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ENZYMATIC HYDROLYSIS COUPLED TO HOT WATER EXTRACTION FOR DETERMINATION OF FLAVONOIDS IN PLANTS



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by

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Abstract <p>The word sustainability is more and more frequently used within the field of chemistry and in many other fields. Green or sustainable chemistry was introduced during the 90's as a field of chemistry where the environmental and health impacts of processes and products are evaluated and improved. Within analytical chemistry the awareness of sustainability has also increased, which has resulted in optimisation and development of new techniques and methods.</p> <p>The easiest way to improve the sustainability of an analytical method is to decrease and/or replace the use of hazardous chemicals with ones known to have less negative impact on the environment. This thesis reports on a study of the extraction and hydrolysis of quercetin glucosides from onion using sustainable solvents and catalysts. Water at elevated temperatures has been used as a green extraction solvent and, to catalyse the hydrolysis reaction, a thermostable β glucosidase expressed from <i>Thermotoga neapolitana</i>, TnBgl1A has been used. It is shown that the use of water as extraction solvent and enzyme as catalyst have less environmental impact than the conventional method where aqueous methanol and a high concentration of HCl are used as extraction solvent and catalyst, respectively.</p> <p>In studying the rate of hydrolysis, it was found that glucose bound at the 3 position of quercetin was slower to hydrolyse than glucose bound at 4'-position when TnBgl1A was used as catalyst. Different amino acids in the active site of TnBgl1A were mutated and one mutant, TnBgl1A_N221S/P342L, showed an increased hydrolysis rate of glucose at 4'-position, but also of glucose at 3 position, compared to the wild type enzyme. TnBgl1A_N221S/P342L was then immobilised to support materials to allow reuse and also to be used in a continuous flow system.</p> <p>The combination of extraction and hydrolysis of quercetin glucosides from onion in a continuous flow system was not as trivial as originally expected. The total time was quite long, 2 hours, due to slow extraction at optimal hydrolysis conditions. However, by using an on-line method, manual work was reduced and no extra separation step of extract and immobilised enzyme was needed. Molecularly imprinted polymers were developed to accomplish the step after extraction and hydrolysis, i.e. isolation of quercetin from the onion extract. The imprinted polymers were tested for isolation of quercetin from aqueous yellow onion extract, and it was found that the binding capacity of quercetin to MIP increased significantly with increasing temperature.</p>		
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ENZYMATIC HYDROLYSIS COUPLED TO HOT WATER EXTRACTION FOR DETERMINATION OF FLAVONOIDS IN PLANTS

Sofia Lindahl



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A doctoral thesis at a university in Sweden is produced as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarises the accompanying papers. These have either already been published or are manuscripts at various stages (*in press, submitted, or manuscript*)

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Abstract

The word sustainability is more and more frequently used within the field of chemistry and in many other fields. Green or sustainable chemistry was introduced during the 90's as a field of chemistry where the environmental and health impacts of processes and products are evaluated and improved. Within analytical chemistry the awareness of sustainability has also increased, which has resulted in optimisation and development of new techniques and methods.

The easiest way to improve the sustainability of an analytical method is to decrease and/or replace the use of hazardous chemicals with ones known to have less negative impact on the environment. This thesis reports on a study of the extraction and hydrolysis of quercetin glucosides from onion using sustainable solvents and catalysts. Water at elevated temperatures has been used as a green extraction solvent and, to catalyse the hydrolysis reaction, a thermostable β -glucosidase expressed from *Thermotoga neapolitana*, *TnBgl1A* has been used. It is shown that the use of water as extraction solvent and enzyme as catalyst have less environmental impact than the conventional method where aqueous methanol and a high concentration of HCl are used as extraction solvent and catalyst, respectively.

In studying the rate of hydrolysis, it was found that glucose bound at the 3-position of quercetin was slower to hydrolyse than glucose bound at 4'-position when *TnBgl1A* was used as catalyst. Different amino acids in the active site of *TnBgl1A* were mutated and one mutant, *TnBgl1A_N221S/P342L*, showed an increased hydrolysis rate of glucose at 4'-position, but also of glucose at 3-position, compared to the wild type enzyme. *TnBgl1A_N221S/P342L* was then immobilised to support materials to allow reuse and also to be used in a continuous flow system.

The combination of extraction and hydrolysis of quercetin glucosides from onion in a continuous flow system was not as trivial as originally expected. The total time was quite long, 2 hours, due to slow extraction at optimal hydrolysis conditions. However, by using an on-line method, manual work was reduced and no extra separation step of extract and immobilised enzyme was needed.

Molecularly imprinted polymers were developed to accomplish the step after extraction and hydrolysis, i.e. isolation of quercetin from the onion extract. The

imprinted polymers were tested for isolation of quercetin from aqueous yellow onion extract, and it was found that the binding capacity of quercetin to MIP increased significantly with increasing temperature.

Populärvetenskaplig sammanfattning

I Sverige produceras dagligen flera ton biprodukter avfall inom jordbruks-, livsmedels- och skogsindustrierna. Detta avfall förbränns, komposteras och går till biogasproduktion samt i vissa fall till djurfoder. Intressanta avfallsgrupper ur ett svenskt perspektiv är lök- morots-, och potatis avfall från jordbruk- och livsmedelsindustrin och bark från skogsindustrin. Dessa avfallsgrupper är intressanta eftersom ur dessa kan värdefulla ämnen extraheras, som t.ex. antioxidanter och olika färgpigment. Dessa värdefulla ämnen kan eventuellt användas som tillsats i olika produkter och ersätta de syntetiska ämnen som oftast används idag i t.ex. livsmedel, kosmetika och läkemedel. I denna avhandling har lök studerats. Gul lök men även vissa andra löksorter, som t.ex. rödlök och schalottenlök, innehåller ett ämne, quercetin. Detta är en antioxidant som skyddar lök och andra växter från påverkan av bl.a. UV-strålning och mögel- och svampangrepp.

För att extrahera quercetin från gul lök och andra växter används vanligtvis lösningsmedel som t.ex. etanol och metanol. Dessa lösningsmedel framställas ofta från fossila råvaror, vilket medför att produktion och destruktion av dem kan belasta miljön. Anledningen att dessa lösningsmedel vanligtvis används för att utvinna quercetin är att quercetin har hög löslighet i dessa lösningsmedel. I detta forskningsprojekt har istället varmt vatten använts som lösningsmedel, då detta varken har negativ effekt på miljön eller på de som arbetar med det, jämfört med de flesta organiska lösningsmedel. Vatten är ett polärt ämne, vilket medför att lösligheten av quercetin är låg eftersom quercetin inte är lika polärt. Genom att öka temperaturen på vatten, minskar dess polaritet vilket medför ökad löslighet av quercetin, vilket i sin tur ökar utbytet av quercetin. Om behållaren trycksätts så höjs vattnets kokpunkt och temperaturer över 100 grader går att använda vid extraktion. Quercetin återfinns oftast i lök och andra växter med inbundna sockergrupper. Dessa gör att quercetins löslighet i vatten ökar, vilket är bra ur ett extraktionsperspektiv. För att förenkla kvantifieringen av quercetin i extraktet har ett enzym använts som tar bort sockergrupperna från quercetin efter extraktionen. Detta medför att det bara finns en sort av quercetin att kvantifiera och inte både quercetin och olika quercetin-glukosider.

Andra skäl att ta bort sockergrupperna är att quercetins antioxidativa effekt är bättre utan inbundna sockergrupper samt att de standarder som behövs för att bestämma mängden av de olika varianterna av quercetin är mycket dyra. Att använda sig av enzym för att katalysera en reaktion, t.ex. hydrolys av quercetin-glukosid till quercetin och glukos, kallas biokatalys. Samma reaktion kan även katalyseras av starka syror, t.ex. saltsyra. Dock medför produktion av saltsyra en större belastning på miljön med avseende på energianvändning, jämfört med enzymproduktion. Det enzym som jag har använt är β -glukosidas och detta enzym finns hos en mängd olika organismer. Även vi människor har detta enzym i våra tarmar där det katalyserar olika reaktioner, bl.a. hydrolys av quercetin-glukosider till quercetin. I denna studie har enzym från *Thermotoga neapolitana* använts, vilket är en bakterie som återfinns i varma källor i Neapel. Då enzymet kommer från en organism som lever vid höga temperaturer, har även enzymet sin optimala aktivitet vid höga temperaturer. Detta är en stor fördel eftersom själva extraktionen av quercetin-glukosiderna görs vid höga temperaturer och ingen nedkylning behövs före hydrolys. Efter extraktion och hydrolys är nästa steg att extrahera/isolera quercetin från löksoppan, som innehåller många andra ämnen. Detta kan göras på olika sätt, t.ex. genom att använda ett material som quercetin kan binda in till. I ett av projekten har sk ”molecular imprinted polymers” (MIP) testats. MIP är en polymer där man gjort avtryck av det ämne man är intresserad av, i detta fall quercetin. MIP kan liknas med ett lås och en nyckel. Det finns många nycklar som bara har små skillnader mellan sig men trots det finns det bara en nyckel som passar i låset. Det svåra med att använda MIP i ett vattenextrakt är att selektiviteten för quercetin minskar.

Forskningen beskriven ovan har studerats och resultat av försöken finns presenterade i **paper I-V**.

Sammanfattningsvis finns det några resultat som kan lyftas ut. Det visade sig att använda vatten och enzym istället för metanol och saltsyra har mindre påverkan på miljön när det gäller utsläpp av växthusgaser och energianvändning (**paper I**). Reaktionshastigheten för hydrolys av quercetin-glukosider berodde på var på quercetin molekylens glukos var inbundet (**paper I**). Dock ökade reaktionshastigheten när aminosyrasammansättningen modifierades i den del av enzymet där quercetin-glukosiderna binder in och glukos tas bort (**paper I och II**) och det var en modifiering som visade sig bättre än övriga testade modifieringar (**paper II**). Modifierat och icke modifierat enzym bands in till olika bärarmaterial (**paper III**), eftersom det då blir lättare att separera enzymet från lökextraktet och att återanvända enzymet. Eftersom höga temperaturer används i extraktions- och hydrolysstegen testades temperaturstabiliteten av bärarmaterialen (**paper III**). Resultatet visade att de inte påverkades av de höga temperaturer som behövs. Då enzymet är inbundet till ett bärarmaterial kan det användas i ett flödessystem där extraktion och hydrolys görs direkt efter varandra och inte i två separata steg

(**paper IV**). Dock visade det sig att det ej var lätt att göra extraktion och hydrolys i en följd då de optimala betingelserna för extraktion och hydrolys inte var lätta att kombinera (**paper IV**). Olika MIP producerades och när dessa studerades i lösningar som innehöll quercetin visade det sig att mer quercetin band in till MIP om en högre temperatur användes (**paper V**).

Det finns mycket kvar att studera när det gäller att utveckla tekniker och metoder för att utvinna antioxidanter och andra intressanta ämnen från industriella biprodukter och avfall, men i denna avhandling visar att det finns goda chanser att utveckla metoder som ger mindre effekt på miljön än de som används idag.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals I-V.

- I. **Lindahl S.**, Ekman A., Khan S., Wennerberg C., Börjesson P., Sjöberg P.J.R., Nordberg Karlsson E. and Turner C. (2010) Exploring the possibility of using a thermostable mutant of β -glucosidase for rapid hydrolysis of quercetin glucosides in hot water, *Green Chemistry*, 12, 159-168, (<http://pubs.rsc.org/en/content/articlelanding/2010/gc/b920195p>)
- II. Khan S., Pozzo T., Megyeri M., **Lindahl S.**, Sundin A., Turner C. and Nordberg Karlsson E. (2011) Aglycone specificity of *Thermotoga neapolitana* β -glucosidase 1A modified by mutagenesis, leading to increased catalytic efficiency in quercetin-3-glucoside hydrolysis, *BMC Biochemistry*, 12 (11), (<http://www.biomedcentral.com/1471-2091/12/11>)
- III. Khan S., **Lindahl S.**, Turner C. and Nordberg Karlsson E. (2011) Immobilization of thermostable β -glucosidase variants on acrylic supports for biocatalytic processes in hot water, *Manuscript submitted to Journal of Molecular Catalysis B: Enzymatic*
- IV. **Lindahl S.**, Liu J., Khan S., Nordberg Karlsson E. and Turner C. (2011) An on-line method for pressurised hot water extraction and enzymatic hydrolysis of quercetin glucosides from onions, *Manuscript in preparation*
- V. Pakade V., **Lindahl S.**, Chimuka L. and Turner C. (2011) Molecularly imprinted polymers targeting quercetin in high-temperature aqueous solutions, *Manuscript submitted to Journal of Chromatography A*

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Contribution by the author to the different papers:

Paper I: The author participated in the planning of experiments, performed major part of the experiments (all hydrolysis experiments using quercetin derivatives and subcritical water extraction) and wrote major parts of the paper.

