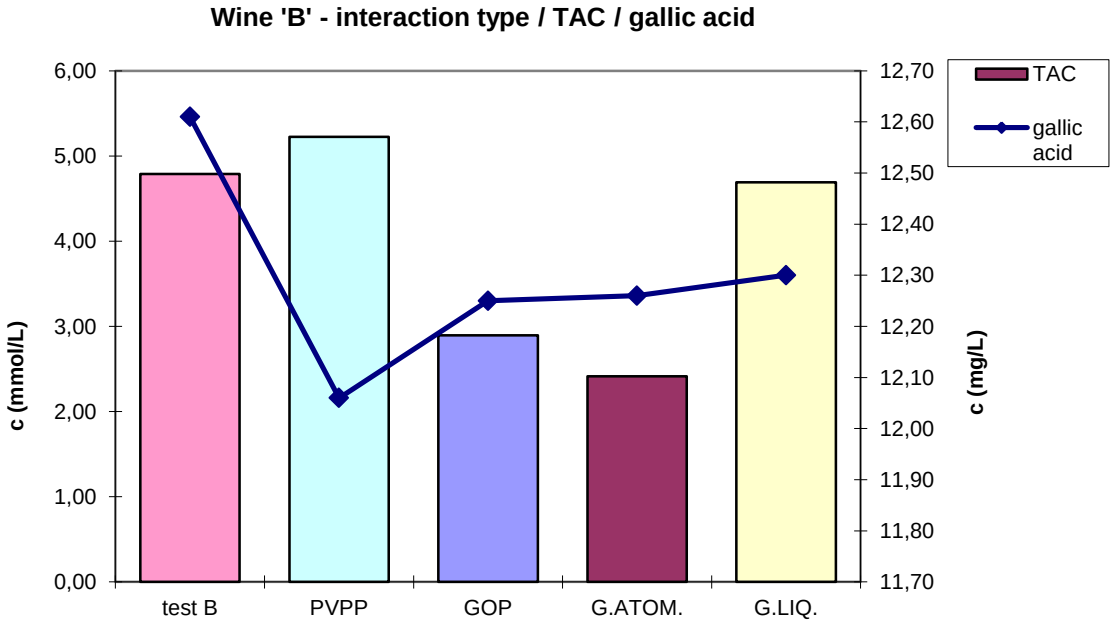
Wine 'B' - interaction type / TAC / gallic acid
6,00
5,00
4,00
3,00
2,00
1,00
0,00
c (mmol/L)
12,70
12,60
12,50
12,40
12,30
12,20
12,10
12,00
11,90
11,80
11,70
c (mg/L)
TAC
gallic acid
test B
PVPP
GOP
G.ATOM.
G.LIQ.

Fig. 35

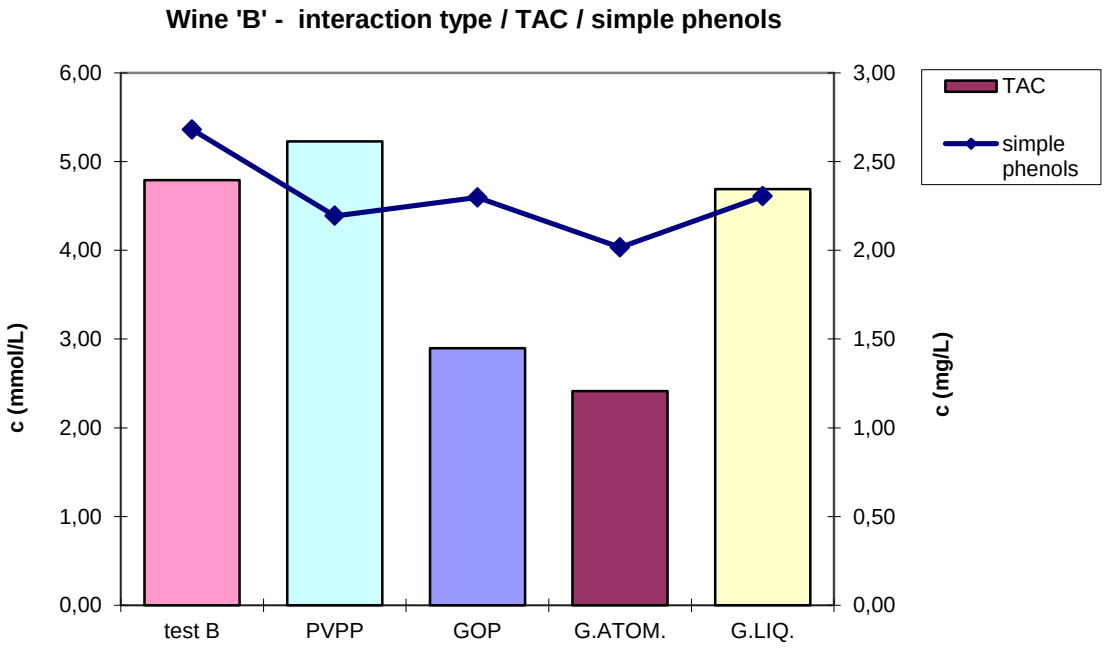

Fig. 36

Graphs from No. 37 to 40 are showing equal evaluation for wine samples C. As it can be seen from the graphs, the TAC doesn´t correspondent with amounts of individual components by anthocyanins (**Figure 38**), gallic acid (**Figure 39**) and simple phenols (**Figure 40**). Again samples treated by PVPP contain largest amount of polyphenols (**Figure 37**), also the TAC is much higher than by smaples treated with gelatin.

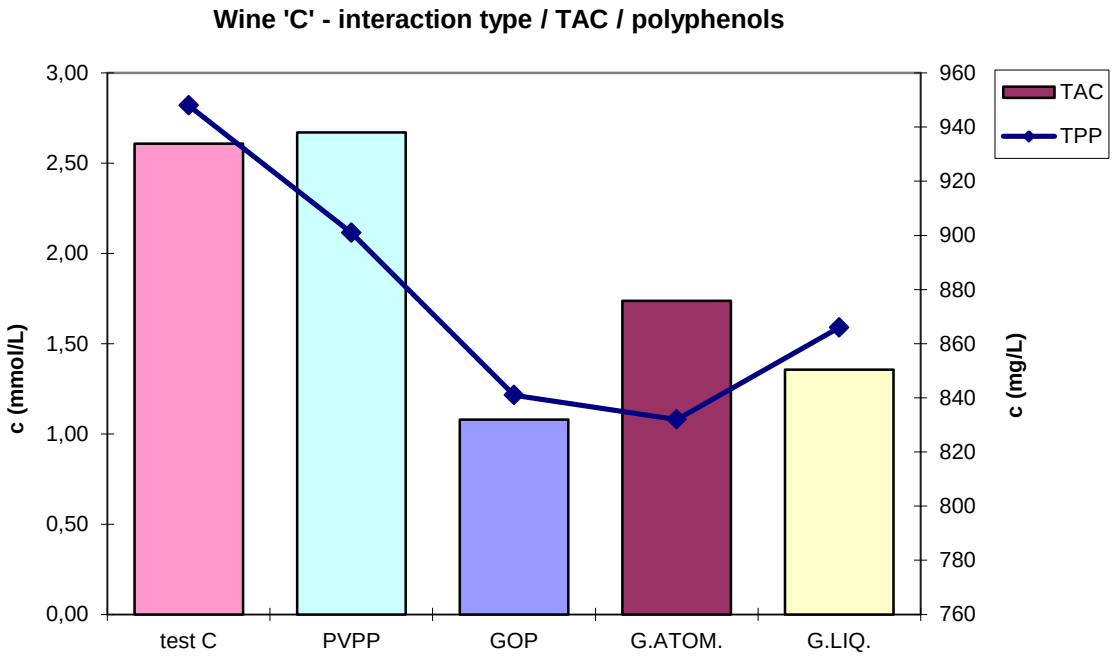
Wine 'C' - interaction type / TAC / polyphenols
3,00
2,50
2,00
1,50
1,00
0,50
0,00
c (mmol/L)
960
940
920
900
880
860
840
820
800
780
760
c (mg/L)
TAC
TPP
test C
PVPP
GOP
G.ATOM.
G.LIQ.

Fig. 37

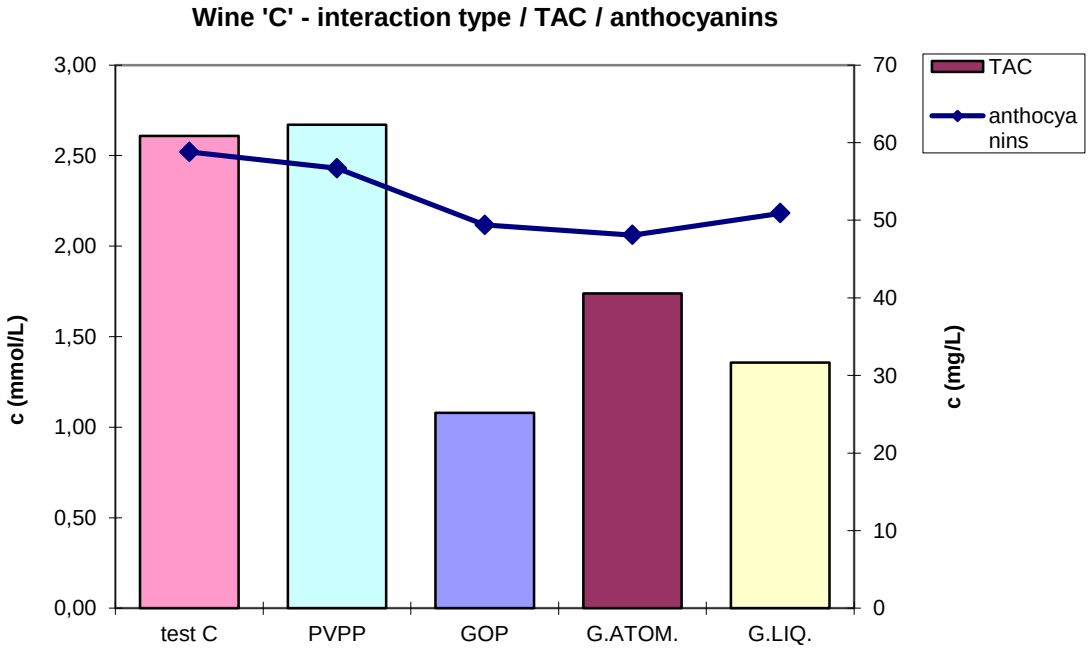
Wine 'C' - interaction type / TAC / anthocyanins
3,00
2,50
2,00
1,50
1,00
0,50
0,00
c (mmol/L)
70
60
50
40
30
20
10
0
c (mg/L)
TAC
anthocya
nins
test C
PVPP
GOP
G.ATOM.
G.LIQ.

Fig. 38

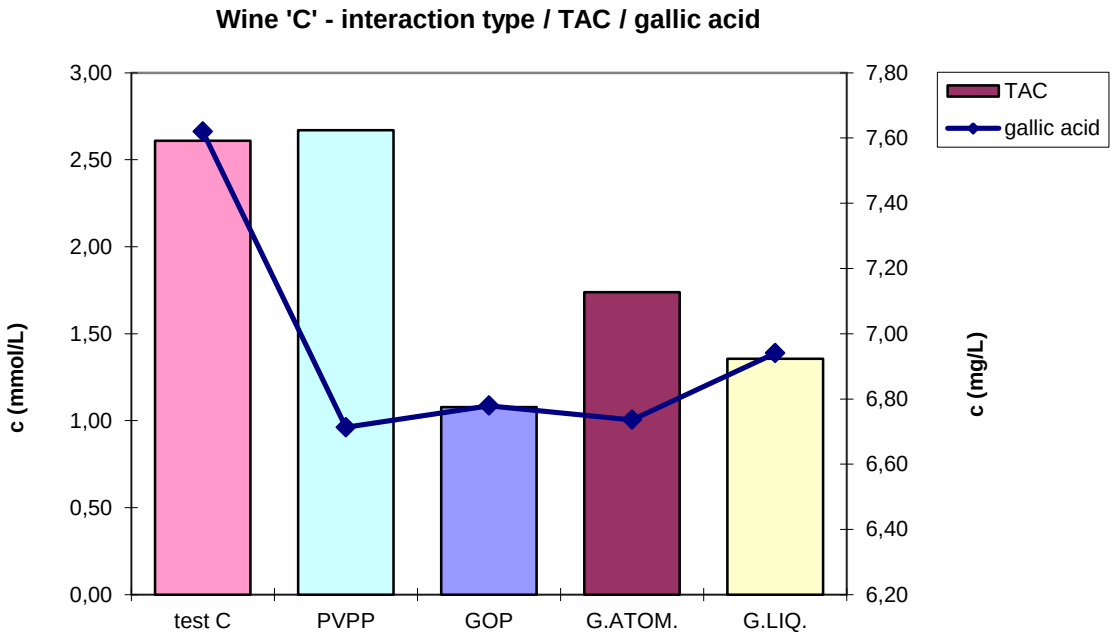
Wine 'C' - interaction type / TAC / gallic acid
3,00
2,50
2,00
1,50
1,00
0,50
0,00
c (mmol/L)
7,80
7,60
7,40
7,20
7,00
6,80
6,60
6,40
6,20
c (mg/L)
TAC
gallic acid
test C
PVPP
GOP
G.ATOM.
G.LIQ.

