Pressurised Fluid Extraction of Bioactive Species in Tree Barks

Analysis using Hyphenated Electrochemical Mass Spectrometric Detection

MICHELLE CO
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Abstract

Analytical chemistry has developed throughout time to meet current needs. At present, the interest in biorefinery is growing, due to environmental awareness and the depletion of fossil resources. Biomass from agricultural and forestry industries has proven to be excellent raw material for different processes. Biorefining valuable species such as bioactive species from biomass, without compromising the primary process of the biomass is highly desirable. Pressurised fluid extraction (PFE) using water and ethanol as a solvent was developed for extracting betulin from birch (Betula pendula) bark. Apart from betulin, stilbene glucosides such as astringin,isorhapontin and piced were also extracted from spruce (Picea abies) using PFE. PFE is an advanced technique that extracts at temperatures above the solvent’s atmospheric boiling point. The applied pressure in PFE is mainly to maintain the liquid state of the extraction solvent. Parameters such as type of solvent, temperature, and time affect the extraction selectivity and efficiency. Therefore it is necessary to comprehend these parameters in order to optimise extraction. The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay was used to determine the antioxidant capacity and activity of the obtained bioactive species. The results showed high antioxidant capacity in bioactive species that were extracted at an elevated temperature, 180°C. Extraction and degradation occur simultaneously during the extraction. Hence, it is crucial to separate these two processes in order to obtain the actual value.

An online hyphenated system of chromatographic separation electrochemical mass spectrometric detection was developed (LC-DAD-ECD-MS/MS). The electrochemical detector facilitates real-time monitoring of the antioxidant capacity and activity of each antioxidant and its oxidation products. This developed LC-DAD-ECD-MS/MS method enabled rapid screening of antioxidants and created a fingerprint map for their oxidation products. Characterisation and molecular elucidation of bioactive species were also performed. Degradation of bioactive species was investigated with the said online system and birch bark extract was compared with birch bark extracts that were hydrothermally treated. The obtained results showed some degradation of antioxidants at 180°C.

In summary, the aim of this thesis was to develop analytical methods integrated with sustainable chemistry for extraction of bioactive species in biomass from the forestry industry. A novel online system using selective and sensitive detectors such as diode-array, electrochemical, and tandem mass spectrometry was developed to rapidly determine the antioxidant capacity and activity of antioxidants. Furthermore, tandem mass spectrometry enables identification of unknown bioactive species without the need of reference samples.

Keywords: Pressurised fluid extraction, antioxidants, DPPH, water, ethanol, antioxidant activity, antioxidant capacity, electrochemical detection, mass spectrometry

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“Science may set limits to knowledge, but should not set limits to imagination”

– Bertrand Russell
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


IV. Co, M., Zettersten, C., Nyholm, L., Sjöberg, P. J.R., Turner, C. **Extraction and degradation of antioxidants from birch bark using water at elevated temperature a pressure.** Manuscript in preparation for *Analytica Chimica Acta* **2010**.

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This doctorate thesis is based on my licentiate thesis, which is entitled High Pressurised Fluid Extraction of Antioxidative Species from Plants. The discussions encountered here in this doctorate thesis are further developed from the licentiate thesis, with new materials.
Author Contribution

I. Planned and performed most of the experiments and wrote the paper.

II. Planned and performed most of the experiments and wrote the paper.

III. Participated in the planning, performed parts of the experiments and wrote parts of the paper.

IV. Planned and performed all of the experiments and wrote most of the paper.

Papers not included in this thesis


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<tr>
<td>APCI</td>
<td>Atmospheric Pressure Chemical Ionisation</td>
</tr>
<tr>
<td>ASE</td>
<td>Accelerated Solvent Extraction</td>
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<tr>
<td>AuxE</td>
<td>Auxiliary Electrode</td>
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<tr>
<td>DPPH</td>
<td>1,1-diphenyl-2-picrylhydrazyl</td>
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<tr>
<td>EC50</td>
<td>Half Maximal Effective Concentration</td>
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<td>ESI</td>
<td>Electrospray Ionisation</td>
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<tr>
<td>ET</td>
<td>Electron Transfer</td>
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<tr>
<td>FCR</td>
<td>Folin-Ciocalteu reagent</td>
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<td>HAT</td>
<td>Hydrogen Atom Transfer</td>
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<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
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<td>LCA</td>
<td>Life Cycle Assessment</td>
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<td>MS</td>
<td>Mass Spectrometry</td>
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<td>Tandem Mass Spectrometry</td>
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<td>M&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Molecular Weight</td>
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<td>m/z</td>
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<td>PHWE</td>
<td>Pressurised Hot Water Extraction</td>
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<td>Pressurised Fluid Extraction</td>
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<td>PLE</td>
<td>Pressurised Liquid Extraction</td>
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<td>PSE</td>
<td>Pressurised Solvent Extraction</td>
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<td>RE</td>
<td>Reference Electrode</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>SFE</td>
<td>Supercritical Fluid Extraction</td>
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<td>Subcritical Water Extraction</td>
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<td>TEAC</td>
<td>Trolox Equivalent Antioxidant Capacity</td>
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<td>Q3G</td>
<td>Quercetin-3-glucoside</td>
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<td>Q4’G</td>
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<td>Q3,4’G</td>
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1 Introduction

The industrial revolution gave prosperity and drastically raised the standard of living for mankind. However, the price was unbelievably high; chemical pollution on a large scale that led to horrible effects for human health and environmental damage, for example, chronic diseases, DNA mutation and reduced immunity to infection and diseases. In addition, we have the extinction of plant species, fresh water pollution, acidification and excess nutrification of water bodies and emissions to the atmosphere that in turn led to the green house effect. The chemical damage is severe and the list is long. The environmental impact has to be minimised or avoided. From the analytical chemist’s point of view, this can be achieved by sustainable awareness and actions such as avoiding or minimising the use of toxic chemicals, employing catalysts, developing processes that better utilise biomass to obtain valuable products, developing new processes that require less energy, utilising less polluting solvents and developing improved methods for analysing of chemical pollutants. Sustainable development should be incorporated in every discipline, ranging from social science to natural science. One of many definitions of sustainable development is perhaps well known. However, it is worth emphasising one more time; “ability to make development sustainable - to ensure that it meets the needs of the present without compromising the ability of future generations to meet their needs”

This thesis addresses current developments in society, which are driving us towards a sustainable society with environmental awareness. The fossil fuels that our society depends on are limited resources. Biorefinery of biomass from agricultural and forestry industries is an alternative that can be used to obtain new fuels and other beneficial species for human health, as well as bulk chemicals for industries. Therefore, it is desirable to develop new environmentally friendly techniques to minimise and avoid the use of toxic chemicals and expensive raw materials.

The general definition of analytical chemistry is identification, characterisation, and quantification of different types of chemicals. It uses methods that involve chemistry, physico-chemistry, physics and biology or a combination of all these to produce signals that can be processed and interpreted to give qualitative, quantitative and/or structural information about a sample. In analytical chemistry, sample collection and sample pre-treatment are crucial steps that have to be performed and planned carefully
to achieve reliable values that reflect the whole sample and not only part of the sample, especially when it comes to quantitative determination. The analytical chemist’s work is to improve an already established method and/or to extend new samples to existing methods, as well as to develop new methods to quantify and identify chemical phenomena.

**Papers I and II** focus on developing extraction techniques that use environmentally sustainable solvents such as water, ethanol and supercritical carbon dioxide to extract valuable species from forestry biomass. **Paper III** aimed to develop a reliable and efficient online system to characterise and identify antioxidative species, since conventional methods of determining antioxidants lack efficiency and specificity. An online hyphenation system including electrochemical mass spectrometric detection was therefore developed and successfully employed to rapidly characterise and identify the antioxidants in the model sample, onion extract. **Paper IV** linked all the knowledge from **Papers I-III** together to investigate the degradation of antioxidants extracted at elevated extraction temperature that was encountered in both **Papers I and II**.

In summary, the work presented in this thesis shows the whole chain of the analytical process from sample collection to final analysis. In addition, sustainable development was integrated in the analytical process, where toxic and hazardous chemicals were replaced and biomass was used as raw material. The developed techniques proved to be efficient in refining biomass to give highly valuable species.
Our industrial civilisation is mainly based on using fossil fuels to supply energy and also to produce bulk chemicals. The fossil fuels are scarce and therefore lately an interest in the integration of sustainable awareness into industrial processes has gained ground, with the foremost focus on biorefinery. Biomass is produced in thousands of tonnes per annum from agricultural and forestry industries (Figure 1). The use of biomass as a raw material gives increased economic growth for industries without compromising the environment. These in combination also increased the socioeconomical value in terms of new R&D that leads to more employment and new products that are beneficial for human health. Furthermore, developing new technologies that are more sustainable and where hazardous chemicals and solvents are avoided results in less strain on the environment.

One way to increase the value of biomass is to retrieve valuable species but still not affect the suitability of the material as an energy source or raw material for other processes. For instance, Sweden is the world’s second largest producer of forest-processed products and hence also generates a vast amount of forestry biomass, which is often incinerated to produce energy. Therefore it is of interest to retrieve valuable species such as primary and secondary metabolites (e.g. terpenoids, stilbenoids and flavonoids) from biomass, in order to increase the value of the biomass.

Figure 1. Voluminous birch bark waste from the forestry industry.
Paper I that is presented in this thesis is a study of pressurised fluid extraction (PFE) of betulin and antioxidants from birch (Betula pendula) bark, a voluminous by-product from the paper pulp and forest industry. Birch bark is reported to contain efficient species against a wide range of diseases\textsuperscript{2-5}. Hence, the aim was to extract the “high-value” species from the bark. The definition of a “high-value” species in this thesis is a species that has increased the value of its raw material (for more detailed discussion see chapter 3). The extraction solvents used in the experiments were ethanol and water, of which water is recognised as the most environmentally friendly solvent. An assessment of different solvents that are commonly used in chemical industries demonstrated that simple alcohol solvents such as methanol and ethanol are environmentally preferable solvents to dioxane, acetonitrile, formaldehyde, etc. In addition, ethanol compared to methanol is a slightly more environmentally friendly solvent\textsuperscript{6}.

There are many tools that can assess the environmentally friendliness of a chemical such as life cycle assessment (LCA) and environmental, health and safety method (EHS)\textsuperscript{7, 8}. For instance, LCA can be applied to calculate the total environmental impact for the complete chain of ethanol production, ranging from the cultivating and harvesting of raw materials in ethanol production to the distribution of ethanol to the different industry sites and its final destruction\textsuperscript{9-11}. The total emissions of gases such as CO\textsubscript{2}, SO\textsubscript{2} and NO\textsubscript{x} are then determined, together with liquid and solid emissions, and compared with values obtained from methanol or acetonitrile production\textsuperscript{12-14}. Furthermore, it is important to consider the production of ethanol and compare the different methods. For instance, if the used ethanol is a by-product from an existing process then it is more environmentally sustainable than if farmlands are sacrificed to produce ethanol instead of grain.

Paper I is a demonstration of combining sound analytical chemistry with sustainable chemistry. After extraction, the birch bark can still be incinerated to produce energy or to be used as feedstock for animals. The developed methods, although on a laboratory scale, will hopefully inspire industries to employ or integrate them into their processes.

The forestry industry invests a great deal of money and effort into fending off unwanted herbivores from newly planted seedlings, using many methods, both mechanical and chemical. Despite this, none of the current methods are long-lasting and cost-effective. Extracting birch bark with ethanol as a solvent has proven to be effective in repelling herbivores\textsuperscript{15}. The secondary metabolites extracted from birch bark might repel herbivores. Since this is a mixture of different species, the animal might not adjust to liking it. This work is not included in this thesis, but it is an interesting application for birch bark, particularly as it uses biomass from the forestry industry to solve a current problem.