Paper II: The author performed minor parts of the experiments (Q3 hydrolysis of selected mutants) and helped in writing (minor assistance) and revising the paper.

Paper III: The author performed minor parts of the experiments (testing the thermal stability of the support materials and application towards onion sample) and helped in writing (minor assistance) and revising the paper.

Paper IV: The author participated in the planning of experiments, performed the major part of the experiments (optimisation of hydrolysis (cryogel), on-line and conventional extraction and hydrolysis experiments) and wrote major parts of the paper

Paper V: The author participated in the planning of the experiments, performed minor parts of the experiments (TGA analysis and adsorption with time at 84 °C) and helped write (minor parts) and revised the paper.

Papers not included in this thesis:

Heiskanen A., Spégel C., Kostashe N., **Lindahl S.**, Ruzgas T. and Emnéus J. (2009) Mediator-assisted simultaneous probing of cytosolic and mitochondrial redox activity in living cells, *Analytical Biochemistry*, 384, 11–19

Khan S., Mahmood T., Kulkarni T.S., Sundin A., **Lindahl S.**, Turner C., Logan D. and Nordberg Karlsson E. (2011) Comparison of the active site mutants of *Thermotoga neapolitana* β -glucosidase 1A for hydrolysis of *pNPGlc* and quercetin-3-glucosides, *Manuscript in preparation*.

Ekman A., Campos M., **Lindahl S.**, Co M., Börjesson P., Nordberg Karlsson E. and Turner C. (2011) Addition of value in bioresource utilization by sustainable technologies in new biorefinery concepts. *Manuscript in preparation*

Andersson J., **Lindahl S.**, Turner C. and Rodríguez-Meizoso I. (2011) Hot water extraction with particle formation on-line by supercritical fluid technology. *Manuscript submitted to Food Chemistry*.

Pakade V., Cukrowska E., **Lindahl S.**, Turner C. and Chimuka L., (2011) Molecular imprinted polymer for solid-phase extraction of quercetin from *Moringa Oleifera* leaf extracts. *Manuscript in preparation*.

Abbreviations

ASE	accelerated solvent extraction
CE	capillary electrophoresis
DAD	diode array detector
DPPH	1,1-diphenyl-2-picrylhydrazyl
GC	gas chromatography
GH	glycoside hydrolases
GHG	green house gases
GWP	global warming potential
HPLC	high performance liquid chromatography
LC	liquid chromatography
LCA	life cycle assessment
MAE	microwave-assisted extraction
MIP	molecularly imprinted polymer
MS	mass spectrometry
NIP	non-imprinted polymer
NMR	nuclear magnetic resonance
PFE	pressurised fluid extraction
PHWE	pressurised hot water extraction
PLE	pressurised liquid extraction
PSE	pressurised solvent extraction
Q3	quercetin-3-glucoside
Q3,4'	quercetin-3,4'-diglucoside
Q4'	quercetin-4'-glucoside

RPLC	reversed phase liquid chromatography
RSD	relative standard deviation
scCO ₂	supercritical carbon dioxide
SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
SHWC	super heated water chromatography
SLE	solid-liquid extraction
SPE	solid phase extraction
SWE	subcritical water extraction
TGA	thermogravimetric analysis
THF	tetrahydrofuran
TLC	thin layer chromatography
UAE	ultrasound-assisted extraction
UPLC	ultra high performance liquid chromatography
UV	ultraviolet
WEPO	water extraction and particle formation on-line

Table of Contents

Abstract.....	v
Populärvetenskaplig sammanfattning	vii
List of Papers.....	x
Abbreviations	xii
Table of Contents	xiv
1 Introduction.....	1
2 Objectives	3
3 Sustainable Chemistry.....	4
3.1 Green Analytical Chemistry	5
3.2 Life Cycle Assessment.....	7
4 Flavonoids.....	12
4.1 Quercetin.....	13
4.1.1 Quercetin in onion	14
4.2 Analysis and quantification of flavonoids and quercetin.....	15
5 Extraction	20
5.1 Solvent	20
5.2 Temperature and Pressure.....	21
5.3 Time	22
5.4 Static extraction vs continuous flow extraction	23
5.5 Sample pre-treatment of plant material prior to extraction.....	25
5.6 Extraction of flavonoids.....	26
5.6.1 Conventional solid-liquid extraction (SLE).....	27
5.6.2 Pressurised Fluid Extraction (PFE).....	28
5.6.3 Pressurised Hot Water Extraction (PHWE).....	28
5.6.4 Other enhanced extractions techniques.....	29
5.7 Extraction of quercetin from onion.....	30
5.8 Solubility of quercetin	32
6 Hydrolysis of flavonoids	34
6.1 Hydrolysis of quercetin glucosides.....	34

6.2 β -glucosidase from <i>T. neapolitana</i>	35
6.3 Quercetin glucoside specificity of <i>TnBgl1A</i>	36
6.4 Immobilisation of the enzyme	39
7 Hyphenation	42
7.1 On-line hydrolysis.....	42
7.2 Molecularly Imprinted Polymers (MIP)	43
8 Conclusions and future perspectives.....	49
Acknowledgments	52
References.....	54

1 Introduction

In Sweden several tons of by-products and residues (waste) are produced daily by agricultural, food and forest industries ¹. These by-products are used today for example in biogas production, animal feed and incineration. The value of such by-products can be increased by using them as raw material in other processes before their final use as for example biogas, a process that applies the concept referred to as biorefinery ² (Fig. 1).

Examples of by-products are fruit and vegetable waste such as onions, carrots, apples from the food and agricultural industries, and bark from the forest industry. These materials contain high-value compounds in low amounts, which could increase the market value of a by-product for an industry if such compounds were isolated and sold. Since the high-value compounds are usually present in low amounts, the extraction from the by-product will not change the energy value of the material significantly. As a result, it can still be used for example for biogas production or animal feed.

Flavonoids, an example of a high-value compound that can be extracted from fruits and vegetables, are of particular interest because many of the flavonoids present in fruits and vegetables are antioxidants. Antioxidants are compounds that slow down or inhibit the oxidation of other compounds and thus synthetic antioxidants are commonly added to food, cosmetics and pharmaceuticals products today to prolong their shelf life. In addition to their antioxidant properties, some flavonoids, for example anthocyanins, can also be used as colorants, since they absorb light of certain wavelengths.

Flavonoids are usually present as glycosides in plants, i.e. sugar attached to the aglycone part. These sugar groups can be removed to simplify qualitative and quantitative determination and to increase the yield of the aglycone, which is usually a stronger antioxidant. The sugar can be removed in hydrolysis reaction where strong acids or bases can be used, but a more environmentally sustainable alternative to use is enzymes ³. Once the sugars are removed, the next step would be to isolate the compound(s) of interest from the extract. There are different techniques for doing this, for example solid phase extraction ⁴.

The synthetic antioxidants used as additives in products, for example cosmetics, might be replaced by natural antioxidants extracted from plant by-products.

Example of a flavonoid that can be extracted from fruit and vegetable by-products is quercetin from onions/apples/grapes⁵. While there is research on the extraction and isolation of high-value compounds from plant material, the novelty of our work lies in the development of methods that have less environmental impact than the conventional methods.

The research about developing environmentally adapted methods belongs to the field of sustainable chemistry. In 1998 Anastas and Warner published the Twelve Principles of Green Chemistry⁶ and these can also be implemented within analytical chemistry. The simplest way to improve the sustainability of an analytical method is to decrease the amount of hazardous chemicals used or change to non-hazardous chemicals⁷. In the case of extraction of flavonoids from plant material, the easiest way to improve the greenness of the method is to change the extraction solvents used. Examples of more benign and environmentally adapted solvents are water, ethanol and supercritical CO₂ (scCO₂). Several methods are available for studying the environmental impact of a method, for example life cycle assessment (LCA), E-factor, and atom economy⁸⁻⁹.

In the papers included in this thesis, pressurised hot water extraction (PHWE) has been used in combination with enzyme-catalysed hydrolysis of quercetin glucosides from onion.

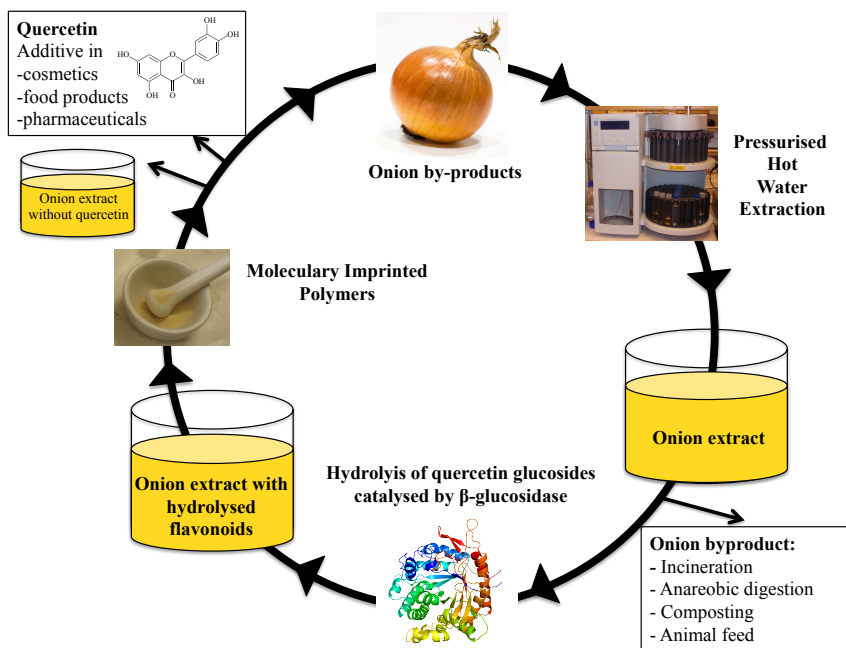


Figure 1. The cycle of onion, from by-product in the industry to a quercetin product.

2 Objectives

The aims of the research reported in this thesis are:

- To develop environmentally sustainable methods for extraction and hydrolysis of quercetin derivatives from onion.
- To study the use of a thermostable enzyme, β -glucosidase, to catalyse the hydrolysis of quercetin glucosides so as to enable a simplified quercetin analysis.
- To combine hot water extraction with enzyme catalysed hydrolysis of quercetin glucosides from onion.
- To use molecularly imprinted polymers to isolate quercetin from aqueous onion extract.

3 Sustainable Chemistry

One of the aims of the research presented in this thesis is to develop environmentally sustainable methods for the extraction and hydrolysis of quercetin glucosides from onion. Green, or sustainable, chemistry was first introduced in the beginning of the 1990s and one of the definitions of Green Chemistry is “*design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances*”¹⁰. In 1998 Anastas and Warner published the 12 principles of Green Chemistry⁶, see Fig. 2, which are principles that chemists and others can use to improve the sustainability of an already existing process or to develop new processes¹⁰.

1. Prevention*
2. Atom economy
3. Less hazardous chemical synthesis
4. Designing safer chemicals
5. Safer solvents and auxiliaries*
6. Design for energy efficiency*
7. Use of renewable feedstock
8. Reduce derivatives*
9. Catalysis
10. Design for degradation
11. Real time analysis for pollution prevention*
12. Inherently safer chemistry for accident prevention*

Figure 2. The 12 principles of Green Chemistry⁶. *Principles that can mainly be applied in analytical chemistry⁷.

Winterton *et al.*¹¹ have published *twelve more green chemistry principles* that can be used as a complement to the 12 principles of Green Chemistry in the design of a green process, for example parameters to think about in a future up-scaling of the

process. In the next section, the application of green chemistry principles in analytical chemistry is described.

