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Comparison of liquid chromatographic methods and spectrophotometric assays for polyphenol analysis – validation and quantification in selected food

MASTER'S THESIS

to achieve the university degree of

Diplom-Ingenieur

Master's degree programme: Biotechnology

submitted to

Graz University of Technology

Supervisor

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Institute of Biochemistry

Graz, October 2017

AFFIDAVIT

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Acknowledgement

At first, I would like to thank my supervisor Ao.Univ.-Prof. Dipl.-Ing. Dr.techn. Michael Murkovic for providing this topic, his support during my laboratory work and for the excellent coffee each and every morning.
Furthermore, many thanks to Univ.-Prof. Dipl.-Ing. Dr.techn. Macheroux for allocating the needed facilities and the work at the Institute of Biochemistry at Graz University of Technology as well as Priv.-Doz. Mag. Dr. Willibald Wonisch, for providing food samples.

Special thanks go also to the whole team „Chemie funktioneller Lebensmittel", especially to Maria Hulla and Tamara DeZuani for their help in the laboratory.

Last but not least, I would like to state my sincere gratitude to my parents Wolfgang and Eva-Maria and my sister Anna-Kristin, for being a rock in the surf throughout all the years of study.

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Abstract

Polyphenols are secondary plant substances that significantly contribute to many plant characteristics, for instance stability, colour or flavour. They are known to exert benefits on human health due to their interaction with free radicals (reducing properties). Therefore, they are thought to be beneficial in cancer protection and for prevention of cardiovascular diseases. Due to this, consumers and food industry likewise have become increasingly interested into this class of chemical compounds and analytical methods for determining polyphenols have improved during the last decades which are commonly employed in food science and research. Researchers are interested in both, the amounts of polyphenols in selected groceries as well as their identity and therefore their influence on human health.

In this thesis, different analytical strategies were investigated regarding their suitability for polyphenol analysis in food samples (e.g. grain samples, coffee, extracts of *Pinus cembra*, etc.). Amongst the most commonly employed methods are such that generate a sum parameter e.g. total antioxidant capacity (TAC) or total polyphenol content (TPC). Analytically, methods are separated into spectrophotometric (e.g. Folin Ciocalteu assay) assays which can be performed in 96-well plates to increase sample throughput and chromatographic techniques e.g. thin layer chromatography for method development where e.g. a DPPH staining can be used. Here, the DPPH radical reacts with antioxidants and therefore not only a qualitative statement regarding the number of separated compounds and their retention factor (Rf value) can be made, but also signal intensity can be read either directly or fluorometrically.

For identification of individual antioxidative substances, mass spectrometry is the most commonly used strategy. Here, ions are generated following separation by a chromatographic (for polyphenols high performance liquid chromatography is most commonly used) system, whose mass over charge ratio is determined and additionally the molecule is fragmented and based on the fragmentation pattern, the molecular structure can be derived.

Kurzfassung

Polyphenole sind sekundäre Pflanzenstoffe die maßgeblich zu vielen Pflanzencharakteristiken beitragen, beispielsweise zur Stabilität, Farbe oder Geschmack. Aufgrund der Interaktion von freien Radikalen mit diesen Molekülen, geht man davon aus, dass jene Stoffe zur Vorbeugung von Krebs und kardiovaskulären Erkrankungen beitragen. Deswegen sind sowohl Verbraucher als auch Industrie an diesen chemischen Komponenten interessiert, wodurch sich entsprechende Analyseverfahren zur Bestimmung von Polyphenolen in den letzten Jahrzehnten stetig verbessert haben. Forscher sind hierbei sowohl an den Mengen einzelner Polyphenole in ausgewählten Lebensmitteln als auch an der Bestimmung und deren Einfluss auf die Gesundheit interessiert.

In dieser Arbeit werden unterschiedliche Analysemethoden untersucht und deren Einsatzmöglichkeit zur Polyphenolbestimmung in Lebensmittelproben (z.B. Getreide, Kaffee, Zirbenextrakte etc.). Unter den üblichsten Methoden sind jene die Parameter wie etwa die totale antioxidative Kapazität (TAC) oder den totalen Polyphenolgehalt (TPC) generieren. Analytische Methoden werden hierbei in spektrophotometrische Assays (z.B. Folin Ciocalteu) und chromatographische Techniken wie beispielsweise HPTLC-DPPH unterschieden. Hier reagiert das DPPH Radikal mit Antioxidantien, weshalb nicht nur eine qualitative Aussage betreffend die Anzahl der separierten Komponenten und deren Retentionsfaktor gemacht werden kann, sondern auch eine quantitative Aussage anhand der Signalintensität.

Zur Identifizierung der einzelnen antioxidativen Substanzen eignet sich Massenspektrometrie am besten. Hier werden nach chromatographischer Separation Ionen gebildet, welche nach Masse zu Ladung bestimmt und weiters fragmentiert werden. Anhand des Fragmentationsmusters kann die molekulare Struktur bestimmt werden.

Aim

The aim of this thesis was to investigate different methods used for the quantification of polyphenols and, for this purpose, different food samples. For instance, fruit, cereals, alcoholic beverages and coffee were tested using different analytical methods to compare the results of these. Spectrophotometric assays, such as Folin Ciocalteu, DPPH (2,2-diphenyl-1-picrylhydrazyl), hydrogen peroxide and reducing power assays were amongst the analytical methods used during the practical part the research. Chromatographic techniques, for instance high performance thin layer chromatography with DPPH detection and HPLC-MS measurements were also performed at the Medical University Graz, in order to validate the results of total polyphenol quantification with data that also aided the identification of the single molecules.

1 Introduction

1.1 Food chemistry

Food products are complex mixtures that are composed of a multitude of naturally occurring chemicals such as proteins, carbohydrates, fats (lipids), vitamins and organic acids. In recent years food trends have shown that consumer awareness is increasing and therefore only a thorough analysis of a food´s major components (e.g. protein content) and undesired by-products (e.g. pesticides) are demanded but the public is interested increasingly in the presence of compounds that (are supposed to) have beneficial effects on human health; polyphenols are amongst the most relevant classes of these chemicals. This trend has boosted the search for naturally occurring food sources that are rich in beneficial molecules and has even given rise to production of functionalised food e.g. by selective breeding of plants or enrichment of dishes with so called superfoods [1].

1.2 Secondary Metabolites

Secondary metabolites are produced by many organisms, and especially by plants which exist in multicellular communities. Secondary metabolites are not essential for the survival of an organism, as opposed to the case of primary metabolites, both organisms benefit by providing protection during antagonistic interactions with other organisms or against environmental stress. Many secondary metabolites are known to have effects on human health e.g. antibiotics [3], and have evolved over millions of years to enable communication

of different species in diverse eco-systems. They can also act as defensive mechanisms by inhibiting competitor growth [4].

1.3 Polyphenols

Polyphenols are produced by different plant species and can be further classified into flavonoids (flavonols, isoflavones, proanthocyanidins, etc.) phenolic acids (hydroxy-benzoic or -cinnamic acid) stilbenes, and lignans based on their molecular structure and chemical properties [5]. Polyphenols are characterized by the presence of multiple aromatic groups, but have diverse structures and functions, and their molecular mass ranges from less than 100 Da to highly polymerized molecules with molecular weights exceeding 30000 Da. Many polyphenols are thought to aid the prevention of multiple diseases by interacting with enzymes and cell receptors, owing to their antioxidant properties and are, therefore, the active substance of many plants used in phytomedical applications. Polyphenols also modulate sensory properties e.g. anthocyans as colouring agents and tannins that cause bitterness [1].

Phenolic compounds have anticancer and cardio protective properties and a positive effect on age related diseases [6]; their antioxidative effect is due to the phenolic group, which makes them excellent for quenching radical activity. Adjacent molecular structures such as hydroxyl groups, conjugated double bonds, and glycosylation, influence their reduction potential and therefore predict the action of the respective polyphenols as stronger or weaker antioxidants [7].

1.4 Alimentary antioxidants

Antioxidants are generally understood to be molecules in food, which decrease the quantity of free radicals, allowing them to serve as molecules with helpful biological and chemical functions. Cell damage is decreased to a minimum, making antioxidants of interest in preventing human diseases [8]. De facto, this definition is incomplete since antioxidants are important in the fields of biochemistry, food chemistry and polymer chemistry, for example [9]. Nevertheless, the focus in this study is on alimentary antioxidants and, in this case, the most important micronutrients are vitamins and secondary plant products, including polyphenols and carotenoids.

L-Ascorbic acid, known as vitamin C, and tocopherols respectively tocotrienols, known as vitamin E, both function as antioxidants; Olson showed that these micronutrients are related inversely to indicators of oxidative stress [10]. Therefore, these two compounds are briefly described in the next section.

1.4.1 Vitamin C: Ascorbic Acid

Vitamin C refers to compounds related to L-ascorbic acid, including the oxidized form dehydroascorbic acid (DHAA), and more than 80% of vitamin C in human diet especially originates from citrus fruits, berries, potatoes and green vegetables. The content of micronutrients can vary considerably between different samples of one type, since it is influenced by the degree of ripeness, climate or other environmental influences during cultivation [11]. Ascorbic acid has antioxidant and anti-inflammatory effects, and large amounts of vitamin C are present in fruit juices especially orange juice, which has been studied widely in relation to its antioxidative properties, and the beneficial effects it exerts on consumer health [6].

Quantification of vitamin C is usually performed using chromatographic, spectrophotometric (based on reduction of cupric ions) or titrimetric methods (e.g. reduction of 2,6-dicholorophenolindophenol). All of these methods have advantages and disadvantages: titrimetric methods are less expensive but there are interferences when used with coloured foods; chromatographic methods are more expensive and require well trained personnel; spectrophotometric methods are the most accurate and sensitive [12].

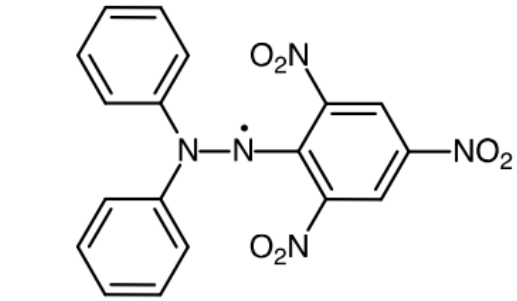
1.4.2 Vitamin E

Vitamin E is class of fat soluble vitamins with antioxidant properties, which also participate in signalling and gene regulation. Different forms of vitamin E are involved in gene regulation and the mechanism of these functions is independent from their function as an antioxidant. As a signalling molecule vitamin E participates in tissue development in animals and humans. Vitamin E also functions as an immuno-homeostatic factor modulating responses of immune cells e.g. in allergic diseases. The most common form of vitamin E is α-tocopherol, which exerts cyto-protective effects and prevents inflammatory liver processes during its exposure to a range of xenobiotics, environmental pollutants and dietary factors [13].

1.5 Free Radicals

During metabolism and biological processes, free radicals are produced and are usually controlled by enzymes as and by the aforementioned antioxidants. During recent decades, research efforts have been stimulated regarding antioxidants, particularly polyphenols, because laboratory evidence showed that oxidative stress in form of free radicals could cause severe medical problems [14]. Free radicals are characterised by the presence of an unpaired electron, which has high reactivity, and therefore they react quickly with other matter (mostly biomolecules e.g. unsaturated fatty acids contained in the cell membrane in the case of physiological reactions) from which they can obtain an electron, in other words they act as oxidising agents. If a biomolecule is oxidised by a free electron, the paired electron it possessed, is donated to the radical, which becomes an electron pair, whilst the biomolecule changes to a radical, with an unpaired electron inducing a cascade of reactions. This phenomenon is referred to as oxidative stress and is associated with many common disease, such as cancer or coronary heart disease [15].