To conclude, biorefinery of biomass to obtain products other than energy is highly desirable. The extracted species are natural and hence reduce any
side effects that are commonly encountered in synthetic antioxidants (e.g. butylated hydroxyanisole and butylated hydroxytoluen)\textsuperscript{16, 17}. In the long term they might reduce diseases that are related to synthetic chemicals.
3 “High value” species

The definition of a “high-value” species in this thesis is a species that has added values compared to its raw material. To date, different types of “high-value” species have been extracted from plant-based material, such as quercetin from onion waste\textsuperscript{18}, anthocyanins from red cabbage/red onion\textsuperscript{19} and carotenoids from carrot waste\textsuperscript{20,21}. The retrievable species from forestry biomass are for example: lignins, tannins, carbohydrates, terpenoids, and stilbenoids. Common for many of these “high-value” species is that they can prevent or reduce a wide range of diseases, e.g. cancer and neurodegenerative diseases\textsuperscript{22-25}. This thesis predominantly focuses on the extraction and analysis of different bioactive species (e.g. terpenoids and stilbenoids) in the bark of birch and spruce (Papers I and II). In addition, in Paper III, four different bioactive species (flavonoids) were used as model solutes to develop a rapid online system, which included separation coupled to three different detectors to characterise and identify the bioactive species. A case study of quercetin and its glucosides in onion\textsuperscript{26} was also carried out (Paper III).

3.1 Terpenoids

A diverse range of chemicals is produced in the plant kingdom. The produced chemicals are classified into two categories: (i) primary metabolites and (ii) secondary metabolites. Primary metabolites are defined as those species that are common to all species, whereas secondary metabolites are in general known as “natural products”. The commonly encountered secondary metabolites are terpenoids, alkaloids, stilbenoids, flavonoids, shikimates and polyketides. The backbone of terpenoids is isoprene, which contains five carbon atoms (Figure 2) so therefore the number of carbon atoms in terpenoid is a multiple of five. The isoprene skeleton of terpenoids may be folded to form rings. Oxygen or other heteroatoms are easily introduced into terpenoids. The simplest terpenoid is monoterpane, which contains two isoprene units. Diterpene contains four isoprene units and triterpene contains six isoprene units. Terpenoids are ubiquitous in the plant kingdom and have an essential role for the plant survival, taking care of plant defences and communication\textsuperscript{27}. Intensive studies have proved that terpenoids have antifungal, antibiotic, antioxidant,
and allelopathic properties\textsuperscript{28, 29}. Tree bark is a rich source of terpenoids, but also small amounts of low molecular phenolic constituents are present. The polyphenolic species found in trees in general occur as glucosides, but this behaviour is rarely found in terpenoids. The terpenoid that was studied in \textbf{Paper I} included betulin.

![Figure 2. The isoprene skeleton.](image)

### 3.1.1 Betulin

The phytochemicals in tree bark have always been of interest for researchers. In 1788, Lowitz succeeded in isolating a species from birch bark. The isolated species was named betulin (1, lup-20(29)-ene-3, 28-diol), which is a triterpene that is mainly found in white-bark birches that belong to the family of \textit{Betulaceae}. Betulin is a molecule (Figure 3) that gives the white colour of the birch bark and it is abundant, approximately 30 weight % of the bark\textsuperscript{30}. It is important to keep in mind that betulin content varies in different birch species and also depends on the geographic location\textsuperscript{30, 31}. Betulin has been studied for many years and it is already recognized and used in the pharmaceutical and cosmetic industries, for instance in skincare and hair products. For a long time in the pharmaceutical industry, betulin was principally used for the synthesis of its more active derivative, betulinic acid, which is in a limited amount in plants\textsuperscript{32, 33}. Betulinic acid can be used for the treatment of certain types of cancer and HIV\textsuperscript{5, 34-36}. However, lately studies of betulin have proven that betulin also exhibits cell apoptosis\textsuperscript{3, 37, 38}. This finding increases the value of betulin remarkably, especially when it comes to in optimisation and developing new extraction techniques.

The most general approach to obtaining betulin is to extract it from birch bark using different extraction techniques and several companies have successfully achieved this. \textbf{Paper I} used pressurised fluid extraction (PFE), which is presented in more detail in section 4.5.3, which is an environmentally sound technique for betulin extraction. The highest betulin yield obtained with PFE was around 26 weight %, using ethanol as the extraction solvent at 120 °C, 50 bar and 15 minutes total extraction time. Betulin is renowned for having anti-inflammatory and anti-bacterial effects\textsuperscript{30, 39}, but it does not exhibit any antioxidant activity, according to the DPPH
(1,1-diphenyl-2-picrylhydrazyl radical) assay (results not published). This finding is in good agreement with the chemical structure of betulin, which consists of five rings that are not conjugated and with predominantly methyl groups attached. Betulin is therefore not an electron-rich molecule that can easily react with other molecules.

Figure 3. The molecular structure of betulin in birch bark, $M_w = 443$ g/mol.

3.2 Stilbenoids

Other interesting phytochemicals that also belong to the family of secondary metabolites are stilbenoids. The backbone of stilbenoids is a 1,2-diphenylethylene unit, which is closely related to phenylpropanoids. Hence stilbenoids exhibit similar characteristics as phenylpropanoids, for example, antimicrobial properties$^{40, 41}$. Stilbenoids are effective against pathogens and herbivores and therefore classified as phytoalexins$^{42}$. The most general and well-known stilbene is resveratrol, which is commonly found in grapes. Many stilbenes are derived from trans-resveratrol (Figure 4), for instance piceid, astringin and rhapontin. In Paper II, these compounds were extracted with PFE using water and ethanol as solvent, and identified with tandem mass spectrometry. It was also demonstrated that using DPPH (1,1-diphenyl-2-picrylhydrazyl radical) assay piceid, isorhaponitn and astringin exhibited antioxidant properties.
3.2.1 Resveratrol

The IUPAC nomenclature of resveratrol is 3,4′,5-trihydroxystilbene and it can be found in red wines, since red grapes contain a high amount of resveratrol. Hence, intensive studies have been conducted on the health benefits of resveratrol, especially to explain the French paradox, low mortality caused by coronary heart disease despite an unhealthy way of living with a high consumption of cigarettes and dietary saturated fat. The regular consumption of red wine by the French might save them from cardiovascular diseases, because resveratrol and other polyphenolic species are found in red wines. Resveratrol exists in two forms, cis and trans configurations (Figure 4). Recently, resveratrol has been found to inhibit the proliferation of various cancer forms: breast, prostate, stomach, colon and pancreatic cancer\textsuperscript{43, 44}. However, the obtained results were obtained from animal tests, hence little is known about the effects of resveratol in humans. Glucosidation of resveratrol is also common and produces piceid, which is resveratrol-3-O-beta-glucoside. Piceid also exhibits the similar property as its sibling and is found in many tree species. Beside glucosidation, it is also common with methylation of stilbenoids. It is however, still under speculation as to whether the methoxy group increases or decreases the antioxidant activity of the species.

In Paper II, different extraction techniques to extract antioxidative species from spruce bark were compared. PLE using water and ethanol as solvents proved to be the best technique. The optimal PLE condition using water was 15 min, 160°C and 50 bar. The extraction temperature for PLE using ethanol was higher, 180°C, but the extraction time and applied pressure were the same. Apart from the antioxidants, three stilbene glucosides: piceid, isorhapontin, and astringin were also extracted with PLE using water and ethanol, respectively. The identity of the extracted stilbene glucosides: piceid, isorhapontin, and astringin were confirmed with nuclear magnetic resonance spectrometry (NMR) and tandem mass spectrometry.
(MS/MS). The backbone of these stilbenoids is resveratrol, and hence they all exhibit antioxidant activity. Moreover, terpenoids were also observed in the obtained MS/MS spectra.

3.3 Flavonoids

Flavonoids are polyphenolic species, and they are commonly found in the plant kingdom e.g. in flowers, trees, fruits and vegetables. These species provide defence against herbivores and microbial attacks from fungi and parasites, hence they are essential for plant survival. It has been demonstrated that a human diet containing flavonoids can affect long term health and reduce the risk of chronic and degenerative diseases. Products such as olives, vegetable oils, citrus and other fruit juices, chocolate, tea, coffee and wine are all plant-derived, and so we are encouraged to include these food-stuffs in our daily diet. Interest in flavonoids has increased markedly the last decade and as a result more than thousands of naturally occurring flavonoids have been reported. The significant feature of these species is that they all include a ring structure, designated A, B and C, with one or more hydroxyl groups attached, as well as other functional groups (Figure 5). The generic structure of flavonoids is shown in figure 5. Flavonoids make up one big family and due to structure variations, it can be further subdivided into four smaller families: flavonol, flavon, flavanol, and isoflavone (Figure 6).
Figure 6. The molecular structure of the four subfamilies of flavonoids: (A) flavonols, (B) flavone, (C) flavanol, and (D) iso-flavone.

The radical scavenging activity, or as it is more commonly known, the antioxidant activity of the different flavonoids, depends on the structural arrangement and the number and positions of the hydroxyl groups. Studies have shown that with hydroxyl groups in the orto-3', 4'-position (orto-dihydroxy structure) in the B-ring gives flavonoids with high antioxidant activity. Furthermore, the arrangement of the orto-dihydroxy structure in the B-ring, the 2,3-double bond in combination with both the 4-keto group and the 3-hydroxyl group in the C-ring, enhances electron-delocalisation and therefore gives species with high antioxidant activity (see Figure 5). For instance, the high level of quercetin that is found in the peel of yellow onion exhibits high antioxidant activity (Figure 7). Glucosidation is also commonly found in flavonoids, and it affects their antioxidant property as well as their water-soluble property. It has been proved that flavonoids with one or more glucosides exhibit low antioxidant activity, whereas their water solubility increases. Glucose is the most common sugar molecule that binds to the aromatic ring system, although other sugar molecules such as rutinose, galactose, xylose and rhamnose are also seen.\textsuperscript{50, 51} Flavonoids without the sugar moiety are called flavonoid aglycones. Flavonoids often encountered in the Swedish diet are, for example, quercetin and kaempferol in onion, catechin in apple and tea, resveratrol in wine.\textsuperscript{52-54} These species were identified and characterised in \textbf{Paper III}, using a recently developed method, online liquid
chromatography coupled to electrochemical and electrospray ionisation tandem mass spectrometric detection, LC-DAD-ECD-MS/MS.

Figure 7. The molecular structure of the aglycone quercertin, M<sub>w</sub> = 302 g/mol.

3.4 Value and function of antioxidants

The living standard of mankind has increased since the last century and as a result, the average lifetime has also increased. Hence, age-related diseases, e.g. Alzheimer’s, Parkinson’s and Amyotrophic lateral sclerosis, are more commonly encountered. The cause of these diseases is still unclear, but studies have shown that an intake of antioxidants might reduce the risk of developing these neurodegenerative diseases. In-vitro studies of antioxidants are also reported as preventing cancer and other oxidative stress-related diseases. Apart from this, antioxidants are already added in the production of various types of skincare and age-preventive products in the cosmetic industry. In the food industry, antioxidants are used as functional food or as additives to prolong the shelf-life of food. In summary, the functional and economic value of antioxidants is high. Antioxidants are used in other industrial products such as polymers and papers to prevent unwanted oxidative reactions. Furthermore, antioxidants extracted from natural materials are in many cases preferable to chemically synthesised ones (e.g. butylated hydroxyanisole), which can give various side effects and form unwanted isomers. It is in our interest to study (identify and characterise) the antioxidants that are extracted from natural materials.
3.5 Antioxidant reactions

"Antioxidants are defined as any substance that, when present at low concentration compared with that of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate" - Halliwell and Gutteridge, 1990

It is important to understand antioxidant reactions in order to apply the right analytical chemistry method to determine and evaluate antioxidant activity and capacity. Antioxidant activity of an antioxidant is defined as its strength to reduce an oxidation process. A strong antioxidant has high antioxidant activity towards oxidation processes. The antioxidant capacity of an antioxidant refers to how much of the antioxidant it is needed to reduce an oxidation process. It is important to distinguish between antioxidant activity and capacity when dealing with antioxidants, since many papers mix these two concepts. Before discussing the different antioxidant reactions, it is essential to understand how oxidation processes can occur. Oxidation is the most fundamental chemical reaction and occurs continuously around us and within us, caused predominately by oxygen in the air and sunlight. Oxygen is a stable molecule in its ground state, with two unpaired electrons with parallel spins. It becomes reactive when excited, when the unpaired electrons have opposite spins. The reactive oxygen reacts easily with other species to form a superoxide anion radical (Equation 1), which in turn transforms to hydrogen peroxide and oxygen in aqueous solution (Equation 2).

$$O_2 + e^- \rightarrow O_2^- \cdot \quad \text{(Eq. 1)}$$

$$O_2^- \cdot + O_2^- \cdot + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(Eq. 2)}$$

The generated hydrogen peroxide is not a strong oxidant compared to its sibling, the hydroxyl radical (OH\(^-\)), which is formed through oxidation by oxygen in the air and in vivo homolytical cleavage in the presence of a metal catalyst e.g. the Fenton reaction (Equation 3), where the iron ion shifts its oxidation state between 2+ and 3+.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH \cdot \quad \text{(Eq. 3)}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2 \cdot + H^+$$

The hydroxyl radical and the superoxide anion radical are strong oxidants that are generally categorised as reactive oxygen species (ROS). These species are responsible for degenerative diseases in humans and rancidity and deterioration in food. The function of polyphenolic antioxidants is to
Antioxidants can have an effect on oxidation processes in several ways:  

1. Radical scavenging – stabilising the ROS via either hydrogen atom transfer or electron donation.  
2. Prevention of transition metal formation – avoidance of the Fenton reaction that generally generates ROS.  
3. Additive effect – interaction with other antioxidants, which leads to a cooperative antioxidant effect toward oxidants.