3.1 Green Analytical Chemistry

The 12 principles of Green Chemistry can be applied in the design of new analytical methods or in improving existing methods to decrease their impact on the environment and on human health ⁷. Of the 12 Green Chemistry Principles, it is mainly principles one, five, six, eight, eleven and twelve that are most applicable in the field of analytical chemistry ⁷, see principles in Fig. 2 with asterisks. Normally there are eight steps in the analysis of a sample; (i) planning, (ii) sample collection, (iii) sample preparation, (iv) separation, (v) detection, (vi) evaluation, (vii) interpretation and (viii) validation, Fig. 3. In many of these steps, hazardous chemical substances are commonly used and sometimes the chemicals used and the waste produced are more toxic than the analyte in the sample ⁷. It is mainly in steps *iii-v* where the “greenness” can be improved by for example reducing or changing the solvents and other chemicals used, and by automation and/or miniaturisation and/or hyphenation of techniques ¹². In this thesis the focus has been on the change to environmentally sustainable extraction solvents and catalysts, but also on hyphenation of different techniques.

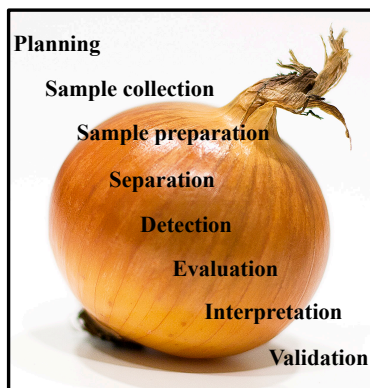


Figure 3. Eight steps in the analysis of a sample.

One way to improve the greenness of a method is to replace hazardous chemical substances with substances known to be less toxic for the environment and/or for humans. There are a number of publications where solvents have been ranked regarding their impact on the environment and/or health that can be used in the selection of solvents, for example Capello *et al.* ¹³, Demou *et al.* ¹⁴ and

Henderson *et al.*¹⁵. Examples of solvents to avoid include chlorinated solvents (e.g. dichloromethane, carbon tetrachloride), tetrahydrofuran (THF) and formaldehyde, whereas examples of more sustainable solvents are water, ethanol and scCO₂. However, the effect of the change in the method on the results of the analysis has of course to be evaluated with respect to the measurement uncertainty of the method.

One of the steps in sample preparation, which consumes a great deal of solvent, is the extraction step, where analytes are extracted from the sample matrix. In the extraction from solid matrices, for example from plant material, conventional solid liquid extraction (SLE) is commonly applied using organic solvents such as methanol, ethanol and ethyl acetate⁴. Alternative techniques that are regarded as more sustainable than SLE are, for example, microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE) and pressurised fluid extraction (PFE) (see section 5 *Extraction*)^{7, 12}. In MAE, UAE and PFE, organic solvents are still used, but the solvent-sample ratio is usually lower than in SLE, and the time for the extraction is usually shorter. In **papers I, III-V**, PFE was used with water as extraction solvent (PHWE) to extract quercetin glucosides from yellow onion. The extraction step took 20 minutes when static extraction was used, compared to the conventional method, where aqueous methanol is used, which takes 2 h.

In the separation step in the analytical chain, two commonly used separation techniques are gas chromatography (GC) and liquid chromatography (LC). GC is a quite environmentally adapted separation method since harmless gases are used as mobile phase. Sometimes sample derivatisation is necessary before GC-analysis to improve the separation and detection. Derivatisation should be avoided according to principle eight of the Green Chemistry Principles (Fig. 2), since derivatisation results in the use of extra hazardous substances and extra waste might be generated¹⁰.

In reversed phase LC (RPLC), organic solvents are used in the mobile phase, and commonly used solvents are acetonitrile and methanol, where methanol is a greener alternative¹³. Another alternative green solvent to use is ethanol¹³, but ethanol-water mixtures have a high viscosity and problems can arise with the backpressure in the LC-system. There are a number of ways to decrease solvent consumption in the LC-analysis. The easiest and least expensive way is to optimise the separation method, with respect to the analysis time, because then the amount of organic solvent consumed per sample could be minimised. The optimisation can be carried out, for example, using gradient elution, changing the temperature of column and changing the pH of the mobile phase¹⁶. When gradient elution is used the equilibration time also has to be included in the calculation of amount of organic solvent used. Another way to decrease the consumption of

solvent in LC separation is to use shorter columns with smaller particle diameters, reduce the internal diameter of the column and/or change the type of stationary phase ¹⁶. However, the use of smaller particles and internal diameter will result in a higher back-pressure and then an ultra high pressure LC system (UPLC) may be needed. In **papers I-V**, a UPLC system was used for analysis of some of the samples, and methanol mixed with water was used as mobile phase. During the projects reported on in this thesis, an attempt was made to change to a shorter column with smaller particle size, (from 100 mm to 50 mm and from 3.5 micron to 2.7 micron), with a different stationary phase, to speed up the separation and decrease the amount of methanol used. The separation was faster but there were clogging problems when the extracts were analysed, so the longer column with larger particles was kept.

Other separation techniques that could have better environmental performance than HPLC include supercritical fluid chromatography (SFC) ¹⁷, super heated water chromatography (SHWC) ¹⁸ and capillary electrophoresis (CE) ¹⁹. In these techniques no or low amounts of organic solvents are used in the mobile phase.

3.2 Life Cycle Assessment

Sometimes, concluding statements in the literature describe a newly developed method as a “greener” alternative compared to a conventional method based solely on the fact that a less environmentally adapted solvent was replaced with a more environmentally adapted one. However, merely changing to another solvent does not ensure that an alternative is more sustainable than existing ones. Instead, newly developed methods have to be studied in detail and the entire process chains of the existing and new methods must be compared, from raw materials to waste handling.

A tool for this kind of quantitative analysis and comparison of methods is Life Cycle Assessment (LCA) and the LCA methodology is described in ISO standard 14040:2006 ²⁰ and 14044:2006 ²¹. An LCA can be divided into four steps; *(i)* definition of goals and scope, *(ii)* inventory analysis, *(iii)* impact assessment and *(iv)* interpretation of the results, see Fig. 4.

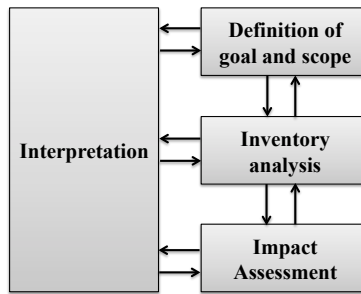


Figure 4. The four steps of an LCA. ²⁰

In the definition of goals and scope, the product or process is described, the aim and objective of the LCA stated and the intended use of the final results is described ²². In the second step, i.e. inventory analysis, data collection and calculations for inputs and outputs in the product system are performed ²²⁻²³. In the impact assessment step, the impact of the results of the inventory analysis is described. In the last step, interpretation of, for example, the result of the LCA is analysed and evaluated, and conclusions and recommendations are discussed ²²⁻²³.

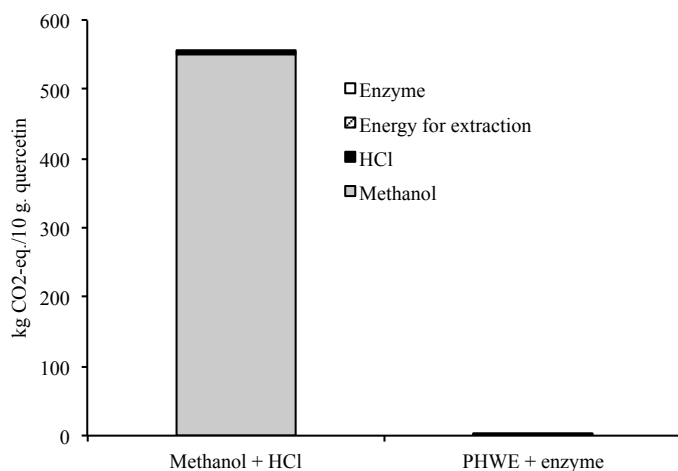
An LCA can for example be used to compare the environmental impact of two different processes for the same product or to identify which part of a life cycle of a product or process has the largest environmental impact ²³. In the impact assessment step, the three main environmental impact categories that can be included and studied in LCA are the use of natural resources, health effects and ecological effects ²³. In the category use of natural resources, energy use and the use of land required, for example, are studied; in health effects, toxic effects in and outside the work environment are studied, and in ecological effects the emission of green house gases (GHG), eutrophication, acidification and photochemical smog formation are possible aspects studied ²³.

In an LCA, a part of or the whole life cycle of a process/product can be studied from a cradle-to-grave, cradle-to-gate or gate-to-gate perspective. In a cradle-to-grave perspective, all steps in the life cycle of the product are included, from starting raw materials to disposal of the used product. In the cradle-to-gate perspective, the LCA stops at the gate of the factory, i.e. use and disposal of product are not included. In a gate-to gate perspective, the environmental assessment is only conducted for the production of the product.

In **paper I**, LCA was used to compare the differences in environmental impact between two methods for the extraction and hydrolysis of quercetin glucosides from onion and a gate-to-gate perspective was used. This perspective was selected since the two methods use the same raw material (onion) and result in the same

product (quercetin). In the LCA two environmental impact categories were studied, energy use and global warming potential (GWP), i.e. emission of GHG. In Fig. 5, the amount of energy needed and GHG emission for extraction and hydrolysis of 10 g of quercetin is shown.

(a)



(b)

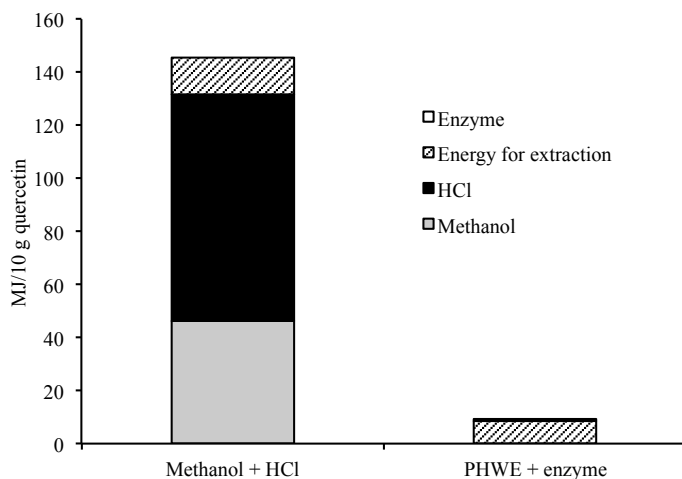


Figure 5. (a) Total contribution to the GWP expressed as kg CO₂-equivalents per 10 g of quercetin, for the two extraction methods and (b) as total primary energy consumption, expressed as MJ per 10 g of quercetin, for the two extraction methods. Modified from **paper I** ³.

In the environmental impact assessment comparing the two methods (see Fig. 5a), it is seen that, for the conventional method, it is methanol that contributes the most to the emission of GHG (expressed in CO₂-equivalents). Methanol is a fossil-derived solvent, from natural gas, and it is both during the manufacture and the disposal phase of methanol that the GHG are emitted. In the new method, on the other hand, water is used as extraction solvent and in the calculations it was assumed that the environmental impact of water was insignificant. The different catalysts used for the two methods, HCl (conventional) and enzyme (new), did only have a marginal contribution to GWP, compared to the methanol (Fig. 5a).

There was only a small difference in the energy consumption of the two different extraction techniques used (Fig. 5b). The new method (PHWE) was performed at a higher temperature (120 °C vs 80 °C), but the extraction time was shorter, 15 min vs 2 h. As for the emission of GHG, the energy used for the manufacture of methanol is higher than for water (Fig 5b). The two different catalysts, HCl and enzyme, resulted in different energy consumption, where the process for manufacture of HCl is much more energy-consuming compared to the enzyme (Fig. 5b).

The overall conclusion of the LCA was that the new method was energy efficient and had a much lower contribution to the GWP. However, these calculations were performed on a linear up-scaling of an analytical process, from some few milligrams up to gram scale, which adds an uncertainty to the results obtained. The energy usage and GWP regarding the conventional method might be decreased if a solvent based on a renewable feedstock is used instead of a fossil-derived solvent, for example bioethanol. In the literature one can find other LCA or environmental impact assessments on the use of biocatalysts²⁴⁻²⁶. Skals *et al.*²⁵ studied five different possible enzyme applications in the pulp and paper industry and the result was that the energy use and environmental impacts were lower compared to conventional methods.