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are amongst the most commonly studied free radicals, which are formed in the mitochondria during cellular respiration. Commonly non radical forms of oxidants are also classified as ROS and RNS, but are less reactive although they may also induce oxidative stress on biological molecules. Common free radicals, apart from ROS and RNS, are superoxide ($O2^{\bullet -}$), hydroxyl (OH•), or nitric dioxide ($NO_{(2)}\bullet$) groups [16]. The most important free radical regarding this research is DPPH, chemical name, π-radical 1,1-diphenyl-2-picrylhydrazyl, a purple coloured radical, which can be measured spectrophotometrically [17]. DPPH was discovered by Goldschmidt and Renn in 1992 and is extremely stable for a radical molecule, owing to steric crowding around the nitrogen atom, and the additional induced captodative (push-pull) effect, in which electrons are donated by the diphenylamino group and accepted by the picryl groups located on each side of the divalent nitrogen atom [18].



1,1-Diphenyl-2-picrylhydrazyl radical
(DPPH radical)

Figure 1: DPPH Structure modified from [19]

1.6 Analytical Methods

1.6.1 Chromatographic Methods

Chromatography is defined as the separation of analytes, based on their differential interaction with a stationary phase, whilst being transported in a mobile phase. In this work, the two techniques used for analysing polyphenol contents are described in more detail.

Food and plant product fingerprinting is commonly performed by HPLC or GC, which are capable of conducting quantitative and qualitative analysis of different constituents. However, these instruments are expensive to purchase and to maintain, owing to the large quantities of solvents/gasses used as mobile phases/carrier gas and a high level of expertise is required to implement the systems, especially if mass spectrometry is used as the detection method. The benefit of TLC is that it is easy to use, and it is well established for qualitative analysis and because TLC has become more sophisticated (HPTLC), it now has the potential to also provide quantitative data [2], as well to perform Automated Multiple Development (AMD).

1.6.1.1 HPTLC

High Performance Thin Layer Chromatography (HPTLC) evolved from Thin Layer Chromatography (TLC), which is assisted by instrumental devices. The basic principle of TLC was established almost 100 years ago by Nikolai A. Izmailov at the Institute of Experimental Pharmacy (State University Kharkhof), and employed for the rapid analysis of galenic pharmaceutical formulations. However, the technology stagnated for decades until

instruments for automated sample application that guided the transition from Thin Layer Chromatography to High Performance Thin Layer Chromatography were developed. HPTLC and TLC are both characterised by a thin layer of stationary phase on a carrier material, and are commonly used for chromatographic applications, in order to reduce the analysis time [20]. The main benefit of (HP)TLC is that very small amounts of solvent are required, and that the stationary and mobile phases can be easily changed during method development. In order to increase resolution of thin layer chromatography, over pressurised TLC was developed, in which the imitating factor of chamber saturation is overcome and mobile phase is delivered using a pump [20].

1.6.1.2 HPLC- MS

HPLC-MS is a generic term for different strategies in which High Performance Liquid Chromatography (HPLC) is employed to separate analytes prior to analysis by Mass Spectrometry. Most commonly, MS/MS spectra are generated when the analyte is ionized prior to entering the mass spectrometer, and more energy is applied to the gaseous analyte molecule, which induces its fragmentation. This technique is also called tandem mass spectrometry, and is very useful for structural elucidation since fragmentation patterns are highly dependent on the functional groups of an analyte molecule. When combined with exact mass analysis in the MS1 dimension, this method is very useful in screening for unknown secondary metabolites, including discovery driven approaches of polyphenol research [21], [22].

In this study HPLC-MS was used as an additional method to confirm the results of the research and, although the measurements were not available when the thesis was written, a short description of the method is included, in order to provide better understanding of the entire scope of the study, and the variety of methods that are commonly used to characterise polyphenolic substances in food samples.

1.6.2 Assays for Analysing Antioxidative Potential

Studies of antioxidant properties are classified into two groups, based on the reaction mechanisms. The methods which rely on transferring hydrogen atoms (HAT), are generally implemented using a competitive scheme, in which the antioxidant analyte and a substrate are competing peroxyl radicals, which are generated thermally from azo-compounds. The second group are electron transfer (ET) methods, which analyse the reduction of an oxidant by the antioxidant resulting in a change of the colour of the solution, correlated with the concentration (calibration is performed by measuring serial dilutions of known concentration) [6].

1.6.2.1 Total Polyphenol Content (Folin Ciocalteu Assay)

Many methods use spectrophotometric strategies for detection, in which the intensity of light is decreased by an analyte or a coloured analyte reaction product, and the light decrease is proportional to the concentration of the sample. The most commonly employed strategy for spectrophotometric analysis of total polyphenol content is the Folin Ciocalteu assay, which is used in polyphenol analysis (but also for other applications such as protein quantification) and can also be used as a spray reagent for chromatographic applications (e.g. HPTLC). In basic pH conditions, electrons are transferred and the constituent reagent phosphomolybdic/phosphotungstic acid complexes of are reduced to form molybdenum complexes with the element in its reduced oxidation state; the absorbance of these chromogens is between 550 nm and 750 nm [23]. However, the absorbance can be affected by the presence of compounds other than polyphenols, which also have electron transferring groups that can reduce the oxidation state of the molybdenum [24].

1.6.2.2 DPPH Assay

During the DPPH assay, radical scavenging capacities are investigated by reducing the DPPH chromogen radical (purple) with an antioxidant or reducing substances to a pale-yellow hydrazine compound. This reaction is photometrically monitored at 515–520 nm (absorbance maxima of different DPPH forms are shown in Figure 2). Classical solution based assays and dry reagent arrays exist, which are less limited by the decomposition of reagent solutions over time [17].

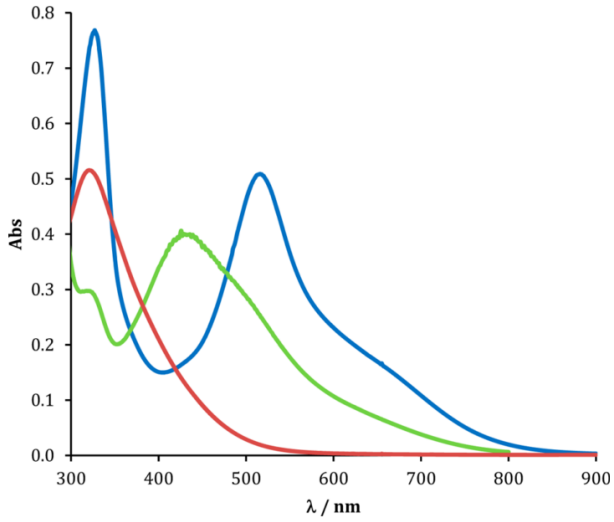


Figure 2: ***UV−vis spectra of DPPH• shown in blue, DPPH−H shown in red, and the anion DPPH⁻ shown in green***

Polyphenol determination using DPPH can either be performed offline using the spectrophotometric approach, or by using an online coupled HPLC–MS–DPPH assay. In this thesis, the sample is separated chromatographically and split post column; one part is investigated by mass spectrometry to obtain structural information about the analytes and the other part is subjected to post column derivatisation using DPPH which is delivered using an additional pumping system. The polyphenol that has reacted with DPPH can then be analysed by spectrophotometric methods [25].

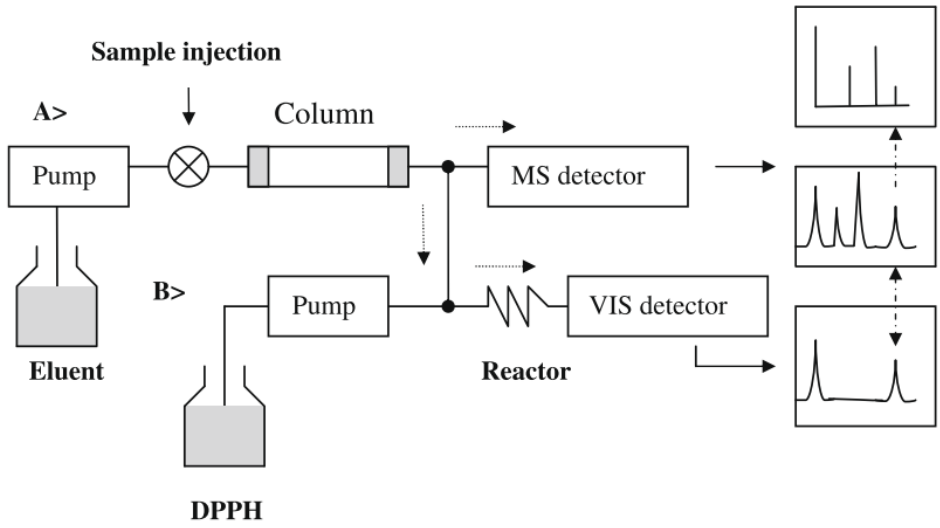


Figure 3: On-line coupled HPLC–MS–DPPH assay [25]

1.6.2.3 Hydrogen Peroxide Assay

The hydrogen peroxide (H_2O_2) assay measures the peroxide scavenging activity, by measuring the disappearance of H_2O_2 spectrophotometrically at a wavelength of 230 nm (UV range). However, phenolic substances also absorb in this region of the spectrum, therefore the methods used in this case are supplemented by assays in which a colorimetric reaction is characteristic. This reaction produces a chromogen which absorbs in a different spectral region, and in which interference can be minimised. In the case of very low concentrations of sample material being available and when interference is not an issue, the more traditional approach by Ruch et al. can be used, as described in the materials and methods section. The simultaneous measurement of reference standards, not treated with hydrogen peroxide, can also be used to obtain information about the sample material absorbance [26].

1.6.2.4 Reducing power assay (FRAP)

In the Ferric Reducing Antioxidant Power assays, antioxidants are measured based on their reducing properties, and performed in a linked redox-colorimetric assay, Iron (III) is used as an easily reduced oxidant. Whilst Fe (III) forms a colourless complex, the tripyridyltriazine complex formed by iron (II) has an intense blue colour, which can be measured photometrically; the absorbance is proportional to the antioxidant reducing power in a reaction in which electrons are donated [27]. Therefore, this assay is suitable for a wide range of applications ranging from medical applications to food science. The wavelength at which the measurements for the reducing power assay are performed is specific, which is a consequence of the compounds contained in the reaction mixture. As a consequence, different methods for measuring TAC have wavelengths at which the complex used exerts maximum absorbance. In order to determine this wavelength, UV-vis spectra of the sample are conducted on the appropriate reaction mixture; for the Fe (III) Reducing Antioxidant Power (FRAP) assays, the detection wavelength of the reduced compound is 595 nm [28]–[30].

2 Materials and Methods

2.1 Folin-Ciocalteu:

2.1.1 Sample Preparation

The samples referred to as 22, 23, 29, 30, 31, 32, 33 and 34 required solid phase extraction (SPE) prior to analysis, in order to remove any substances that might interfere with the Folin-Ciocalteu assay. The stationary phase was conditioned using 5 mL MeOH and 2 mL water so that the solid phase extraction could take place, subsequently 4 mL of the sample were added to bind it, and it was washed on the stationary phase with 2 mL water and 2 mL 20% MeOH to remove any substances that were not bound. The sample was finally eluted using 500 µL MeOH and then 1000 µL MeOH.