Fig. 39

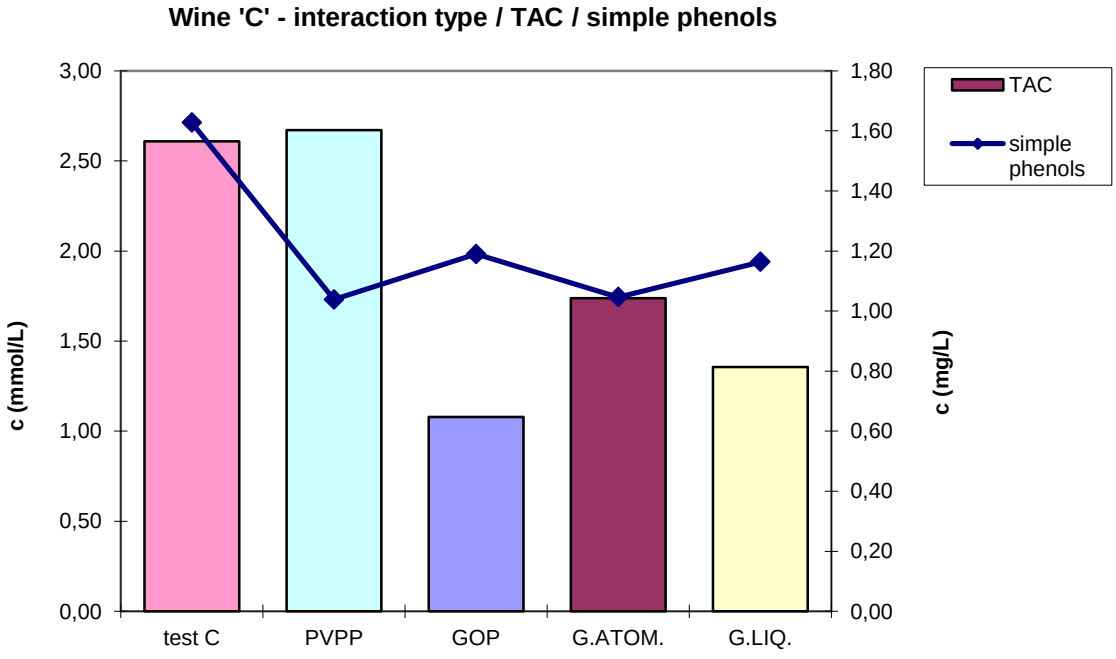
Wine 'C' - interaction type / TAC / simple phenols
3,00
2,50
2,00
1,50
1,00
0,50
0,00
c (mmol/L)
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
c (mg/L)
TAC
simple phenols
test C
PVPP
GOP
G.ATOM.
G.LIQ.

Fig. 40

Next four graphs summarize the results for all three types of wine together, treated by each clarifying agent but independently on its dosage again. The first one (**Figure 41**) refers to polyphenols. The values of the quantity of polyphenols in samples reply for the size of the TAC, so that the maximum amount of polyphenols remains in the wine after treatment by PVPP, as well as the TAC is kept, followed by GL, GOP, and finally GA.

For anthocyanins (**Figure 42**) on the other hand, rapid drop-off in values of PVPP samples is obtained, as well as for GL, on the contrary by samples with lower TAC (treated by GOP and GA) the amount of anthocyanins remains higher. It can be assumed that the cleanup of these compounds does not have an impact on the final value of TAC.

The content of gallic acid (**Figure 43**) declines in samples treated by PVPP, GOP and the GA of about 8%. For samples treated by GL is the final amount slightly higher. Quantity of gallic acid then does not have a direct impact on the TAC.

The value of simple phenols (**Figure 44**) after the treatment is falling on average by 25%, in the case of PVPP and the GA is the decrease slightly bigger than for the GOP and GL.

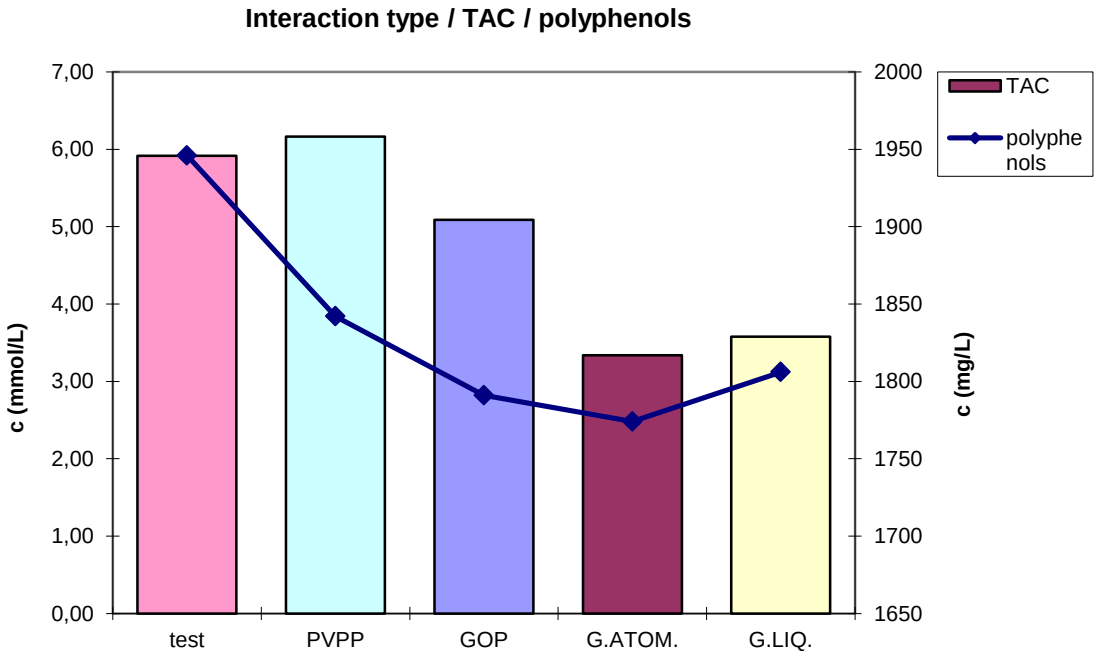

Fig. 41

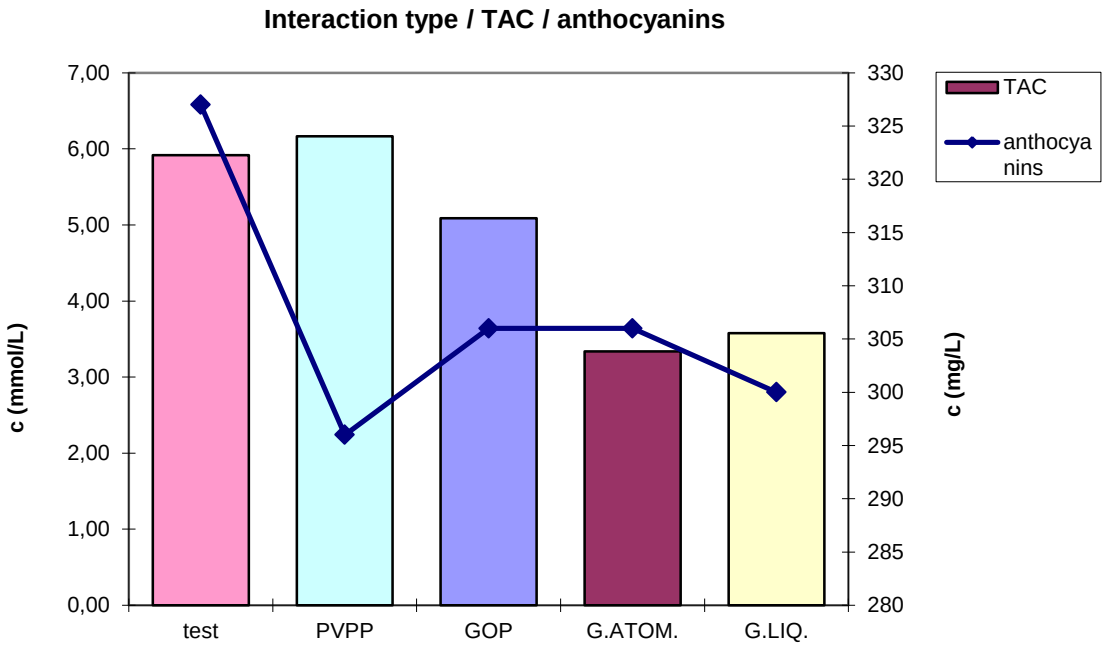
Interaction type / TAC / anthocyanins
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
c (mmol/L)
330
325
320
315
310
305
300
295
290
285
280
c (mg/L)
test
PVPP
GOP
G.ATOM.
G.LIQ.
TAC
anthocya
nins

Fig. 42

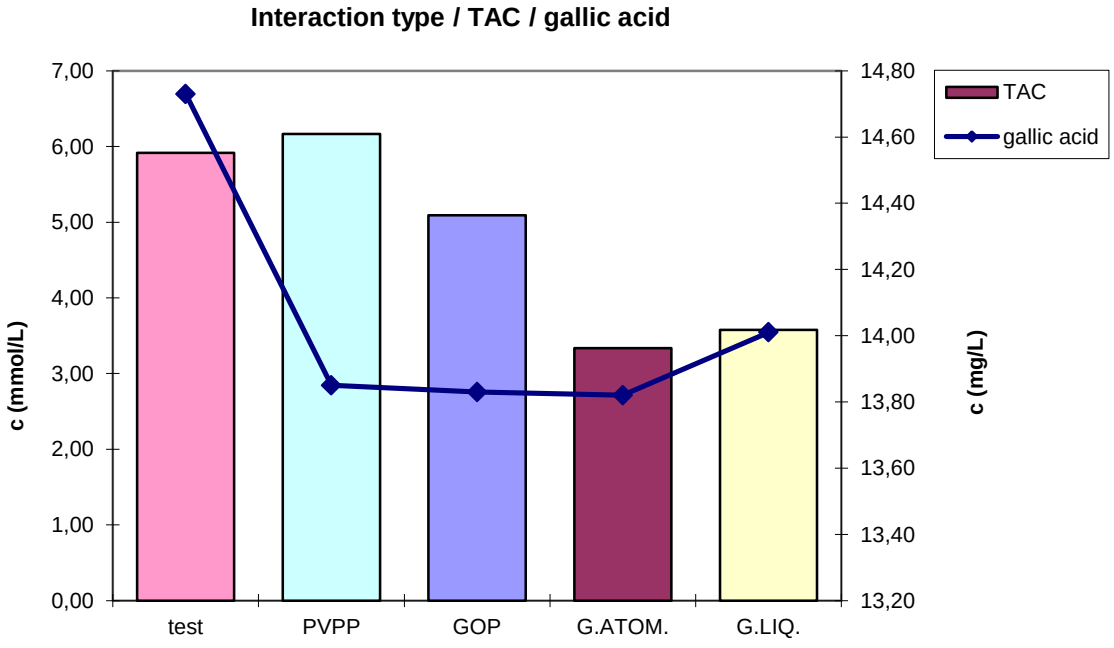
Interaction type / TAC / gallic acid
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
c (mmol/L)
14,80
14,60
14,40
14,20
14,00
13,80
13,60
13,40
13,20
c (mg/L)
test
PVPP
GOP
G.ATOM.
G.LIQ.
TAC
gallic acid

Fig. 43

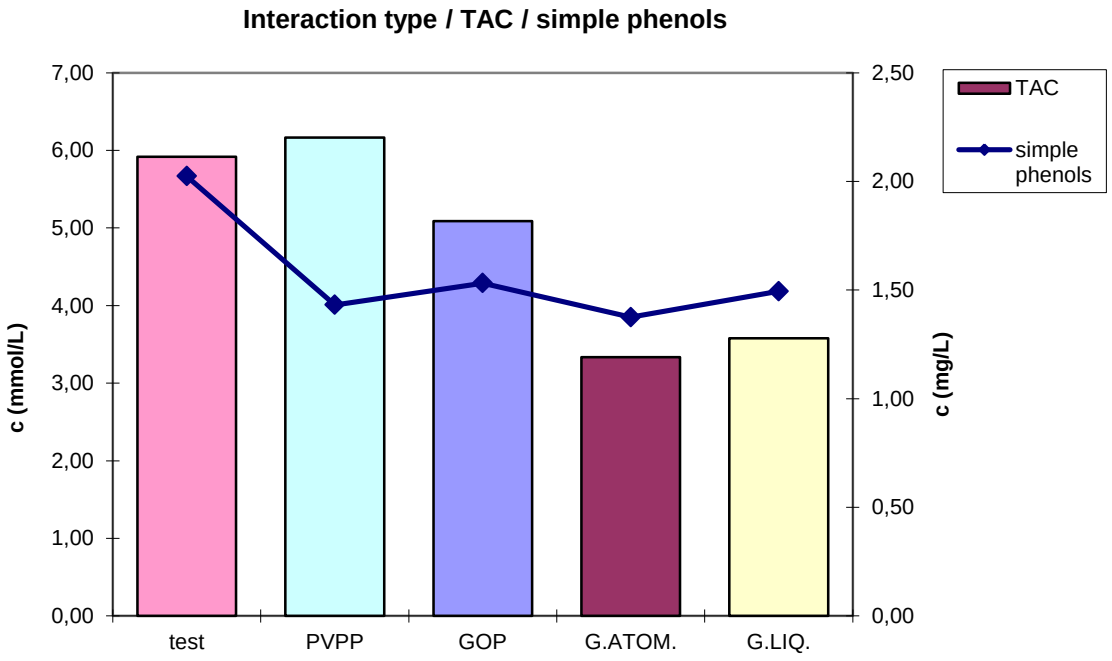

Fig. 44

Following several graphs are showing changes in content of the various antioxidant components and also the TAC for each concentration of clarifying agents, regardless of what specific type it is.