Furthermore, LCA studies can instead show that the newly developed method is not a better alternative from an environmental impact perspective compared to the conventional used method. Within the research group, preliminary studies have shown that the replacement of hexane with ethanol for extracting carotenes from carrots was not a better alternative (unpublished data) from an environmental perspective. The reason was that the amount of ethanol that was needed per kg of carrots was much higher than the amount of hexane needed, because the solubility of carotenes is relatively low in ethanol compared to hexane. However, a better solvent could be scCO₂, which has solubility properties similar to hexane.

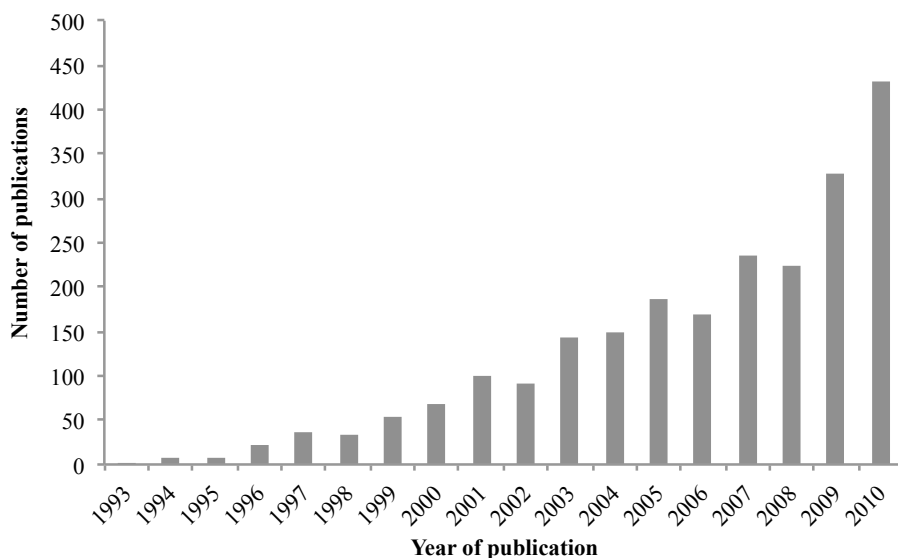


Figure 6. Bar chart over publication of LCA ²⁷.

In Fig. 6 the number of publications on LCA between 1993-2010 is shown ²⁷. It is clear that there is an exponential trend in the increase of publications about LCA, but very few of them are in the field of analytical chemistry. Many LCA studies are conducted on industrial processes in large scale, for example biofuel production, and not on analytical techniques. Analytical techniques do not consume as large amounts of chemicals as do industrial process since the scale is much smaller and the aim is not to produce products, rather to do qualitative and quantitative analysis of samples. However still analytical techniques do consume chemicals and when you have the opportunity to use different kinds of techniques it would be of interest to know which alternative is better to use from an environmental and health perspective. Another reason why it can be of interest to conduct an LCA of analytical techniques is if there is an interest to up-scale the technique in a later stage and this was why an LCA was done in **paper I**. One problem in performing LCA on analytical methods is that the availability of inventory data for fine chemicals are scarce, compared to bulk chemicals. Alternative tools to use instead for LCA are for example E-factors and atom economy ⁹. These tools are more simple to use, compared to LCA, but they might be too general and for example important environmental impacts can be missed ⁹.

4 Flavonoids

A polyphenolic compound is a compound with aromatic rings with several hydroxyl groups and there are several thousands of polyphenolic compounds present in plants. Polyphenolic compounds are secondary metabolites in plants and these are divided depending on their structure into different classes for example phenolic acids, flavonoids, stilbenes and lignans²⁸. In the projects behind this thesis, the focus has been on quercetin derivatives, which belong to the flavonoids. The general structure of a flavonoid is shown in Fig. 7a and more than 6500 flavonoids have been identified. Flavonoids can be further divided into subclasses, and examples of subclasses are flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (Fig. 7b-g).

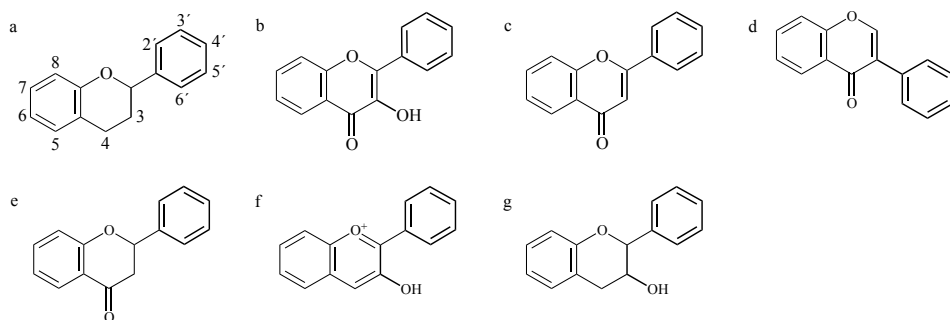


Figure 7. General structure of (a) flavonoid and subclasses (b) flavonols, (c) flavones, (d) isoflavones, (e) flavanones, (f) anthocyanidins and (g) flavanols.

The role of the flavonoids and other phenolic compounds in plants is for example to protect the plant from UV-radiation and pathogens²⁹⁻³⁰. Flavonoids can be present as free aglycones or substituted at different positions at the backbone, for example, it can be glycosylated or methylated^{29, 31-32}. Glycosylation is common, and different types of sugars can be attached to the different hydroxyl groups of the backbone, for example, glucose, rhamnose and galactose, whereas the most common derivative is glucose at the 3-position³². The aglycone can be associated with one or several monosaccharides and/or oligosaccharides, for example

quercetin-3,4'-diglucoside or kaempferol-3-robinoside-7-rhamnoside. Glycosylated flavonoids are more polar than its aglycone, which increase the water solubility, but the compound is also less reactive; i.e. it exhibits lower antioxidant activity^{29, 33-34}.

The most common flavonoids present in plants are quercetin and kaempferol derivatives²⁸ (Fig. 8) and the average daily human intake is from 3 to 70 mg/day, depending on the diet³⁵.

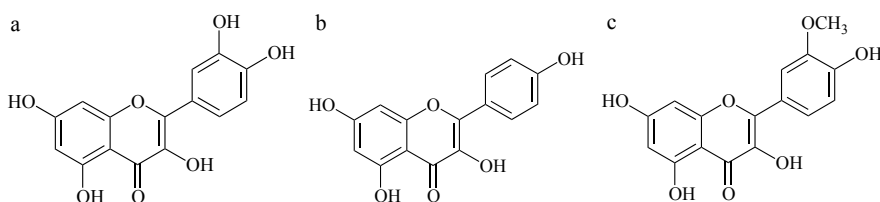


Figure 8. Structure of (a) quercetin, (b) kaempferol and (c) isorhamnetin.

Flavonoids and other antioxidants are thought to have a positive impact on our health. A number of epidemiologic studies have shown a correlation between flavonoid intake through the diet and decreased risk of for example coronary artery disease³⁵⁻³⁶. Flavonoids are present in many different plants, i.e. they are consumed through eating vegetables and fruit. One of the plants that contain the highest amount of flavonoids per gram of plant material is onion²⁸. The amount of onion by-products produced only in Sweden yearly is 1000-5000 ton¹. Onion is the plant used in **papers I and III-V** to study the flavonoid quercetin.

4.1 Quercetin

Quercetin, is not only present in onion but also in other plants, for example, apples, berries, grapes, and tea³⁷. Quercetin is an antioxidant and the average daily dietary intake of quercetin is around 2.6-38.2 mg/person/day³⁵. Quercetin is mainly present as different glycosides in plants, and the most commonly substituted sugars to quercetin are glucose and rhamnose. However, other sugars such as galactose and arabinose are also present³⁷. The glycosylation of quercetin is most common at the 3-position, but glycosylation can also occur at the 7 and 4'-positions, while it is rare at the 5-position³⁷⁻³⁸, (Fig. 6a and 8a).

At least 180 different quercetin glycosides have been identified³⁸. The level of glycosylation of quercetin and the amount of quercetin derivatives can differ throughout a plant. In onions, for example, the amount of quercetin derivatives is

higher in the skin than in the bulb³⁹⁻⁴⁰. Furthermore, the relative amount of quercetin is higher in the skin compared to quercetin glucosides, but in the bulb the relative amount of quercetin glucosides is higher than quercetin³⁹. In humans, quercetin and its glucosides are absorbed in the intestine^{38, 41} and thereafter the absorbed glucosides can be hydrolysed by intracellular β -glucosidases and other enzymes^{28, 41}. Quercetin is an antioxidant and can therefore scavenge radicals such as reactive oxygen and nitrogen species, which can damage, for example, DNA⁴¹. Other positive effects of quercetin shown by *in vitro* studies are that it has anti-inflammatory and anti-bacterial properties⁴¹. However, when quercetin is oxidised, quercetin quinones are formed, and these may have a toxic effect in the body⁴¹.

4.1.1 Quercetin in onion

As mentioned above, onion is a by-product of the food and agricultural industries in Sweden, and yellow onion was thus selected as the main plant material for study throughout the projects described here. Onion, as mentioned above, is one of the plants that contain the highest amounts of the flavonol quercetin. In yellow onion, the main glucosides present are quercetin-3,4'-diglucoside (Q3,4'), quercetin-4'-glucoside (Q4') and quercetin-3-glucoside (Q3) (Fig. 9), but other glucosides and the aglycone are also present^{3, 39, 42-43}. Other flavonoids are also present in yellow onion, such as isorhamnetin and kaempferol (Fig. 8) derivatives⁴³. In **paper IV**, red onion and shallot onion were also studied, and the main quercetin glucosides reported in the literature are also Q3,4' and Q4'⁴⁴.

The quercetin content reported in the literature for yellow onion has a quite large range. Slimestad *et al.*⁴³ have summarised some of the published quercetin content of onion, and in yellow onion the total quercetin content could vary from around 20 mg/kg fresh weight up to almost 2000 mg/kg fresh weight. The reason for this wide range depends on the type of cultivar, and maybe also which type of extraction method was used.

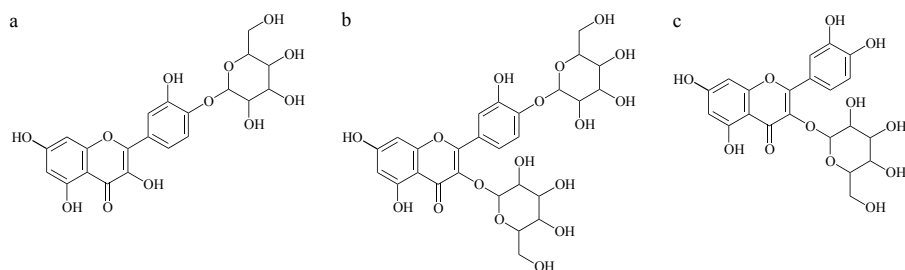


Figure 9. Structure of (a) quercetin-4'-glucoside, (b) quercetin-3,4'-diglucoside and (c) quercetin-3-glucoside.

That the quercetin content varies among different onion cultivars and from onion to onion can be a problem in the development and optimisation of analytical methodologies for extraction and analysis of quercetin derivatives because it might be difficult to determine whether an increase in the quercetin yield depends on the method used or that different onions were used in the different experiments. To avoid this problem, a large batch of onions should be used throughout a project, because this minimises difference in yield due to difference in quercetin content, see further discussion in section 5 *Extraction*.

Since the range of the reported amount quercetin in onion is large, it is difficult to compare results of own experiments with other published results. However, if one wants to compare the extraction yield using a newly developed technique you can do the extraction using a conventional technique and compare the extraction yield results. This was done in **paper IV**, where the on-line hot water extraction and enzymatic hydrolysis was compared to conventional extraction and hydrolysis using aqueous methanol and HCl.