2.1.2 Dilution of Samples 1-34:

The samples were diluted as prior to measurement: samples P4 and P7 were diluted 1:10 with ethanol (300 µL sample + 2700 µL EtOH); Samples 22, 23, 29, 30, 31, 32, 33, 34 were purified using SPE and diluted 1:3 with methanol

2.1.3 Food Samples

1 mL of the extracted sample solution was pipetted into a volumetric flask, approximately 60 to 70 mL distilled water were added, and the flask was swirled to mix the components. Distilled water was added to the contents of the flask up to the 100 mL mark and mixed well by inversion, then 5 mL of the Folin-Ciocalteu phenol reagent mix (Sigma Aldrich P/N F-9252) were added to the sample solution, mixed well. After between 1 minute and 8 minutes had elapsed, 15 mL sodium carbonate solution (20 g in 100 mL water) were added the time was set at zero. The solutions were mixed and diluted to 100 mL with distilled water, and again mixed by inverting the flask several times. After 2 hours, the spectrophotometric measurement (using the Varian Cary 50 photometer) was performed at the maximum absorbance (range of wavelength scan = 550-850 nm) of 765 nm

2.1.4 Standards

For calibration of the reducing power assay, a gallic acid stock solution was prepared by weighing 2 mg gallic acid into a 2.5 mL volumetric flask and adding 1.5 mL 40 % EtOH. The mixture was left standing until it had cooled down, and then 40 % EtOH was added to bring solution to the 2.5 mL mark on the volumetric flask; homogenization of the solutions was accomplished by shaking the flask. A series of diluted samples was prepared: 1:2, 1:4, 1:8, 1:16 and 1:32 the solvent used was 40 % EtOH as previously

2.2 DPPH Assay

2.2.1 Extraction

All extractions of *Pinus cembra* samples were performed for approximately 45 minutes at a temperature of 45 °C and the following parameters were altered between the six different extractions' samples 1-3 were ground using an IKA A11 basic, and were less fine than samples 4-6. The effect of different solvents on the extraction was investigated using 96 % EtOH (sample 1), pure distilled water (sample 2) or 40 % EtOH (sample 3).

The more finely ground samples (4-6) were also analysed (all extracted using 40 % EtOH); sample 5 and 6 were skin only and sample 6 was soaked for 72 hours before analysis (maceration).

2.3 Thin layer Chromatography

Thin layer chromatography was performed using a series of equipment: plates spotted with a CAMAG Automatic TLC Sampler 4 (ATS 4) and developed using a double chamber, a horizontal chamber or an AMD 1 for automated multiple development (all CAMAG). A densitometric slit scanner (CAMAG TLC Scanner) was used to visualise the plates and a Hetotrap CT 110 to cool them during their automated development.

The plates were:

- Machery-Nagel POLYGRAM SIL G/UV254 0.20 mm 4×8 cm (Number: 805021)
- HPTLC plate (Merck, Number 5641) 20×10 cm

The solvents used for HPTLC analysis:

Solvent Name	Manufacturer	Purity
Acetone	Chem-Lab NV / Promochem	minimum 99.5 %
Chloroform	Riedel-de Haën (Sigma-Aldrich)	99 - 99.4 %; stabilized with up to 1 % EtOH
Dichloromethane	Sigma Aldrich	minimum 99.9 %
Ethanol	J.T. Baker	minimum 99.9 %
Ethyl acetate	Roth	minimum 99.9 %
Formic acid	Roth	minimum 98 %
Glacial acetic acid	J.T. Baker	99 – 100 %
Methanol	Chem-Lab NV / Promochem	minimum 99.9 %
Toluol	Roth	minimum 99.5 %

A 0.05 mM DPPH solution was prepared in MeOH and its measured absorbance was approximately 0.4. Then 20 mL of this solution was poured into the separation chamber in order to coat 20×10 Merck silica 60 F254 plates with the detection reagent. Samples 1-34 were spotted onto the plates undiluted; a spotting volume of 10 µL was chosen and a 20 µL sample was another option that could be used if the concentration proved to be too low. A mixture of 14:1:5 butan-2-ol, acetic acid and water was initially used as a mobile phase, but this mixture did not result in separation of the individual compounds into spots, therefore tests were continued using different mobile phase compositions. A mobile phase composition of 9:1 chloroform: methanol was also investigated, which also failed to separate the spots. In this case, detection was performed using two different types of DPPH solution. 150 mL of 0.2 mM solution by dissolving 15.77 mg in 200 mL of methanol and 50 mL of 0.1 mM solution were prepared by dissolving 7.88 mg DPPH in methanol. Both solutions were used for spectrophotometric detection and, as described above 20 mL, were poured into the separation chamber; the samples were mixed 1:1 with DPPH solutions and the absorbance was recorded at a wavelength of 517 nm.

Standard solutions were prepared at concentrations of: 0.05 mg/ml; 0.025 mg/ml; 0.0125 mg/ml; 0.00625 mg/ml and a blank was also prepared using sample solvent only. Ascorbic acid standards were used for the DPPH measurement at concentrations of 1 g/l, 0.5 g/l, 0.1 g/l, 0.05 g/l and 0.01 g/l.

Prior to conduction HPTLC-DPPH, samples 22, 23, 30, 31, 32, 33 and 34 were purified using SPE, and measured purified (+SPE) and unpurified (+SPE). The radical scavenging activity determined using this method was calculated using the formula:

$$\frac{Ac - (Ai - Aj)}{Ac} \mathbf{x\ 100}$$

A_c: Control (DPPH-Solution)

A_i: Sample plus DPPH-Solution

A_j: Sample only

The standard solutions were used at a concentration of 1 g/L, to further optimise the HPTLC method. The following standard substances were used: catechin, gallic acid, gallotannins, caffeic acid, ascorbic acid, pooled standards and naringin. During these tests small volumes were spotted by the autosampler (max. 5 µL) because the binding capacity of plates was found to be too small for spotting 15 µL. A 60:40:4 mixture of chloroform: methanol: acetic acid was used as a mobile phase, and was prepared by mixing 11.5 mL chloroform, 7.7 mL MeOH and 0.77 mL acetic acid. The separation of samples could not be achieved on this occasion either, because spotting 2 µL of standard solutions at a concentration of 1 g/L was insufficient and, therefore, the sample volume employed was adjusted to 7 µL.

In order to improve the method further, the 20×10 Merck silica 60 F254 plates were treated with methanol prior to use, so as to remove any potential contamination; they were subsequently dried to remove any residual water and activate hydroxyl groups. To investigate the effect of plate preconditioning, a comparison of plates treated with methanol and untreated plate was performed.

Finally, the thin layer chromatograms were developed using a mixture of toluene: ethyl acetate. formic acid = 4:7:1 (V/V) for less polar compounds [= chromatographic system 1 (CS1)] and ethyl acetate: water: formic acid (17:2:2 (V/V) for medium and highly polar compounds [chromatographic system 2 (CS2)].

The separation chamber Twin Trough Chamber (CAMAG) was saturated for 20 minutes assisted by using filter paper, the development distance was set to 80 mm and post-development drying time to 2 min. The plates were subsequently heated for 3 min at 100 °C on a TLC Plate Heater III (CAMAG) and, after drying, the plates were instantly dipped in a 0.5 % solution of NST in ethyl acetate for 1 s, using Chromatogram Immersion Device III (CAMAG). In order to enhance and stabilise the fluorescent zones, after 5 minutes of air drying, the plates were immersed in a 5 % solution of PEG 400 in dichloromethane for 1 second; image capture was accomplished at 366 nm with CAMAG video documentation system and Reprostar 3 (CAMAG).

2.4 Hydrogen Peroxide Assay

The hydrogen peroxide assay measures the ability of a sample to scavenge hydrogen peroxide, and was performed as described by Ruch et al. and Keser et al. A phosphate buffered (pH 7.4) hydrogen peroxide solution (concentration = 40 mM) was prepared, and extracts of samples were made in distilled water at a concentration of 100 µg/mL (=100 that were analysed mg/L). These were added to the buffered hydrogen peroxide solution (0.6 mL) and, after an incubation time of 10 minutes, the absorbance was analysed at a wavelength of 230 nm against a blank value (only phosphate buffer without H_2O_2). The percentage hydrogen peroxide scavenged extracts and standard substances could be calculated, based on the absorbance, using the formula [31], [32]:

$$\%\, Scavenged\, [H_2O_2\,] \;=\; [(AC - AS)/AC]\; x\; 100$$

AC= absorbance of the control; AS= absorbance of extract or standard solutions

2.5 Reducing Power Assay (FRAP)

The reducing power assay was performed using ascorbic acid standards in a concentration range of 0.1 to 1 g/L as calibrant, and establishing a calibration function. A 300 mM acetate buffer was prepared by weighing 3.10 g sodium acetate trihydrate and adding glacial acetic acid (16 mL) to adjust the pH of the solution to 3.6, and then adding deionized water to make 1000 mL of solution. 40 mM Hydrochloric acid was prepared by diluting 3.4 mL of concentrated HCl to 100 mL using deionized water.

On the day when the analysis was conducted several reagents were freshly prepared:

- 10 mM TPTZ:
    - 3.123 g TPTZ were made up to 1000 mL using 40 mM hydrochloric acid
- 20 mM ferric chloride:
    - 5.406 g $FeCl_3$ * $6H_2O$ were weighed into a 1000 mL volumetric flask and deionized water was added to produced 1000ml solution
- FRAP reagent:
    - The standard solutions were prepared using a 10:1:1 mixture of acetate buffer: TPTZ:H_2O
    - The samples were prepared using a 10:1:1 mixture of acetate buffer: TPTZ: ferric chloride solution
- For the cuvette method, a 0.001 M ferric sulphate solution was prepared by weighing 2.78 g $FeSO_4$ *$7H_2O$ into a 1000 mL volumetric flask and adding deionized water to make 1000 ml solution, which was subsequently diluted 1:10.

	1	2	3	4	5	6	7	8
H_2O [µL]	1000	985	970	940	880	820	760	700
1 mM Std [µL]	0	15	30	60	120	180	240	300
FRAP [mL]	2	2	2	2	2	2	2	2
Std. concentration [µM]	2	5	10	20	40	60	80	100

The blank was prepared by mixing 2 mL FRAP reagent with 1 mL water, and samples were measured by mixing 100 µL sample with 900 µL distilled water and 2 mL FRAP reagent in a cuvette, and incubating the mixture in the dark for 30 minutes. The spectrophotometer readings were taken at a wavelength of 593 nm.

The reducing power of samples was calculated as follows:

$$FRAP\ values\ (samples)[\mu M] = \frac{Absorbance\ (samples) \times FRAP\ value\ (standards)[\mu M]}{Absorbance\ (standard)}$$

3 Results

The results of the different analytical strategies employed to determine polyphenols in food samples are shown below. To obtain a better comparability of the methods used, the results of this study are sorted by the analysis method used.

3.1 Folin-Ciocalteu Assay for assessing total phenolic content (TAC)

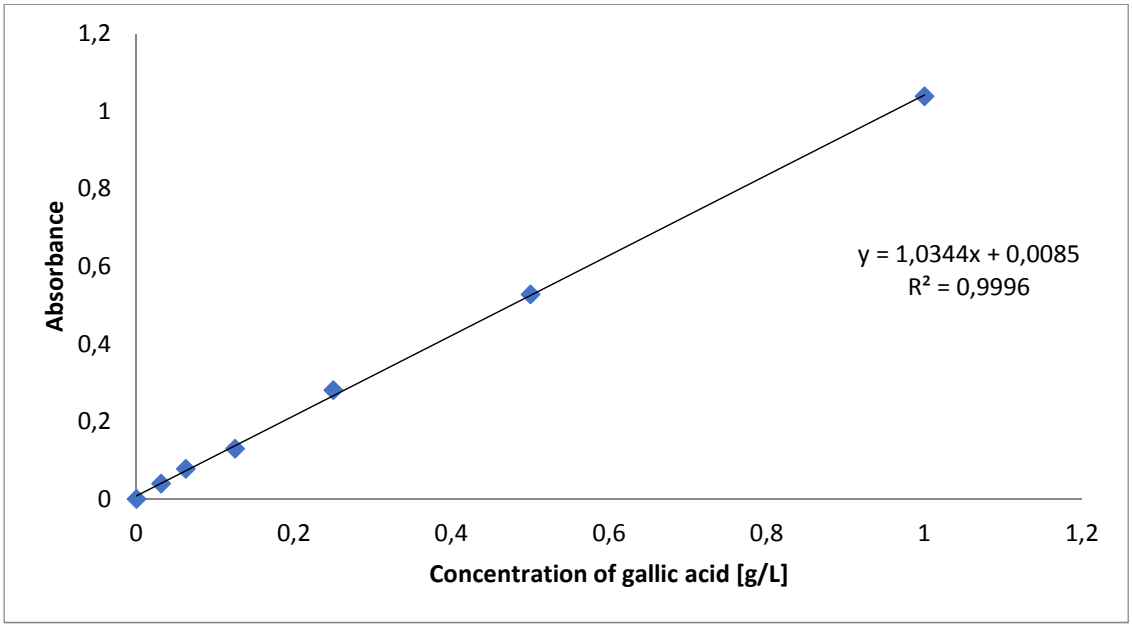


Figure 4: Folin-Ciocalteu Assay - Calibration with Gallic Acid

The calibration for the Folin-Ciocalteu Assay was performed using gallic acid standards, the correlation with absorbance was in a linear dynamic range, with a correlation coefficient of 0.9996 and the equation function used to calculate concentrations from absorbance values is shown in Figure 4.

