Optimization of extraction conditions of total phenol compounds and antioxidant activity in grain wheat

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ABSTRACT:

Wheat grain is a rich source of antioxidants. The concentration of polyphenols in wheat grain is affected by many factors, e.g. genetic, environmental and technological ones. Therefore, it is important to choose optimal conditions for the extraction of polyphenols from wheat grain. The purpose of these studies was to select the most optimal conditions for the extraction of total phenolic compounds from winter wheat grain. For this purpose the Folin-Ciocalteu method was used. The material studied was winter wheat seed Muszelka (Plant Breeding and Acclimatization Institute (IHAR) – National Research Institute in Radzików). The extraction of polyphenols was carried out with methanol and its 90%, 80% and 75% aqueous solutions. 10 grams of shredded wheat weight was covered with 50 cm3 of extraction. Subsequently, it was treated with ultrasound in 3 variants of 10, 20 and 30 minutes. After being filtered in the vacuum, the supernatant was evaporated on a rotary evaporator, dry extracts were transferred by HPLC to the vials and evaporated to dryness under a stream of nitrogen. Polyphenol content was analyzed by UV-Vis spectrophotometry method (Spectronic 200 Thermo Scientific) at λ = 760 nm wavelength. Total phenol content expressed in mg/kg was calculated as gallic acid. A spectrophotometric confirmation (λ = 760 nm). An ABTS solution was used to determine the antioxidative activity. The antioxidative activity of polyphenols was expressed in µmol of the Trolox / 100 g sample, relative to the calibration curve. The data on antioxidant activity vary according to the variant of the sample. The best parameters were obtained during a 30 minutes ultrasonically assisted extraction with methanol. Total phenolic content of methanol using ultrasound-assisted extraction for 20 and 30 minutes was the highest whereas the extraction performed over 10 minutes period in all solvent variants was the least effective.

1. INTRODUCTION

Being an important source of nutritious and bioactive substances, wheat grain is widely used in feed and food industries. The antioxidant compounds it contains belong to a large group of natural substances. Among them polyphenols, which vary according to the basic carbon skeleton structure. These are phenolic acids and flavonoids as well as other bioactive compounds. (Fig.1).

A characteristic feature of the abovementioned compounds is that, at low concentration, they delay or prevent oxidation. The anti-oxidation activity of polyphenols mentioned here consists in, among others, the direct reaction with free radicals, which is so-called scavenging of free radicals. Free radicals are atoms or particles which possess an unpaired electron in valence orbital, and their active forms are shown in Table 1.

Table 1 Types of free radicals

ACTIVE OXVGEN	FORMULA
Superoxide anionradical	$O^{\cdot}_2$
Hydroperoxide radical	HO'_2 OH'
Hydroxyl radical	OH'
Singlet oxygen	$^1O_2$
Ozone	O_3
Hydrogen peroxide	H_2O_2

Free radicals may be generated during biochemical oxidoreduction in a plant cell or be a result of biotic and abiotic factors' activity. The presence of the free radicals mentioned may lead to metabolic disorders. Plant cells, however, are equipped with defence systems against active oxygen: an enzymatic one based on suitable enzymes, and non-enzymatic one connected to antioxidants' activity (Tab. 2) [1].

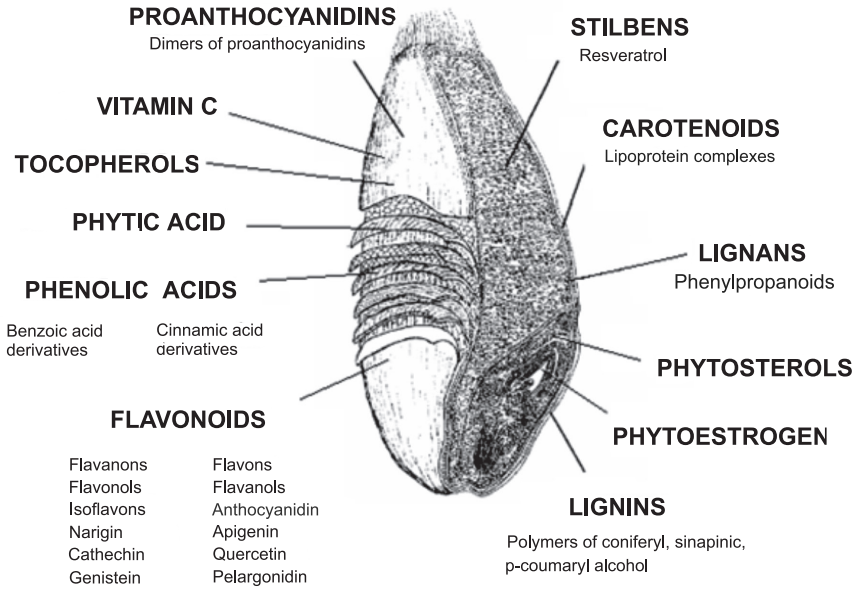
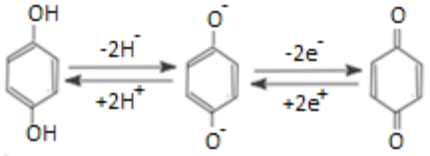