4.2 Analysis and quantification of flavonoids and quercetin

The two common ways used today to determine the flavonoid content in plant extracts are either spectrophotometric assays or (chromatographic) separation combined with different spectrometric detection methods ^{4, 45}. Electrochemical detection can also be used. Before the analysis of a plant extract, some sample pre-treatment is necessary, especially before chromatographic analysis. A plant extract may contain particles, from the plant itself or of analytes that precipitated after sample collection. In the case of chromatographic analysis, it is important to

avoid injecting particles into the system, since this can result in clogging of the tubing and/or column. In the case of precipitation of analytes, the sample can be diluted before analysis and for other particles the sample can for example be filtrated or centrifuged ⁴⁶. In the case of filtration it is important to select a filtration medium that the analytes of interest do not adsorb to, since this can affect both the qualitative and quantitative analysis of the sample. To avoid adsorption of analytes to the filtration medium, centrifugation can be used instead. When onion extract was analysed in **papers I and III-V**, the samples were centrifuged before HPLC-UV analysis.

A frequently used spectrophotometric assay is the Folin-Ciocalteu colorimetric method, which determines the total phenolic content of a sample based on electron transfer between antioxidants in the plant extract and the FC-reagent (oxidant) ⁴⁷. The disadvantage of this and other assays is that they are not specific; that is, other non-phenolic compounds present in the extracts can be detected, and, in addition, no information is given about which antioxidants are present in the extract. On the other hand spectrophotometric assays can be used to study the antioxidant capacity of different antioxidants, by measuring them one by one. One such example is the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) assay ⁴⁸. For example, the antioxidant capacity of different derivatives of a compound can be studied, if the different substituted groups on the aglycone, for example glucose, increases or decreases the antioxidant capacity of a compound. As mentioned in **paper I**, we used the DPPH assay to determine that the antioxidant capacity of the quercetin aglycone is higher than those of the quercetin glucosides.

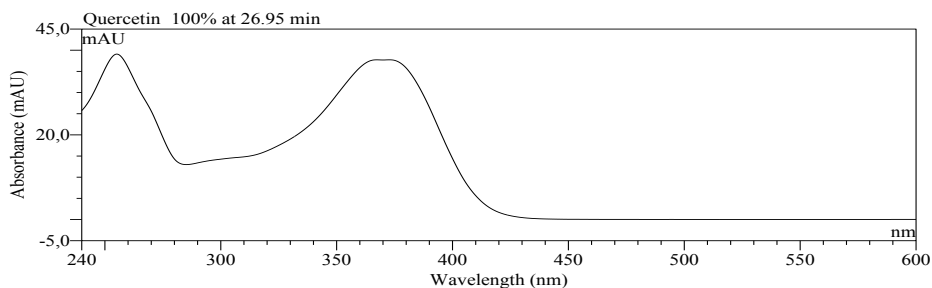
For qualitative and quantitative analysis of specific analytes in a plant extract a separation method has to be employed before detection. Different types of chromatographic separation have been used in the analysis of flavonoids, including GC, thin layer chromatography (TLC), HPLC and CE ^{4, 29, 49}.

HPLC is the most frequently used method for quercetin and flavonoid analysis. The most common separation in HPLC is based on polarity differences between the mobile phase and the stationary phase and the difference in distribution of the analytes between the stationary phase and the mobile phase. HPLC with reversed phase (RP-HPLC) is used for separation of flavonoids, and the most commonly used stationary phase is octadecyl silica; i.e. silica particles, which surface has been modified with C18 chains ⁴. Throughout **papers I-V** a Zorbax SB-C18 column (2.1x100 mm, 3.5 micron) from Agilent Technologies was used. This column withstands pH down to 0.8, which is advantageous since low pH in the mobile phase is needed to avoid unwanted interaction between quercetin and the stationary phase. Initially, 0.1% of formic acid was used in the mobile phase but, due to severe tailing of the quercetin peak, the amount of formic acid was increased to 0.5 % which improved the shape of the peak. That the column

withstands lower pH is also an advantage in the analysis of plant extracts hydrolysed with a high concentration of acid.

The most commonly used detector for flavonoids combined with HPLC is the UV/Vis detector. Flavonoids have two absorptions maximum within the UV-region ⁴. The absorptions maximum differs between the flavonoid classes and also between aglycones and glucosides. In Fig. 10 absorptions spectra of quercetin and Q3,4' can be seen. In **paper I**, kinetics for the hydrolysis of Q3 and Q4' catalysed by *TnBgl1A* and *TnBgl1A_N221S/P342L* were studied. The hydrolysis kinetics of glucose catalysed by β -glucosidases is commonly measured using *para*-nitrophenyl- β -D-glucopyranoside (*p*NPGLc) as substrate (**paper II**). The product *p*NP absorb light at 405 nm and since the substrate does not absorb any light at this wavelength the hydrolysis can be measured in real time in a spectrophotometer. The hydrolysis kinetics using quercetin glucosides as substrate can not be measured in real time since the absorptions spectra of substrate (quercetin glucoside) and product (quercetin) overlap (Fig. 10). In **paper I**, the amount of substrate and product at different time points was instead measured using HPLC-UV at 350 nm.

(a)



(b)

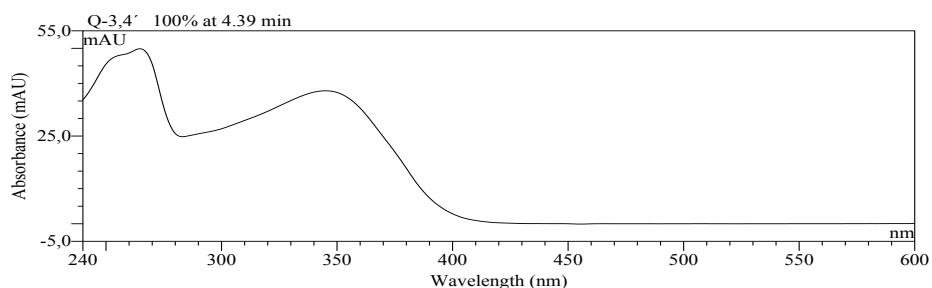


Figure 10. Absorption spectrum of (a) quercetin and (b) Q3,4'.

If the extract of interest contains many different flavonoids, detection at multiple wavelengths may be necessary, since different flavonoids may not absorb light at the same wavelengths. The diode array detector (DAD) provides an alternative, approach, since it can monitor multiple wavelengths simultaneously and can usually collect absorption spectrum for each peak. The absorption spectrum can thus be used for identification of analytes⁵⁰, and also to evaluate the purity of a peak, i.e. if a peak contains only one or more than one analyte.

Flavonoids can be identified and quantified using UV/Vis detection by comparing retention time, absorption spectrum and peak area with standards. Plant extracts may contain many different flavonoids and other polyphenolic compounds. This can be a problem in the identification and quantification step, since the price and lack of availability of standards can slow down and hinder identification and quantification. This is especially true for the derivatives of the aglycone, for example glycosides, since the relevant standards are not commercially available or are very expensive. This problem can be partly solved, by hydrolysing the glycosides to the aglycones. The hydrolysis can be catalysed using high

concentrations of acid ⁵¹⁻⁵³ or by employing enzymes ^{39, 52, 54-55}. After hydrolysis the total amount of the aglycone can be quantified but not the individual glucosides. The hydrolysis step also decreases the number of analytes, which results in faster HPLC analysis since there are fewer analytes to separate. In **papers I-V**, a UV/Vis detector has been used in combination with HPLC and standards were used to identify and quantify analytes of interest in samples and onion extracts.

Two other detection techniques that can be used for identification and/or structural elucidation of flavonoids in combination with HPLC are mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) ⁵⁶.

The advantage of working with yellow onion extracts compared to other plant extracts is that there is not that many different flavonoids present, which simplifies the analysis, see Fig. 11.

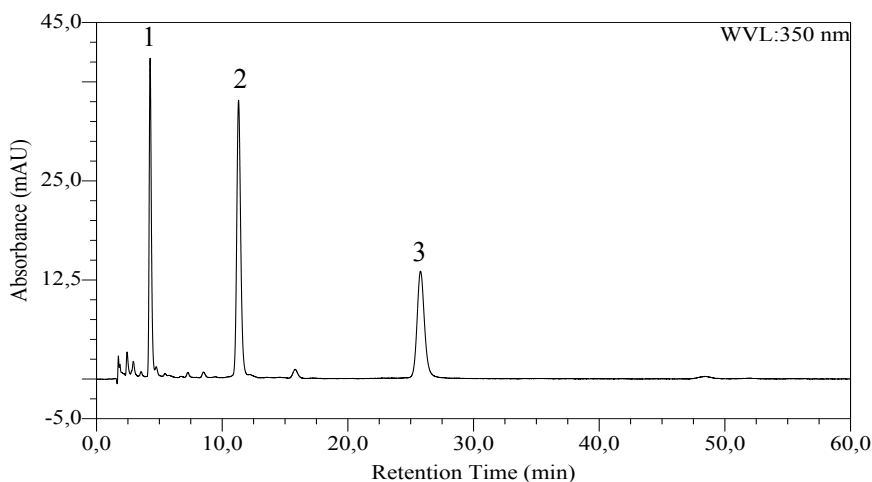


Figure 11. HPLC-UV chromatogram of yellow onion extract, $\lambda=350$ nm. 1=Q3,4', 2=Q4' and 3=quercetin. The other non-identified minor peaks are probably other quercetin derivatives and/or isorhamnetin and kaempferol derivatives.

5 Extraction

In this section some extraction theories are discussed, along with different parameters to study in processes for the extraction of flavonoids from plant material. An extraction process can be divided into five steps; *(i)* wetting of the matrix, *(ii)* desorption of the analyte from the matrix, *(iii)* solvation of the analyte in the extraction solvent, *(iv)* diffusion of the analyte out of the matrix and *(v)* diffusion of the analyte through the stagnant solvent layer around the matrix out into the solvent ⁵⁷. These different steps are limited by different parameters, such as the selection of extraction techniques as well as extraction solvent and temperature, pressure, time and flow rate used in the extraction. There is a number of different techniques for the extraction of flavonoids. Here some commonly used techniques will be presented along including the one used in this PhD project.

5.1 Solvent

Selection of extraction solvent is a crucial step in the design of the extraction process. The solvent selected should have a high solubility of the analyte but low solubility of the matrix and other non-interesting compounds in the matrix; i.e. the solvent should be selective. If one selects a solvent that dissolves “everything”, the extraction is not selective and an extra post-treatment step might be necessary before analysis of the sample. If solubility data of the analyte is available for different solvents, it can be used in the selection of extraction solvent ⁵⁸. If no solubility data is available some general guidelines/tools can be applied. As a first approximation, if the analyte is polar, a polar solvent should be used. There are also commercially available software to estimate the solubility, for example Hansen solubility parameters ⁵⁹. The solubility of standards in different solvents can be studied by an initial screening experiment. However, the fact that the analyte is easily dissolved in a certain solvent does not ensure that the extraction method will be efficient. The solvent used also has to be able to penetrate the matrix and disrupt bonding/interactions between the analyte and the matrix.

A mixture of different solvents can also be used in the extraction process to increase the yield and efficiency of the process. In the extraction of anthocyanins, water-ethanol mixtures ⁶⁰ and water-ethanol-formic acid mixtures ⁵⁰ have been used. In the extraction processes, the pH of the extraction solvent can be an

important factor and in such cases, acids or bases are added to the extraction solvent. The reasons for controlling the pH of extraction solvent can be to avoid degradation or to increase the solubility of the extracted analytes, but may also be used to disrupt the interaction between matrix and analyte.

In **paper IV** aqueous buffer with different concentrations of ethanol was used as extraction solvent. A buffer was used with the optimal pH for the hydrolysis reaction using an immobilised enzyme, since the extraction and hydrolysis was performed in an on-line system. Ethanol was added to the extraction solvent to improve the extraction efficiency and also to avoid precipitation of quercetin due to the decreased solubility of quercetin in the extract collected at room temperature. Another factor that can be considered in selection of solvent, as discussed in section 3 *Sustainable Chemistry*, is the environmental impact and the toxicity of the solvent used¹³. Other important properties of the extraction solvent in the extraction process are surface tension and viscosity.