The results of Folin-Ciocalteu measurements of *Pinus cembra* samples are recorded in Table 1; the measurements were performed three times (average values and standard deviation were calculated) and visually depicted in Figure 5.

Table 1: *Pinus cembra* Sample Measurements

	Concentration [Mg/L]				
Sample number	**Measurement 1**	**Measurement 2**	**Measurement 3**	**Average value**	**Standard deviation**
1	732	744	738	738	4.90
2	34	47	40.5	40.5	5.31
3	614	675	644.5	644.5	24.90
4	931	862	896.5	896.5	28.17
5	1782	1282	1532	1532	204.12
6	1228	1142	1185	1185	35.11

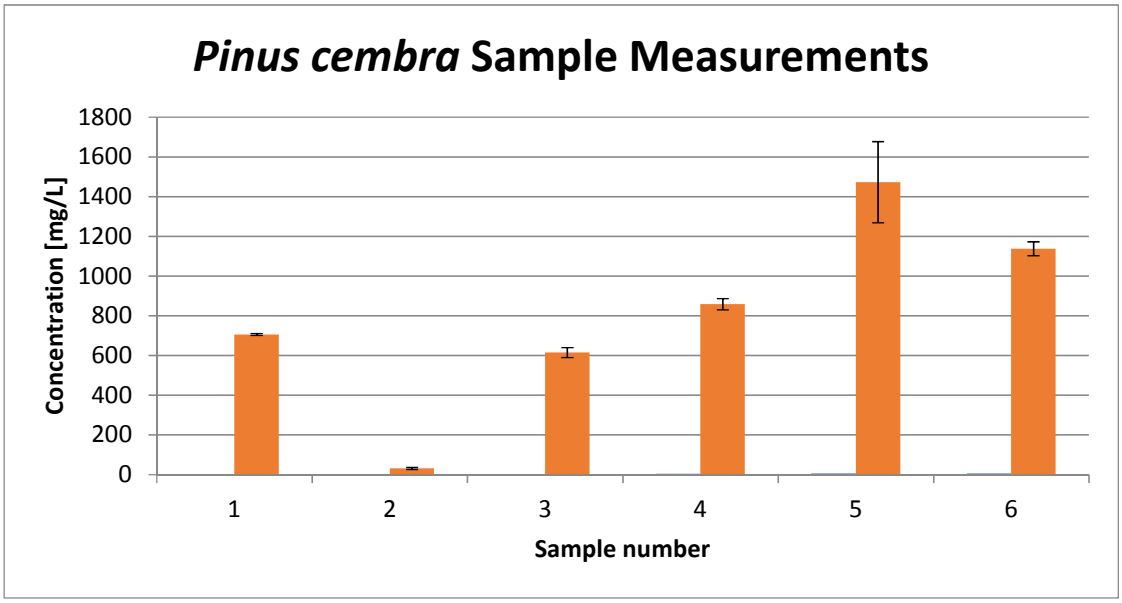


Figure 5: Folin-Ciocalteu Assay - *Pinus cembra* Sample Measurements

The analysis of polyphenols using the Folin-Ciocalteu assay was repeated three times. Therefore error bars are evident in Figure 5, which indicated the reproducibility of measurements. The standard deviation in sample 5 is the highest, which is probably related to the calculated concentration of sample 5 (and also sample 6 but there the standard deviation is not as high), which is higher than the highest standard of gallic acid used during the calibration procedure, and therefore whether the method is still in the linear dynamic range and capable of accurately measuring the absorbance, cannot be definitely ascertained In contrast to sample 5, sample 6 was only 185 mg/L above the highest calibrator and,

therefore, the standard deviation is only 35.11 mg/L (in contrast to sample 5 where it is 204.12 mg/L).

3.1.1 Crop Samples

The Folin-Ciocalteu assay was also conducted to analyse the TPC values at the medical university and at the TU Graz, in order to perform a cross laboratory validation of the results; two experiments were performed in this laboratory, and one at the medical university Graz. The results data in Table 2 was also transformed into graphs for graphical representation.

Table 2: Comparison of TPCs of Crop Samples

	TPC MedUni [mg/ml]	**TPC TU [mg/ml]**	**TPC TU number 2 [mg/ml]**
Integral bianco	51.2	59.2	53.5
Ib r-960	78.9	79.0	81.4
Koerngold flour finely ground 480	44.1	46.5	47.3
Flour with antioxidants	112.2	120.1	118.9
Finis Feinstes wheat flour	59.7	60.3	59.6
IB pizza flour 28.2.2016	43.1	47.2	43.7
Quinoa-wheat flour	120.5	131.5	125.7
Spilt whole grain flour finely ground	43.8	44.5	43.8
Spilt whole grain flour gritty	61.1	64.2	62.2
Spilt bread (crust)	23.7	25.0	25.2
Spilt bread (inner parts)	25.7	29.2	27.0
Quinoa wheat bread (crust)	40.9	42.4	42.0
Quinoa *wheat bread (inner parts)*	18.1	21.2	18.0
Wheat bread neutral (crust)	32.4	35.8	33.6
Wheat bread neutral (inner parts)	16.1	17.3	15.7
Spilt bread with quinoa (crust)	41.5	43.9	42.1
Spilt bread with quinoa (inner parts)	18.2	19.4	18.9
Bread integral bianco (crust)	32.4	33.2	33.6
Bread integral bianco (inner parts)	25.0	26.0	25.7
Boiled noodles	9.8	10.1	9.1
Noodle water	20.4	20.4	20.5

The results of TPC analysis, Figure 6, are visually displayed as a bar chart, which demonstrates that the results of this research are in accordance with those generated in a different laboratory, Figure 7.

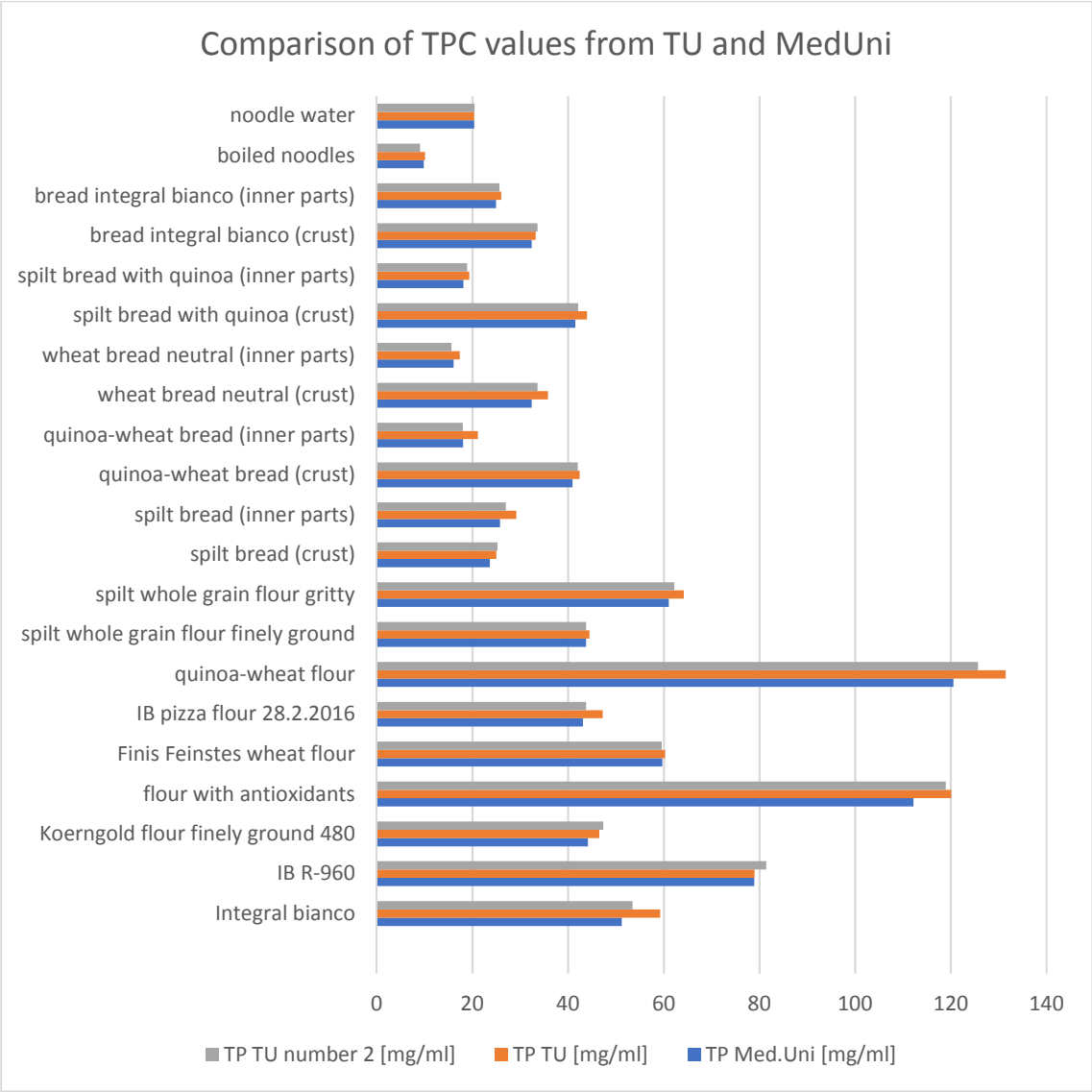


Figure 6: Comparison of TPC values obtained at the medical university (n=1) and the TU (n=2) Graz

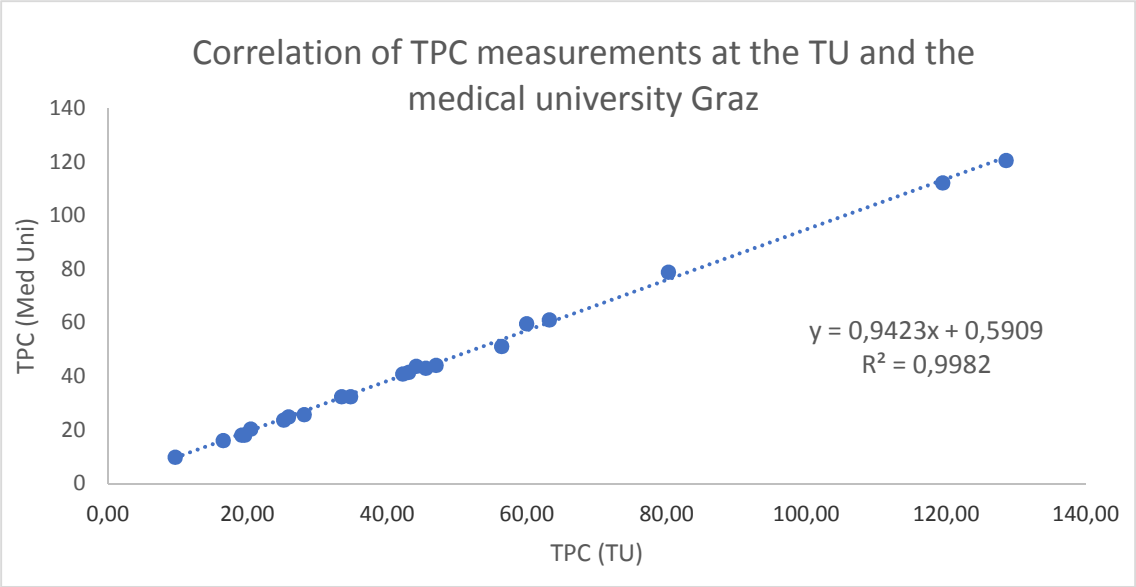


Figure 7: Correlation of TPC measurements at the TU and the medical university Graz

A strong correlation between TPC measurements at the TU and the medical university Graz is evident in Figure 7: The data obtained at TU Graz (average of both measurements) were plotted on the x-axis and these obtained at the medical university on the y-axis. The linear correlation is evident and, a trendline added to obtain a linear function shows that the correlation coefficient R^2 has a value of 0.9982 which means that the linear model is an optimum fit for the selected data.