Antioxidants are often generalised into two categories: (i) primary chain-breaking antioxidants and (ii) secondary or preventive antioxidants. Chain-breaking antioxidants compete with superoxide anion to prevent the formation of ROS, and thereby to break the oxidation chain. The removal of available radicals slows down the oxidation process until it finally stops. Quinones and various types of vitamins (e.g. vitamin K and C) are classified as chain breaking antioxidants. The preventive antioxidants prevent or inhibit oxidation processes in such a way that no ROS or other free radicals are generated (e.g. inhibition of Fenton reaction). Thus, these antioxidants are generally iron-sequesterants. One important aspect worth of note is that antioxidants’ behaviour in vitro and in vivo differs greatly. The antioxidant activity (easy to oxidise) and capacity (amount) in vivo are more complex than in vitro. Several factors play a major role, e.g. antioxidant concentration, localisation, the presence of an antioxidant-derived radical and synergistic effects of different antioxidants. These factors complicate the evaluation, thus when comparing results obtained from in vitro and in vivo studies, care has to be taken to avoid misinterpretation.

### 3.5.1 Are antioxidants dangerous for human health?

We often read about the good effects of antioxidants in our daily magazines and newspaper. General opinion encourages us to include food that contains high number of antioxidants in our daily diet to prevent or avert degenerative diseases. However, new reports have demonstrated that an over intake of antioxidants fails to protect against diseases and could instead have an opposite effect. For example, quercetin given to early-stage diabetes mellitus rats accelerated the development of kidney cancer, if not inducing the disease. Another study on α-tocopherol, commonly known as Vitamin E, showed effects against atherosclerosis and neurodegeneration in mice. Thus, the overall health benefits of antioxidants are still unclear and uncertain.

Antioxidants can give pro-oxidant effects, which means that antioxidants can stimulate oxidative damage through creating reactive oxygen species.
For example, oxidative DNA damage was studied in human volunteers; they were given mixtures of ascorbate, β-carotene and α-tocopherol. However, the obtained data varied greatly and did not provide conclusive evidence as to whether the studied antioxidants were pro-oxidants.

There are also suggestions that antioxidants have a protective effect before absorption, for example, within the stomach, intestines and colon. The gastrointestinal tract is continuously exposed to reactive species, which are present in our daily diet, e.g. food and beverages. This gives rise to speculation that flavonoid-rich food might protect against gastric and colonic cancer.

In summary, antioxidants proved have an antioxidant effect \textit{in vitro} in many test systems. Nevertheless, antioxidants can also act as pro-oxidants in other tests. How antioxidants behave \textit{in vivo} has still not been fully clarified. There is still a great deal to explore about the action of antioxidants and whether they are harmful or beneficial for us.
4 Sample preparation

Many steps are involved in an analysis and sample preparation is frequently neglected. It is necessary to understand the different steps involved in sample preparation in order to minimise or avoid bias and the propagation of error. The sample preparation processes, which are included in this thesis, are sample collection, sample pre-treatment, and extraction. Each of these processes is discussed below to explain their importance and how they can affect the results.

4.1 Sample collection

The sampling procedure is decisive and affects the outcome of the whole analysis to a great extent. Most often, energy and effort are put into the analysis step and the sampling procedure is neglected. Ignorance of the importance of the sampling procedure often leads to incorrect results that do not reflect true values or, in the worse case, leads to complete misinterpretation. The significant parameters that affect the outcome are the representative sample and the sampling technique. It is important to clearly define these before performing the sampling procedure. The complexity of the solute location and distribution in the matrix are also significant. Most samples are heterogeneous and constitute a challenge compared to homogenous samples. Therefore it is important to plan and perform sampling in such a way that the collected sample is representative for the sample under study\textsuperscript{83}. In addition, a number of replicates of the sample are necessary to ensure reliable statistical values.
One practical aspect that is easy to neglect is storage, which is also included in the sampling procedure. Plant materials usually have a high water content. For this reason, prior to storing, plant materials are often air-dried in a fume cupboard to remove the water content. An alternative way of removing water content in plant materials is freeze-drying. Samples should be stored in a -20°C freezer directly after collection to minimise solute degradation, induced either by oxygen or light. Sometimes temperature of -20°C is not enough to prevent degradation, hence a -80°C freezer is used instead and it is often used for biological samples such as blood plasma, body fluids or different types of proteins.

Polyphenolic antioxidants from natural plants are distributed differently (e.g. leaf, bark and root) within the plant. This thesis predominately focuses on the qualification and quantification of antioxidants in tree barks. The geographic location, season of the year, the technique used to harvest the bark and the definition of bark (e.g. the thickness of bark) are important parameters that should be considered before analysis. The definition of bark, defined in the Encyclopaedia Britannica is; “tissues external to the vascular cambium (the growth layer of the vascular cylinder)”85. The more popular definition of bark is the outside covering of stems and roots. Bark is divided into two categories; the outer layer and the inner layer. The outer layer is dead tissue consisting of cork and cork cambium. The inner layer of bark consists of living phloem. The different parts of bark are clearly described in Figure 9.

![Figure 9. Simplified schematic picture of the different layers of bark](image)

In Paper I, birch bark was studied and differentiated before analysis. The distinctive appearance of birch bark is interesting; with a white and light
brown outer part and a reddish brown inner part (Figure 10). Polyphenolic antioxidants with high antioxidant activity and capacity are in general colourful e.g. anthocyanins in blueberries and other colourful berries, fruits and vegetables. Thus, the highest antioxidant activity and capacity should presumably be obtained from the inner part of the bark. In order to verify this assumption, the sampling procedure of bark was carefully performed when it came to separation of outer and inner bark into two separate containers before conducting the PFE experiments. The obtained result proved to be contrary to the initial assumption, with the white outer bark exhibiting the highest total antioxidant capacity (Table 1). A lower EC50 value implies a higher antioxidant capacity, as further explained below (5.1). In order to correctly evaluate the obtained antioxidant capacity of bark, a homogenous sampling of bark is necessary.

![Image of white outer bark and darker inner bark of birch](image)

Figure 10. The white outer bark (A) and the darker inner bark of birch (B).

4.2 Sample pre-treatment

The sample pre-treatment involves a variety of mechanical and chemical techniques such as grinding to reduce the particle size, mixing with sand or hydro-matrix to get rid of the water content in the sample, hydrolysing the sample or adding chemicals that can enhance the disruption between the species and the sample matrix. Common for all is to improve the extraction efficiency and selectivity.

4.2.1 Particle size

It is important to determine the particle size of the sample, since it significantly affects the extraction. Studies have shown that reducing the particle size of the sample results in significantly improved extraction efficiency. This observable fact is mentioned in Paper I (Table 1), where different sizes of bark (big bits, long strips and finely ground
particles) were extracted with PFE. The results favoured extracts with small particle size origin. Small particle samples have a larger total surface area than bigger particle samples, which affects the contact area between solute and solvent. A large contact area gives short diffusion distances, resulting in increased mass transfer rate of solute in solvent.

High extraction efficiency and recovery is obtained with samples of small particle size. The most common process used to achieve small particle size is either homogenisation with a mixing device or mechanical grinding with a mortar. Apart from the particle size, the mechanical process also helps the loosely bound solute to be disrupted from the sample matrix more easily. Homogenisation or grinding has to be performed carefully, since loss of volatile solutes or enhanced degradation of thermally-labile and light sensitive solutes might occur.91

Table 1. Effect of sample particle size on antioxidant capacity found in extracts from inner and outer layers of birch bark. Ethanol was the extraction solvent and the extraction conditions were 130°C, 50 bar and an extraction time of 3×5 min.

<table>
<thead>
<tr>
<th>Experiment # (n=3)</th>
<th>Birch layer type</th>
<th>Particle size (mm)</th>
<th>EC50 value (μg bark/μg DPPH)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Inner</td>
<td>10 × 10</td>
<td>57</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Inner</td>
<td>Finely ground</td>
<td>37</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Outer</td>
<td>110 × 10</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>Outer</td>
<td>Finely ground</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

n = number of replicates, RSD = relative standard deviation

4.3 Extraction

4.3.1 Fundamental concepts of extraction

In biological samples, the solutes are commonly found inside the cell; hence the cell membrane has to be disrupted before extraction of interesting solutes. Disruption of the cell membrane is in general by chemical treatments or mechanical forces (e.g. centrifugation). Temperature and pressure can also facilitate disruption of cell membranes. This process does not describe the extraction procedure fully, therefore a household waste
particle is used here to give a full picture of the different steps included in extraction.

Solute incorporation in the matrix in various ways, and this must be taken into consideration when sample pre-treatment and extraction are performed. Solutes that are easily extracted from the matrix are in general loosely bound to the matrix surface, or loosely integrated in the pores of the matrix (Figure 11). The biggest challenge in extraction concerns solutes that are strongly incorporated and/or chemically attached in the deepest pores. Another challenge is to avoid co-extraction of unwanted species.

Figure 11. A schematic picture of solutes differently bound/incorporated in a matrix: (1) adsorbed on the surface of the matrix, (2) dissolved in the pore solvent and/or adsorbed on the pore surface, (3) dissolved in micro/nano pores or adsorbed on the walls of the micro/nano pores, (4) chemically bonded to the matrix, and (5) dissolved in the bulk solution.

The extraction process used to a solute involves many steps. The first step is diffusion of the solvent into the matrix, in other words wetting the matrix properly. The second step is important and sometimes also decisive for the whole extraction. It includes desorption of the solute from the matrix. The third and fourth step is solvation of the solute into the extraction solvent followed by diffusion out from the matrix. The last step also involves diffusion, taking the solute through the Nernst layer and into the bulk solvent.

Mass transfer is defined as the net movement of mass from one location to another. Consequently, the mass transfer of the solute out to the extraction solvent depends on several parameters, such as how well the solute is desorbed from the matrix and the diffusion rate of the solute. It is important to understand these parameters in order to estimate both the extraction efficiency and time for any extraction processes.
Interaction between the solute and the matrix must be broken before solvation of the solute into the extraction solvent can occur. There are various procedures that can be utilised for the disruption of chemical and physical interactions, (i) selection of an appropriate solvent according to the solute property, (ii) addition of energy in the form of increasing the temperature, (iii) mechanical disruption such as stirring and ultra-sonication, and (iv) the use of pressure to force the solvent into the pores.

An optimal extraction should be gentle, use small amounts of solvent, be rapid, selective and give a high yield in a short time. However, no such universal extraction technique is available, thus compromises are unfortunately necessary.

4.3.2 Extraction solvent

The choice of an extraction solvent is a critical factor in the extraction process. It has to have the right chemical properties, such as polarity and pH, to break the intra- and intermolecular forces between the solute and matrix. A good solvent should wet the matrix properly, be selective for the target solute and also be appropriate for the final analysis or product. A general rule of thumb says that if the solute and solvent have the same polarity, then the solute will be dissolved in the solvent. Nevertheless, it is a general assumption and hence has to be applied critically. As mentioned (see 4.3.1) the solutes are incorporated differently in the matrix. Solutes that are loosely bound to the sample matrix are usually surrounded by some kind of solvent molecules (i.e. water molecules). In this case the partition coefficient of the extraction solvent has to be considered in order to achieve selective and efficient extraction. Sometimes a small amount of water in the extraction solvent enhances the extraction efficiency drastically. The water in the extraction solvent interacts with the water molecules around the solute and so allows extraction of the solute out to the extraction solvent.