A low surface tension of the solvent will improve the wettability of the matrix, and a low viscosity will improve the transport of analyte out of the matrix. Some extraction solvent properties such as density, viscosity, surface tension and polarity can be affected by varying temperature and pressure. In section 5.6 *Extraction of flavonoids* the different extraction techniques and different kinds of solvent used for extraction of flavonoids from plant material are described.

5.2 Temperature and Pressure

Temperature is an important factor in the extraction process. An increase in temperature usually results in a faster extraction process because the desorption of the analyte from the matrix is improved due to the extra energy added. In addition, the viscosity and surface tension of the extraction solvent decrease, whereas the diffusivity increases, all of which improve the wetting of the matrix and the transport of the analyte out of the matrix⁵⁸. For example, in a study by Turner *et al.*³⁹, the extraction temperature was varied and the extraction yield of quercetin from yellow onion increased with increasing temperature. In the case of polar solvents, for example water, an increase in temperature also decreases the dielectric constant of the solvent, i.e. the “polarity” of the solvent. This effect is for example used in pressurised fluid extraction (PFE), which is further discussed below (5.6 *Extraction of flavonoids*). Furthermore, desorption of analyte from the matrix is promoted with higher temperature since the intermolecular interactions that binds the analyte to the matrix are reduced.

Though an increase in temperature may increase the solubility of the analyte, it can also increase the solubility of other compounds and the matrix, which results in a less selective extraction. Rodríguez-Meizoso *et al.*⁶¹ showed that by

increasing the temperature in PHWE between 25 °C and 200 °C the dry matter extraction yield increased from 29% to 54%. Therefore it is important to find the optimal temperature for the extraction of an analyte so as to avoid loss in selectivity.

Using high temperatures is a disadvantage if the analytes of interest are thermolabile, because then degradation of the analyte can occur during the extraction step. An example of flavonoids that are sensitive to heat is anthocyanins. Quercetin, the compound studied in **papers I-V**, is less temperature sensitive. Tests using quercetin standard dissolved in citrate-phosphate buffer at 95 °C for 1 hour showed no sign of degradation (unpublished data). Ko *et al.*⁶² noticed a decrease in quercetin extraction yield from yellow onion when temperatures above 165 °C were used, an effect which could have been due to the degradation of quercetin.

Pressure itself does not have a great effect on the extraction efficiency in PFE when for example water⁶³ or a mixture of dichloromethane and acetone⁶⁴ were used as extraction solvent. Pressure has a low effect when water is used as extraction solvent, since the compressibility of liquids is low. However, even though the pressure only has a minor effect, it may help to force the solvent into the pores of the matrix⁶⁴⁻⁶⁵. On the other hand, when scCO₂ is used as extraction solvent, the pressure is an important factor, since by changing the pressure the density of the scCO₂ is drastically modified, leading to a dramatic change in the solvating power and solubility of the analyte of interest.

5.3 Time

The optimal time for extraction differs depending on the type of analyte, matrix and extraction techniques used. In the development of an extraction method, the extraction yield of the analyte can be studied as a function of extraction time, to find the time when no more analyte is extracted under the applied conditions. However, if there are strong interactions between matrix and analyte that are not disrupted during the extraction there might still be analyte left in the matrix, even though nothing or very low amounts are detected.

Conventional solid-liquid extraction can take several hours, and even up to a day, whereas PFE is usually complete in less than one hour⁶⁶. Most extraction processes can be performed quantitatively in a few cycles, where the extraction solvent is partly or completely replaced between each cycle. The extraction yield may be higher when the extraction is carried out in several cycles where the solvent is replaced in-between, since the extraction will not halt due to saturation of the analytes in the extraction solvent. Turner *et al.*³⁹ optimised the extraction of quercetin from yellow onion using PHWE, and the highest extraction yield was

achieved using two or three cycles instead of one. However, since both total extraction time and amount of solvent used differed between the experiments, it is difficult to conclude whether the increased extraction yield was due to longer extraction time or to the fact that the solvent was replaced ³⁹. In **paper IV** extraction profiles of quercetin, Q3,4' and Q4' from yellow onion were collected to see how much time was needed with extraction solvents of different composition, and after 90 minutes of extraction only negligible amounts of quercetin derivatives were detected in the eluting extract when a flow rate of 3 ml/min was used. However, when the flow rate was 1 ml/min the extraction time needed for complete extraction was 240 min. These results are further discussed in *5.7 Extraction of quercetin from onion*.

Finally, it has to be noted that interactions between different extraction parameters might occur in the extraction process. For example an increase in temperature might decrease the extraction time necessary. To find the optimal conditions in the development or improvement of an extraction method, there are many parameters to vary, including type and amount of solvent, temperature, pressure, time, pH, and particle size and different possible pre-treatment steps. The list can be long, which results in the need to perform an enormous number of experiments if one parameter is changed at a time. To decrease the number of experiments and also to study the effects of the interactions between different factors, an experimental design can be done ⁶⁷.

5.4 Static extraction vs continuous flow extraction

An extraction can be performed either in a static or in continuous flow mode. For each mode, different instrument set-ups are necessary and the optimal conditions may differ. In static mode the extraction solvent is not continuously replaced, but is partly or completely replaced after some time if more than one extraction cycle is used ⁶⁶. In a continuous flow system, there is a constant flow of extraction solvent through the extraction cell ⁶⁶. In Fig. 12, a schematic drawing of a static and a continuous flow extraction system is shown. In **papers I-III** and **V** a static extraction method was used, whereas in **paper IV** a continuous flow extraction method was used.

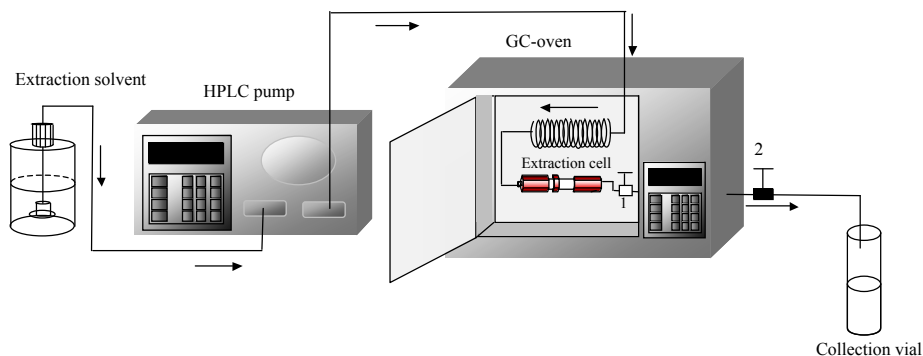


Figure 12. Schematic figure over static or continuous flow extraction equipment. 1 = on/off valve and 2= heated back-pressure regulator. In case of static extraction, 1 is closed during extraction and in case of continues flow extraction 1 is open during extraction.

In a continuous flow system another parameter to study in addition to the ones above is the flow rate. In a continuous flow system, the extraction process is either solubility controlled or desorption controlled ⁶⁸. If the extraction is solubility controlled, higher flow rates will improve the extraction yield per time unit, whereas if it is desorption controlled the extraction yield per time unit will not change with the flow rate. Of course the extraction can first be solubility controlled and then desorption controlled, depending on where in the matrix the analyte(s) are located and how they interact with the matrix. In **paper IV**, extraction profiles of total quercetin (Q3,4', Q4' and quercetin) yield was studied using two different flow rates, 1 ml/min and 3 ml/min. In Fig. 13 it can be seen that the quercetin yield is higher when the flow rate was 3 ml/min compared to 1 ml/min. A reason why the total quercetin extraction yield is lower using 1 ml/min compared to 3 ml/min, even though very similar total volumes of solvent was used, might be due to degradation or adsorption of the quercetin derivatives. Compare for instance the accumulative yield after 80 min (3 ml/min) with 240 min (1 ml/min) in Fig. 13.

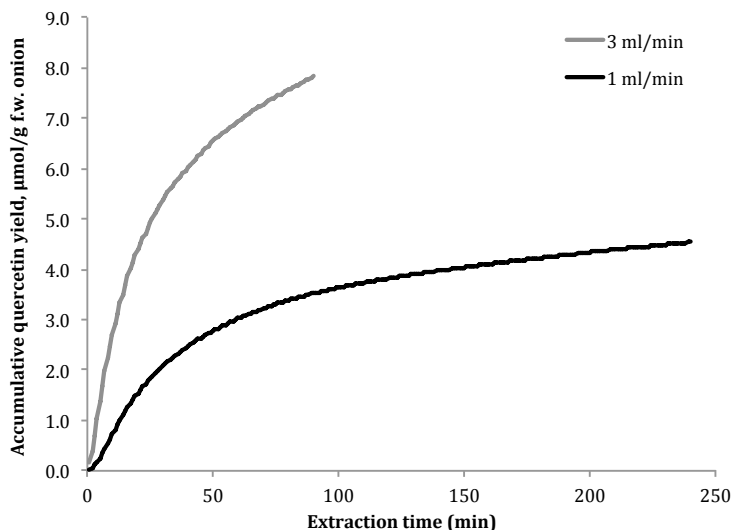


Figure 13. Extraction profiles of total accumulative quercetin derivatives (Q3,4', Q4' and quercetin) yield as function of time using a flow rate of 1 ml/min or 3 ml/min. f.w. = fresh weight. From **paper IV**.

5.5 Sample pre-treatment of plant material prior to extraction

Before extraction, the plant material can be pre-treated in different ways, for example, drying by air or by freeze-drying, and by reducing particle size. The extraction can also be carried out on fresh or frozen plant material. In freeze drying a sample is frozen and then the frozen water within the sample is removed by applying vacuum, and the water goes from solid state to gas, in the so-called sublimation process. A reason for freeze-drying or freezing the plant material is that the material can be stored for a long time without causing any change in the plant material composition. Another reason to freeze dry the plant material is that water is removed which otherwise can disturb the extraction process if scCO_2 or other non-water miscible solvents are used.

Freeze-drying or freezing the material allows one to work with the same material throughout a project, i.e. one can chop up many onions and freeze them in a large batch, and then take samples from the same batch throughout the project (**paper IV**). Working with the same sample throughout a project eliminates variation in results due to the use of different samples. In the case of onion in **paper IV**, we wanted to ensure that any difference in for example extraction yield was not due to

different batches of onion being used, especially since the quercetin content of onions can differ quite a lot. However, it is important to be aware that there might be a change in the composition of the material overtime even when it is stored in a freezer.

Pre-treatment by freezing or freeze-drying may also affect the extraction yield. Pérez-Gregorio *et al.*⁶⁹ compared the extraction yield of Q3,4' and Q4' from red onion using fresh, frozen or freeze-dried material and found no significant effect on the extraction yield when frozen onion was used instead of fresh onion⁶⁹. However, when the results between freeze-dried and fresh onion were compared, there was a significant increase in the extraction yield for the freeze-dried sample when methanol-water-formic acid was used as extraction solvent. Of course, this kind of pre-treatment depends on what kind of compounds are being studied, because the pre-treatment must not be one that degrades the compounds

The particle size of the plant material may have an effect on the extraction efficiency of the compound studied⁷⁰⁻⁷³. Usually, the smaller the particle size, the better the extraction efficiency. This effect is for example due to larger surface area per gram of plant material and to the fact that cell walls can be disrupted and that compound trapped within cells are more easily extracted. In **papers I and III-V** the onions used were either chopped using a kitchen knife or an “onion cutter”. The advantage of using the onion cutter (**paper IV**) is that onion pieces used throughout the project were of the same size. If the sample size of the analysed plant material is the same throughout the project, the effect of particle size can be excluded from the discussion about differences in extraction yield in different experiments. In **paper IV**, the extraction was quite slow, and it is possible that the extraction time could have been shorter if smaller onion pieces had been used.

As discussed in section 3 *Sustainable Chemistry*, it is important to take into account the environmental impact of each extra step in the production chain. If the aim is to develop large-scale processing, addition of many pre-treatment steps before the extraction might increase the environmental load, which may not be worth a small increase in extraction yield.

5.6 Extraction of flavonoids

In this section different extraction techniques used today to extract flavonoids from plant material are presented. The technique used throughout **papers I and III-V**, pressurised hot water extraction, will be described in detail.