Figure 1 Compounds of antioxidant properties in wheat grain [own elaboration]

Table 2 Defence systems against active oxygen

DEFENCE SYSTEM		FACTOR	REACTION
ENZYMATIC	Superoxide dismutase	Superoxide dismutase enzyme	$2O_2^{\bullet-} + 2H+ \rightarrow H_2O_2 + O_2$
	Catalase	Hydrogen peroxide	$2H_2O_2 \rightarrow 2H_2O + O_2$
	Peroksidaze	Ascorbate peroxidase Glutathione peroxidase	$H_2O_2 + AH_2 \rightarrow 2H_2O + A$
NON-ENZYMATIC	Scavenging of oxygen free radicals	Anthocyanidins Flavonoids Phenolic acids Ascorbate acid Terpenoids	

Polyphenols' ability to 'scavenge' oxygen free radicals is closely connected to their structure. Anthocyanidins and flavonoids, which contain numerous -OH groups in B ring, are considered to be the most active ones.

Phenylpropanoids have a similar antioxidative activity. The antioxidant activity of polyphenolic acids also grows with an increasing number of –OH groups attached to the ring (Fig. 2).

Wheat grain, as well as its extract, is a complex analytic matrix due to the presence of numerous bioactive compounds as well as nutrients and non-nutrients which can mask one another's presence making it hard to detect.

Therefore, it is important to choose suitable extraction parameters and analytical ones. Currently, many different methods of antioxidant extraction from wheat grain are used, with solvent extraction as the most widespread technique [2]. Solubility of polyphenols is linked to solvent polarity, the degree of depolymerisation, the interaction between polyphenols and other wheat components or the creation of insoluble complexes. Flavonoid glycosides dissolve both in water and ethyl and methyl alcohol while aglycones are soluble in organic solvents. In the case of total phenolic compounds extraction, polar solvents are applied: methanol, ethanol and their mixtures with water, hydrochloric acid or weak organic acids, such as acetic or formic acid. The extraction time for polyphenols varies and may reach even 24 hours, depending on grain fineness and wheat variety, whereby it may be shortened by applying an ultrasonic bath. Extraction efficiciency is also affected by the ratio of the sample weight to the solvent. Most frequently it is from 1 to 5÷10 (m/v) respectively. Phenolic compounds occurring in a bound form should be subjected to the alkaline or acid hydrolysis. Another solvent extraction method is the continuous extraction with a Soxhlet extractor (SOE), during which methylene chloride is used for extraction, followed by the application of methanol to extract the phenolic fraction [3]. An alternative for phenolic compounds extraction from plant material is the extraction with a solvent, assisted by ultrasounds (UAE) or microwave radiation (MAE) [4, 5]. The phenomenon of microwave energy absorption by chemical compounds may be used for the extraction of analytes. Process effectiveness results from a solvent variety (methylene chloride or a mixture of acetone and hexane) and temperature. Another method used is the accelerated extraction with a solvent (ASE), which is based on using solvents in higher temperature (a range of 100 do 180°C) and under increased pressure (to 140 atm). These conditions facilitate matrix penetration with a solvent, the desorption of compounds, which results in the accelerated solubility of analytes. Another example is supercritical water extraction (SFE). It is characterized by high selectiveness, high performance and a shortened process time [6]. A next technique is the extraction with a solvent from a sample mixed with a filler (MSPD), which consists in the isolation of polyphenols by means of the flow of solvent through the solvent mixture. It triggers extraction and extract separation into a liquid phase (solvent), and solid phase (sorbent) [7]. One of further examples is solid-phase extraction (SPE). It consists in the transfer of analytes appearing in the liquid phase to the solid one. SPE characteristic features are high selectiveness and the possibility of fast purification of the substance [8]. Following the sufficient extract preparation, quantity and quality analyses are conducted to assess the amount of antioxidants. These analyses may include chromatographic methods (e.g. TLC, HPLC, UPLC), spectrophotometric, less and less frequent colorometric ones as well as electrochemical techniques, such as cyclic voltammetry and spectroelectrochemistry, or electron paramagnetic resonance (EPR).