The most common extraction solvents for polyphenolic species are acetone, diethyl ether and methanol. These solvents are usually encountered in the extraction of plant-based materials. With today’s environmental awareness in mind, these above mentioned solvents have to be replaced by more environmentally friendly ones such as pure water or supercritical carbon dioxide (SC-CO\textsubscript{2}). Papers I and II optimised pressurised fluid extraction (PFE) of polyphenolic species with more environmentally friendly solvents, and the obtained recoveries were satisfactory.

The general approach in extraction is to use a mono-solvent, but at times it is not efficient enough. Then it is more appropriate to use a two-component solvent. Yilmaz et al. and Pinelo et al. suggested a combination of water with an organic solvent for the extraction of phenolic species from plants, instead of using a mono-component solvent. The two-component solvent gave increased efficiency for the studied species. Karacabey et al
studied resveratrol and other phenolic species from milled grape canes with a different ethanol and water mixture. Their study reported maximum solubility using ethanol concentrations of between 50-70%\textsuperscript{99}. \textbf{Paper I} did not involve a two-component system because of the objective - to investigate the solvent selectivity for antioxidants. In addition, the advantage of using a mono-solvent system is to obtain a pure extract, and the used extraction solvent could be recycled without further treatment (i.e. separation of two different solvents).

4.3.3 Temperature

The extraction temperature is clearly one of the most crucial parameters to be determined in an extraction method. It is well-known that a high extraction temperature increases extraction efficiency, rate and recovery significantly\textsuperscript{100}. The solute and matrix interaction as well as the extraction solvent’s properties are affected by high extraction temperature.

At elevated temperature, the solute solubility is enhanced markedly, since the thermal kinetic energy is raised. The applied energy facilitates the rupture of the intra- and intermolecular forces, such as van der Waal’s forces, and the hydrogen bonding and dipole attraction that exist between the solute and the matrix.

High temperature affects the physical properties of the solvents in terms of decreased viscosity and surface tension, allowing better contact and penetration of the solvent into the sample matrix\textsuperscript{101, 102}. These properties in turn improve the diffusion coefficient of the solvent and thereby enhance the extraction efficiency. Furthermore, the “wetting” of the matrix is also improved by the low viscosity and surface tension of the solvent.

In summary, elevated temperature improves the extraction efficiency in terms of better mass transfer of solute out to solvent. However, extraction at elevated temperature also has its disadvantages. The probability of solute degradation increases with increased extraction temperature and the physical property of the matrix is also affected by temperature. The matrix might alter in such a way that unpredicted reactions occur.

It is important to pay attention to the issue of degradation and it is further discussed below (4.4), in which a set of experiments was conducted in order to investigate degradation of antioxidants in birch bark.

4.3.4 Solubility

The definition of solubility found in IUPAC is; “The analytical composition of a saturated solution, expressed in terms of the proportion of a designated solute in a designated solvent, is the solubility of that solute”. The more simple description of solubility is how much of a solute can be
dissolved in a solvent. There are three intermolecular forces to be accounted for in the dissolving process. The forces are: (i) solute-solute interaction, (ii) solvent-solvent interaction, and (iii) solute-solvent interaction. The intermolecular attraction of solute-solute, as well as of solvent-solvent has to be broken before forming new intermolecular attraction between solute-solvent. The breaking of old bonds and forming of new bonds occurs simultaneously. The solute is solubilised in a solvent if the solute-solvent attractions are greater than the combined solute-solute and solvent-solvent interaction forces. In other words, Gibbs’ free energy of the systems has to be negative to favour the solubility of a solute in a solvent\(^9\). However, there are factors that affect solubility. High temperature tends to increase solubility, since energy is applied to break the intermolecular forces. The polarity of the solute and the solvent is significant. In general, polar solutes are readily solubilised in polar solvents. The size of the solute molecule is also decisive for its solubility, as large molecules are more difficult to solubilise than small molecules. In order to dissolve a solute molecule, the solvent molecules have to surround the solute, and the ease with which they surround a solute decreases with increased molecule size.

Many methods can be used to predict and determine the solubility of a solute in a specific solvent. These methods are more or less based on internal pressure and cohesive energy density\(^1\). The most widely known methods are: Hildebrand’s, Burell’s, Scatchard’s, and Hansen’s solubility parameters. Below, the Hildebrand and Hansen’s solubility parameters are discussed in more detail.

### 4.3.4.1 Solubility parameter

The solubility of a solute in a solvent is roughly a combination of the solvent and the temperature. This means a less suitable solvent can be more appropriate at higher temperature. There are many theories for the prediction of solvent or solute solubility and two of the most well-known were posed by J. Hildebrand\(^1\) and C. Hansen\(^1\). In 1936 Hildebrand produced an equation to predict the solubility of a solute in a solvent or how to overcome intra- and intermolecular forces to separate the molecules. The intra- and intermolecular forces within and around a molecule are van der Waals forces, hydrogen bonding, dipole-dipole, dipole-induced dipole and dispersive bonding. Hildebrand’s theory involves the calculation of the cohesive energy density of the solvent, that is the energy required to break intra- and intermolecular forces. Hence, the Hildebrand solubility parameter is based on the cohesive energy density of the solvent, which in turn is derived from the heat of vaporization (Equation 4).
δ is ascribed as the solubility parameter, which is the same as the square root of the cohesive energy density of a solvent. ΔH is the heat vaporization in J/mol, R is the gas constant in J/K mol, T is the temperature in Kelvin and \( V_m \) is the molar volume of the solute.

Thirty years after Hildebrand, Hansen derived his theory, which is an extension of Hildebrand’s. The Hansen’s solubility parameter (Equation 5) gives a better prediction for polar and hydrogen systems compared to the former, which is better for estimating a non-polar system. Hansen also made use of the cohesive energy density of the solvent and modified it into three components: (i) polar, (ii) dispersion and (iii) hydrogen bonding.

\[
\delta^2 = \delta_p^2 + \delta_d^2 + \delta_h^2 \quad (\text{Eq. 5})
\]

Where \( \delta_p \), \( \delta_d \) and \( \delta_h \) are the polar, dispersion and hydrogen bond component, respectively, \( \delta \) the solubility parameter that in turn is the square root of the cohesive density of the solvent.

In Paper I, water was used to extract betulin, which is a non-polar molecule and therefore immiscible with water at ambient temperature. However, the polarity of water decreases with increased temperature (see 4.5.3) and for that reason water was investigated as an alternative extraction solvent than the more commonly used organic solvents. Despite the high temperature tested (180 °C); no detectable amount of betulin was seen. In order to investigate this further, the solubility parameter for betulin, water and ethanol were calculated using a modified Hansen’s solubility parameter\(^{103,108-110}\).

Figure 12 shows the modified solubility parameters as a function of temperature at 50 bar. For two species to be completely miscible with each other, the differences of their curves should be less than four units apart\(^110\). The obtained diagram indicated no mutual miscibility between betulin and water at any of the temperatures below 250 °C. In the case of betulin and ethanol, the diagram shows mutual miscibility starting at room temperature until around 220 °C. In conclusion, ethanol is a suitable extraction solvent for betulin up to 220 °C, although caution has to be taken when too high temperature is used, since the betulin might decompose.
4.3.5 Extraction time

General extraction technique is exhaustive extraction, which means that the recovery is favoured by long extraction time. Therefore, extraction time is possibly the second crucial parameter to be optimised after the temperature. Extractions are either performed in a static or a dynamic manner, and the extraction yield as a function of time looks different for each. In static mode, the extraction recovery increases linearly with time in the beginning until it reaches a plateau, which indicates that the partition equilibrium of the solutes in the solvent has been reached. The reached plateau may also reflect that all of the solutes have been extracted out to the solvent, at least those solutes extractable under set conditions. Dynamic extraction (flow-mode) on the other hand never reaches equilibrium, due to the continuous flow. In this case, the accumulated solute concentration increases linearly with time in the beginning until all easily available solutes have been extracted (i.e. solubility controlled), then the more tightly bound solutes are slowly extracted in a non-linear fashion, (i.e. diffusion controlled extraction)\textsuperscript{111}.

When sensitive solutes are extracted out into the solvent, they start to degrade without the protection of the matrix. Therefore, to avoid this short extraction time is preferred for sensitive solutes. Direct cooling of the obtained extract possibly averts or at least retards degradation. Petersson et al.\textsuperscript{112} using PHWE found that the kinetic model for extraction of anthocyanins from red onion was of the first order reaction, which means
that the extraction recovery is linear with time. In their study, they also concluded that degradation of anthocyanins occurred simultaneously with the extraction process. This complex issue of degradation counteracting extraction is dealt with in section 4.4. In summary, for degradable solutes it is advantageous to use dynamic extraction, since it prevents further reaction with other accumulated extractives, and simplified collection and storage of the solute directly after extraction.

In Paper I we optimized the extraction time of betulin using a multivariate response design, starting from 5 minutes to 10 min and 15 min. For birch bark extracts using ethanol as solvent (Table 2), it was clearly shown that the extraction time was not significant at any of the temperatures tested (80, 130 and 180 °C); which was confirmed by statistical analysis. Water as extraction solvent was not able to extract betulin even at the highest tested temperature, which is discussed above in section 4.3.4.1. In summary, the obtained result from the multivariate analysis showed that the extraction time using ethanol as a solvent was not significant at 95% confidence level, with the p-value larger than 0.05.

Table 2. Effect of temperature and extraction time using pressurized hot ethanol for betulin extraction.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Coefficient</th>
<th>t-test</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>7.378</td>
<td>6.531</td>
<td>0.001</td>
</tr>
<tr>
<td>C</td>
<td>2.555</td>
<td>2.261</td>
<td>n.s</td>
</tr>
<tr>
<td>T x C</td>
<td>-5.802</td>
<td>-3.424</td>
<td>0.014</td>
</tr>
<tr>
<td>C x C</td>
<td>0.283</td>
<td>0.167</td>
<td>n.s</td>
</tr>
<tr>
<td>T x C</td>
<td>-1.925</td>
<td>-1.391</td>
<td>n.s</td>
</tr>
</tbody>
</table>

SD = 2.767, R-Square adjusted (%) = 83.9
n.s. = not significant, T = temperature, C = extraction time
4.4 Degradation

The universe is heading towards chaos (disorder), or slightly exaggerated in chemical expression; the second law of thermodynamic rules the universe. The law simply states that everything around us decays with time.

Degradation of species can occur easily, either from the influence of surroundings or because the species itself is not stable. Light, pH and oxygen are often the causes of degradation. As a result, species are stored in dark, cold places (e.g. a freezer). Oxygen transforms easily to the more reactive superoxide anion radical and hydroxyl radical (see 3.5). The formed radicals react further with the solute of interest and form new species. Subsequently, oxygen and acid free surroundings are necessary to prevent an uncontrolled reaction.\(^{113,114}\)

Before extraction, the matrix protects a solute, but after extraction, the solute is floating freely in the solvent. Without the matrix as its protector, the solute might not be stable. Furthermore, the chance for the solute to react with other extractives in the solvent is higher, and it eventually leads to solute degradation.

Extraction at elevated temperature gives high recovery in a short time. However, the possibility of degradation also increases, since most solute cannot sustain too high temperatures. Most often the extraction process and degradation coexist and counteract each other during the extraction. It is therefore crucial to separate these processes from each other in order to evaluate their individual contribution.