5.6.1 Conventional solid-liquid extraction (SLE)

The conventional way to extract flavonoids from plant material is SLE using organic solvents such as ethanol and methanol, either pure or mixed with water^{4, 49, 74}, although ethyl acetate, acetone and hexane have also been used. Sometimes the extraction solvent is acidified to catalyse the hydrolysis of the glycosylated flavonoids; this approach will be discussed in section 6 *Hydrolysis of flavonoids*. The choice of solvent or mixture of solvents to use during the extraction depends on which compounds are to be extracted and also on whether the solvent selected will increase or decrease the selectivity of the extraction method. The disadvantage of the conventional SLE is the use of the organic solvents, which are harmful both for the environment and for the persons working with them. Interest in replacing the organic solvents has thus increased in recent years and other extractions techniques such as PFE, SFE and MAE have started to become more popular. With these techniques an organic solvent may still be used, but in much lower amounts. **Paper IV** presents a comparison of the quercetin extraction yield from yellow, red and shallot onion when using SLE (aqueous methanol with HCl, 2 h 80 °C) and continuous flow extraction and hydrolysis (water and enzyme, 2 h, 84 °C), see Table 1.

Table 1. Amount of extracted quercetin from yellow, red and shallot onion using conventional extraction and hydrolysis method (μmol/g f.w.) f.w. = fresh weight. Modified from **paper IV**.

Amount (μmol/g f.w. onion)	Yellow onion	Red onion	Shallot onion
SLE (Methanol-HCl)	1.4 ± 0.3 ²	2.5 ± 0.5 ¹	1.6 ± 0.5 ¹
Continuous flow extraction and hydrolysis	3.2 ± 0.2 ¹	3.8 ± 1.3 ¹	3.2 ± 1.2 ¹

¹ n=3, ² n=6

As seen in Table 1, the extraction yield was lower when using SLE. This effect may be due to the degradation of the extracted quercetin due to the harsh conditions (1.2 M HCl)^{51, 53}.

The temperature used in SLE is limited by the boiling point of the extraction solvent used. Another limitation of SLE is the extraction times used, which can be quite long, up to several days⁶⁶. In addition, since SLE is a batch extraction technique, it is important to replace the solvent with fresh solvent during the extraction. Another disadvantage of SLE is that a filtration step is needed before further analysis to separate the extract from the plant material and to remove fine particles⁶⁶.

5.6.2 Pressurised Fluid Extraction (PFE)

In PFE higher temperatures than in SLE can be used, since by applying pressure temperatures above the boiling temperature of the solvent can be used and the solvent is still maintained as a liquid^{58, 64, 75}. PFE is also called pressurised liquid extraction (PLE), pressurised solvent extraction (PSE) and accelerated solvent extraction (ASE). In PFE, more polar solvents such as water and alcohols can replace non-polar solvents such as hexane by using a higher extraction temperature, which increases the solubility of non-polar compounds. As mentioned above, PFE extraction is a more sustainable alternative than SLE, even if organic solvents are used, since the amount of solvent per gram of sample is usually less than with SLE and also the extraction time is shorter^{66, 76}. For extraction of flavonoids from plant materials, there are a number of published PFE methods and commonly used solvents are water, methanol and acetone⁷⁷. When water is used as extraction solvent, the method is called pressurised hot water extraction (PHWE). An advantage of using PFE is that the extract can be filtered before collection by the addition of a filter in the outlet of the extraction cell. In both the static and continuous flow system used in **papers I and III-V**, the onion extract passed through a glass fibre filter and/or a stainless steel filter.

5.6.3 Pressurised Hot Water Extraction (PHWE)

Pressurised fluid extraction using water as extraction solvent above its boiling point can be called subcritical water extraction (SWE) or pressurised hot water extraction (PHWE), but PLE, PFE, PSE and ASE are other names used in publications. The fact that one technique has many different names is a drawback in the literature. In literature searches, publications can be missed if one does not know all the different names used, and in the case of publishing, authors may not reach readers because of the choice of name used.

In this thesis the abbreviation PHWE will be used when water was the extraction solvent. In PHWE, the water used as extraction solvent has a temperature of 100 to 374 °C^{65, 78-79}, i.e. between the boiling point and the critical point of water. The water is kept as a liquid by applying pressures between 10 and 80 bar⁷⁹. If temperatures and pressures above 374 °C and 221 bar are used, the water is in its supercritical state. The use of supercritical water as extraction solvent is not common since the water is then usually extremely corrosive, which can be bad for both the analytes extracted and the instrument. PHWE has been used to extract flavonoids from different plant materials, for example quercetin from onion³⁹, quercetin-3-galactoside, kaempferol and isorhamnetin from seabuckthorn leaves⁸⁰ and dihydrokaempferol and naringin from aspen knotwood⁸¹. The advantage of using water as extraction solvent is that it is non-toxic, non-flammable, environmentally friendly and easily available. Water is a polar solvent at ambient conditions, which results in low solubility of organic compounds such as

flavonoids. However, as already mentioned, the “polarity” of water can be decreased by increasing the temperature⁶³. The dielectric constant, ϵ , of water at ambient conditions is 90, but at 100 °C it is around 65 and at 250 °C, around 35⁶³. The dielectric constant of water at 250 °C is the same as for methanol at ambient conditions⁶³. The most important factor controlling the extraction in PHWE is the temperature, which has been discussed above in section 5.2 *Temperature and Pressure*. Sometimes an organic modifier such as ethanol or methanol is added to the water to improve the extraction yield by enhancing the solubility of the analytes in the extraction solvent^{65, 79} and by improving the wetting of the matrix. In **paper IV**, the extraction profiles of quercetin derivatives were studied using only aqueous buffer or aqueous buffer with 5% of ethanol as extraction solvent, see discussion in section 5.7 *Extraction of quercetin from onion*.

Commercial pressurised fluid extraction systems are currently available, but it is also possible to build PFE systems in the lab. To do so, a solvent reservoir, a pump, a heater, an extraction vessel/cell, a pressure regulator and a collection vessel are needed^{65, 79}. Before extraction it is important to degas the water to remove the dissolved oxygen, which otherwise may destroy the instrument at high temperatures and/or oxidise the extracted analytes. In **papers III-IV** an extraction system was built consisting of a gradient HPLC-pump and a GC-oven. Since the extraction solvent was not preheated before being pumped into the system it was important, to have a sufficiently long pre-heating coil before the extraction vessel (Fig. 12). In the home-built system used in **papers III-IV**, no backpressure regulator was needed, since temperatures below the boiling point of water were used.

5.6.4 Other enhanced extractions techniques

Three other extraction techniques, which are referred to in the literature as more sustainable alternatives, are supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE).

Supercritical Fluid Extraction (SFE) is an alternative extraction technique, and the most commonly used supercritical fluid being scCO_2 ⁷⁸. A supercritical fluid has a liquid-like density, gas-like viscosity, high diffusivity and zero surface tension. Supercritical CO_2 is obtained by applying a pressure above 72.9 atm and a temperature above 31.2 °C. The advantage of using scCO_2 extraction is that scCO_2 is non-toxic and non-flammable. There are a number of publications reporting on the use of scCO_2 to extract flavonoids from different types of plants, one example being the extraction of (+)-catechin, (-)-epicatechin, rutin, quercetin and trans-resveratrol from grape skin⁸². Another advantage of using scCO_2 is that after extraction the system is depressurised and the scCO_2 turns into gas leaving a dry extract behind, i.e. the extraction solvent is easily removed compared to other extraction techniques. This is also an advantage from an energy efficiency and

environmental point of view, since transport of a dry extract is more efficient than it is transporting the high-value compound dissolved in low concentration in a solvent. scCO_2 is a non-polar fluid, so for extraction of medium polar to polar compounds an addition of a co-solvent is commonly used to increase the polarity of the extraction solvent and the solubility of the extracted compounds ⁷⁸.

In microwave-assisted extraction (MAE) the extraction solvent is directly heated by the microwaves, a method which is much more efficient than conventional heating, where the solvent absorbs heat from the extraction vessel ⁸³. The solvent is heated due to ionic conduction and the dipole rotation of the solvent molecules. In MAE, the use of polar solvents is preferable since non-polar solvents, for example hexane, may not absorb the microwave energy and commonly used solvents in MAE are thus ethanol, methanol and acetone ⁸³⁻⁸⁴. Parameters studied in MAE are for example solvent type and volume, temperature and time. The advantages of using MAE are that it is fast and lower volumes of solvents are needed compared to SLE ⁸³⁻⁸⁴. The disadvantages are that a clean-up step is necessary before further analysis and only solvents that can absorb microwaves can be used ⁸³⁻⁸⁴. MAE has been used for the extraction of flavonoids from plant material, for example anthocyanins from grape skin ⁸⁵ and quercetin from onion ⁸⁶.

Another green extraction technique is ultrasound-assisted extraction (UAE). When a sample is exposed to ultrasound, bubbles are formed within the solvent and these bubbles grow. When they burst, local high pressure and temperatures are created ⁸⁷⁻⁸⁸. When a plant material is exposed to ultrasonic waves, cell walls and membranes may burst, which results in better penetration of solvent into the material and quicker transport out of analytes to the extraction solvent ⁸⁸. The advantages of using UAE are also that it is quick, compared to SLE, and cheap. A disadvantage is for example the risk of degradation of compounds due too the ultrasonic irradiation. In addition, the same solvent to sample ratios as with SLE are used and a clean-up step may be necessary before further analysis to remove particles. UAE has been used to extract flavonoids from plants, for example hesperidin from the citrus Penggan ⁸⁹ and naringin, rutin and others from strawberries ⁹⁰.

5.7 Extraction of quercetin from onion

Onion is a plant material that is widely used for quercetin extraction since it contains high amounts of quercetin. SLE using methanol ^{42, 51, 53, 91} or ethanol ^{55, 92-94} is often used for extraction of quercetin from onions. The extraction may also be combined with hydrolysis of extracted quercetin glucosides using high concentrations of acid ⁵¹⁻⁵³. The hydrolysis step will be further discussed in section 6 *Hydrolysis of flavonoids*. Alternative methods, as discussed above, are for

example PFE ⁹⁵, PHWE ^{39, 62, 96}, MAE ⁸⁶ or SFE ⁹⁷, which have all been used for extraction of quercetin from onion. Turner *et al.* ³⁹ found that the optimal extraction conditions for PHWE of quercetin from onion were, 120 °C for 15 min, whereas Ko *et al.* ⁶² had highest extraction yield at 165 °C and 15 min. Turner *et al.* used the whole onion, whereas Ko *et al.* only looked at the onion skin. In the preparation of onion extract using static extraction described in **papers I and III-V**, the method of Turner *et al.* ³⁹ was used. In **paper IV** a lower temperature was used along with a continuous flow system, since the extraction was combined with enzymatic hydrolysis, and too high temperatures can inactivate the enzyme. In **paper IV** extraction profiles of quercetin from yellow onion were studied using a 20 mM citrate-phosphate buffer of pH 5.5 or 2.6, with 0% or 5% ethanol in a continuous flow system with a flow rate of 3 ml/min. In Fig. 14 the extraction profiles of total quercetin yield are shown for the different conditions. It is clear that the quercetin extraction yield is more than twice as high using 5% of ethanol compared to using 0% of ethanol in the extraction solvent. The increase in quercetin yield is probably due to the increased solubility of quercetin in the extraction solvent. Even higher concentrations of ethanol in the extraction solvent might have improved the extraction yield of quercetin derivatives. However, since the optimisation of the hydrolysis reaction showed that 5% ethanol was optimal for the hydrolysis, this was not further studied. Fig. 14 also shows that the extraction yield at pH 5.5 is higher than at pH 2.6. This difference might be due to the fact that more number of quercetin molecules are charged at higher pH, which would increase the solubility of quercetin in water solutions, see discussion about quercetin solubility in section 5.8 *Solubility of quercetin*. The difficulties of combining extraction and hydrolysis are further discussed in section 7 *Hyphenation*.