Graphs No. 45 - 48 show the results for the samples of wine A. While the amount of polyphenols (**Figure 45**) is falling the most when 10 g / hl of fining agent is added, the treatment can be observed significantly on values of TAC after adding 40 g / hl. Similar stuff can be also applied to anthocyanins (**Figure 46**).

From **Figure 47** you can see that the amount of gallic acid decreases with increasing amount of clarifying agent almost linearly, for simple phenols (**Figure 48**) again, the biggest drop occurs for concentration of 10 g / hl, with the increasing amount of agent the decrease is no longer so dramatic.

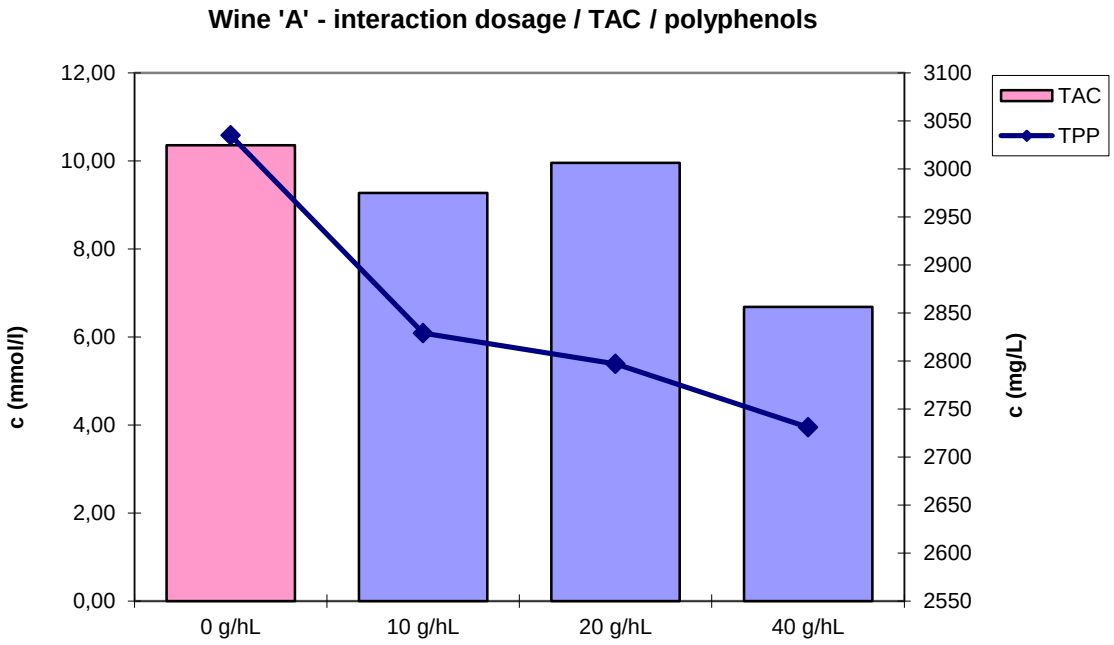
Wine 'A' - interaction dosage / TAC / polyphenols
12,00
10,00
8,00
6,00
4,00
2,00
0,00
c (mmol/l)
3100
3050
3000
2950
2900
2850
2800
2750
2700
2650
2600
2550
c (mg/L)
TAC
TPP
0 g/hL
10 g/hL
20 g/hL
40 g/hL

Fig. 45

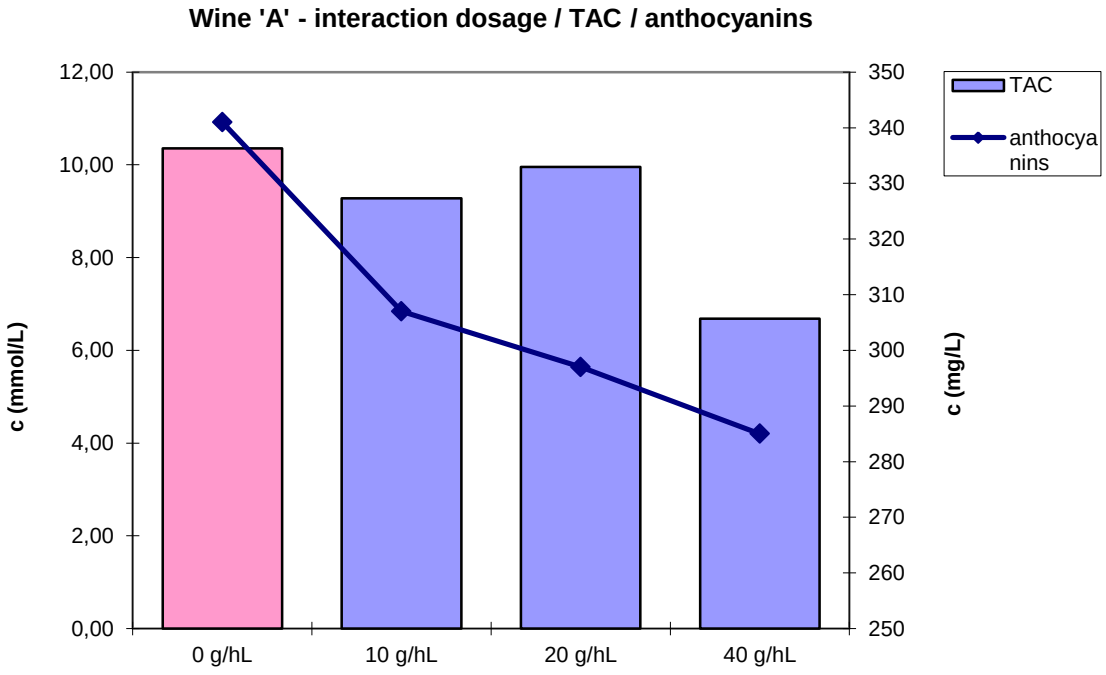
Wine 'A' - interaction dosage / TAC / anthocyanins
12,00
10,00
8,00
6,00
4,00
2,00
0,00
c (mmol/L)
350
340
330
320
310
300
290
280
270
260
250
c (mg/L)
TAC
anthocya
nins
0 g/hL
10 g/hL
20 g/hL
40 g/hL

Fig. 46

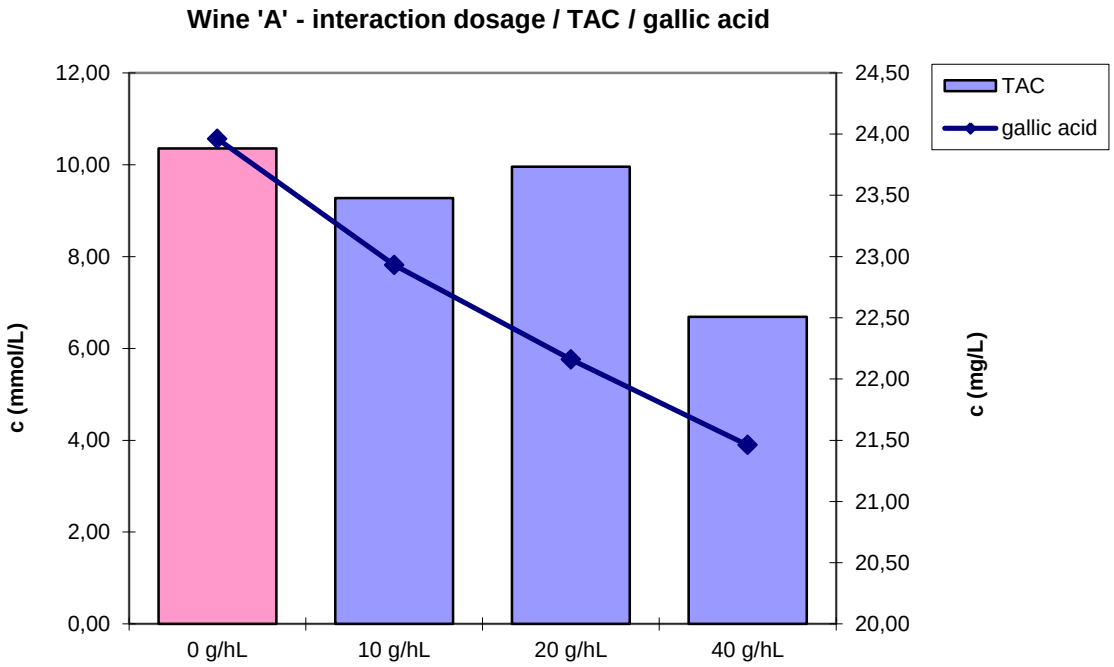

Fig. 47

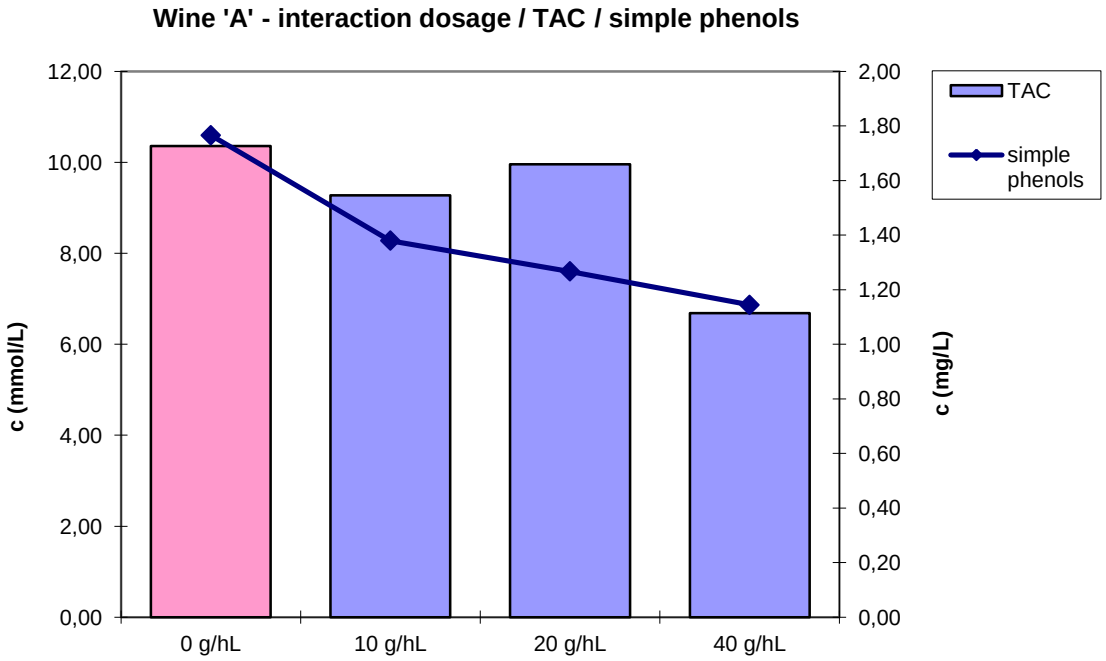

Fig. 48

By B samples, the quantity of each antioxidant component decrease gradually with increasing quantities of fining agent in the sample, but the total TAC haven´t shown a similar addiction.

In the graph showing amount of polyphenols (**Figure 49**) , the content has decreased at a concentration of 10 g / hl to 90%, while by the concentration which was four times higher only by a further 5%.

Situation was similar with anthocyanins (**Figure 50**), but the fall in the value by addition of 10 g/hL correspond to a decrease after addition of another 30 g/hl.

In the case of gallic acid (**Figure 51**), the biggest decline in the quantity of component occured by measuring samples containing 20 g/hl.