The obtained results in both Paper I and Paper II were similar; high antioxidant capacity was obtained in extracts that were extracted at the highest temperature tested, \(180^\circ C\). Nevertheless, at a high temperature some degradation\(^{112,115}\) of the solute would occur, particularly when using water as a solvent, as it can easily cause hydrolysis\(^{116,117}\). Degradation might, however, increase antioxidant concentration or create antioxidants that exhibit higher antioxidant activity, for instance by deglycosylation. Another explanation can be the formation of Maillard reactive products\(^{118}\). As a result, studies on the degradation of antioxidants at high temperature were started. In Paper III, an online hyphenated system was developed in order to rapidly screen and characterise, as well as identify, species with antioxidative properties. The developed technique was applied in Paper IV to study possible degradation at elevated temperatures. The results showed no degradation occurrence at \(80^\circ C\). However, at \(180^\circ C\), degradation of some antioxidants did occur. Degradation of antioxidants was observed in results obtained from three different detectors: a diode-array, an electrochemical, and a mass spectrometric detector. Details of the different detectors are dealt with in chapter 6. At high temperatures, epimerisation of compounds was seen. Catechin, which is commonly found in trees, conformed to its diastereroidomers at high temperatures. Catechin has two chiral centers that
give four different diastereoisomers: (+)-catechin, (-)-catechin, (-)-epicatechin, and (+)-epicatechin (Figure 13).

![Figure 13. The four diastereoisomers of cathechin found in birch bark: (A) (+)-catechin, (B) (-)-catechin, (C) (-)-epicatechin, and (D) (+)-epicatechin.](image)

4.5 Extraction techniques

The crude samples/sample particles prior to the analyses are often different in shape and size. Choosing an appropriate extraction technique is difficult, since the assortment is multitude. The final analysis has to be considered prior to selecting a sample preparation technique to minimize contamination, bias, and/or losses of solute. The common aim for all sample preparation techniques is to extract the solute from its matrix as efficiently as possible and to avoid co-extraction of other unwanted species. As mentioned, there are a large variety of extraction techniques, most of which are exhaustive. However, this thesis mainly focuses on the different techniques that were applied in the different papers.

4.5.1 Solid-liquid extraction (SLE)

Solid-liquid extraction is by far the most commonly used extraction technique for solid samples. Plant materials are in general extracted with organic solvents such as methanol, propanol and ethanol\(^{119}\), of which the
latter is often more preferable than methanol, which is dietary toxic. The simplest way to perform SLE is to immerse the crude sample in a solvent for a certain length of time at room temperature (leeching). The method is similar to the tea-making procedure; the teabag is lowered into a cup of warm water and after a certain time, the tea aroma is extracted out to the solvent. Advantages of the technique are many: it is easy to perform, there is no need to use sophisticated instruments, there is a minor risk of degradation if low extraction temperatures are used, and it is applicable for almost all solid samples. The disadvantages of the method are lengthy extraction time, and the utilization of large amounts of organic solvents that leads to dilution of the sample.

SLE was employed in Paper II to extract antioxidants from spruce bark. Two solvents were used, water and ethanol, and the extractions were kept overnight at room temperature. Comparing the different extracts gave a slightly higher solid yield in the water extract. Extraction with PFE and supercritical fluid extraction (SFE) were also carried out and compared with SLE. The outcome demonstrated that SLE at room temperature overnight was not as efficient as PFE using the same solvent at 80, 130 and 180 °C.

4.5.2 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) is a high diffusion fluid extraction technique. In SFE, the solvent temperature is above its critical temperature and pressure. Above the critical point, the fluid does not exhibit any physicochemical properties similar to gas or liquid, instead it is a mixture of both (Table 3).

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Density (g/cm³)</th>
<th>Viscosity (g/cm s)</th>
<th>Diffusion coefficient (cm²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas</td>
<td>(0.6-2)10⁻³</td>
<td>(1-3)10⁻⁵</td>
<td>0.1-1.0</td>
</tr>
<tr>
<td>Supercritical fluid</td>
<td>0.2-0.9</td>
<td>(1-3)10⁻⁴</td>
<td>(0.1-5)10⁻³</td>
</tr>
<tr>
<td>Liquid</td>
<td>0.6-1.6</td>
<td>(0.2-3)10⁻³</td>
<td>(0.2-3)10⁻⁵</td>
</tr>
</tbody>
</table>

A supercritical fluid has zero surface tension and it is easy to control the solvent strength by varying the temperature and pressure. One important aspect of SFE is that the species must be soluble in the supercritical fluid.
Otherwise, the extraction cannot occur. The solubility of the solute increases by merely changing the temperature, pressure and flow rate, since the viscosity and the surface tension of the solvent are lowered when the temperature and pressure are raised. The extraction yield can be improved by varying the flow rate of the supercritical fluid if the sample is solubility-controlled, which was discussed in detail above (4.3.5). However, if the sample is more desorption-controlled, then a few percent of an organic modifier can be added to help break the chemical bonds between solutes and the matrix. Apart from extraction, supercritical fluid is also used in chromatographic separation of non-polar species, in reactions and for cleaning, as well as for particle formation.

Supercritical CO₂ (SC-CO₂) is the most common fluid used in SFE, mainly due to its low critical temperature, 31 °C, and pressure, 73.9 bar. It is also rather inexpensive compared to other supercritical fluids and it gives solvent free products. Furthermore, SC-CO₂ is gentle when thermally labile species are extracted. These beneficial features of SC-CO₂ can be used to carry out particle formation in combination with extraction. SC-CO₂ is easily recycled back into the system, which makes it an environmentally friendly solvent.

Only non-polar solutes can be extracted by SC-CO₂ due to its non-polar feature. Nevertheless, the polarity of the fluid can be improved by adding small amount of modifier (co-solvent). The added modifier also helps the species to be desorbed from the matrix. However, some loss in extraction selectivity occurs because of the added co-solvent. The obtained extract is not solvent free; hence separation of the extraction solvent is needed to obtain a pure solute.

Another technique using carbon dioxide worth mentioning is liquid CO₂. The conditions for liquid CO₂ are a temperature of around 15-20°C and pressures of 50-100 bar. The features of liquid CO₂ are similar to SC-CO₂, which means that it is suitable for extracting non-polar species. The physical parameters of liquid CO₂ such as viscosity, surface tension and diffusion coefficient are larger than for supercritical CO₂. Despite this, it is still efficient as an extraction solvent. The pressure and temperature of liquid CO₂ are less demanding than in the supercritical state, so the equipment costs are lowered significantly. Besides extraction, liquid CO₂ is used today in the dry cleaning industry.

In Paper II, spruce bark was extracted by using pure SC-CO₂ and SC-CO₂ together with ethanol as co-solvent. Extraction with ethanol improved the antioxidant recovery significantly compared to using pure CO₂. The reason behind this is that the added ethanol improved the polarity of SC-CO₂. In spite of this, SC-CO₂ together with a co-solvent was not as effective as PFE using pure water for extracting antioxidants from spruce bark. This finding indicates that the extractable antioxidants in spruce bark are polar.
species. It would be interesting to try pure liquid \( \text{CO}_2 \) or a mixture of liquid \( \text{CO}_2 \) and water to extract spruce bark.

4.5.3 Pressurized fluid extraction (PFE)

PFE was introduced in 1995 and it is a solid-liquid extraction technique compatible with a wide range of different solvents. The main feature of PFE is extraction at elevated temperatures and applied pressure. The pressure used in PFE is to maintain the solvent in a liquid state. The applied pressure also helps to force the solvent into the matrix pores and thus enhances the extraction efficiency and also helps to solubilise the air bubbles in the samples. Extraction at elevated temperatures has many advantages, as discussed above (4.3.3). Studies have demonstrated that the efficiency of PFE is comparable with conventional techniques (e.g. SLE and Soxhlet)\(^{129-131}\). Furthermore, it is easy to conduct method development, since the important factors that affect the solute recovery are predominately temperature and the type of solvent used.

The tuning of temperature in PFE affects the chemical/physical properties of the solvent, for example, the dielectric constant, also called the relative permittivity (\( \varepsilon \)), which is defined as the ability of a material to resist the formation of an electric field within. The dielectric constant varies with temperature in such a way that it decreases with increasing temperature\(^{102,132,133}\). The solvent polarity is tightly correlated with the dielectric constant, i.e. a polar solvent becomes less polar at higher temperatures since the dielectric constant of the solvent decreases. Consequently, the extraction selectivity can also be tuned by controlling the temperature.
Figure 14. The dielectric constant of pure water at different temperatures, ranging from 25 - 250 °C, at 50 bar. The dielectric constant of methanol/water and acetonitrile/water mixtures at 25 °C and ambient pressure are also demonstrated in the diagram\textsuperscript{102}.

The dielectric constant change for water is more significant than for other solvents. PFE using water as extraction solvent is often entitled as subcritical water extraction\textsuperscript{134} (SWE) or pressurised hot water extraction (PHWE). Polyphenolic species with antioxidant properties from natural plants of various types such as rosemary\textsuperscript{135}, medicinal plants\textsuperscript{136, 137}, fruits, etc. have been successfully extracted with PHWE.
5 Separation

In general, a sample mixture contains several different solutes and it is necessary to employ a particular kind of separation technique in order to get quantitative and qualitative information of the individual solute. Many separation techniques are available on the market and the choice of an appropriate separation technique depends on the property of the solute and the stated objective. If the solute is in a gas phase then gas chromatography (GC) is more appropriate, and if the solute is charged and non-volatile then capillary electrophoresis (CE) is better. The most common separation technique for liquid samples consisting of uncharged solute is high performance liquid chromatography (HPLC), also known as high-pressure liquid chromatography (HPLC). The majority of HPLC used today is reversed phase liquid chromatography (RPLC) with a non-polar stationary phase and an aqueous-organic solvent mixture as the mobile phase. The opposite is normal phase liquid chromatography (NPLC) that uses a polar stationary phase and a non-polar mobile phase. However, the hazardous organic solvent in NPLC has led to the reduced practice of the technique. Another disadvantage of NPLC is the poor reproducibility of retention times, since water or other protic organic solvents change the stationary phase.

5.1 High-performance liquid chromatography (HPLC)

The fundamental principle of HPLC separation is based on solute interactions (i.e. distribution) with both the stationary and the mobile phase. The stationary phase is commonly packed in a stainless steel column, and the particles are mainly micro porous spherically modified with functional groups. In principal, the functionality of the side-chain is selected to suit the solutes. The mobile phase in HPLC is a liquid that competes with the stationary phase over the solute. The commonly used mobile phases are purified water mixed with organic modifiers; e.g. acetonitrile and methanol that are used in HPLC to elute the solute in the stationary phase. For instance, an increased quantity of the organic modifier in the mobile phase leads to faster elution of the solutes. Sometimes, a buffer with determined pH is used as the mobile phase to protonate or deprotonate the solutes in order to obtain good, effective separation. Additionally, an ion-pairing agent is sometimes an option for achieving sufficient separation, despite changed
buffer pH and the quantity of organic modifier. Ion-pairing agents are ionic compounds, which contain a hydrocarbon chain that imparts a certain hydrophobicity that can be retained on a reversed-phase column. Ion-pairing agents are commonly added to positively charged solute.

The most general stationary phase employed in RPLC is octadecylsilane (ODS)\textsuperscript{138}, and columns containing this packing are called C18-columns. These columns are highly lipophilic, with particles ranging between 2 and 10 µm in diameter with a pore size of a few Ångstrom, 100-300 Å\textsuperscript{138}.

Factors that influence an optimised separation are the number of theoretical plates (N), the separation factor (α) and the retention factor (k). Below is the equation (Equation 6) of the resolution between two neighbouring peaks; it is derived with the assumption that the average of the two peaks is identical to the peak width of the second peak. This equation is well used to determine the efficiency of the separation. The resolution between two neighbouring peaks has to be larger than 1.5 in order to separate the peaks.

\[
R_s = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_2}{1 + k_2}
\]  
\text{(Eq. 6)}

Where \( R_s \) is the resolution, \( N \) is the number of theoretical plates, \( \alpha \) is the separation factor and \( k_2 \) is the retention factor of the second peak.

It is important to consider the size and uniformity of the particles packed in the analytical column when selecting a column for separation. Small particle size gives rise to a large surface area and high loading capacity, which in turn gives better separation. Too small particle size generates high back-pressure, which in turn limits the flow rate. The correlation between particle size, flow rate, resistance to mass transfer, longitudinal and eddy diffusion are well described by the famous van Deemter curve\textsuperscript{138}.

Column technology has improved during the last decade, resulting in a large assortment of columns\textsuperscript{139, 140}. New columns have improved reproducibility, selectivity, and mechanical strength. In addition, new columns give faster mass transfer kinetics, better temperature endurance and they can be used over a broad range of pH.