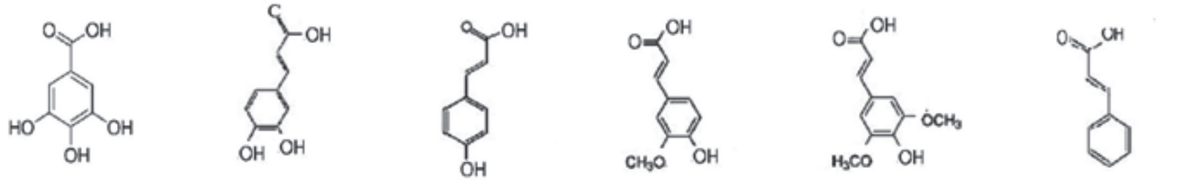


Figure 2 Activity series of polyphenolic acids

1.1 Antioxidant activity analysis

Quality and quantity determination of phenolic compounds and their antioxidant activity in extracts after preliminary treatment may be also performed with the spectrophotometric analysis. There are numerous spectrophotometric methods used for activity determination, and the most common ones are presented in Table 3. The spectrophotometric method consists in the absorption or emission of electromagnetic radiation by the extract obtained from the tested wheat. The method is used to measure the intensity of passing radiation as a function of wavelength. Energy transition taking place in particles as a result of electromagnetic radiation in UV – VIS – IR range is used for analyses. Quanity analysis is carried out with spectrophotometric UV-VIS method, based on the measurement of the tested solution absorbance at a specific wavelength and the application of Lambert-Beer law. The rule used here is that each chemical compound possesses the ability to absorb radiation at the strictly defined wavelength, which allows its identification and *ipso facto* the quality analysis. Another technique is the application of various chromatographic methods for the quantity and quality analyses. To analyse phenolic compounds, the spectrophotometric detector is most frequently used as phenolic compounds absorb electromagnetic UV radiation, and flavonoids absorb visible light as well. Besides, fluorescent and electrochemical detectors are in frequent use. More rarely - mass spectrometer (MS), nuclear magnetic resonance (NMR) and infrared spectroscopy with Fourier transformation (FT-IR). The methods to determine single phenolic compounds use combined techniques, such as, for example, HPLC/MS or HPLC/MS/MS [8, 11].

1.2 The analysis of free phenolic acids

The Folin-Ciocalteu (F-C) [10, 12] method is most often applied to determine the total content of polyphenols in wheat grain. Marking is based on a reversible reaction of reducing molybdenum (VI) by polyphenols in the alkaline environment (pH 10) to molybdenum (V) contained in the Folin-Ciocalteu reagent. The product of these reactions is a blue compound showing the maximum absorption at λ = 745÷750 nm wavelength.

Table 3 Spectrophotometric methods used for testing antioxidant activity in wheat grain extracts