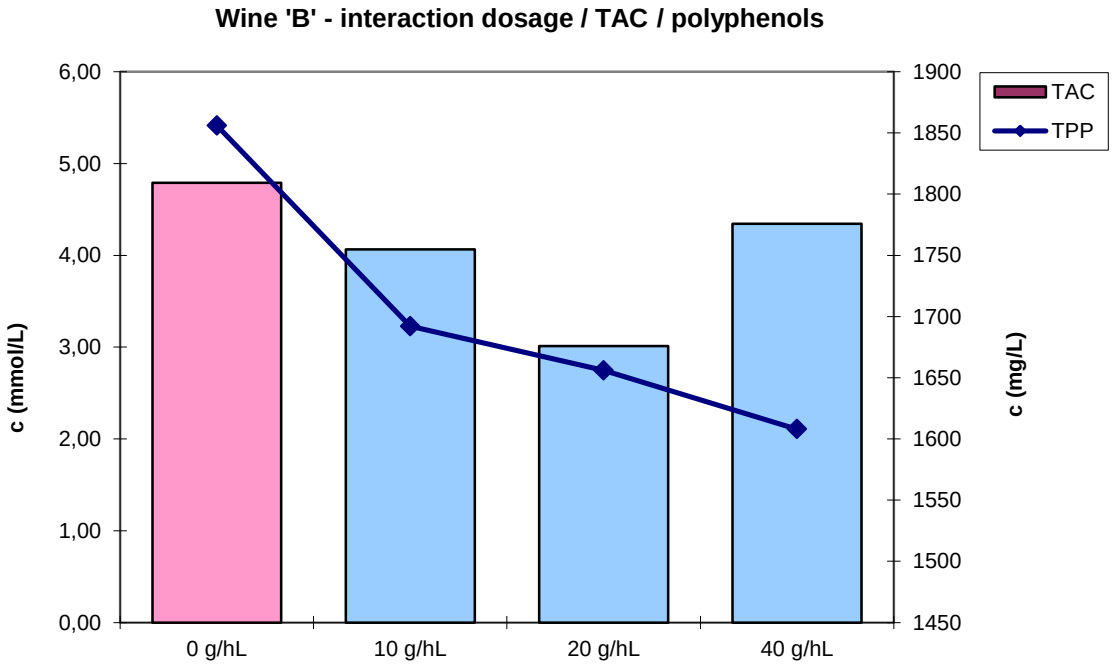

Fig. 49

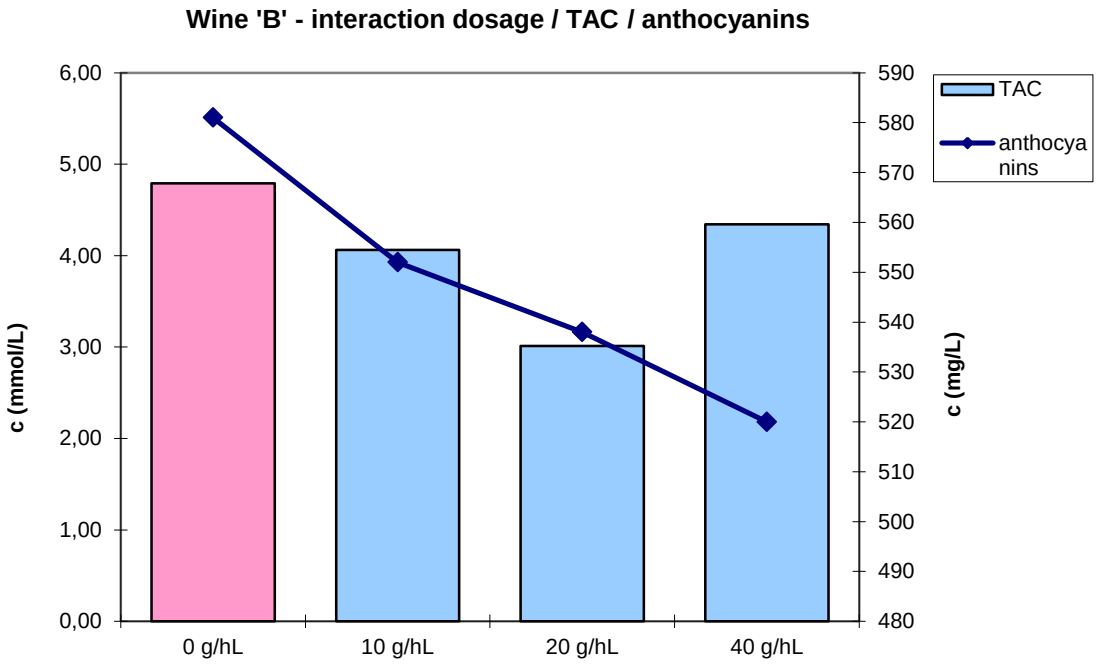
Wine 'B' - interaction dosage / TAC / anthocyanins
6,00
5,00
4,00
3,00
2,00
1,00
0,00
c (mmol/L)
590
580
570
560
550
540
530
520
510
500
490
480
c (mg/L)
TAC
anthocya nins
0 g/hL
10 g/hL
20 g/hL
40 g/hL

Fig. 50

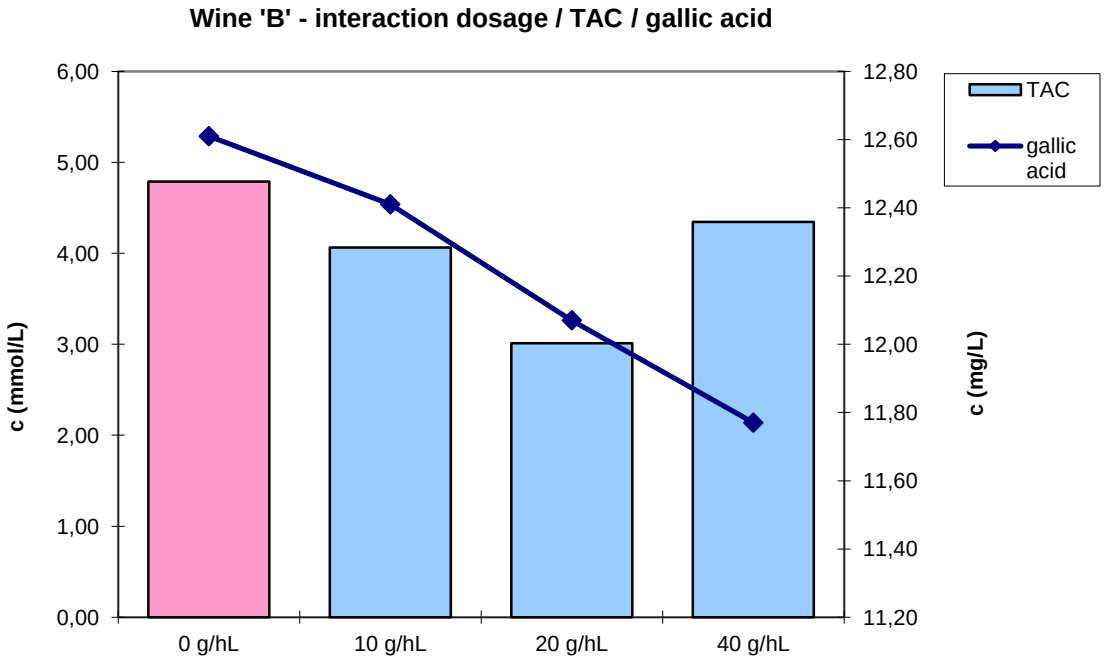
Wine 'B' - interaction dosage / TAC / gallic acid
6,00
5,00
4,00
3,00
2,00
1,00
0,00
c (mmol/L)
12,80
12,60
12,40
12,20
12,00
11,80
11,60
11,40
11,20
c (mg/L)
TAC
gallic acid
0 g/hL
10 g/hL
20 g/hL
40 g/hL

Fig. 51

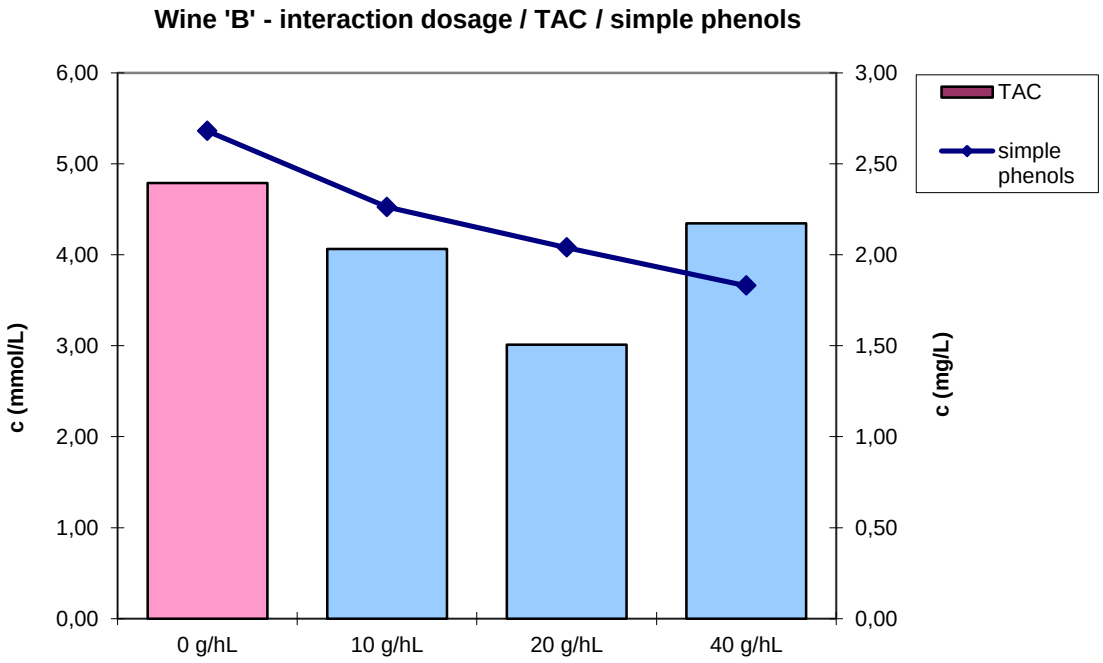

Fig. 52

Graphs No. 53 - 56 show the results for the C samples. The TAC (decreased the most after adding 10 g/hL of agents, while with increasing amounts of finings has stayed practically on the same level) imitated behavior of anthocyanins (**Figure 54**), gallic acid (**Figure 55**) and also simple phenols (**Figure 56**). On the other hand content of polyphenols (**Figure 53**) was falling almost linearly with increasing amounts of finings.

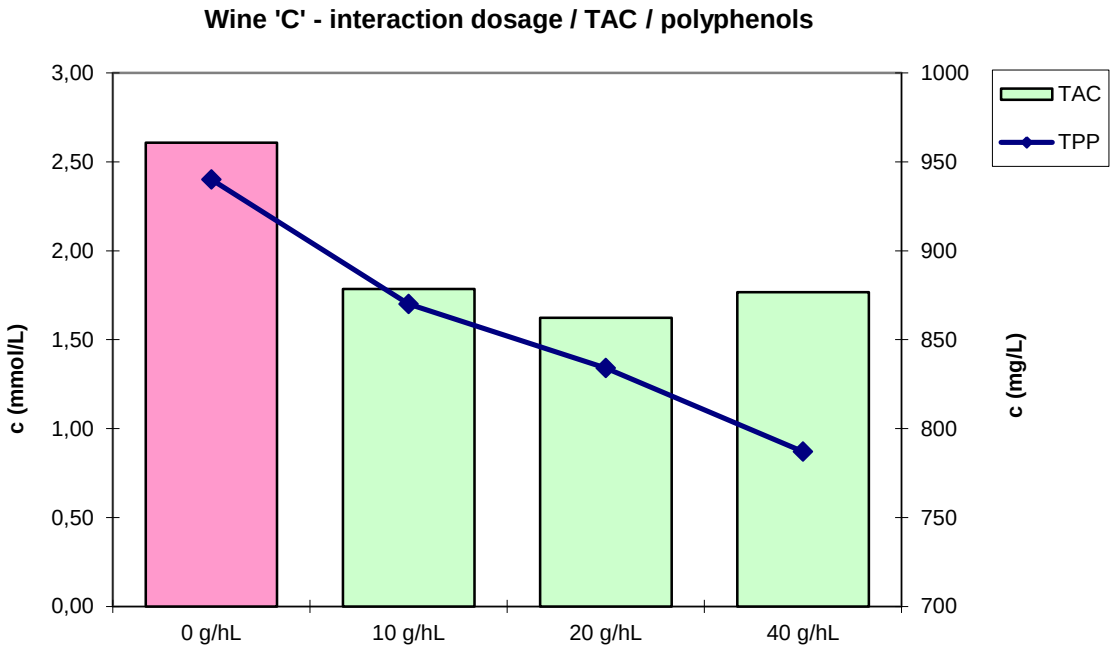
Wine 'C' - interaction dosage / TAC / polyphenols
3,00
2,50
2,00
1,50
1,00
0,50
0,00
c (mmol/L)
1000
950
900
850
800
750
700
c (mg/L)
TAC
TPP
0 g/hL
10 g/hL
20 g/hL
40 g/hL

Fig. 53

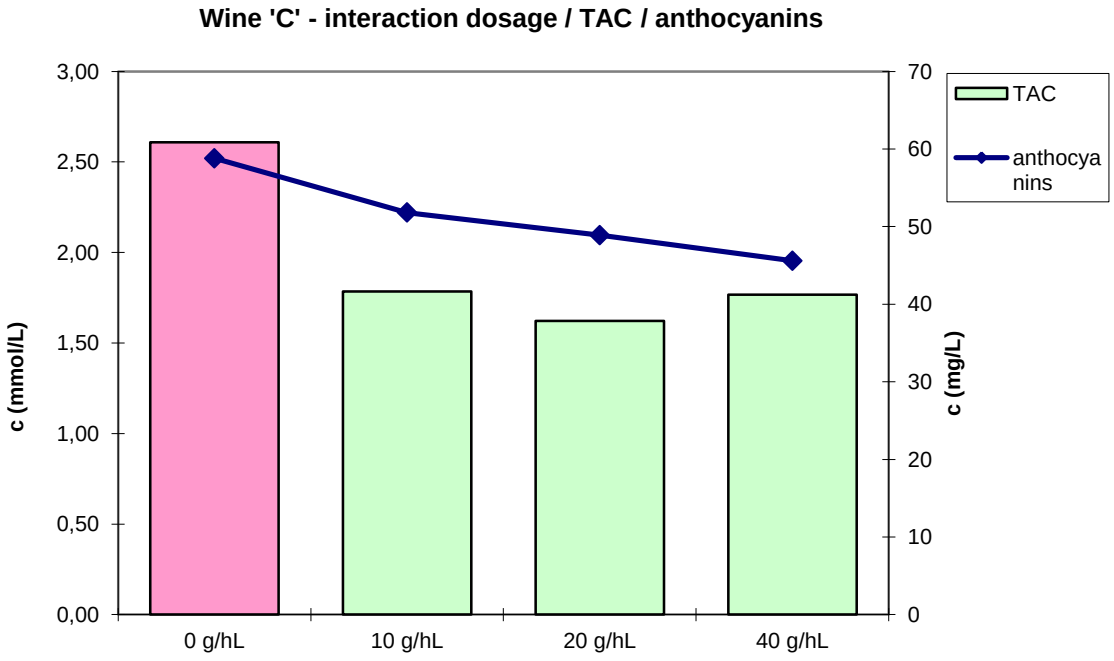
Wine 'C' - interaction dosage / TAC / anthocyanins
3,00
2,50
2,00
1,50
1,00
0,50
0,00
c (mmol/L)
70
60
50
40
30
20
10
0
c (mg/L)
TAC
anthocya
nins
0 g/hL
10 g/hL
20 g/hL
40 g/hL