3.1.2 Sample 1-34

The results of determining the total polyphenol content of samples 1-34 are shown in Table 3 below; note that sample 30 had an absorbance value of 3.654, which is probably no longer in the linear range of the instrument. Therefore, interpretation of the absolute value of this sample is required to consider this aspect. The absorbance value of sample 24 was so low that a negative concentration of TPC was calculated, therefore the absorbance value but no concentration is shown.

Table 3: Absorbance and TPC of Samples 1-34

Sample Number	Absorbance	Concentration of Total Polyphenol Content [mg/L]
1	0.036	26.6
2	0.100	88.5
3	0.027	17.9
4	0.018	9.18
5	0.050	40.1
6	0.053	43.0
7	0.023	14.0
8	0.027	17.9
9	0.066	55.6

10	0.034	24.7
11	0.041	31.4
12	0.050	40.1
13	0.018	9.2
14	0.035	25.6
15	0.018	9.18
16	0.046	36.3
17	0.031	21.8
18	0.022	13.1
19	0.023	14.1
20	0.059	48.8
21	0.018	9.2
22	0.423	401
23	0.041	31.4
24	0.003	
25	0.023	14.0
26	0.070	59.5
27	0.194	179.3
28	0.506	481
29	0.038	28.5
30	3.654	3520
31	0.332	313
32	0.423	401
33	0.184	170
34	0.704	672

The data shown in Table 3 is graphically represented in Figure 8 and Figure 9 below; the data was separated into two graphs because the polyphenol content of different samples varied greatly, for instance sample 30 has a TPC more than ten times higher than the average dataset.

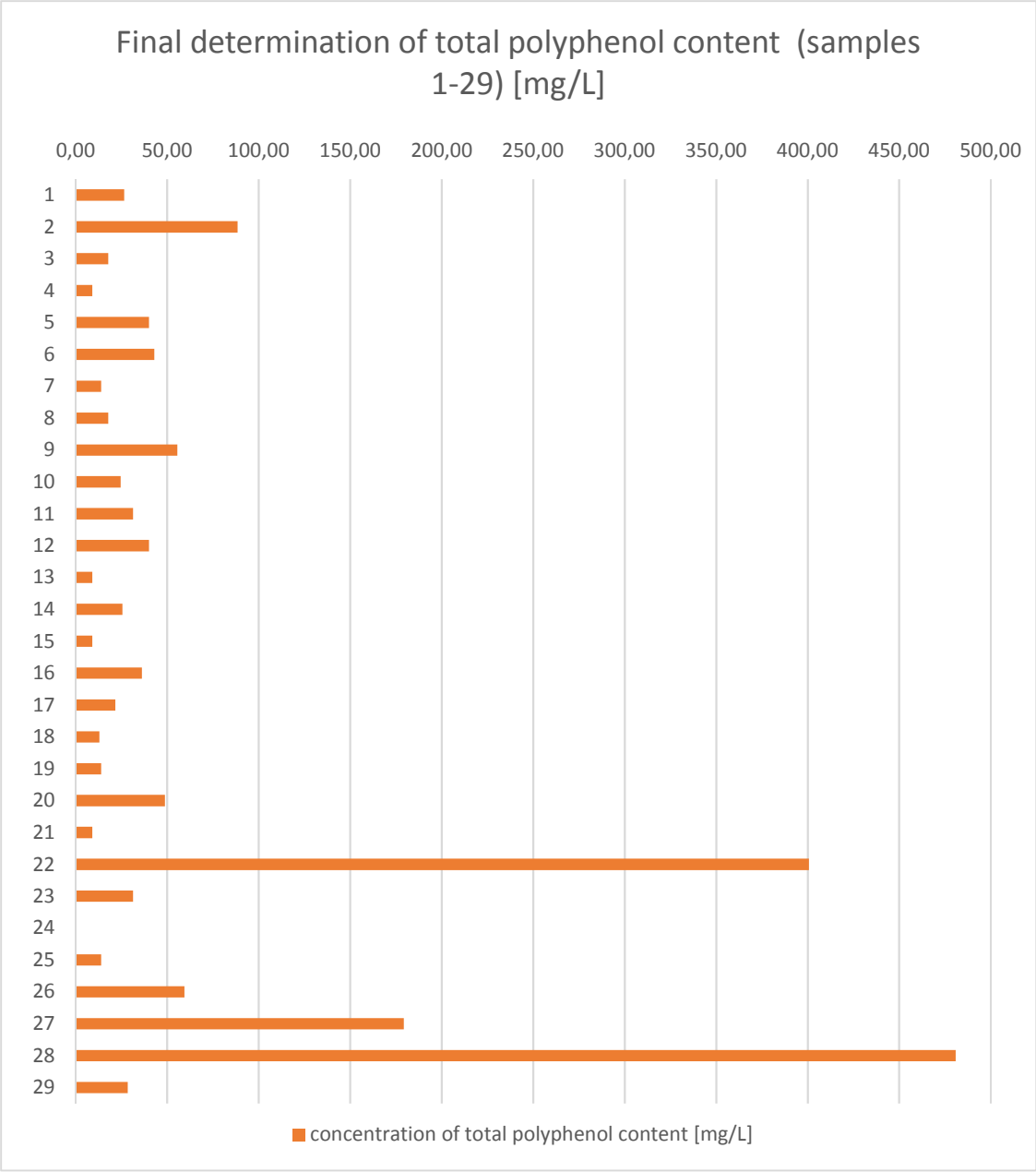


Figure 8: Total polyphenol content of samples 1-29

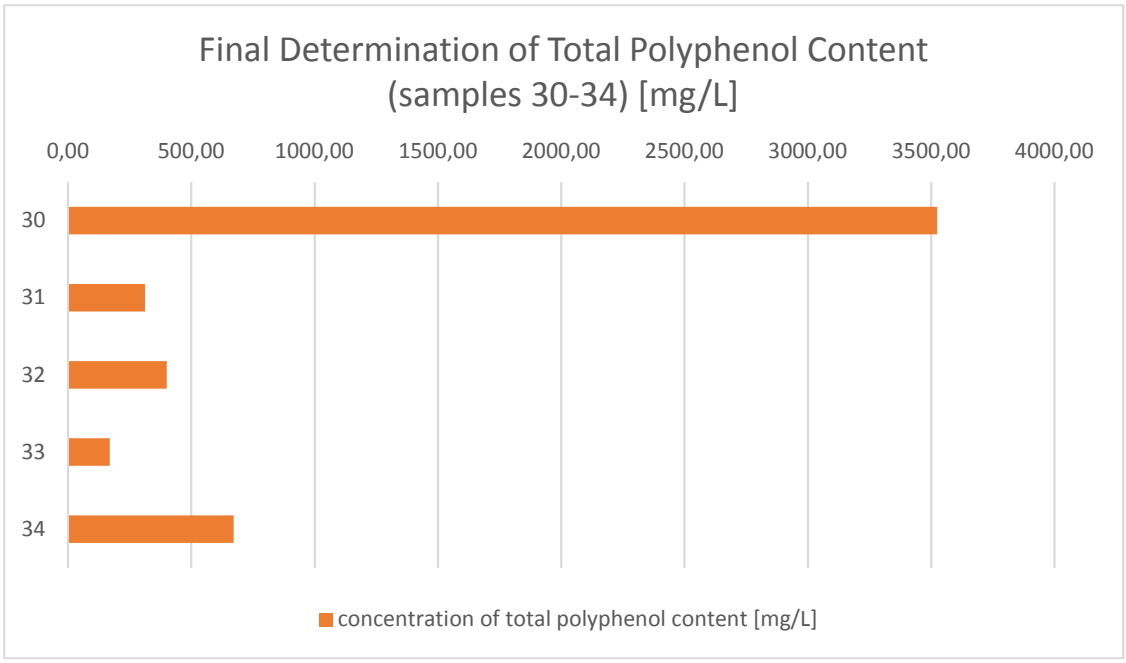


Figure 9: Total polyphenol content of samples 29-34

3.1.3 Food Samples

Four different food samples were also analysed for their total polyphenol content Figure 10.

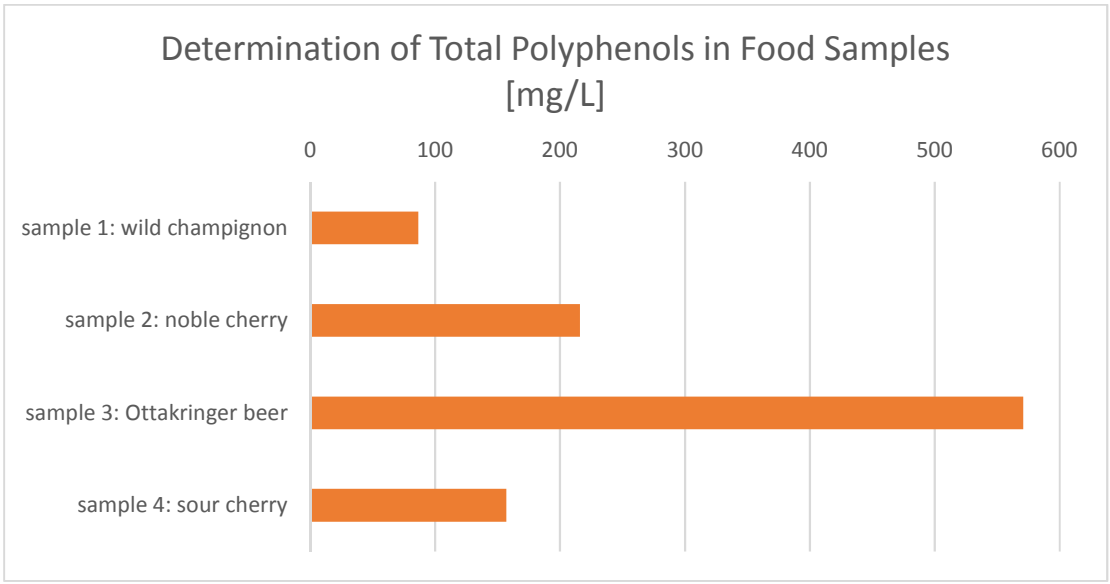


Figure 10: Determination of total polyphenols in food samples 1-4

3.2 Analysis of Peroxides in Grain Samples

Grain samples were analysed for peroxidases and peroxides; the assay to determine the total content of peroxides was performed in this study and additional samples were measured at the Medical University Graz, in order to validate the results. The data listed in Table 4 are not normalised, and the figures in the last column are the sample weights used for the analysis and for normalisation of data, which was the basis of subsequent calculations.