METHOD	COMPOUNDS DESIGNATED	PROCES PRINCIPLEE	REAGENTS	WAVELENGTH [nm]	LITERATURE
Christa-Műller	Flavonoids	Colour intensity measurement	Antimony trichloride Zirconium oxychloridE Uranyl acetate Beryllium nitrate Boric acid Aluminum chloride	λ = 750÷784	[8, 9]
Folina-Ciocalteu	Flavonoids	Colour reaction Colour of the complex: green-blue Na_2WO_4/Na_2MoO_4 + phenol → (phenol-$MoW_{11}O_{40}$) $^{-4}$ Mo(VI) (yellow) + ē → Mo(V) (blue)	Folin-Ciocalteu reagent	λ = 750÷784	[8, 10]
ABTS, DPPH, DMPD	Polyphenols	Decoloring Blanking of syntetic free radicals		λ = 734 λ = 515 λ = 505	[8, 10]
FRAP	Polyphenols	pH-dependent reactions, proceeding with metal ions	2,4,6-tripyridyl-s--triazine (TPTZ)	λ = 593	[8, 9, 10]
CUPRAC	Polyphenols	$X^{\bullet} + AH \longrightarrow X^{-} + AH^{\bullet+}$ $AH^{\bullet+} \xleftrightarrow{H_2O} A^{\bullet} + H_3O^{+}$ $X^{-} + H_3O^{+} \longrightarrow XH + H_2O$ $Me(III) + AH \longrightarrow AH + Me(II)$	Cu^{2+}	λ = 450	[8, 9, 10]

The Folin-Ciocalteu reagent is a mixture of sodium tungstate ($Na_2 WO_4$), sodium molybdate (Na_2MoO_4), lithium (lithium sulfate $Li_2 SO_4$), bromine water and concentrated chydrochloric and phosphoric acids.

$$Na_2WO_4/Na_2MoO_4 + phenol \rightarrow (phenol\text{-}MoW_{11}O_{40})^{-4}$$

$$Mo(VI)\ (yellow) + \bar{e} \rightarrow Mo(V)\ (blue)$$

This method uses a polyphenols' ability to start colour reaction with Folin-Ciocalteu reagents, and the absorbance measured at λ = 756 nm wavelength is proportional to the total content of phenolic compounds in the sample being tested.
Due to the content of other than polyphenols compounds in wheat grain, such as reducing sugars, aromatic amines, sulphur dioxide or ascorbic acid, numerous interactions between these compounds and phenols are possible.
To achieve reliable measurement results, it is necessary to maintain the proper ratio of base quantity to the Folin-Ciocalteu reagent and to optimize reaction time and temperature. Gallic acid is used a model. A less frequent spectrophotometric method which allows the determination of total polyphenol content is the one that uses the ability of phenolic compounds to reduce iron (III). The reduced iron(II) we obtain reacts with Fe^{3+} ions introduced as $FeCl_3$ solution, which leads to results in a blue complex being used as the basis for determination. Prussian blue is applied for this analysis. Absorbance measurement is performed at λ = 700 nm wavelength. Another rare spectrophotometric method used for the determination of total polyphenols is the one using 1,10-phenanthroline. It takes advantage of a polyphenol ability to reduce iron (III) to iron (II), which both react with 1,10-phenanthroline and in consequence a red complex is obtained, the basis for the determination at λ = 510 nm wavelength [10, 13].
The aim of this study was choosing the most optimal conditions for carrying out the extraction of total phenolic compounds from winter wheat grain so as to quantify total polyphenols with the Folin-Ciocalteu method. Such research on wheat grain has not been conducted yet.

2. EXPERIMENTAL PART

2.1 Material tested

The material studied was winter wheat seed of *Muszelka* variety obtained from Plant Breeding and Acclimatization Institute (IHAR) – National Research Institute in Radzików). 8 separate grain samples in 3 replications were collected for the analysis. Methanol, analytical grade, and acqueous methanol in the following proportions: MeOH:H_2 O : 90:10, 80:20 and 75:25 were used for the extraction of phenolic compounds. (Fig. 3).