Fig. 54

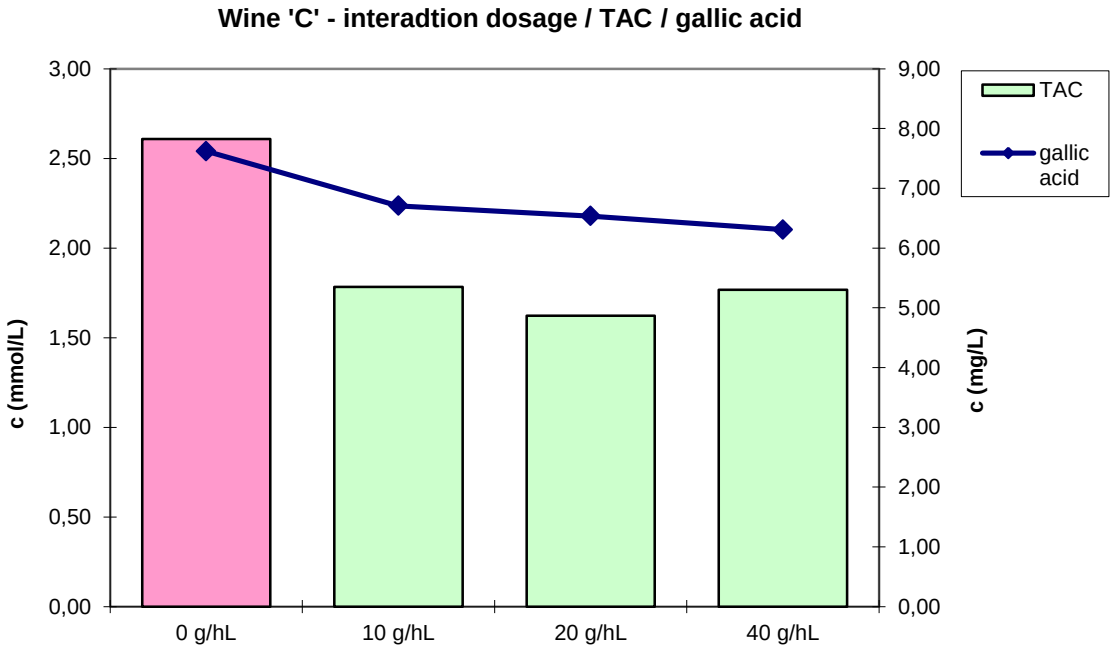

Fig. 55

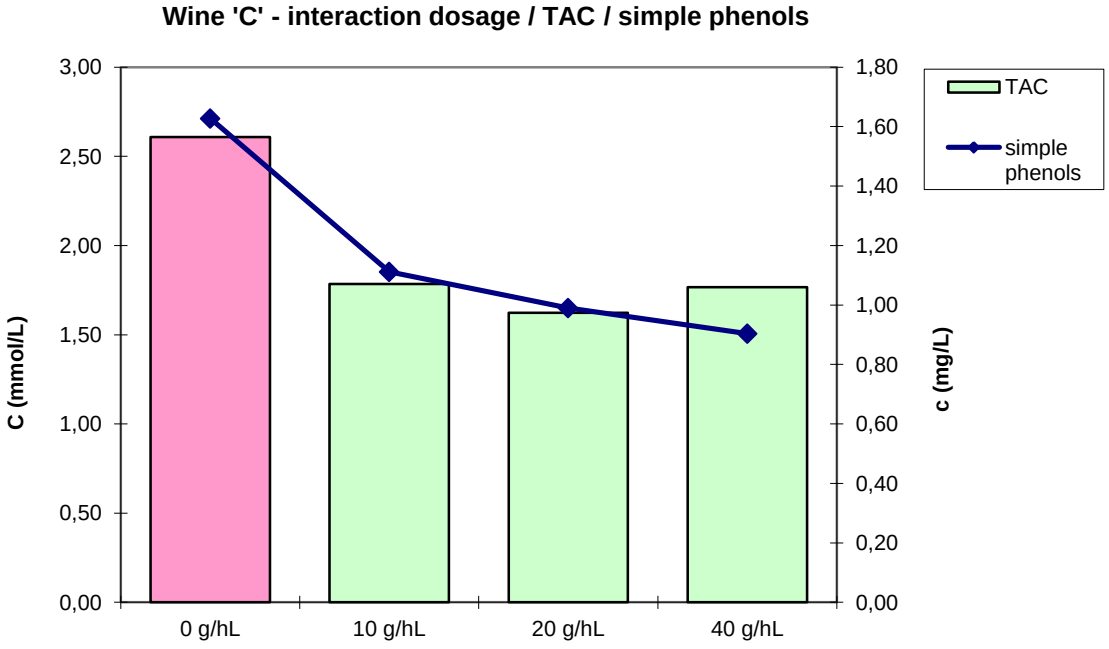

Fig. 56

Next four graphs summarize the results for all three types of wine together, treated by different dosages independently on its type again. From all the four graphs the decline in both the content of antioxidant components and also the

increasing TAC content of finings is apparent. For polyphenols (**Figure 57**), anthocyanins (**Figure 58**) and gallic acid (**Figure 59**) the decreases of the individual antioxidant components are steeper than in the case of TAC. The differences for simple phenols (**Figure 60**) are very small.