Table 4: Results of Food Sample Analysis (absolute results)

Sample number	Sample name	Anti-oxidative capacity	Peroxides	Peroxidases	Peroxides (Meduni)	weight [g]
M1601	Integral bianco	-0.10	5.80	12.16	51.21	3.45
M1602	Ib r-960	0.13	8.06	9.21	78.92	3.3
M1603	Koerngold flour glatt 480	-0.23	9.62	4.83	44.13	3.85
M1604	Flour with antioxidants	0.62	198.42	4.84	112.21	0.5
M1605	Finis Feinstes wheat flour	0.18	9.33	6.57	59.70	3.7
M1606	IB pizza flour 28.2.2016	0.02	1.70	2.02	43.10	3.6
M1607	Quinoa-wheat flour	1.13	118.61	5.06	120.54	2.5
M1608	Spilt whole grain flour finely grinded	-0.03	11.60	9.50	43.77	3.6
M1609	Spilt whole grain flour gritty	0.06	-5.80	4.63	61.06	3.45
B1610	Spilt bread (crust)	0.29	-13.57	0.18	23.69	4.75
B1611	Spilt bread (inner parts)	0.55	-30.26	0.06	25.75	4.55
B1612	Quinoa wheat bread (crust)	0.15	5.09	0.16	40.95	4.6
B1613	Quinoa wheat bread (inner parts)	0.06	30.97	0.07	18.08	4.9
B1614	Wheat bread	0.21	-3.39	0.15	32.41	4.35

	neutral (crust)					
B1615	Wheat bread neutral (inner parts)	0.13	9.47	0.07	16.07	4.55
B1616	Spilt bread with quinoa (crust)	0.86	42.00	0.14	41.52	3.8
B1617	Spilt bread with quinoa (inner parts)	0.37	6.79	0.06	18.16	4.05
B1618	Bread integral bianco (crust)	0.15	-2.55	0.12	32.41	4.65
B1619	Bread integral bianco (inner parts)	0.33	7.21	0.07	24.96	4.5
B1620	Boiled noodles	0.39	-5.37	0.11	9.85	8
B1621	Noodle water	0.04	2.55	0.11	20.41	

Data obtained during analysis of the grain samples which normalised on the sample weight, Table 6.

Table 5: Calculation of Water and Dry Matter Content

	Weighed portion [g]	Dry weight [g]	Water content [%]	dry matter content [%]
Spelt bread cut	43.8	26.7	39.0	61.0
Wheat and quinoa bread cut	24.0	16.8	30.0	70.0
Wheat bread neutral	22.1	15.3	30.8	69.2
Spelt and quinoa bread cut	43.0	26.1	39.3	60.7
Bread Integral bianco cut	37.0	25.0	32.4	67.6

Table 6: Results of Grain Sample Analysis (Normalised on Sample Weight)

	Peroxides/ g (TU)	Peroxides /g (MedUni)
Integral bianco	1.68	14.84
Ib r-960	2.44	23.92
Koerngold flour finely ground 480	2.50	11.46
Flour with antioxidants	396.84	224.42

Finis Feinstes wheat flour	2.52	16.14
IB pizza flour 28.2.2016	0.47	11.97
Quinoa wheat flour	47.44	48.22
Spilt whole grain flour finely ground	3.22	12.16
Spilt whole grain flour gritty	-1.68	17.70
Spilt bread (crust)	-2.86	4.99
Spilt bread (inner parts)	-6.65	5.66
Quinoa wheat bread (crust)	1.11	8.90
Quinoa wheat bread (inner parts)	6.32	3.69
Wheat bread neutral (crust)	-0.78	7.45
Wheat bread neutral (inner parts)	2.08	3.53
Spilt bread with quinoa (crust)	11.05	10.93
Spilt bread with quinoa (inner parts)	1.68	4.48
Bread integral bianco (crust)	-0.55	6.97
Bread integral bianco (inner parts)	1.60	5.55
Boiled noodles	-0.67	1.23

This normalised data was used to show the correlation of the data from this study with that generated at the Medical University Graz; the highest values were measured in flour with antioxidants and flour with quinoa, as Figure 11.

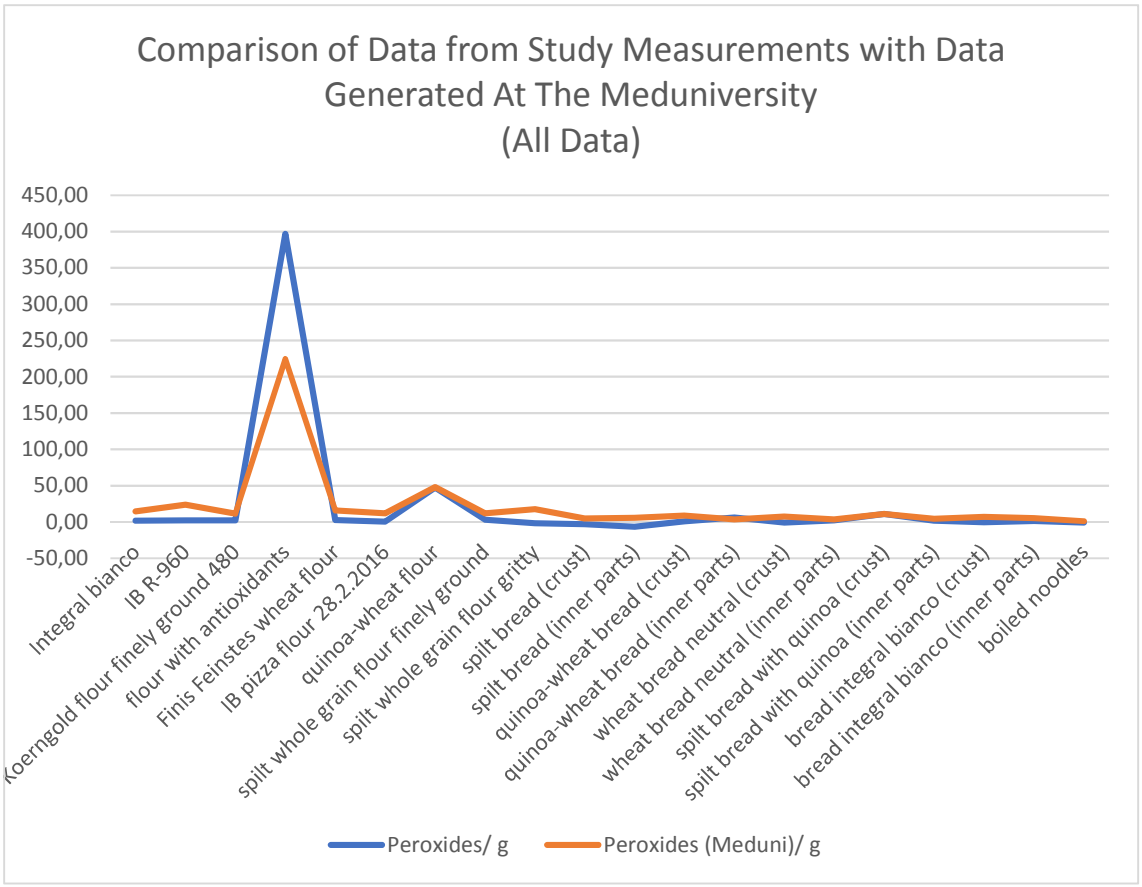


Figure 11: Comparison of Peroxide Measurements: MedUni and TU Graz

The results of measuring peroxides in this study and during confirmation analysis at the medical university are similar, Figure 11. To obtain better graphical representation of the data, it was split into two groups for the following graphs: The two highest samples (flour with antioxidants and quinoa – wheat flour) are shown in a different graph because otherwise the axes scales would not permit smaller values to be well represented.

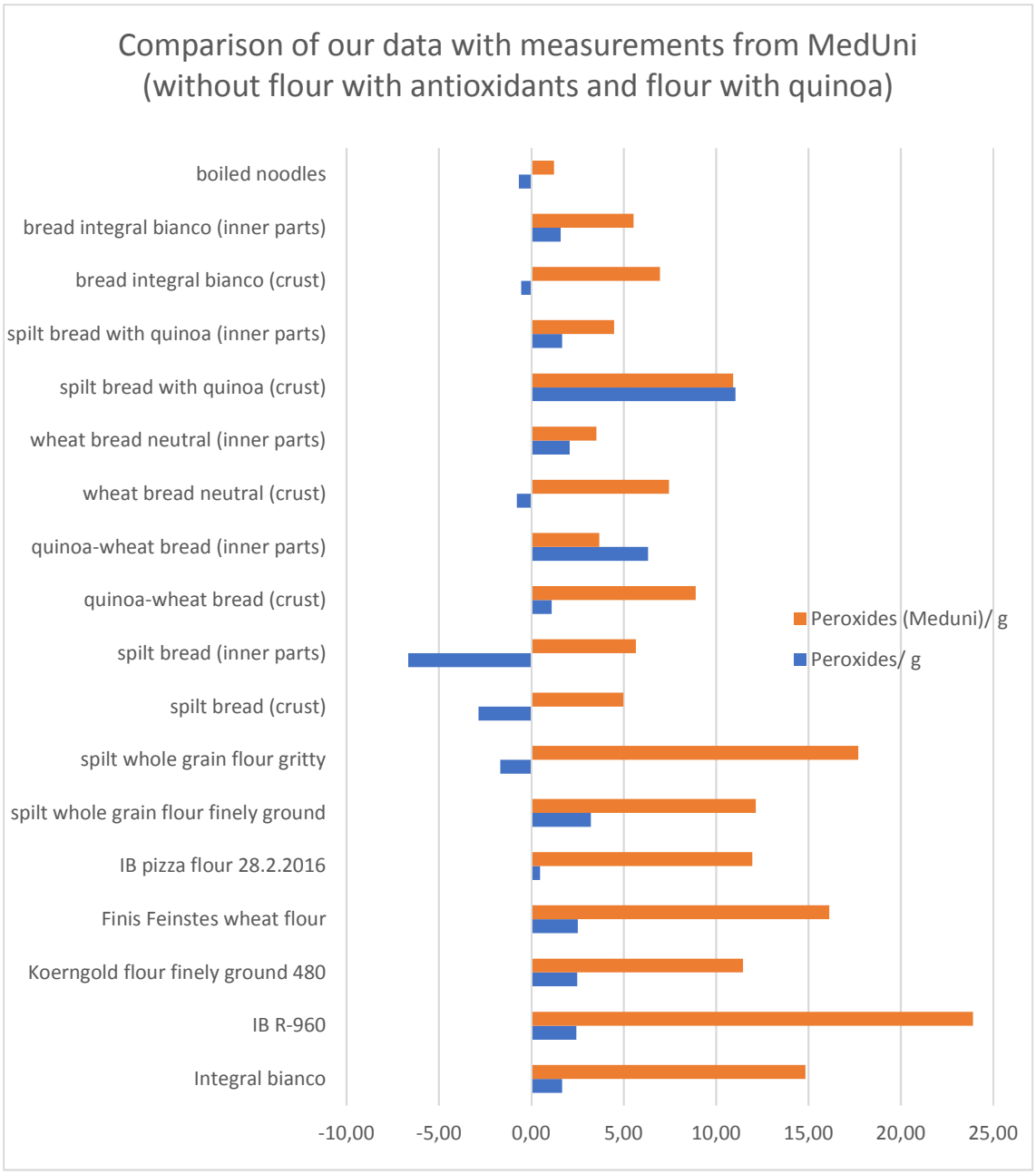


Figure 12: Comparison of peroxides measured at TU and medical university Graz (flour with antioxidants and flour with quinoa were excluded in this graph for scaling reasons)

The graph below shows the two data points which were excluded above.