In \textbf{Paper I}, it took a long time to finally achieve sufficient separation of birch bark extract. Birch bark extract was extracted with water as the solvent; hence the extracted solutes were polar in their characteristic. Many of the solutes were co-eluted in the beginning with a C18 column with a particle size of 3.5 µm in diameter. A C18 column interacts predominantly with hydrophobic interactions and the analysed solutes were rather hydrophilic. For this reason, co-elution of the solutes and poor resolution were observed. The solutes from the birch extract were assumed to be
mainly polyphenolic species, with aromatic ring structure, which has hydrophilic properties. A column with these properties was desired. Separation was then tried with a C6-phenyl column with slightly smaller particle size, 3 µm. The new column had Λ-Λ interactions, due to the phenyl groups, which might have interacted with polyphenolic species\textsuperscript{141-143}. The results obtained with the C6-phenyl column were much improved so the column was used in \textbf{Paper IV}.
6 Detection

The market is overloaded with varieties of detection apparatus that has moderate sensitivity to ultra high sensitivity with limits of detection down to ppb level. There is no universal detector, thus the choice of detector depends on the solute and the stated objectives, e.g., detection that is quantitative and/or qualitative, and if information about electro-activity, bioactivity and chemical structures is of interest. If the solute is in low concentration and demands certain sensitivity, then an ultra-violet detector (UV) is appropriate for the task, presupposing that the solute can absorb the UV-light. UV is sensitive to certain solutes, has good linearity and is reliable, which makes it the most common detector in HPLC-systems. There are two types of UV-detector: one with one fixed wavelength, and one with variable wavelengths. The latter is called a diode array detector (DAD). Instead of one wavelength, DAD can monitor many wavelengths in a fast scan, which makes it more versatile when monitoring several species that have different absorbance profiles. Bio-active species are not always UV-active, thus fluorescence or chemiluminescence detectors can be more suitable for detection. An electrochemical detector (ECD) is both sensitive and versatile and can detect all species that undergo redox reactions, hence the use of ECD has increased markedly in various fields. If the objective is to reveal the molecular structure of a solute, the detectors suitable for this purpose are mainly the mass spectrometric detector (MS) and the nuclear magnetic resonance detector (NMR). In addition, titration of the solute, to monitor the colour changes, is another detection technique, which can easily quantify the character and concentration of a solute. DPPH assay is a titration technique, which was used to detect the antioxidants in Papers I-IV. Below, details of the said technique are discussed.

6.1 The DPPH assay

Antioxidants in natural plants have been thoroughly discussed (see 3.5) and many methods can be used to determine their ability to quench oxidation reactions. The antioxidant activity and capacity of a solute is mainly determined by chemical decolouration assays such as the DPPH assay (1,1-diphenyl-2-picrylhydrazyl radical), the FCR assay (Folin-Ciocalteu reagent) and the TEAC assay (Trolox equivalent antioxidant capacity). One major
drawback of the chemical decolouration assays is that it is hard to compare them to each other, due to their different reaction mechanisms: (i) the hydrogen atom transfer (HAT) reaction, and (ii) the single electron transfer (ET) reaction.

The DPPH assay is by far the most recognized assay used to determine the total antioxidant activity and capacity of polyphenolic antioxidants. The total antioxidant capacity of a sample is here expressed as an EC\textsubscript{50} value, and a low EC\textsubscript{50} value implies high antioxidant capacity. The EC\textsubscript{50} value is defined as half maximal effective concentration and represents the concentration of a compound where 50% of its maximal effect is observed. DPPH is commercially available as an organic nitrogen radical that has its maximum absorption at 515 nm. The DPPH assay is similar to a titration method, where the changed colour of a sample is measured. Unreacted DPPH dissolved in methanol gives a dark purple solution that is reactive to any radical scavengers (e.g. antioxidants). Reacted DPPH solution turns to yellow. The colour change of the DPPH solution indicates that the DPPH molecule gained a hydrogen atom from a scavenger (Figure 15).

\begin{center}
\includegraphics[width=0.5\textwidth]{figure15.png}
\end{center}

Figure 15. Reaction of DPPH with AH (antioxidant) that results in transferring of a hydrogen atom from AH to DPPH.

Various antioxidants react immediately with DPPH, others need a few minutes and there are those that need hours. This difference in reaction times is due to the antioxidant activity and capacity of the solute, as well as the sterical hindrance of the solute. The sterical hindrance of the solute affects the reaction kinetic, and is important to take it into consideration. For example, small molecules have better access and react more easily with DPPH molecules than with big molecules. In addition, it is important to determine the reaction time and the concentration of the solute when using the DPPH assay. If the molecular structure of the antioxidant is known, one can always roughly predict the stoichiometric ratio of the DPPH molecule and the antioxidant. Then the antioxidant concentration can be approximately calculated to suit the used DPPH assay. In Papers I and II the identity of the antioxidants was unknown and therefore it was not
possible to establish stoichiometric assumption. In order to find out the appropriate extract concentration for the used DPPH assay, the trial and error method was applied. In Papers I and II the DPPH reaction was maintained for 4 hours to assure that the reaction was completed. The obtained absorbance reading values for the samples were constant after 4 hours’ reaction with DPPH. This indicates that the reaction is completed with no more antioxidants to react with DPPH.

The DPPH is also oxygen and light sensitive, so the solvent should be properly degassed prior to use, to remove oxygen, and then stored in a dark place to avoid degradation.

In summary, it is fairly tedious to perform chemical assays for antioxidant activity and capacity determination including DPPH and they have many disadvantages. The chemical decolouration assays can only determine the total antioxidant activity and capacity of a sample mixture and not the individual species within it.

However, if DPPH is used online after an analytical column, the antioxidant activity of the different species within a mixture could be qualitatively determined. Despite this, online-DPPH assay is still not able to quantitatively determine the antioxidants. Electrochemical detection in combination with separation can overcome this problem. Therefore, an online system was developed in Paper III to rapidly screen antioxidants and determine both their antioxidant capacity and the antioxidant activity of a specific solute.

6.2 Electrochemical detection

Electrochemistry is everywhere and within our body. The electrochemical processes in our body are occurring at this moment without us noticing them. The electro-chemical process is mainly a process where electron transfer occurs from one species to another. Electron transfer occurs mainly during two reactions; oxidation and reduction. Oxidation is defined as a process where the species loses one or several electrons and reduction is the opposite process, gaining one or several electrons. This electron transfer process is called the redox reaction. The chemical reaction cannot occur without a complementary electron donator or acceptor. A system that consists of an electron donator, electron acceptor, electrolyte and a salt bridge is called an electrochemical cell. There are two types of electrochemical cells, galvanic cells and electrolytic cells.
6.3 Electrolytic cells

Electrolytic cells consist generally of three electrodes, which are in contact with each other through the electrolyte. Each electrode has a function and is made from different material. The first interesting electrode is the working electrode (WE) where the electrochemical reaction occurs. The second necessary electrode is the reference electrode (RE). The most used and common reference electrode is the Ag/AgCl electrode. In some cases the Ag/AgCl reference electrode is replaced by a quasi electrode (QRE), which is not a true reference electrode. Instead, it is a pseudo-reference electrode and has to be calibrated against a true reference electrode (e.g. Ag/AgCl electrode) to obtain the actual potential of QRE. The function of a reference electrode is principally to measure the potential exhibited from the working electrode against a reference potential. The third electrode in an electrolytic cell is the auxiliary electrode (AuxE), commonly called a counter electrode. AuxE current supports the WE and ensures there is no current through the RE.

Both oxidation and reduction reactions occur in electrolytic cells and they each correspond to a half-cell reaction. The sum of two half-cell reactions is equal to the complete electrochemical reaction, and the Nernst equation describes this relationship well. However, care has to be taken, given that it only works for reversible reactions and for dilute solutions.

\[
E_{eq} = E^{o}_{ox/\text{red}} + \frac{RT}{nF} \times \left[ \frac{[\text{ox}]}{[\text{red}]} \right]
\]  

(Eq. 7)

Where \( E_{eq} \) is the potential when the chemical reaction is in equilibrium, \( E^{o}_{ox/\text{red}} \) is the standard potential vs. a standard hydrogen electrode (SHE), R is the gas constant, T is the temperature in Kelvin, n is the number of electrons transferred, F is Faradays constant, and \([\text{ox}]\) and \([\text{red}]\) are the concentrations of the involved species.

6.3.1 Amperometric flow cells

An amperometric thin-layer flow cell was used in Paper III to study antioxidants and their by-products. Flow cells are convenient when it comes to high sample throughput and have been established as robust and versatile detection techniques in many fields. The surface area of the WE is often large and the conversion efficiency of the cell can be designed with regard to the purpose. The conversion efficiency of a flow cell can be adjusted by the surface area of the WE and the flow rate. Large surface area and low flow rate give high conversion efficiency. The principle of an amperometric flow cell is simple; the current is measured while a constant potential is
applied. The observed current is proportional to the concentration of the electrolysed species. A potentiostat was used in the experiments in Papers III and IV to ensure constant potential over the flow cell.

Figure 16 shows a picture of the thin-layer amperometric flow cell that was coupled to the online system studied in Papers III and IV. The flow cell used contained a WE, which was a PEEK block with a dual working electrode system. The stainless steel block that confined both the spacer gasket and WE block worked as AuxE. The spacer gasket was made of thin Teflon™ and it defined the volume of the flow channel. The reference electrode used was a QRE and it was put in a small opening on top of the stainless steel block. The species that were separated in the LC column passed through the flow cell and were oxidised by the applied potential set by the potentiostat. It is important to float the whole system to avoid destroying the potentiostat when it is connected to the mass spectrometer. The reason behind this is that the high voltage in the interface of a mass spectrometer causes back-currents and electrical shocks if an insulation transformer is not used.

6.3.2 Antioxidant activity and capacity determination with amperometric detection

Antioxidants are electro-active species and therefore can be readily oxidised or reduced in the amperometric thin-layer flow cell. The potential applied over the flow cell can be used to study the activity and capacity of the antioxidants. The strength of the solute’s antioxidant activity can be estimated by the redox potential. For instance, an active or strong antioxidant requires low potential to be oxidised and the opposite is true for a less active or weak antioxidant. In other words, a solute with low electro-activity shows
reduced antioxidant activity. The electro-activity of a solute is also commonly associated with its half potential (E$_{1/2}$), which is obtained by plotting the current as a function of the applied potential. Low E$_{1/2}$ indicates a strong antioxidant (high activity) and high E$_{1/2}$ indicates a weak antioxidant. In Paper III, cyclic voltammetry and amperometric detection were used to determine the antioxidant activity of equal amounts of four flavonoids (i.e. quercetin, kaempferol, catechin and resveratrol). The E$_{1/2}$ from cyclic voltammetry and amperometric detection was determined for each of the model solutes. Quercetin had the lowest E$_{1/2}$ value, which made it the most active solute (easy to oxidise) followed by catechin, kaempferol and resveratrol. The findings could also be revealed via the molecular structure of quercetin, which contains a larger number of hydroxyl groups than the other three solutes (see Figure 19). In addition, the position of the hydroxyl groups in quercetin support electron delocalisation (see 3.3). The obtained E$_{1/2}$ value for the four model solutes was in good agreement with the result from the DPPH assay. Nevertheless, if the solute contains several functional groups e.g. hydroxyl groups or any electron-rich group, then it is rather difficult to estimate the E$_{1/2}$ because of the overlapping effect caused by the functional groups. Furthermore, the oxidation potential is pH-dependent; some solutes oxidise more readily at high pH and some at low pH$^{161}$. Therefore, it is important to clarify the pH of the solvent used when comparing methods or potentials.

The antioxidant capacity is defined as the amount of solute that exhibits electro-activity. Therefore, a high current corresponds to high antioxidant capacity. However, the reality is not always that simple. For instance, in Paper III, the same concentration of quercetin (Q) and its glucosides i.e. quercetin-3-glucoside (Q3G), quercetin-4’-glucoside (Q4’G) and quercetin-3,4’-diglucoside (Q3,4’G) were studied at different redox potentials and the obtained results were correlated with results from the DPPH assay. At potential +0.4 V vs. Ag/AgCl, Q exhibited the largest current, which indicated that Q had the highest antioxidant activity. The order of the antioxidants’ activity was Q > Q3G > Q3,4’G > Q4’G. The findings correlated well with results obtained from the DPPH assay. Furthermore, the finding also demonstrated that the redox potential of DPPH is around +0.4 V vs. Ag/AgCl. However, at a potential above 1.6 V, the Q3,4’G gave the largest current. The observation suggested that the oxidation current of Q3,4’G increased significantly at high enough potential. Q3,4’G consisted of larger number of oxidisable hydroxyl groups than Q, hence higher potential was needed to oxidise the remaining hydroxyl groups in Q3,4’G. In summary, the antioxidant capacity of polyphenolic species is particularly dependent on the redox potential. This example clearly demonstrated that it is difficult to correlate a chemical assay with electro-chemical oxidation, since reaction kinetics and mechanisms differ noticeably, as well as the time-
scale, which was longer with DPPH compared to the LC-DAD-EC-MS/MS system.