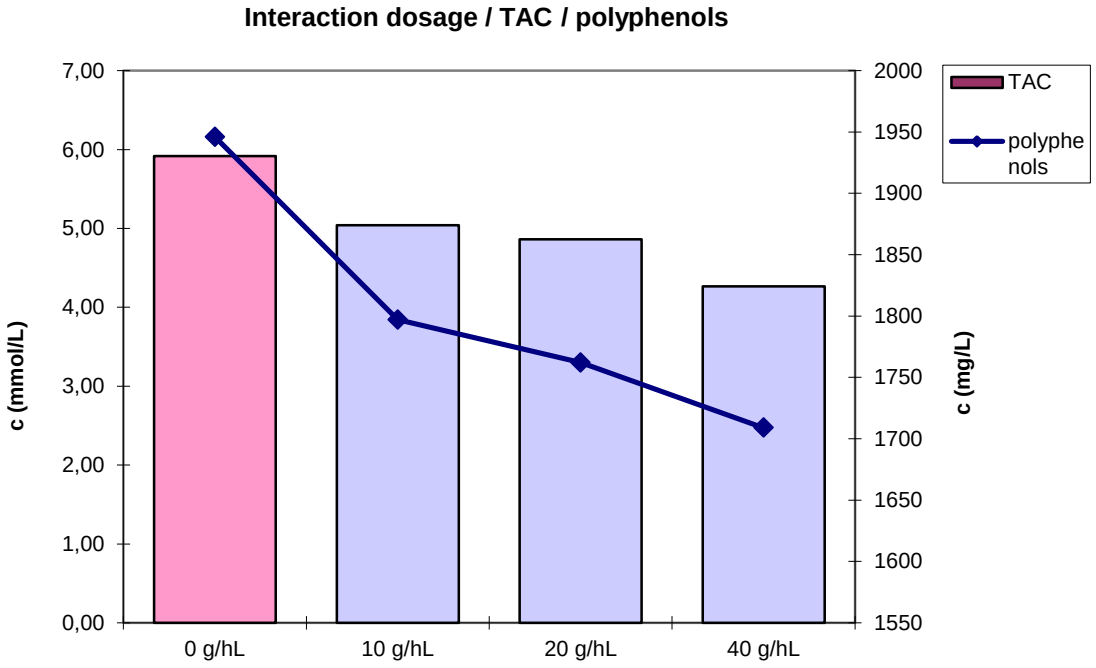

Fig. 57

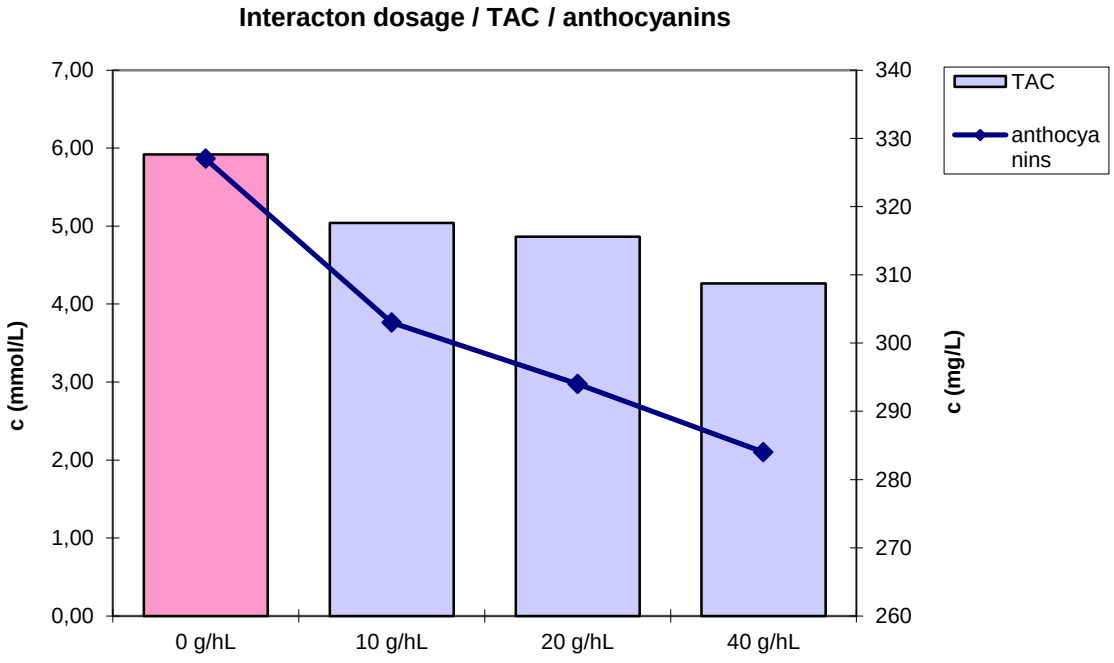

Fig. 58

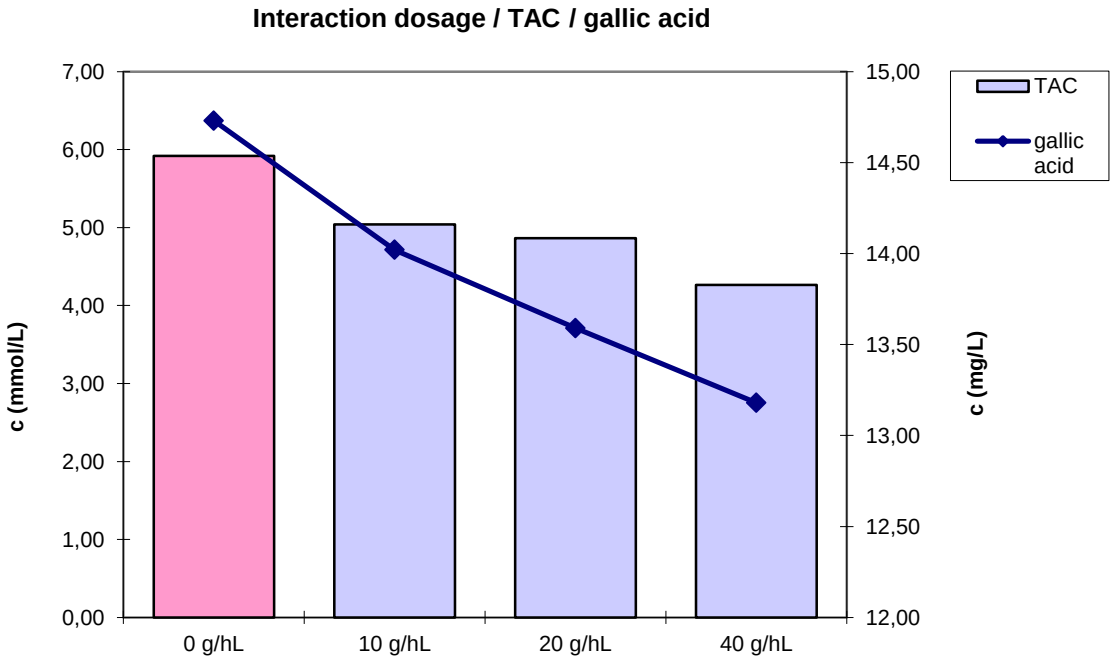

Fig. 59

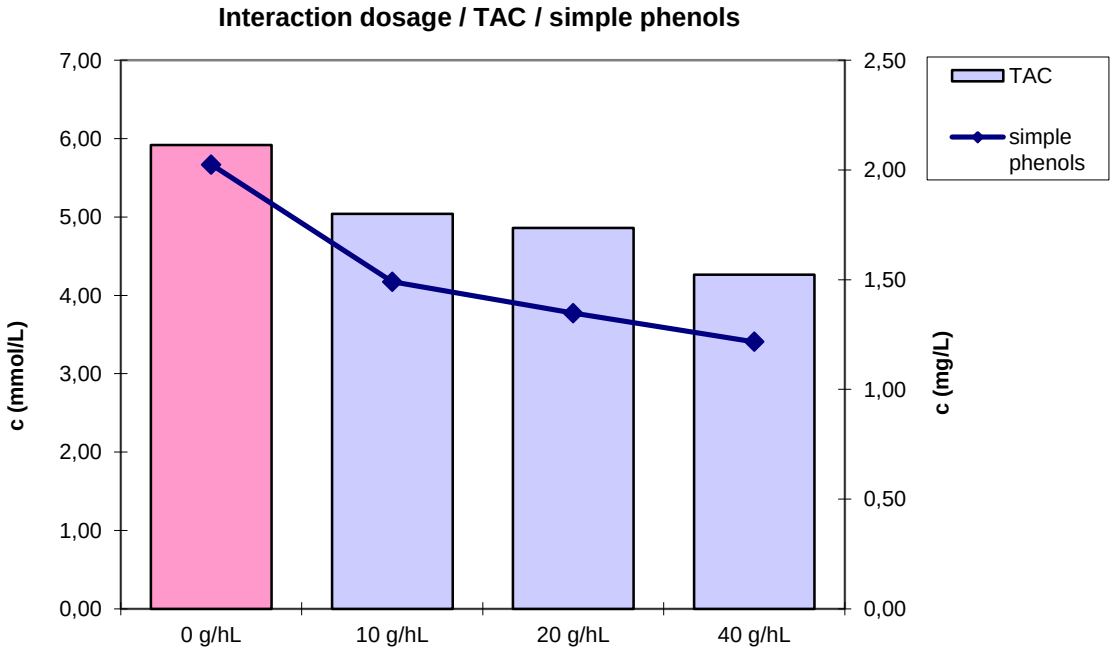

Fig. 60

Following graphs represent interactions between TAC and antioxidant compounds for each type of fining agents and its separate dosages.

First four graphs demonstrate situation of A samples. The greatest amount of polyphenols (**Figure 61**) remain after treatment with PVPP and powder gelatin, also the TAC of these samples is the highest, on the other side GA and GL remove from samples bigger amount of PPT, which is reflected on the final value of TAC. It´s also important to notice, that there is a big difference of PPT contained in test sample, in contrary with all treated samples, which did not manifest in the final TAC.

Differences between amounts of anthocyanins (**Figure 62**) are not as makeable as the TAC. The biggest quantity is kept after GOP treatment, the lowest after GL. The volume of anthocyanins changes in dependence on dosage of clarifying agent only little.

Amount of gallic acid (**Figure 63**) changes very little by samples treated by PVPP, in contrary of other three. Overall by all finings the amount decreases visibly already after treating with 10 g/hL.

In case of simple phenols (**Figure 64**) the amount has been declining with rising dosage of fining agents, but differences between individual agents were insignificant.

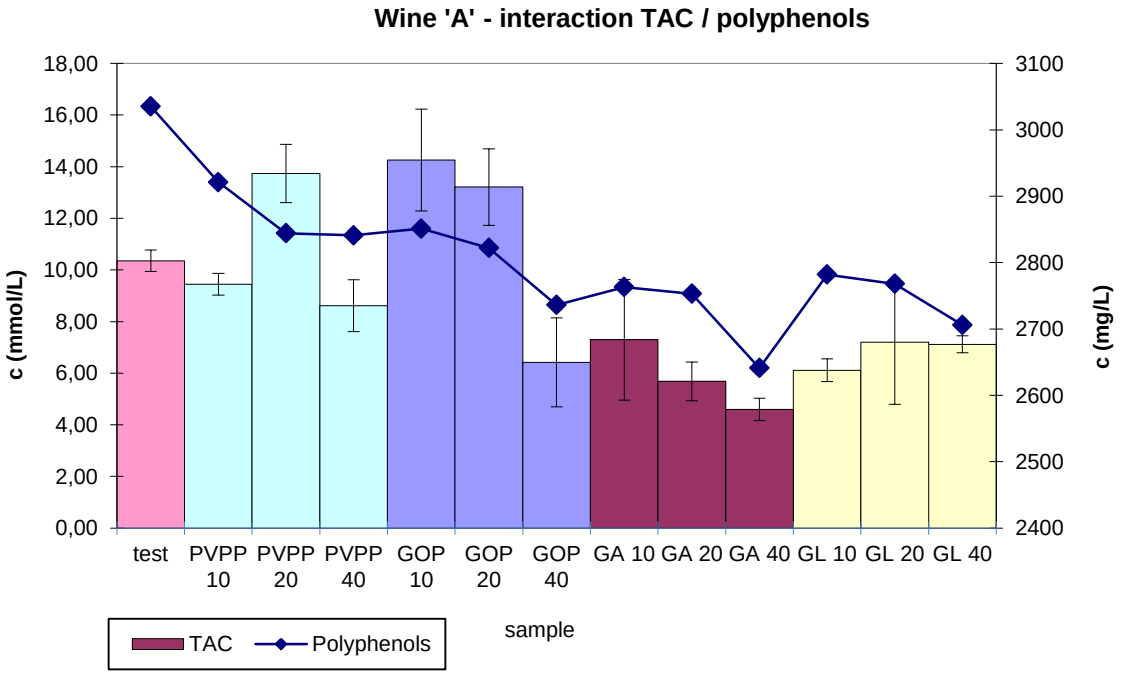

Fig. 61

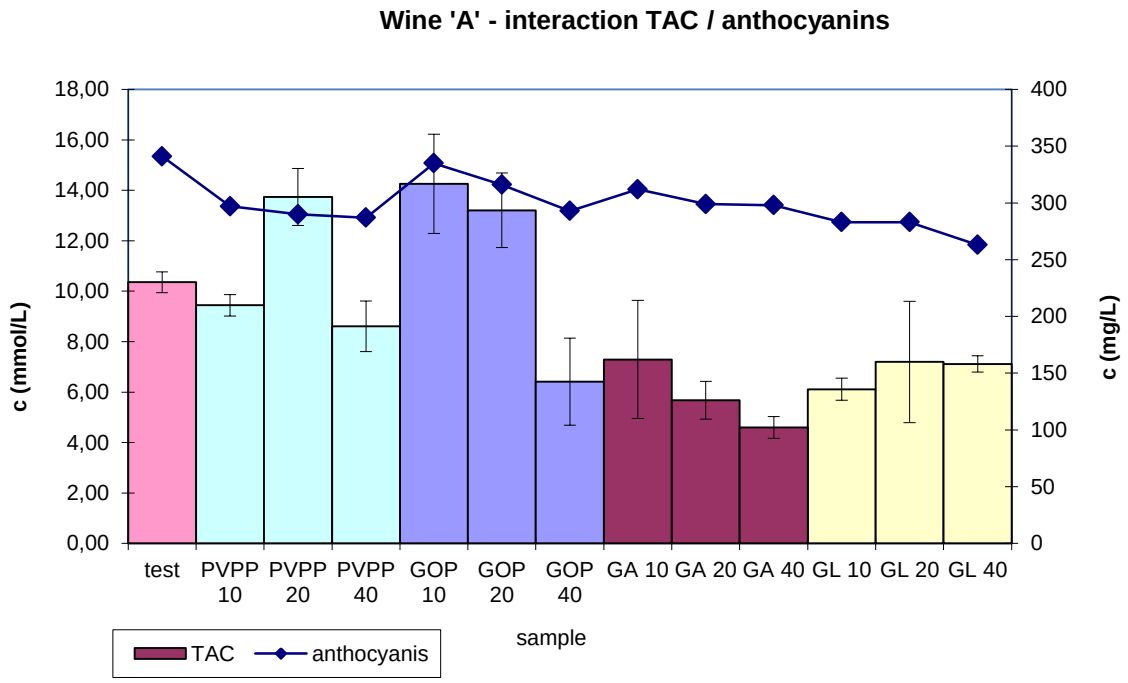
Wine 'A' - interaction TAC / anthocyanins
c (mmol/L)
c (mg/L)
18,00
16,00
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
400
350
300
250
200
150
100
50
0
test
PVPP 10
PVPP 20
PVPP 40
GOP 10
GOP 20
GOP 40
GA 10
GA 20
GA 40
GL 10
GL 20
GL 40
sample
TAC
anthocyanis

Fig. 62

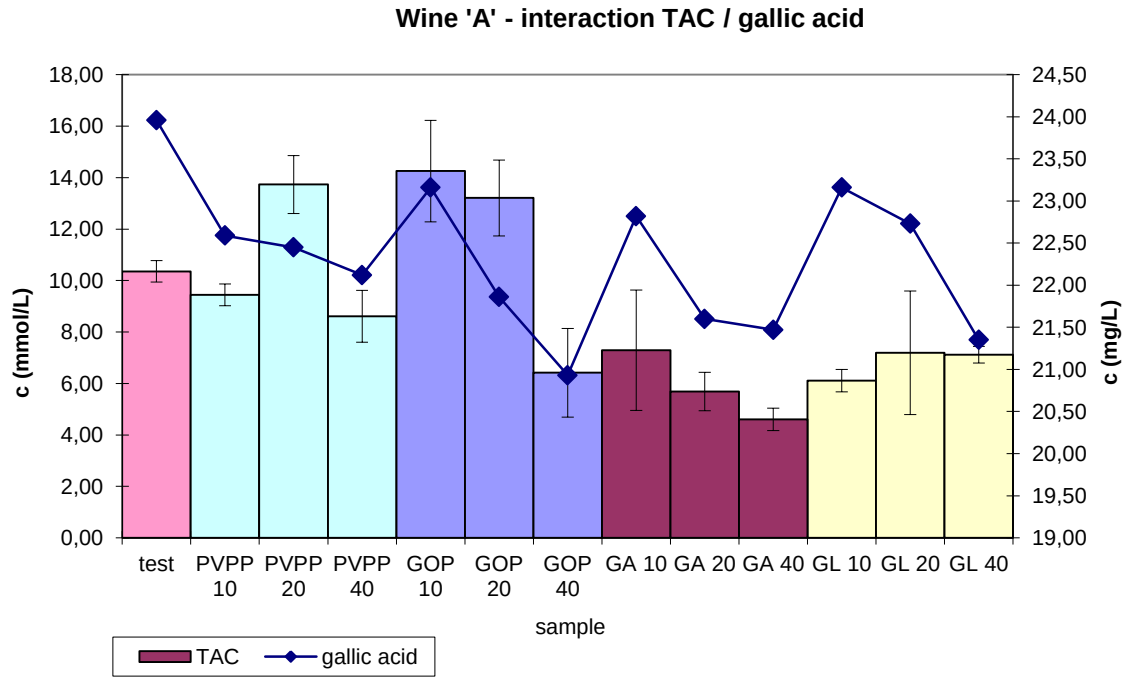
Wine 'A' - interaction TAC / gallic acid
c (mmol/L)
c (mg/L)
18,00
16,00
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
24,50
24,00
23,50
23,00
22,50
22,00
21,50
21,00
20,50
20,00
19,50
19,00
test
PVPP 10
PVPP 20
PVPP 40
GOP 10
GOP 20
GOP 40
GA 10
GA 20
GA 40
GL 10
GL 20
GL 40
sample
TAC
gallic acid

Fig. 63

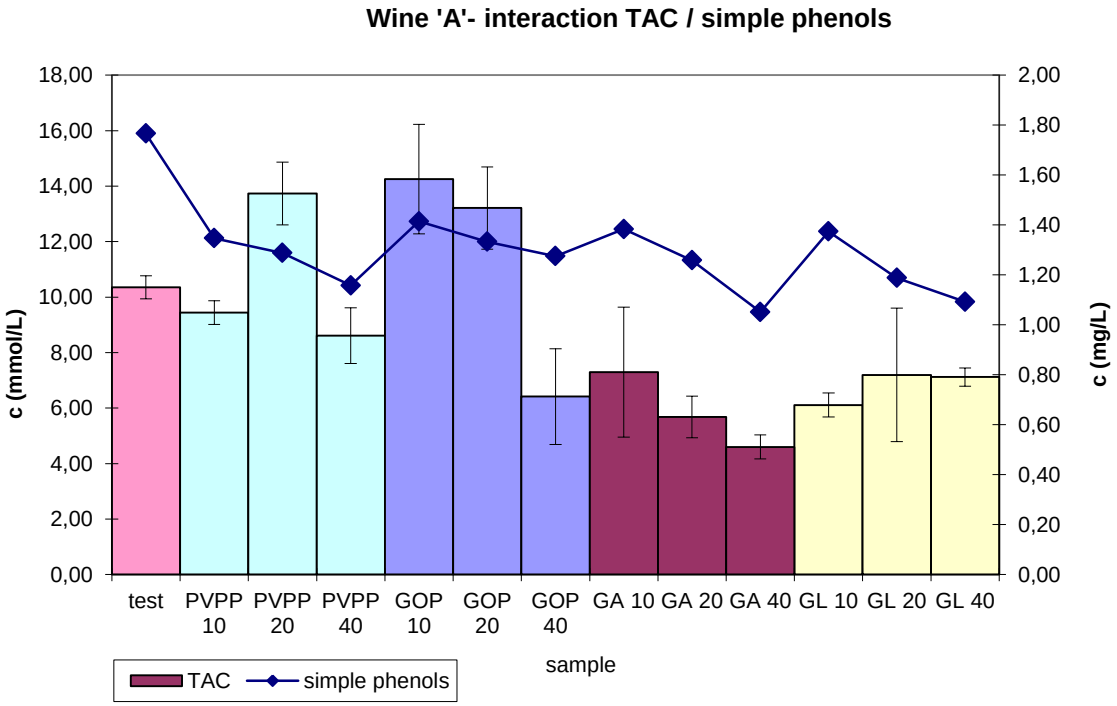

Fig. 64

To assess the situation in the B samples following four graphs were drawn up. Content of polyphenols (**Figure 65**) remains the highest after treatment with GL, followed by PVPP samples, which is also reflected on the values of the TAC. Lower values have been measured for samples containing GOP and GA. In all samples content of antioxidant components has decreased with increasing concentrations of finings,but this dependence did not reflect at the TAC.