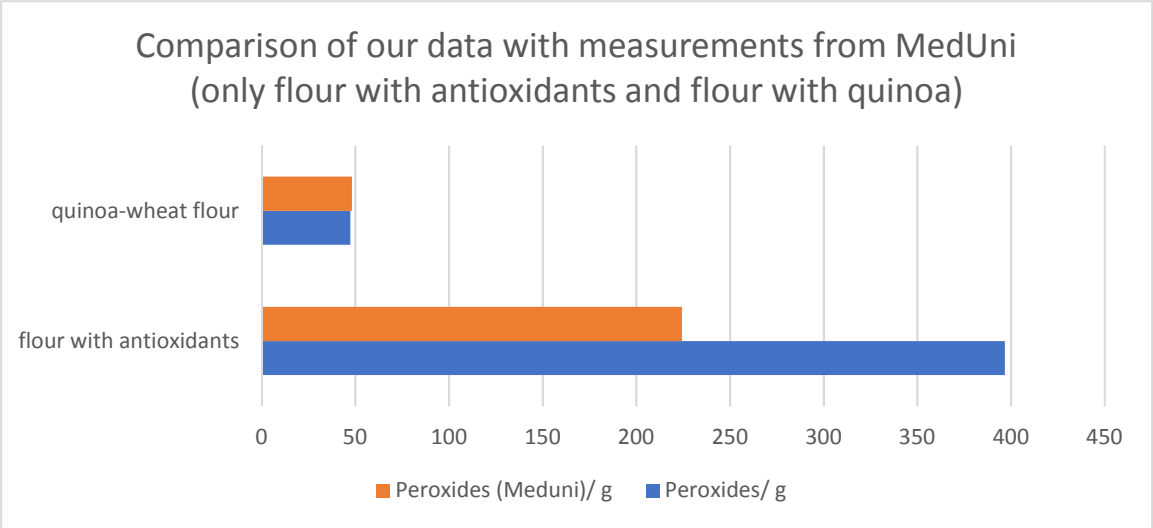


Figure 13: Comparison of TPCs measured at TU and medical university Graz (flour with antioxidants and flour with quinoa are shown alone in this graph for scaling reasons)

For quinoa wheat flour, an excellent consensus of measurement data was shown, however the highest values that were obtained for flour with antioxidants in both test runs are different and this will be addressed in more detail in the discussion. In this research, negative values for TPC were recorded but not in those recorded at the Medical University, Figure 12, as this is not possible, these values are assumed to be zero. Although some values differ greatly, they generally correlated but up to a measurement value of approximately 40, (see absolute values shown in Table 4) the results of this analysis are unreliable (e.g. sometimes showing negative results). Nevertheless, the correlation between both measurements has a correlation coefficient of 0.9829, which confirms that both methods can detect peroxides in the food samples tested.

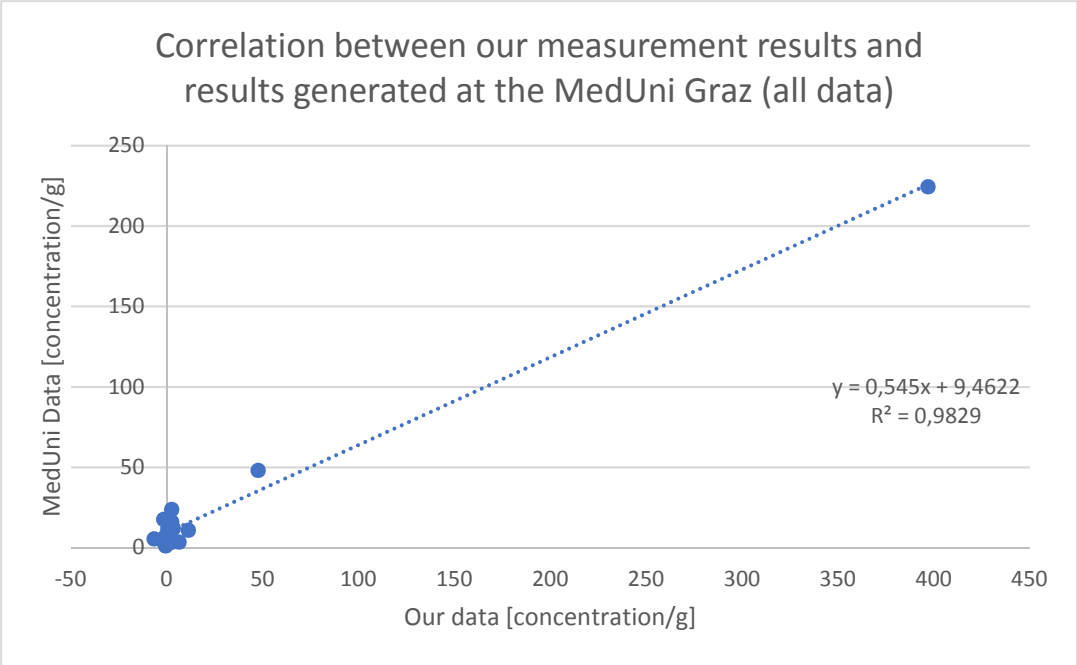


Figure 14: Correlation between our peroxide measurement results and results generated at the MedUni Graz (all data)

3.3 Reducing Power Assay

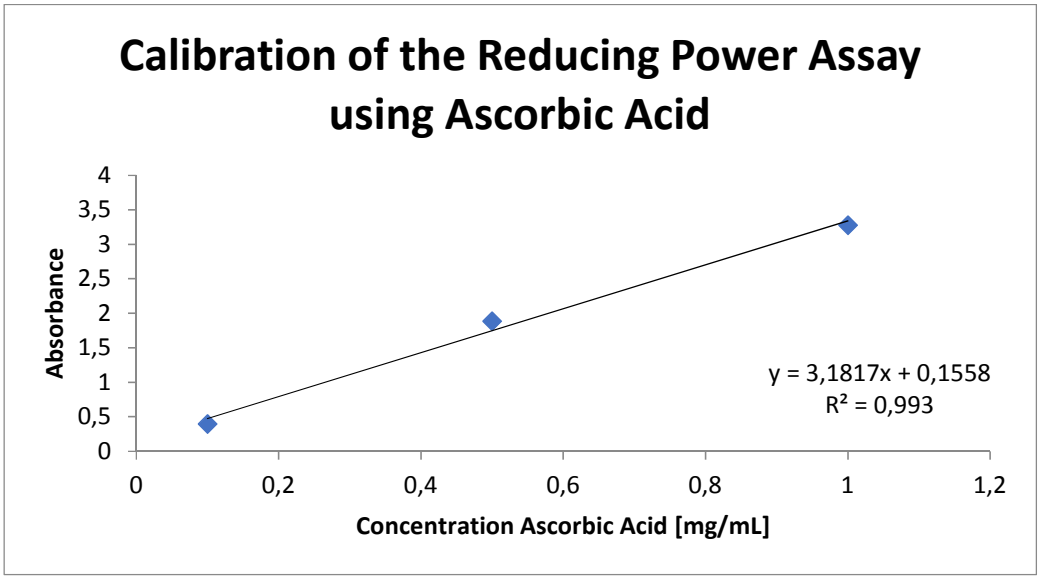


Figure 15: Calibration of the Reducing Power Assay using Ascorbic Acid

The calibration for the reducing power assay was performed using ascorbic acid standards at a concentration range of 0.1 to 1 g/L; the correlation with absorbance was in a linear dynamic range with a correlation coefficient of 0.993. The equation function that was used to calculate concentrations from absorbance values is shown in Figure 15.

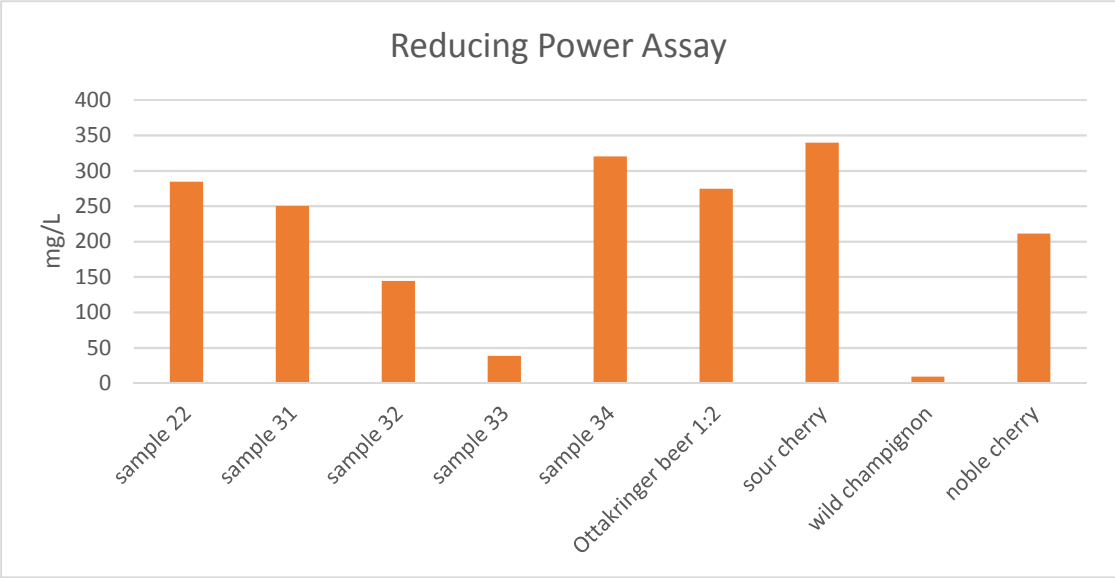


Figure 16: Reducing Power Assay for Samples 22, 31-34 and Food Samples 1-.4

3.4 DPPH Assay for Assessing Radical Scavenging Activity (RSA)

To measure the 6 *Pinus cembra* samples' Radical Scavenging Activity (RSA), a DPPH assay was conducted and the measurements and the calculation of the RSA are shown in Table 7.

Table 7: Sample measurements and calculation of radical scavenging activity (RSA) using the DPPH assay. sample alone= Aj; sample plus DPPH= Ai; Ac= ascorbic acid

Sample Number	**Aj**	**Aj /2**	**Ai**		**Ac (0.1 mM)**	**Ac/2 (0.1 mM)**		**RSA [%]**
			Measurement 1	Measurement 2	Average			
1	0.334	0.167	0.165	0.235	0.200	1.017	0.5085	60.67
2	0.556	0.278	0.173	0.183	0.178	1.017	0.5085	65.00
3	0.468	0.234	0.135	0.144	0.140	1.017	0.5085	72.57
4	0.489	0.2445	0.136	0.154	0.145	1.017	0.5085	71.48
5	0.507	0.2535	0.124	0.147	0.1355	1.017	0.5085	73.35
6	0.478	0.239	0.180	0.124	0.152	1.017	0.5085	70.11

The radical scavenging activity was calculated according to the following formula:

$$Radical\ scavenging\ activity\ [\%] = \frac{\frac{Ac}{2}(0.1\ mM) - A_i\ (sample\ plus\ DPPH)}{\frac{Ac}{2}(0.1\ mM)} \times 100$$

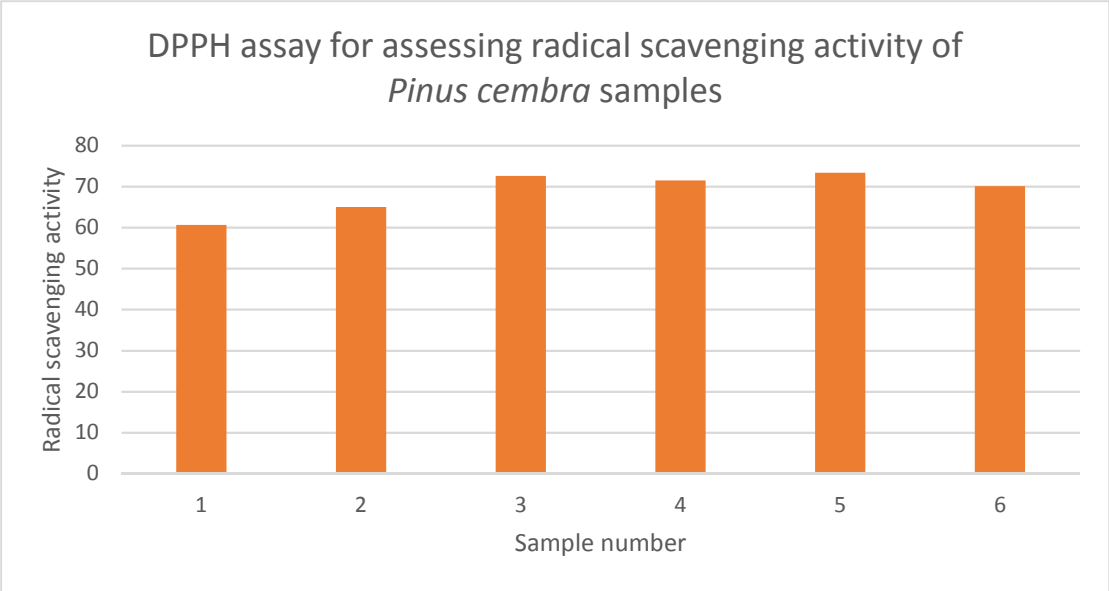


Figure 17: Results of the DPPH assay (sample 1-6) using different extraction procedures

3.5 HPTLC

For the mobile phases 14:1:5 butan-2-ol, acetic acid, water and 9:1 chloroform: methanol no separation of the individual compounds into distinct bands was achieved, therefore, no Rf values for these mobile phases could be calculated as shown in Table 8.