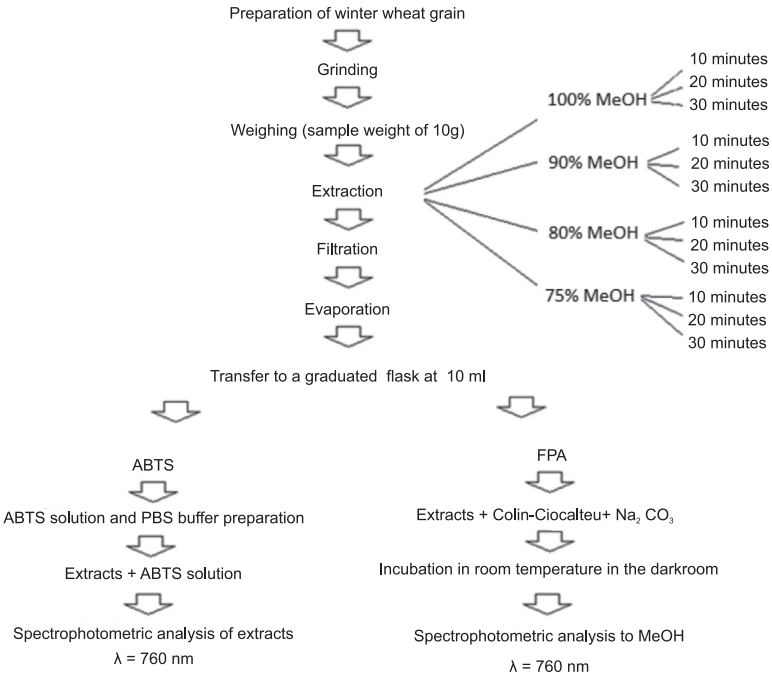


Figure 3 Diagram of analytical processes

For the analysis, a sample weight of 10g, crushed in a laboratory ginder, soaked in 50 cm^3 of extraction mixture (4 varieties) was exposed to ultrasounds in 3 options for a period of: 10 minutes, 20 minutes and 30 minutes. It gave 12 experimental versions. Next, each sample was filtered under vacuum and supernatant was evaporated by means of a vacuum evaporator. Dry extracts were transferred by HPLC-grade MeOH (2 ml) to vials and evaporated to dryness in a stream of nitrogen. Prior to analyzing total phenolic compounds, the extracts were transferred quantatively by MeOH (HPLC) to graduated flasks at 10 cm^3. The subsequent stage was the analysis of $ABTS^{\bullet+}$ antioxidant activity as well as the analysis of FPA total phenolic compounds.

3.DETERMINATION AND RESULTS

3.1 Determination of the $ABTS^{\bullet+}$ antioxidant ability

To indicate the antioxidant activity, the solution of 7 mM of $ABTS^{\bullet+}$ and 2,45 mM of $K_2S_2O_3$ was prepared and then a PBS buffer (phosphate buffered saline), which makes it possible to maintain the constant pH value.

The extracts of appropriate concentration were combined with the buffer solution, and next 100 ml of each dilution was mixed with 1 ml of previously prepared $ABTS^{\bullet+}$ solution. The antioxidant activity was expressed in µmol Trolox / 100 g of the sample, against the standard curve (Fig. 4).

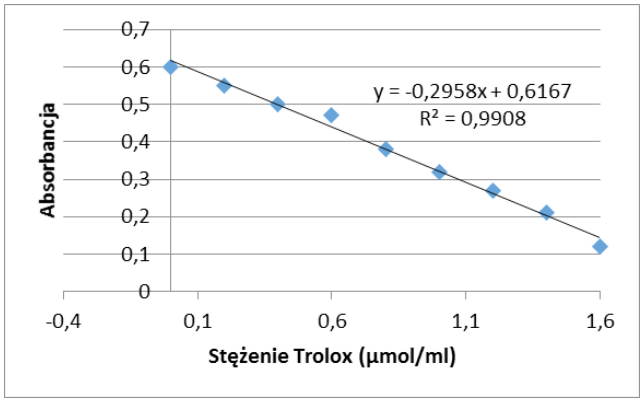


Figure 4 Standard curve for Trolox

3.2 The analysis of total phenolic compounds

Previously prepared extracts were diluted with water and then, 0.2 ml of F-C reagent and 0.5 ml of 7% sodium carbonate were added to 5 ml of each dilution. The incubation was executed in room temperature in a darkroom for 30 minutes, followed by spectrophotometric analysis in comparison to methanol. The total content of phenol compounds was expressed in mg/kg based on gallic acid. The limit of detection was 0.1 mg/kg. 3 working ranges of the method were established: the first one from 0.1 mg/kg to 10 mg/kg (Fig. 5), the second one from 10 mg/kg to 100 mg/kg (Fig. 6) and the third one from 100 mg/kg to 1000 mg/kg (Fig. 7). The coefficient of variation for a series of tests performed under repeatability was 1.6%, and interlaboratory reproducibility – 2.0%. The content of phenolic compounds was analyzed by means of a spectrophotometer UV-Vis (Spectronic 200 ThermoScientific) at the wavelength of λ = 760 nm.