Most of anthocyanins (**Figure 66**) have been removed from the samples treated by PVPP; the other three series had similar results. Amount in the test sample was again the highest and in all samples it has been decreased with increasing quantity of concentration of clarifying agent. This does not apply to the TAC, as it is evident from the graph.

Very different results offer a comparison of the content of gallic acid (**Figure 67**) and the TAC. TAC obviously does not depend on the contents of gallic acid, also quantity of GOP has been not falling with increasing concentrations of fining and for GA higher value than in the test sample was obtained.

Figure 68 shows the content of simple phenols. Amount in all samples has been decreased with increasing quantity of concentration of fining, by samples treated by GOP and GL higher values were found than PVPP- and at the GA-ones . The highest amount was in the test sample again.

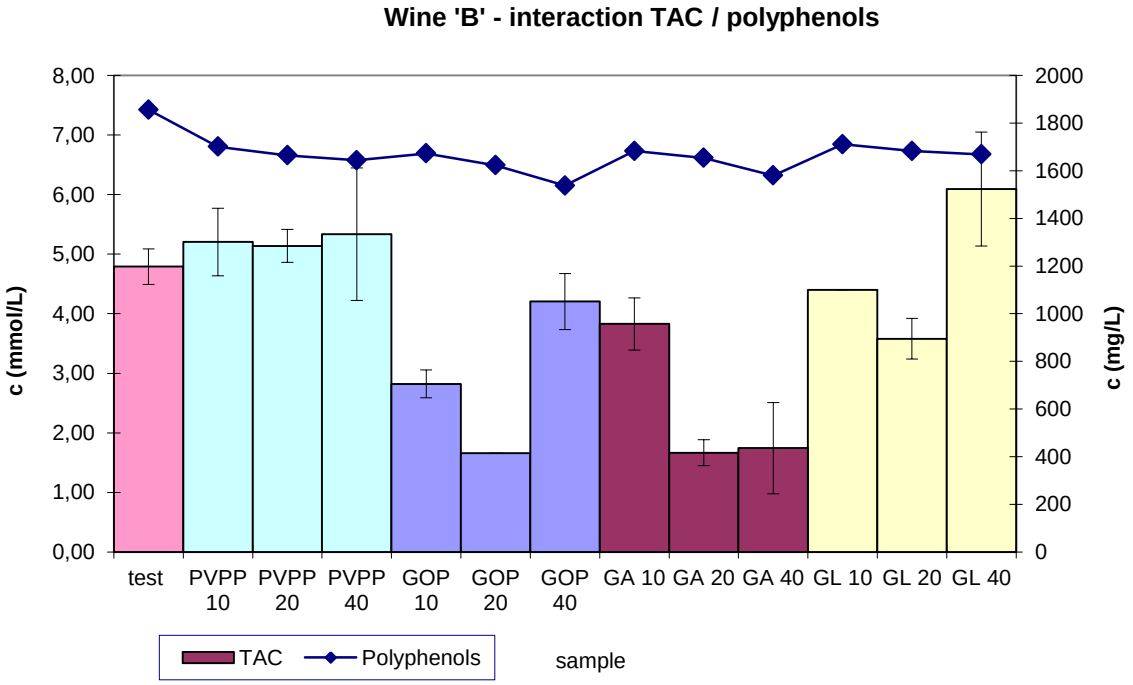

Fig. 65

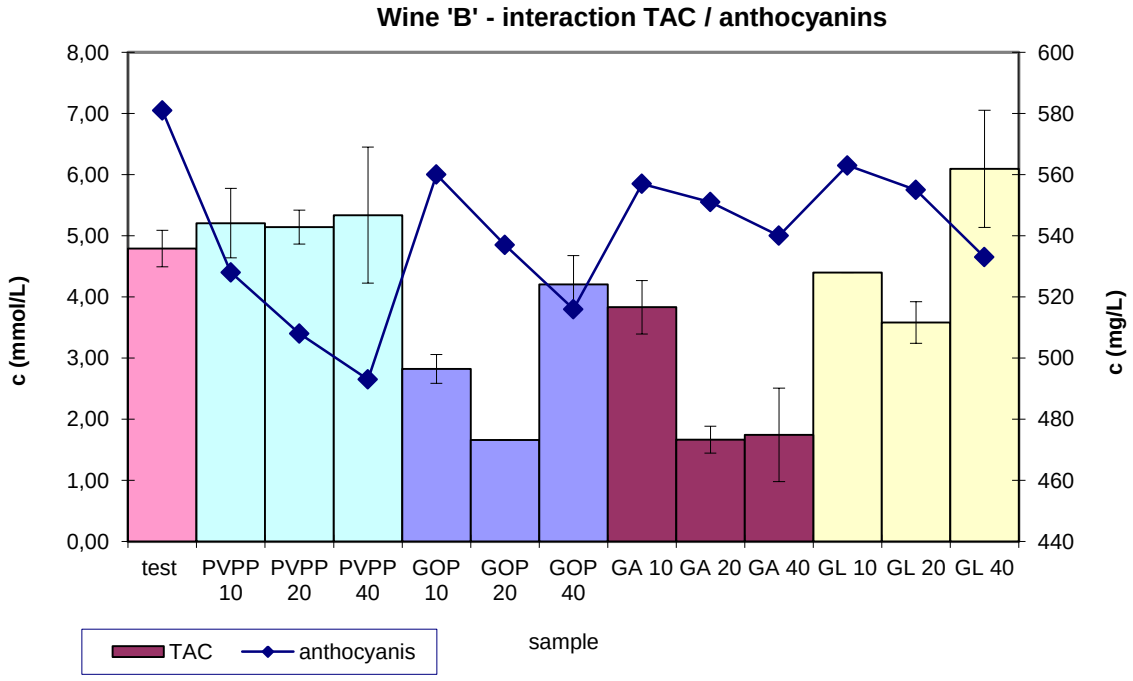

Fig. 66

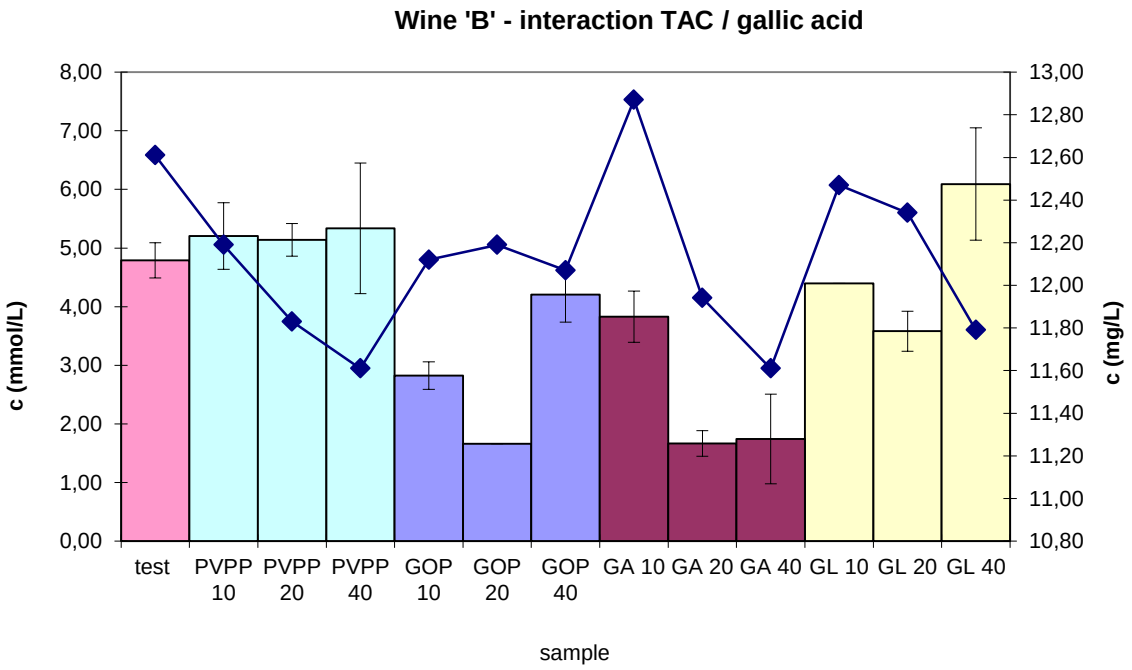

Fig. 67

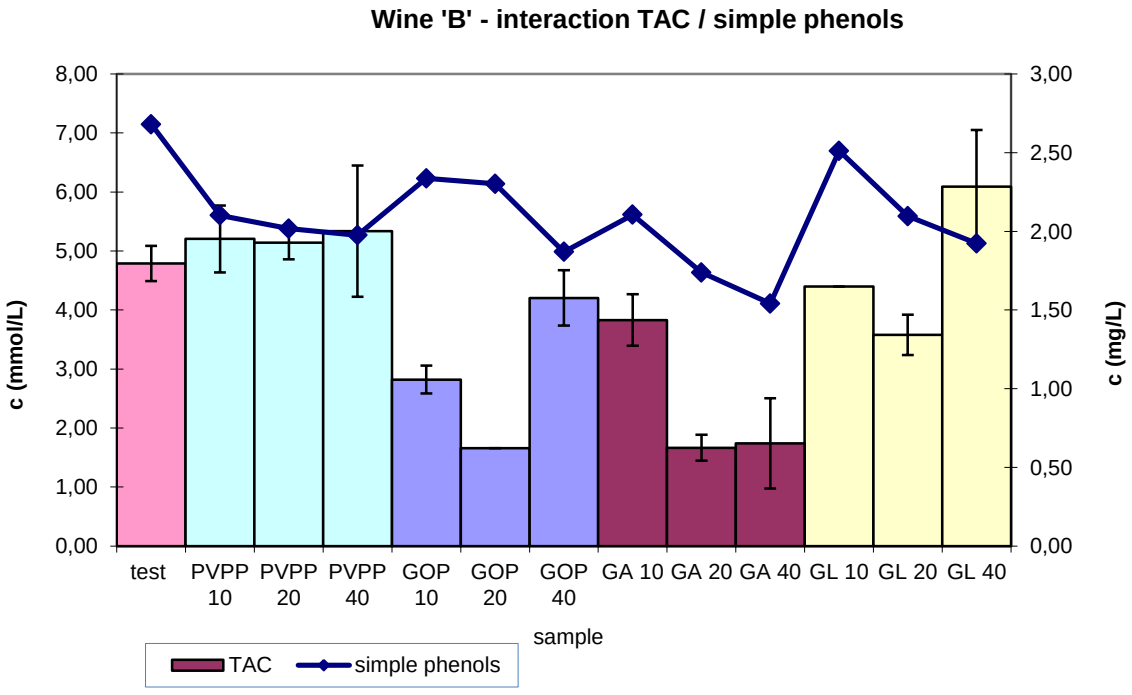

Fig. 68

The last four graphs are related to C samples. The total amount of polyphenols (**Figure 69**) is declining in all samples with increasing concentrations of fining, and

is the highest in the test sample. The top value is clearly observed in the samples with PVPP, also the uppermost TAC was maintained here, but same as by the previous wines, TAC was independent on the concentration of fining and was higher than in the test sample in case of samples with 10 and 40 g/hl.

Also next three graphs did not show dependence of the TAC on the content of individual components. The contents of anthocyanins (**Figure 70**), gallic acid (**Figure 71**) and simple phenols (**Figure 72**) decreased with increasing quantities of finings in the samples. Samples treated by PVPP include larger number of anthocyanins than the other three series, on the other hand, most of simple phenols has been removed. In case of gallic acid there were small differences between samples.