It was also demonstrated that at high enough potential, the antioxidants were oxidised and formed several oxidation products. This was observed in the mass spectra; at high potential decreased ion intensity in the solute and increased ion intensity in the different oxidation products were formed. The capacity (amount of antioxidants) of the solute can be determined by its intensity in the mass spectrum at certain potential.

Figure 17. Molecular structure of quercetin and its glucosides: (A) quercetin, (B) quercetin-3-glucoside, (C) quercetin-4′-glucoside and (D) quercetin-3,4′-diglucoside.

Sample mixtures contain a wide range of solutes. It is therefore important to choose a suitable potential to suit the solute and study objectives. If the objective is to evaluate the individual antioxidant’s activity in a sample mixture and not its oxidation product, then too high potential should be avoided, to minimize the formation of oxidization products. The examples
above clearly showed the critical aspect of the redox potential, which determines the selectivity of the electrochemical detection.

**Paper IV** used the same online system as in **Paper III** to investigate degradation of antioxidants. The redox potential used in the experiments was +0.4 V vs. Ag/AgCl to mimic the DPPH assay. Four different samples of birch bark extract were studied: 80°C extract (E80), 180°C extract (E180), 80°C extract with 80°C hydrothermal treatment (P80), and 180°C extract with 180°C hydrothermal treatment (P180). DAD chromatograms were overlaid with ECD chromatograms to visualise the individual species’ antioxidant properties (Figure 19).

ECD chromatograms of the four different samples were compared in order to verify degradation of antioxidants at high extraction temperature. The ECD of E80 and P80 were identical, while abundant peaks were observed in both E180 and P180. The observation suggested increased recovery of antioxidants at 180°C. Meticulous investigation of the obtained ECD chromatograms demonstrated that although degradation occurred at 180°C, the total antioxidant activity of E180 and P180 was significantly higher than E80 and P80. This can be explained by the fact that degradation at high temperatures creates new species with antioxidant properties.

![Figure 18. Overlaid chromatograms of P180. The DAD was monitored at 280 nm and the redox potential in ECD was +0.4 V vs. Ag/AgCl.](image)

### 6.4 Mass spectrometry

Mass spectrometry is a necessity when it comes to structural elucidation and it is used in almost every analytical laboratory. Since its introduction[^162],
it has been widely used in a wide range of areas, for instance, in forensic analysis\textsuperscript{163}, pharmaceutical analysis\textsuperscript{164, 165}, chemical analysis, environmental analysis\textsuperscript{166} and geological analysis. Mass spectrometry is a powerful tool that is used to quantify, qualify and identify both known and unknown solutes. The structural elucidation of solutes in mass spectrometry is mainly the separation of the ions by mass-to-charge ratio ($m/z$). The ions are then detected in proportion to their abundance. The mass spectrometer is made up of several parts: an ion source for the formation of ions, ion optics and mass analyser to focus the ion beam and then separate the ions by their $m/z$, and a detector that converts the beam of ions to electrical signals\textsuperscript{162}. Commonly, the samples analyzed by the mass spectrometer are often in a liquid state and as the MS separates/detects gas phase ions, the solvent has to be evaporated and the species of interest has to be ionised before it enters the mass analyser. The ionisation step is the key in mass spectrometric detection; it allows the entrance of solutes to the mass analyser. Ionisation suppression is a common issue and should be avoided or minimised to achieve good sensitivity. Ion suppression can be avoided by minimising the number of co-eluting species that are introduced into the ion source by the use of efficient LC separation. The use of high salt content in the mobile phase should be avoided\textsuperscript{167}. Good fragmentation in mass spectrometry can be obtained by choosing a suitable ion source and by the optimisation of acquisition and instrumental parameters.

6.4.1 Ion sources

Many types of ion source are available and they are mainly divided into two groups: (i) hard ionisation, like electron ionisation, which is very energetic and causes extensive up-front fragmentation and (ii) soft ionisation e.g. electrospray (ESI) and atmospheric pressure chemical ionisation (APCI), ionisation sources with gentle fragmentation\textsuperscript{162}. The selected ionisation technique should be suited to the solute and the used solvent. Soft ionisation sources such as ESI and APCI operate at atmospheric pressure and they are appropriate for medium to polar species (see Figure 19). One of the aims of this work was to study the extracted polyphenolic species from biomass. Too extensive fragmentation in MS is not appropriate for identification. For this reason ESI and APCI were used to obtain useful information and both ionisation techniques are suitable for average polar and very polar solutes.
Figure 19. Different ion sources and their different areas of solute polarity and molecular mass.

6.4.1.1 Electrospray ionisation (ESI)

ESI was used in Papers II and III. It ionises the liquid sample and then transfers into the gas phase mainly by dispersion of the solvent droplets that in turn produce single or multiple charged ions. To achieve this, an electric field (± 2-5 kV) is created between the outlet of the spray capillary and the entrance of the mass spectrometer. In positive ESI, a positive potential is applied to the capillary outlet and the created cations then migrate towards the counter electrode. A Taylor cone is then formed because of the charge distribution effect, with positive ion accumulation at the liquid surface. The liquid jet has an access of positive ions that are held together by the surface tension of the liquid. When the surface tension of the liquid is broken by the electric field, small charged droplets are formed that result in a spray of charged ions toward the entrance of the ion source. During their path towards the counter electrode, the solvent evaporation of the charged droplets continues, which eventually leads a reduction of droplet size and an increase in the charge to ratio volume. Negative ESI is achieved in the same way, with the exception of negative potential applied to the capillary outlet. The theory behind the phenomena of ESI is still unclear, even though two theories have been proposed: (i) the charge residue model (CRM) and (ii) the ion evaporation model (IEM).

6.4.1.2 Atmospheric pressure chemical ionisation (APCI)

APCI as an ionisation technique is regarded as slightly harsher than ESI. The principal concept of APCI differs distinctively from ESI. The ionisation in APCI is principally based on a gas-phase ion molecule reaction in atmospheric pressure. The technique is applicable to medium polar and polar, as well as ionic species. In general APCI gives mono-charged ions that often simplify data interpretation.
The liquid sample is introduced to the APCI interface either by HPLC or by direct infusion, with a flow rate between 0.2-2 ml/min. The liquid sample has to be in the gas-phase before reaching the APCI interface. This is achieved by the pneumatic nebuliser, where the nitrogen gas converts the liquid stream into a thin fog, followed by desolvation/vaporation of the liquid in a heated quartz tube. The temperature of the quartz tube is fairly high to effectively vaporise the thin fog sample into the gas-phase. After vaporisation, the hot gas reaches the corona discharger where ionisation takes place. The corona discharger produces primary ions (e.g. N$_2^+$ and O$_2^+$) that collide with solvent molecules to form secondary reactant ions. These secondary reactant ions interact with solute molecules and solute ions are formed. These produced gas ions enter the skimmer towards the mass analyser. APCI is also able to run in positive and negative ion mode. In positive ion mode, the solute ion becomes a cation, through proton transfer. The contrary happens in negative ion mode, with an abstraction of protons that transforms the solute ion into an anion. In summary, APCI occurs in atmospheric pressure and thus gives high collision frequency, which together with the rapid desolvation/vaporisation step gives gentle fragmentation of solutes.

6.4.2 Mass analyser

Ion optics transmits the incoming ions from the ion source to the mass analyser, where they are separated according to their mass-to-charge ratio before they reach the detector$^{162}$. Several features of the mass analyser should be considered when choosing the appropriate mass analyser: (i) the upper mass limit that determines the highest m/z that can be measured, (ii) the transmission, which is the ratio between the number of ions produced and those reaching the detector, (iii) the resolution, which is the ability to distinctively separate two ions with small mass difference, (iv) the accuracy of m/z, and (v) data acquisition speed. The variety of mass analysers is as broad, the most common ones being the quadrupole mass analyser, the ion-trap mass analyser, the Fourier-transform ion cyclotron mass analyser, and the time of flight mass analyser.

6.4.2.1 Quadrupole mass analyser

A Quadrupole mass analyser$^{171}$ acts as a mass filter and it has four parallel rods, either with cylindrical or hyperbolic cross-sections (Figure 20). The rods are either positively charged or negatively charged and together they produce a three-dimensional time-varying oscillating electric field. A factor that affects the trajectory of the incoming ion in the oscillating electric field is the mass-to-charge ratio of the ion. The ratio of the radio frequency to direct current voltage (RF-to-dc voltage) in the analyser constantly varies in such a way that the incoming ions travel differently in the electric field$^{172}$. 
Ions with selected $m/z$ travel through the electric field and arrive at the detector, while other ions collide on the rods and become neutral. Quadrupoles can be added in series with each other in such way that the first quadrupole (Q1) and the third quadrupole (Q3) serve as a mass analyser while the second (Q2) serves as a collision cell. This type of system is generally named tandem mass spectrometry.

![Figure 20. A quadrupole analyser consists of four cylindrical rods, with the ion in the oscillating electric field](image)

In Papers II-IV, a triple quadrupole mass analyser (API 3200™ LC/MS/MS System) with information dependent acquisition (IDA) was used to analyze the antioxidants. The advantage of using IDA is that it can perform many different scan modes such as the precursor scan or the neutral loss scan. The precursor scan or parent ion scan as it is also known, can choose the product ion (daughter ion) and by that determine the precursor ion. The precursor scan is very useful when the $m/z$ of the daughter ion is known, since it can reduce the background and unnecessary/unwanted $m/z$. The neutral loss scan differs from the precursor scan in such a way that both mass-resolving quadrupoles are scanned with a constant mass difference (neutral fragment). Another common scan mode is the selected ion mode scan (SIM) where the selected ion is monitored. SIM was used to monitor quercetin, kaempferol, catechin and resveratrol in Paper III. It was observed that the intensity of the tested solutes decreased with increasing redox potential and new products were formed due to oxidation. The SIM mode is very useful when the identity of the investigated solute is known or the $m/z$ is known. However, it is useless for unknown solutes.

A handful of species in birch bark have been identified but most of the different species are still unknown. The challenge of using MS to elucidate these unknown species is enormous but at the same time intriguing. As mentioned above, many parameters have to be optimised in order to obtain
informative data in MS. Optimisation of acquisition is one major parameter for gathering useful data. Informative data acquisition (IDA) was used in Papers II and IV to allow a wide array of m/z. IDA was performed by first acquiring a mass spectrum of between 150-1500. The two most intense ions were then selected with the first quadrupole and allowed to fragment in the collision cell. The fragments were then separated with the last quadrupole to generate an MS/MS spectrum. The data collected from IDA is huge and the interpretation of them is tedious and time-consuming. Despite this, IDA is the best method for acquiring vast amounts of m/z information of the unknown solutes. Interpretation of the obtained MS data has to be meticulously performed, especially in verifying m/z with its chromatographic profile. In Paper II, three stilbene glucosides were identified using tandem mass spectrometry with ESI in positive mode. From the MS/MS spectrum, fragmentation of glucosides was observed for all three stilbenes: piceid, isorhapontin, and piceid. The MS spectrum for the said stilbenes shows a loss of 162 u, which corresponds to one glucoside moiety after losing a water molecule of 18 u. For instance, piceid has one glucoside attached to it and the loss of one sugar moiety gives its aglycone, resveratrol (Figure 21A), a distinctive signal at m/z 229.2. The same was also demonstrated with astringin (Figure 21B), which loses two glucose moieties to give a signal at m/z 245.2. NMR supported the obtained MS results.
6.4.3 The hyphenation of LC-DAD, EC and MS for studying of antioxidants

Chemical assays only give information about the total antioxidant activity or capacity of a sample mixture and not the individual species within it. Several groups have studied online chemical assays coupled to LC and the steady-state issue is still a recurring problem. It is a major problem when a sample mixture contains both strong and weak antioxidants. By combining electrochemistry with LC-DAD and MS, in this case an amperometric flow cell, the separated species from LC are oxidised immediately in the flow cell in which activity and capacity can be determined without worrying about the time-scale (Figure 22).
By correlating the retention time in DAD and both ECD and MS, the identity of the solute can be revealed, together with its electro-activity. LC-DAD-EC-MS/MS enables rapid screening for a sample mixture containing antioxidative species, instead of having to carry out tedious work of first obtaining the solute by preparative LC and then using chemical assays. This method is time-efficient, solvent-sparing and also avoids environmentally hazardous chemicals.