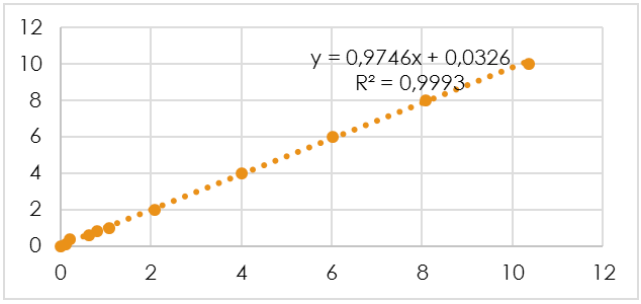


Figure 5 Standard curve for gallic acid
in Folin-Ciocalteu method
for the range of 0.1 mg/kg – 10 mg/kg

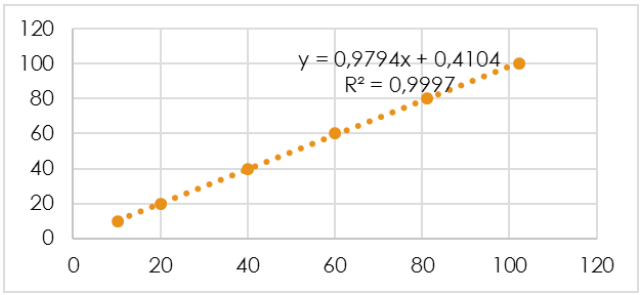


Figure 6 Standard curve for gallic acid
int he Folin-Ciocalteu method
for the range of 10 mg/kg – 100 mg/kg

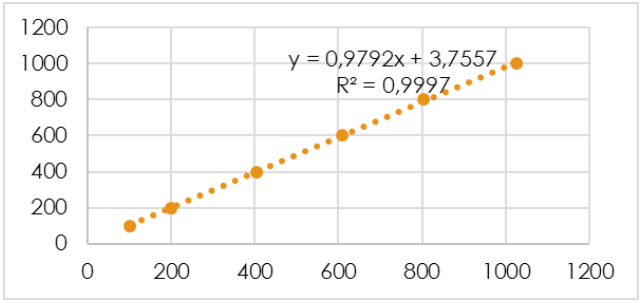


Figure 7 Standard curve for gallic acid
In Folin-Ciocalteu method
for the range of 100 mg/kg – 1000 mg/kg

4. SUMMARY

The study was aimed at the determination of the antioxidant activity (Tab. 4) of phenol compounds in winter wheat grain Muszelka and the comparison of results depending on the applied extraction conditions. As assumed, polyphenols were isolated from winter wheat samples in the process of extraction through methanol and its mixture with water in the following concentrations: 90%, 80% and 75%. The comparison of the results obtained in twelve various variants of the experiment can fully substantiate the assumption that the most effective extraction parameters are: methanol subjected to a 30-minute extraction assisted with ultrasounds, and next, the 90% mixture of methanol and water extracted at the same time. The ultrasound-assisted extraction with aqueous methanol of 80% and 75% concentration is less efficient. The analysed wheat grain extracts obtained with 75% methanol solution, aided with 10- or 20-minute exposure to ultrasounds, demonstrated the lowest efficiency.

The extraction with methanol ultrasonically assisted for 20 and 30 minutes turned out to be the most efficient as far as the total content of phenol compounds (Tab. 5) is concerned whereas the 10-minute extraction was the least efficient in all experiment variants.

Tabela 4 Antioxidant activity of total phenolic compounds [µmol Trolox / 100 g] in winter wheat grain, Muszelka variety, depending on the extraction method applied

Time of exposure to ultrasound (min)	MeOH	MeOH : H_2O 90 : 10	MeOH : H_2O 80 : 20	MeOH : H_2O 75 : 25
10	1638.8	1324.5	1376.3	1266.5
20	1568.8	1255.0	1269.3	1232.5
30	2142.0	1858.8	1782.5	1728.5

Tabela 5 Total phenolic compounds content in winter wheat, Muszelka variety [mg/kg] depending on the extraction method applied

Time of exposure to ultrasound (min)	MeOH	MeOH : H_2O 90 : 10	MeOH : H_2O 80 : 20	MeOH : H_2O 75 : 25
10	509	432	460	458
20	740	513	540	552
30	875	670	630	703

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