Table 8: TLC using mobile phases 14:1:5 butan-2-ol, acetic acid and water or 9:1 chloroform:methanol did not yield in separation of the individual compounds

Mobile phases	14:1:5 butan-2-ol, acetic acid and water or 9:1 chloroform: methanol	Band 1	Band 2	Band 3
Rf Values	**Sample 1**	/	/	/
	Sample 2	/	/	/
	sample 3	/	/	/
	Sample 4	/	/	/
	Sample 5	/	/	/
	Sample 6	/	/	/
	Sample 7	/	/	/
	Sample 8	/	/	/
	Sample 9	/	/	/
	Sample 10	/	/	/

Sample 11	/	/	/
Sample 12	/	/	/
Sample 13	/	/	/
Sample 14	/	/	/
Sample 15	/	/	/
Sample 16	/	/	/
Sample 17	/	/	/
Sample 18	/	/	/
Sample 19	/	/	/
Sample 20	/	/	/
Sample 21	/	/	/
Sample 22	/	/	/
Sample 23	/	/	/
Sample 24	/	/	/
Sample 25	/	/	/
Sample 26	/	/	/
Sample 27	/	/	/
Sample 28	/	/	/
Sample 29	/	/	/
Sample 30	/	/	/
Sample 31	/	/	/
Sample 32	/	/	/
Sample 33	/	/	/
Sample 34	/	/	/

Table 9: Rf factors of HPTLC (polar substances) before DPPH fluorescence

Mobile phase	ethyl acetate: water: formic acid (17:2:2 (v/v)	Band 1	Band 2	Band 3
Rf Values	**Sample 1**	0.34	0.52	0.87
	Sample 2	0.33	/	0.9
	sample 3	0.35	0.5	/

Sample 4	0.33	0.54	0.87
Sample 5	/	0.47	0.92
Sample 6	0.32	0.52	0.88
Sample 7	0.29	0.55	0.94
Sample 8	0.33	/	0.89
Sample 9	0.35	0.50	/
Sample 10	0.35	0.50	/
Sample 11	0.33	0.53	0.86
Sample 12	/	0.47	0.91
Sample 13	0.32	0.51	0.87
Sample 14	0.29	0.55	0.94
Sample 15	0.33	/	0.89
Sample 16	0.32	0.51	0.87
Sample 17	0.29	0.54	0.93
Sample 18	0.29	0.55	0.94
Sample 19	0.34	0.51	0.86
Sample 20	0.33	/	0.89
Sample 21	0.35	0.50	/
Sample 22	0.33	0.53	0.86
Sample 23	0.33	/	0.90
Sample 24	0.29	0.55	0.94
Sample 25	0.33	/	0.89
Sample 26	0.29	0.54	0.93
Sample 27	0.29	0.55	0.94
Sample 28	0.29	0.54	0.93
Sample 29	0.34	0.51	0.86
Sample 30	0.33	/	0.89
Sample 31	0.32	0.51	0.87
Sample 32	0.29	0.55	0.94
Sample 33	0.33	0.53	0.86
Sample 34	0.33	/	0.90

Table 10: Rf factors of HPTLC (less polar substances) before DPPH fluorescence

Mobile phase	toluene: ethyl acetate. formic acid = 4:7:1 (V/V)	Band 1	Band 2	Band 3
Rf Values	**Sample 1**	0.31	0.52	0.84
	Sample 2	0.37	/	0.94
	sample 3	0.28	0.51	/
	Sample 4	0.33	0.44	0.87
	Sample 5	/	0.51	0.93
	Sample 6	0.35	/	0.84
	Sample 7	0.30	0.55	0.94
	Sample 8	0.27	0.51	0.89
	Sample 9	0.28	0.43	0.85
	Sample 10	0.26	/	0.93
	Sample 11	0.27	0.54	0.77
	Sample 12	/	0.49	0.91
	Sample 13	0.32	0.41	/
	Sample 14	0.29	/	0.94
	Sample 15	0.31	0.46	0.94
	Sample 16	0.37	0.51	0.84
	Sample 17	0.28	0.54	0.81
	Sample 18	0.33	0.55	0.84
	Sample 19	/	0.51	/
	Sample 20	0.35	/	0.84
	Sample 21	0.35	0.43	0.90
	Sample 22	0.33	0.46	0.86
	Sample 23	0.31	0.54	0.90
	Sample 24	0.37	0.46	0.94
	Sample 25	0.37	/	0.89
	Sample 26	0.35	0.54	0.93
	Sample 27	0.21	0.43	/
	Sample 28	0.26	0.46	0.93
	Sample 29	/	0.54	0.77

Sample 30	0.26	/	0.93
Sample 31	0.27	0.51	0.81
Sample 32	0.31	0.51	0.84
Sample 33	0.37	/	0.94
Sample 34	0.33	0.51	0.90

4 Discussion

An optimised HPTLC-DPPH method was used to determine the total polyphenol content (TPC) and total antioxidant capacity (TAC) and the results of these assays were compared with standard photometric assays. An HPLC-MS analysis was also implemented by the Medical University of Graz to validate the results obtained in this study. Data from HPLC-MS analysis could show that HPTLC-DPPH and routine photometric assays are exact and robust methods for determining TPC and TAC. HPLC-MS is given a preference in separation and quantification of individual polyphenols compared to HPTLC. This is a consequence of the higher resolution power of high performance liquid chromatography and the potential to obtain additional mass-spectrometric data that reveal structural information for the individual compounds.

This study shows that the different methods tested during this research can exactly and robustly determine the total polyphenol content (TPC) and the total antioxidative capacity (TAC) of different food samples. Photometric assays are recommended for large sample research, because they can be conducted using 96 well plates and a plate reader to obtain absorbance values, which can be transformed into concentrations by using a standard curve that can be measured on the same plate and reduces interference by the analytical system.

The validation of the TPC measurements in this research was accomplished by using test results generated at the Medical University Graz shows that these are correlated. Therefore, the method used for assessing the total polyphenolic content of food samples in this study is very suitable for routine testing, and this strategy can also be implemented for further studies. The TPC measurements of different food samples also revealed that the concentration in different sample types differ substantially and, therefore, a broad linear range of the selected analytical strategy is required to accurately determine the TPCs. By comparing the results with those from the Medical University, the highest value measured was shown to represent a greater difference between the two series of measurement than other samples and, it is assumed that accurate quantification cannot be assured at the highest range of TPC values. Since the signal was higher than the highest standard used, no statement regarding the validity of the linear calibration function can be made for this sample. All methods led to the same results regarding TPC and TAC, confirming both HPTLC and photometry to be suitable for analysis of polyphenols. Coffee and schnapps derived from *Pinea cembra* showed the highest values for TPC and TAC.

Chromatographic techniques are preferred for research purposes in which the sum parameters are relevant, and different substances are to be separated (and possibly also identified). In this research, high performance thin layer chromatography was employed, and is a very important method for development of chromatographic techniques because low solvent volumes are used and solvent parameters can be strongly varied, which is not as easily achievable for HPLC systems. However, HPTLC has the disadvantage that quantification is not as accurate and identification by mass spectrometry can only be performed using compatible staining protocols and offline extraction of analytes from the silica plate material.

If the focus of research is the identification of the different polyphenolic and antioxidative compounds, HPLC coupled to mass spectrometry is the method of choice. Here information dependent acquisition can be utilised, in order to relatively quantify the different substances based on their MS1 generated ion chromatograms, and to identify them based on their fragmentation pattern (MS2). If absolute quantification is to be performed, MRM assays are implemented in which fragmentation parameters are optimised for the selected analytes, and standard curves are measured. In this case, the sensitivity can be increased and interference is reduced, even by highly similar substances, because the quantification of each compound is based on a highly specific transition from a precursor ion to one or more fragment ions (MS2).

The HPTLC method also has benefits. The main advantage is increased flexibility and tolerance to a wider range of mobile phases which cannot be provided by HPLC-MS systems. The parameters can be changed or optimised in order to obtain better separation of the individual compounds for further improvement of the HPTLC methods, for instance: RP-18 plates could be used with methanol as a mobile phase, instead of silica plates, because it is assumed that the reaction between DPPH and silica is stronger than with a stationary phase in which polar groups have been modified with a non-polar group, as is the case with C18 residues. Detection could be performed in fluorescence mode in order to avoid the problem of generating negative peaks. Ultimately, fingerprinting of extracts could be performed by coupling the plate based separation with structural information e.g. generated by HPTLC-MS.

5 Outline

Miniaturization and automation will play an important role in future developments in the sector of polyphenol analysis, based on the general trends in analytical chemistry. Modern research is either focused on quantification of sum parameters e.g. TAC or TPC or on identification of the single polyphenolic molecules and assessing their particular effects on human health. Therefore, future methods used in polyphenol research will focus on accurate quantification of selected polyphenols; developing high throughput chromatographic methods that are capable of simultaneous separation and quantification such as HPTLC, is crucially important.

The extraction of polyphenols from food samples is also strongly influenced by different parameters, including the chemical nature of the analytes, the selected extraction method, storage time and conditions of samples and extracts, as well as the equipment used for separation. In the case of chromatography, particle size and selectivity of the stationary phase are important factors, but interfering substances can influence analytical results and therefore minimising these effects must be resolved. State of the art technologies include HPLC (with UV-Vis detection or mass spectrometry), gas chromatography (GC or GC-MS) and Fourier transformation infrared spectroscopy (FT-IR) or nuclear magnetic resonance (NMR) for structural elucidation [33].

Databases, which describe the chemistry of the individual molecules, are becoming increasingly important for identification of polyphenolic compounds. In order to measure the amount of polyphenols consumed by a certain population, and deriving information regarding their health benefits, detailed information on polyphenol contents of different food types is required. Since polyphenols do not have a distinct molecular structure but are a group comprising variable chemical structures, their content can vary greatly, even in a single food type, making collection of this information difficult. Phenol Explorer was the first web based database giving comprehensive information on polyphenol content in foods; it has more than 37 000 entries from 638 scientific articles and is used to generate representative qualitative and quantitative data for 502 polyphenols in 452 foods. Such databases on food constituents help researchers to obtain a better understanding of how phytochemicals influence the nutritional quality of food. The increasing consumer awareness and the trend towards functionalised foods and superfoods, means that food manufacturers are strongly motivated to develop healthy foods based on ingredients known to have high polyphenol content. Additionally, grains that have high TPCs are selectively bred to obtain even higher polyphenol content and are increasingly purchased by health aware consumers [34].

6 References

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