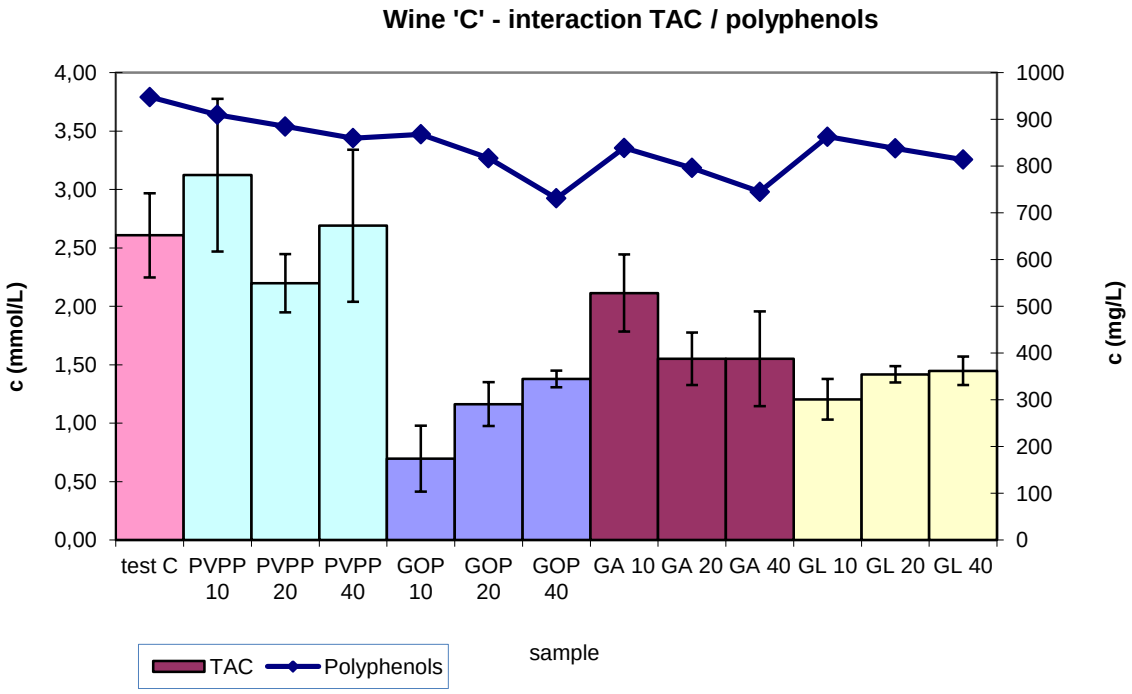

Fig. 69

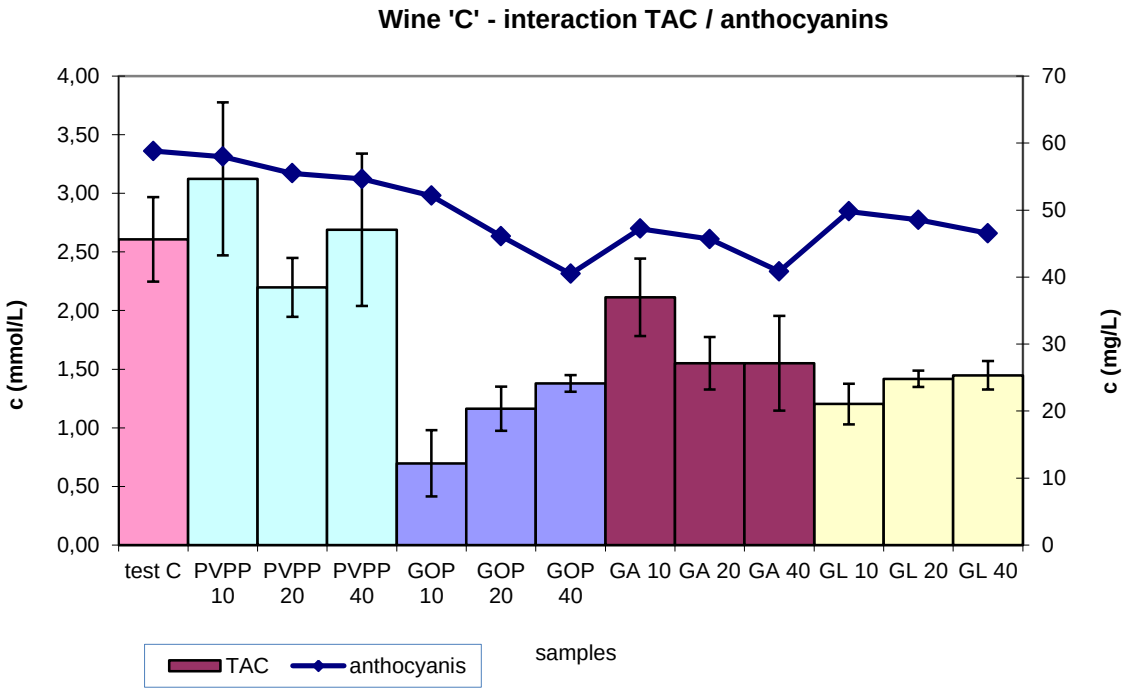
Wine 'C' - interaction TAC / anthocyanins
c (mmol/L)
c (mg/L)
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
70
60
50
40
30
20
10
0
test C
PVPP 10
PVPP 20
PVPP 40
GOP 10
GOP 20
GOP 40
GA 10
GA 20
GA 40
GL 10
GL 20
GL 40
samples
TAC
anthocyanis

Fig. 70

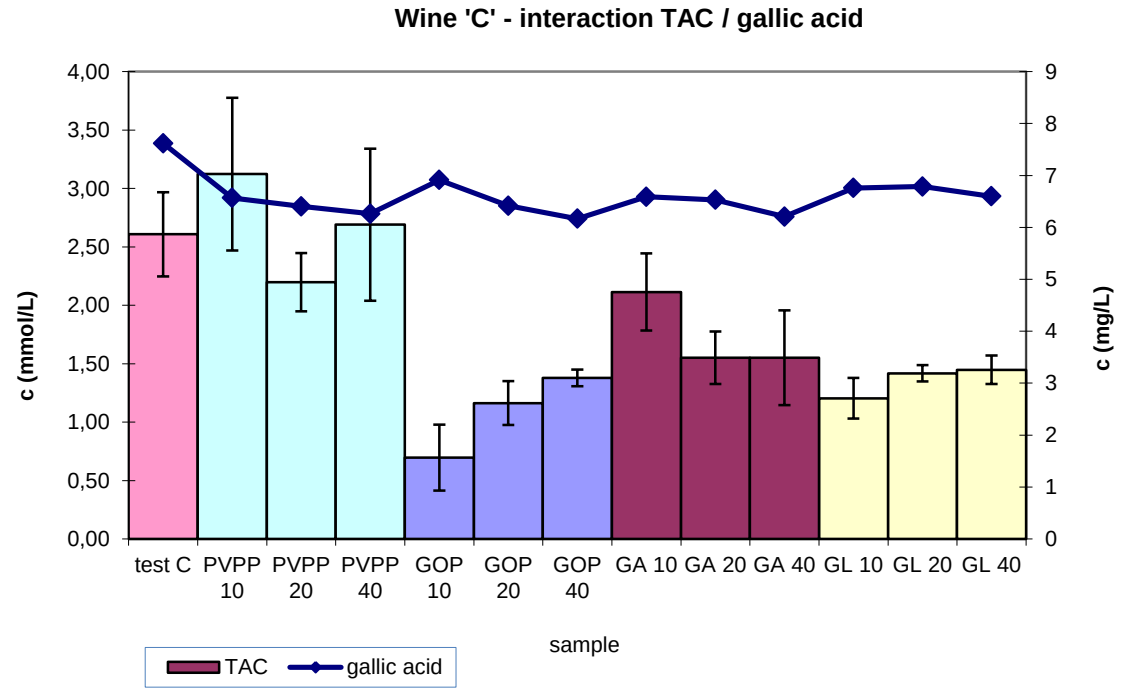
Wine 'C' - interaction TAC / gallic acid
c (mmol/L)
c (mg/L)
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
9
8
7
6
5
4
3
2
1
0
test C
PVPP 10
PVPP 20
PVPP 40
GOP 10
GOP 20
GOP 40
GA 10
GA 20
GA 40
GL 10
GL 20
GL 40
sample
TAC
gallic acid

Fig. 71

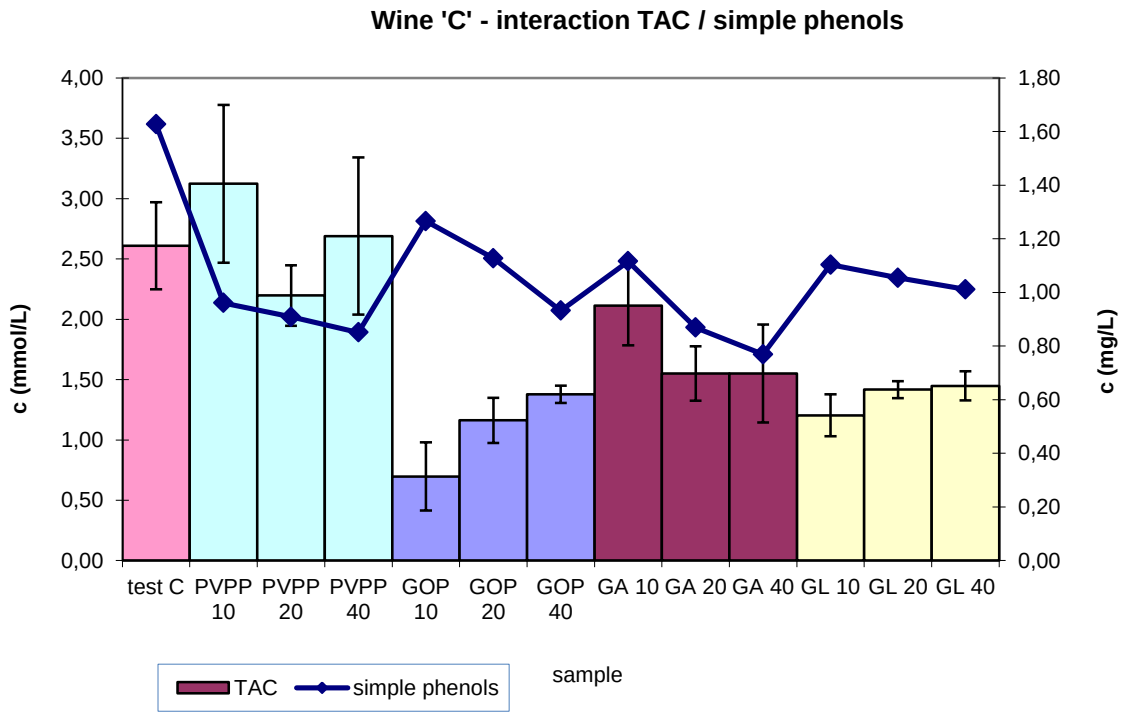
Wine 'C' - interaction TAC / simple phenols
c (mmol/L)
c (mg/L)
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
test C
PVPP 10
PVPP 20
PVPP 40
GOP 10
GOP 20
GOP 40
GA 10
GA 20
GA 40
GL 10
GL 20
GL 40
sample
TAC
simple phenols

Fig. 72

5 Conclusion

This work is concerned with evaluating of total antioxidant capacity (TAC) in red wine. The Trolox equivalent antioxidant capacity assay (TEAC) was used to evaluate it. The method is based on outspread total antioxidant capacity of the sample equivalent concentration of trolox. Samples of three different red wines with different content of antioxidant components are used for the measurements. All wines are clarified with four different agents (polyvinylpolypyrrolidone and three kinds of gelatin – liquid, atomized and powder). Dependence of TAC on the individual antioxidant components included in the wine is also studied; the TAC is compared with the total quantity of polyphenols, with anthocyanins, gallic acid and simple phenols.

According to analysis of the various graphs, which are previously discussed, you can create a general framework for the behavior of wine fined with different agents. As shown by individual values, it should be emphasized the different behavior of gelatin from the PVPP. While the first tends to reduce the parameters like TAC, polyvinylpolypyrrolidone tends to act vice versa. The exceptions are GOP-samples in wine A and GL-samples for wine B.

For PVPP-samples, higher total antioxidant capacity than that of test samples for all three wines was obtained. It is not only because PVPP did not eliminate the large number of polyphenols, but also it protects the wine from oxidative processes and cleans him from small pro-oxidative molecules.

It appears that the gelatin as clarifying agent has a tendency to dramatically reduce the TAC, usually ranging from 40 to 60% compared to test samples but two exceptions were found.

Powder gelatin (GOP) tends to decrease TAC dramatically in the B and C samples, on the contrary of A wine, where the values obtained are higher than in the test samples.

Atomized gelatin reduces TAC of all wines ranging from 40-60%.

TAC was also reduced in samples clarified with liquid gelatin; for wines A and C at about 50%, while B-values remained almost identical to unfined samples.

Comparing samples with different concentrations of fining agents, results showed that the concentrations used didn´t affect the overall TAC, ie. it doesn´t matter if 10, 20 or 40 g/hl of clarifying agent is added into the wine.

It is interesting that when fined samples were measured, there were large fluctuations between the values even of the triplicates (the same samples), unlike test samples, where the standard deviation was minimal even if measured multiple times in different days.

The second part deals with a comparison of the TAC and the content of the various antioxidant components in wine: namely the total amount of polyphenols (PPT), anthocyanins, gallic acid and low-molecular phenols (simple phenols).

The only component, which approximately reflects values of TAC, is the total amount of polyphenols. Values correlate by all three wines A, B and C.

Among others antioxidant components big differences were found and measured amounts do not reproduce TAC values.

Unlike the TAC, concentrations of finings affect the value of components in almost all cases.

Also comparison of test samples showed that the values were strikingly higher than of treated samples. Samples containing PVPP demonstrated large differences between the values for the test samples and those containing 10 g/hl of fining, while the much smaller variations between the samples with 10 and 40 g/hl.

With the view to save the total antioxidant capacity in the wine, PVPP certified itself as a good treatment; even the concentration 40 g/hl retains the values of the TAC and the PPT.

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