A biological sample, onion extract, was studied with this recently developed method in Paper III. Figure 23A shows the ECD chromatogram of onion extract at +1.4 V vs. Ag/AgCl. The four major species in onion, Q, Q3G, Q3,4’G and Q4’G were detected in ECD and UV/Vis and their retention times were also correlated to facilitate further identification with MS/MS. MS/MS elucidation was performed on Q and its glucosides, giving a loss of 162 u after losing a water molecule (-18 u) in all glucosides. These findings corresponded well since the neutral sugar molecule glucose is 180 u. For instance, peak no. 1 corresponds to Q3,4’G, and its MS/MS spectrum is displayed in figure 23B.
Figure 23. LC-DAD-ECD-MS/MS of yellow onion extract, (A) ECD chromatogram, at potential of +1.4 V vs. Ag/AgCl and gradient elution at a flow rate of 200 µl/min was used. (1) = Q3,4′G, 3.3 min, (2)= Q3G, 5.2 min, (3)= Q4′G, 6.5 min and (4)= Q, 8.8 min. (B) MS/MS spectrum of peak no. 1, which corresponds to Q3,4′G, with m/z 625.2[^78].

The main species, Q and its glucosides, were easily identified in onion extract using MS/MS. However, the identity of the oxidation products formed at applied potential is not completely clear, due to the poor MS/MS data obtained. In order to identify the oxidation species, the different parameters in mass spectrometer e.g. collision energy, gas temperature, and other acquisition parameters need to be optimized.

**Paper IV** also used the same online system to study degradation of antioxidants extracted at elevated temperatures using a high-pressure reactor. Three detectors were used, each with different selectivity. DAD is selective towards a solute with chromophore features, hence suitable for detecting polyphenolic species. ECD is selective towards electro-active species and is
hence able to determine which of the polyphenolic species observed in DAD has antioxidant properties. The final detector in the online system is MS/MS, which has high sensitivity and selectivity to a solute that is ionisable. Some solutes are difficult to ionise and need chemical derivatisation prior to introducing them to mass spectrometry. Nevertheless, the degraded species in birch bark were observed in DAD and they also produced electro-activity in ECD. Furthermore, epimerisation of compounds also occurred at a high temperature (180°C). The molecular elucidation determined them to be diastereoisomers of catechin. These findings were supported by DAD spectrum and from the literature\textsuperscript{179, 180}. Figure 24 shows the MS/MS spectrum of catechin, with a high signal at $m/z$ 291 (e.g. mother ion), and loss of a water molecule, which gives a signal at $m/z$ 273.1. A prominent signal at $m/z$ 139.1 was also observed, due to loss of 152 u from $m/z$ 291.2. Catechin consists of an aromatic ring system with A, B and C rings (see 3.3 and 4.4). The signal of $m/z$ 139.1 corresponds to the A-ring after rearrangement and cleavages in the C-ring. Figure 25 describes the fragmentation pathway of catechin. With an oxygen atom in C-1 position in the C-ring, catechin tends to cleave to that position after rearrangement of hydrogen atoms to give $m/z$ 139.1. The reaction is named the retro-Diels Alder reaction (RDA), since it is a reversible reaction of the original Diels-Alder reaction, where conjugated diene and a substituted alkene form a substitute cyclohexene system. Signals at $m/z$ 165.1 and 123.1 were also observed, which corresponded well with the fragmentation pathway of catechin. Online separation coupled to electrochemical and mass spectrometric detection proved very powerful tools for characterising and elucidating the molecular entity of antioxidative species.
Figure 24. MS/MS spectrum of catechin, where loss of 18 u, 152 u and 169 u were observed.

Figure 25. The fragmentation pathway of catechin.
The current atmosphere in society is awareness of the environment, since chemical large-scale pollution has disastrous effects on human health and cause environmental damage. In addition, the fossil fuels that are the backbone of industrialisation are a limited resource. Hence, the interest in biorefinery of biomass from the agricultural and forestry industries has increased. Biomass from the forestry industry is an excellent raw material for obtaining valuable bioactive species. Pressurised fluid extraction has proved to be an efficient technique for retrieving the bioactive species. The quality of the raw material is still unchanged after the PFE process, and therefore still attractive and feasible for its main process. Optimisation of PFE was performed in Papers I and II to extract high-valuable species from tree barks. Betulin from birch (Betula pendula) bark as well as three stilbene glucosides (i.e. piceid, astringin and isorhapontin) from spruce (Picea abies) bark were efficiently extracted with PFE using water and ethanol as solvents. There are many important factors that are involved during an extraction and they have all been thoroughly discussed to give the reader and future user the tools to achieve good selectivity and efficient extraction. For instance, it was demonstrated that small particle size improved the extraction efficiency, and the type of extraction solvent used should be selected with regard to the solute’s property. PFE is an exhaustive extraction technique, which in general facilitates high extraction yield with long extraction time. However, unnecessarily long extraction time is not cost-effective and could also affect the extracted solutes, which are more exposed to degradation without the protection of the matrix. High extraction temperatures in general give a high extraction yield. Then again, the possibility of degradation also increases with increased temperatures, especially, for bioactive species such as antioxidants that were extracted from plant materials. The used PFE instrument is of laboratory scale. However, it is desirable to create a pilot PFE plant on an industrial site, where the different biomass is accumulated. This approach would minimise the emissions caused by transportation and at the same time increase the value of the biomass.

The DPPH assay was used to assess the total antioxidant activity and capacity of antioxidants in the obtained extracts. However, with unknown samples it is difficult to estimate the DPPH concentration needed to react with the antioxidants, as well as the required reaction time. An online hyphenated system with a liquid chromatograph coupled to electrochemical
and tandem mass spectrometric detection (LC-DAD-ECD-MS/MS) was
developed in Paper III. A redox potential applied over the flow cell
oxidised the incoming antioxidants immediately and their electro-activity
was real-time monitored. Strong antioxidants required lower potential than
weaker antioxidants. Consequently, the ECD selectivity correlates with the
employed redox potential. In amperometric detection, the current is
proportional to the concentration of the electro-active compound and the
number of oxidisable groups present on the compound. Tandem mass
spectrometry enables a fingerprint mapping of the analysed antioxidant, in
which the identity of the unknown antioxidant can be revealed by optimising
the ionisation step, different scan modes and acquisition parameters in a
tandem mass spectrometer.

Well-studied polyphenolic compounds such as kaempferol, catechin and
resveratrol and quercetin and its glucosides (i.e. quercetin-3-glucoside,
quercetin-4′-glucoside and quercetin-3,4′-diglucoside) were the model solute
for the online-system and their antioxidant activity was compared to the
conventional DPPH assay. $E_{1/2}$ was determined for each compound and it
was clearly observed that the position and type of functional groups in the
conjugated ring-system reflect the antioxidant activity of compounds. The
$E_{1/2}$ results for the compounds were correlated with the DPPH assay and it
was clearly demonstrated that in order to compare the different methods, is
critical to be determine the redox potential. The factors that affect the
electrochemical measurements are mainly the pH, the flow rate and the
accessibility of the functional groups. With electrochemistry, the compounds
were immediately oxidised and thereby it was possible to determine the
antioxidant activity and the capacity of each individual compound in a
complex sample mixture.

The antioxidant capacity of birch and spruce bark extract was observed to
increase linearly with increased extraction temperatures, which is probably
not only due to higher extraction yield since degradation of compounds
easily occurs at high temperature. The extraction and degradation processes
occur simultaneously, hence the complicated data interpretation and
evaluation of the actual value. Therefore it is crucial to separate these
processes in order to assess the actual extraction efficiency or actual
degradation. In Paper IV, four different samples were processed differently
at two extraction temperatures, 80 and 180°C. Samples that were processed
at the higher temperature exhibited higher total antioxidant capacity. Water
was used as the extraction solvent, hence hydrolysis or oxidation of the
extracted antioxidants by formed peroxide or hydroxide radicals could easily
occur. In order to study the degradation at elevated temperatures, online LC-
DAD-ECD-MS/MS was employed to analyse the extract. By overlaying
chromatograms of DAD and ECD, species with electro-activity could be
characterised as antioxidants. Evaluation of samples that were processed
differently at different temperatures facilitated a conclusion regarding
degradation at elevated temperatures. PFE using water as solvent at elevated temperatures gives some degradation of the investigated birch bark, as well as epimerisation of catechin. Catechin, which is commonly found in trees, was identified and detected. The MS/MS spectra could determine that at 180°C, catechin conforms to its diastereoisomers. On the whole, the total antioxidant capacities of samples obtained at elevated temperatures were significantly higher than at low temperatures. If the objective is to obtain an extract with high total antioxidant capacity then high temperatures should be used for extraction.

It was demonstrated that some species in tree barks were non-polar species, hence it would be interesting to try to extract the said compounds using supercritical liquid carbon dioxide. The condition of liquid carbon dioxide is much milder than PFE using ethanol and water. For this reason degradation of target species could be avoided. The limitation of liquid carbon dioxide is mainly regarding polar compounds. Adding co-solvent to the liquid carbon dioxide might improve the recovery of polar compounds. It would be interesting to pursue this and compare it with PFE.

To find applications for the biorefined species from biomass is another interesting future aspect to pursue. Birch bark contains secondary metabolites, as do other tree barks. In some preliminary results, birch bark extract acts as a repellent agent for herbivores (e.g. fallow deer). It would therefore be interesting to determine the effective compound or compounds in birch bark extract.
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9 Summary in Swedish


Konventionella tekniker som man använder sig av idag kräver oftast farliga kemikalier eller stora mängder organiska lösningsmedel. Hållbar utveckling gynnas om de konventionella teknikerna fasas ut och ersätts med nya tekniker och metoder som eliminerar användningen av farliga kemikalier och ersätter organiskt lösningsmedel med miljövänliga lösningsmedel. De bioaktiva ämnen och antioxidanter som används idag i läkemedel och som tillsats i livsmedel för att förlänga livsmedlens hållbarhet är kemiskt framställda. Den kemiska processen kräver oftast stora mängder kemikalier och lösningsmedel som kan undvikas om ämnena utvinnas naturligt ur växtmaterial, i vårt fall träd bark. Förutom minskning av kemikalier, kan de bioaktiva ämnena och antioxidanter som utvunnits naturligt minska eller eventuellt elimineras biverkningar som oftast följer med de kemiskt framställda ämnena.


Experiment med och utan hydrotermisk behandling utfördes på barkextrakt för att kunna separera extraktionsprocessen från nedbrytningsprocessen, eftersom extraktions- och nedbrytningsprocessen motverkar och samverkar med varandra under extraktionen. Det är därför ytterst viktigt att separera dessa processer från varandra för att möjliggöra identifiering av nedbrytning av antioxidanter vid höga temperaturer.

Vatten och etanol är miljövänliga lösningsmedel och användningen av dem i PFE möjliggör utfasning av konventionella vätskeextraktionsmetoder som oftast belastar miljön med stora volymer organiskt lösningsmedel. Biorefinering av bark med hjälp av PFE gav betulin och andra värdefulla antioxidanter. Detta kan ge mervärde från barken förutom dess värde som energiråvara. Svensk skogsindustri befinner sig i en svår tid med tuffa konkurrenter i Baltikum. Det vore därmed gynnsamt för svensk ekonomi och skogsindustri att utvinna värdefulla ämnena från bark innan den skickas till förbränningen. PFE med ett rent lösningsmedel underlättar dessutom isolationen av de värdefulla ämnena så att de kan användas i läkemedel och som tillsats i livsmedel.
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