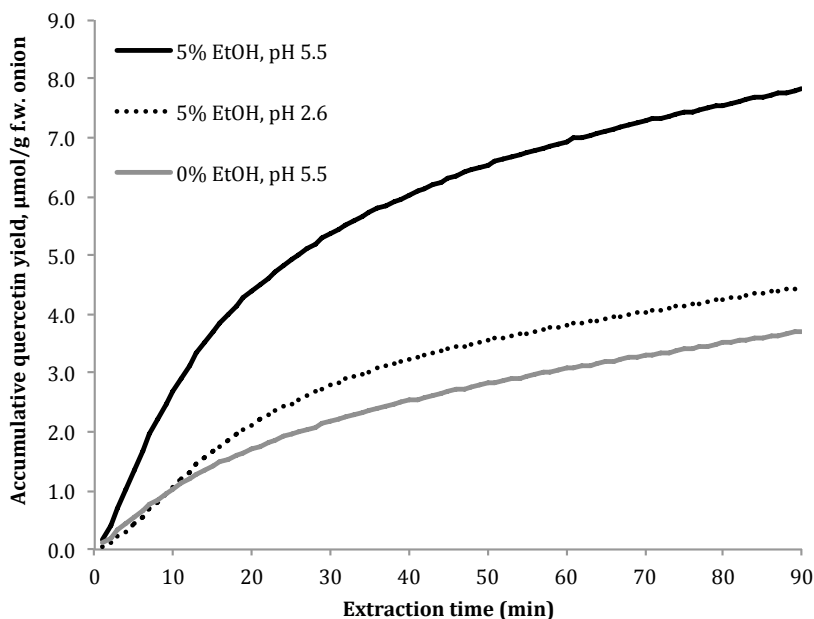


Figure 14. Extraction profiles of quercetin from yellow onion at 84 °C. Accumulative extraction yield of quercetin (Q3,4', Q4' and quercetin) as a function of extraction time with a flow rate of 3 ml/min, (-) pH 5.5, 5% ethanol, (-) pH 5.5, 0% ethanol and (--) pH 2.6, 5% ethanol. From paper IV.

5.8 Solubility of quercetin

Water was used as extraction solvent throughout the projects. However, the solubility of quercetin is very low in water, 0.002 g/l (at 25 °C) ⁹⁸, and the low solubility of quercetin in water was one of the major obstacles throughout the projects described in this thesis. Quercetin has higher solubility in other solvents such as ethanol ⁹⁹, methanol ⁹⁹ and acetone ¹⁰⁰, but we chose to work with water for two reasons: water is a non-hazardous solvent and that the enzymatic hydrolysis would be more difficult in organic solvents. Srinivas *et al.* ⁹⁸ showed that the solubility of quercetin in water increased with increasing temperature, from around 0.002 g/l at 25 °C to 0.7-1.5 g/l at 140 °C. Quercetin derivatives, such as glycosides and sulfates have higher solubility in water than the aglycone, and the solubility is also affected by the position where for example the glucose is attached on the quercetin molecule ¹⁰¹⁻¹⁰². Murota *et al.* ¹⁰¹ showed that the distribution constant (octanol-water) differs among the four quercetin derivatives that were analysed, quercetin, Q3, Q4' and Q3,4', which are the main quercetin derivatives present in yellow onion. The diglucoside, Q3,4', had the lowest

distribution constant (0.005), and quercetin the highest (65.7), but a difference was also seen between Q3 and Q4', where Q3 had a lower constant (5.0) than Q4' (20.7)¹⁰¹.

The solubility of quercetin decreases with decreasing temperature. This can result in precipitation of quercetin in the hot water extract collected, however, there are different ways to avoid such obstacle. In **papers I, III and V** the extraction solvent used was water and the extract collected was diluted with water. In **paper IV** ethanol or methanol was added to the collected extract to avoid precipitation of quercetin. pH is another parameter to play around with to control the solubility of quercetin. Quercetin has ionisable hydroxyl groups and the pKa values reported in the literature differ, depending on which method has been used to determine them¹⁰³. The lower pKa value of quercetin is around 6.6-7.7¹⁰³⁻¹⁰⁵. In the case of extraction with water, it can be favourable to work at a pH where quercetin is charged, since this may improve the solubility of quercetin in the water. Furthermore, in addition to the effect on solubility, pH or ions can also help in the extraction process by disrupting interaction between analyte and matrix.

6 Hydrolysis of flavonoids

Catalysts are used to increase the rate of a chemical reaction without itself being consumed. Acids and enzymes are examples of catalysts and when an enzyme or whole cells are used as catalysts in a reaction, it is called biocatalysis. As mention in section 3 *Sustainable Chemistry*, the LCA in **paper I** showed that the use of an enzyme in the place of an acidic catalyst had reduced environmental impact. In **papers I-IV**, a thermostable β -glucosidase from *Thermotoga neapolitana*, termed *TnBgl1A* (Fig. 15), was used to catalyse the hydrolysis of quercetin glucosides to quercetin. The motivation for having a hydrolysis step are that (i) separation and quantification is simplified since the number of analytes are decreased, (ii) quercetin is a stronger antioxidant without attached sugars and (iii) increased yield of quercetin. In this section, the hydrolysis of quercetin glucosides, the specificity of the *TnBgl1A* enzyme and the immobilisation of this enzyme will be discussed.

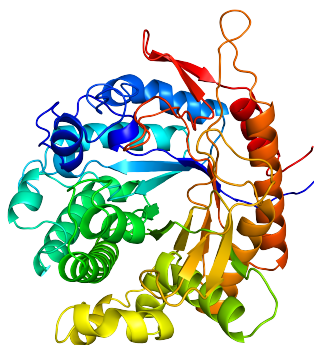


Figure 15. Three-dimensional structure of *TnBgl1A*.

6.1 Hydrolysis of quercetin glucosides

The extraction of flavonoids from a plant material can be combined with the hydrolysis of the glycosylated compounds and, as mentioned before, acids or an enzyme can be used as catalysts. As acid catalyst, high concentrations of HCl are commonly used⁵¹⁻⁵³. The disadvantage of using acid catalysed hydrolysis of flavonoid glycosides is that the conditions are quite harsh, which can result in

degradation of the analytes^{51, 53}. Both Hertog *et al.*⁵¹ and Nuutila *et al.*⁵³ showed that the extracted and hydrolysed quercetin started to degrade if the extraction and hydrolysis time was too long. Another disadvantage of using HCl is that the samples have to be analysed directly after collection to avoid degradation of the flavonoids. If the samples are not analysed directly, inadequate determination of the amount of flavonoids present in the sample can result. When enzymes are used as catalyst, the reaction conditions are not at all as harsh. However, some conditions, for example pH and temperature, have to be controlled to ensure that the enzyme is active in the reaction media.

In **paper IV**, extraction of quercetin glucosides using aqueous methanol with 1.2 M of HCl was used to compare the results when using PHWE and *TnBgl1A_N221S/P342L*, i.e. *TnBgl1A* point mutated at residues 221 and 342, catalysed hydrolysis, see Table 1 in section 5.6.1 *Conventional solid-liquid extraction (SLE)*. The result was that the extraction yield of quercetin was lower using the conventional method. This can for example be due to degradation of quercetin due to the harsh acidic conditions, but less severe for extraction and hydrolysis using red onion (Table 1). A reason for this can for example be that other antioxidants in the red onion were co-extracting with quercetin, which perhaps protected quercetin from degradation. The relative standard deviation (RSD) for the replicates, using conventional extraction, was around 20-30%. This deviation can for example be due to the samples having different waiting times before being injected into HPLC-UV for analysis. When PHWE and enzyme-catalysed hydrolysis was used the RSD of shallot and red onion were higher than for yellow onion, which may be due to that a smaller sample size was used. The RSD of the extraction and hydrolysis, both methods, might have been improved by increasing sample size and number of replicates.

6.2 β -glucosidase from *T. neapolitana*

In **papers I-IV** a β -glucosidase was used as biocatalyst. β -glucosidases are glycoside hydrolases (GH), which are enzymes that under physiological conditions catalyse the hydrolysis of glycosidic bonds between two carbohydrates or between a carbohydrate and a non-carbohydrate¹⁰⁶⁻¹⁰⁷. Under defined conditions β -glucosidases can also catalyse reverse hydrolysis, i.e. synthesis of glycosidic bonds¹⁰⁶⁻¹⁰⁷. GHs are classified under families, in a system based on sequence and structure similarities¹⁰⁸. The glycone and aglycone specificities of different β -glucosidases differ¹⁰⁶. In **papers I-IV** a β -glucosidase from the GH1 family was used to catalyse the hydrolysis of quercetin glucosides to quercetin and glucose. The enzyme used was recombinantly expressed in *Escherichia coli*, and originated from the thermophilic bacterium *Thermotoga neapolitana* (*T. neapolitana*).

Thermophiles belong to a group of organisms called extremophiles. Extremophiles are organisms that live in extreme physical environments, for example at high temperatures, high pressure and high or low pH¹⁰⁹⁻¹¹⁰. Thermophiles live at high temperatures¹⁰⁹ and *T. neapolitana* is a hyperthermophile since it can grow at temperatures above 80 °C¹¹¹. From extremophiles, so-called extremozymes can be expressed that are active at extreme physical conditions of for example pH and temperature¹¹⁰. Since such enzymes are expressed from a thermophile, they are thermostable and active at the same extreme conditions as where the thermophiles grow. The main reason why a thermostable enzyme was used in **papers I-IV** is that the extraction is performed at high temperatures, and as a result no substantial cooling is needed between the extraction and hydrolysis steps. It was thus easier to combine the steps in an on-line continuous flow system (**papers III-IV**). Another important reason is that the reaction will go faster at higher temperature since the mass transport of the substrate and product will be faster.

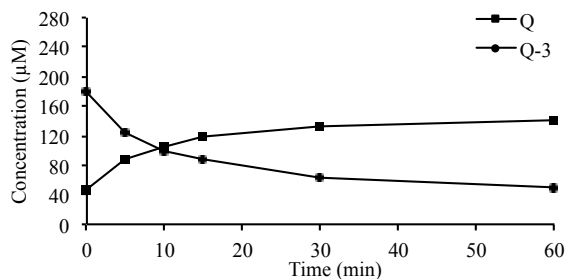
In **paper I**, the hydrolysis of quercetin glucosides standards using *TnBgl1A* and *TnBgl1A_N221S/P342L* were studied at pH 5.0 and 90-95 °C. The temperature was selected from earlier results by Turner *et al.*³⁹ and from the unfolding temperatures in **paper II**, which was around 100 °C. In addition to be active at high temperatures, another advantage of working with thermostable enzymes is that they are almost inactive at room temperature. This property was utilised when the hydrolysis of quercetin glucosides was studied with time, because the samples collected at different time points were directly cooled by addition to a vial at room temperature, which contained a mixture of methanol, water and acid. Thus the enzyme activity between sampling and quantification was limited due to three factors: a decrease in temperature, low pH and the presence of methanol. Another advantage of using thermostable enzymes is that they have a longer shelf life compared to non-thermostable enzymes.

6.3 Quercetin glucoside specificity of *TnBgl1A*

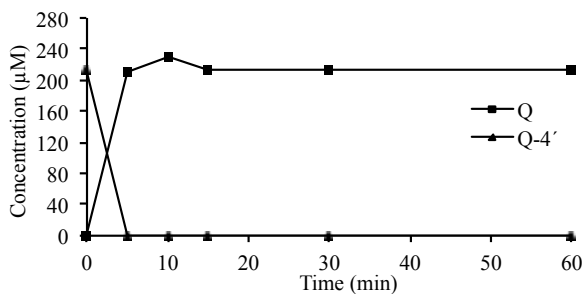
By using enzyme-catalysed hydrolysis, the degradation of the extracted flavonoids can be avoided, due to the resultant mild hydrolysis conditions. The enzyme *TnBgl1A* was selected as biocatalyst since it is thermostable and catalyses the hydrolysis of the glycone glucose, a group commonly substituted to the flavonoids in onion. A disadvantage of using this enzyme for hydrolysis of quercetin glucosides was shown by Turner *et al.*³⁹. There it was reported that the hydrolysis of glucose attached to 3-position of quercetin was slower than glucose attached at 4'-position, using *TnBgl1A*. Similar hydrolysis patterns were also shown when they tested another β -glucosidase named *TnBgl3B* (GH3 family). The reason for the slow hydrolysis was thought to be steric hindrance when the glucose was at

3-position³⁹. In **paper I** the hydrolysis of Q3,4', Q4', Q3 using *TnBgl1A* at 95 °C was studied (Fig. 16).

(a)



(b)



(c)

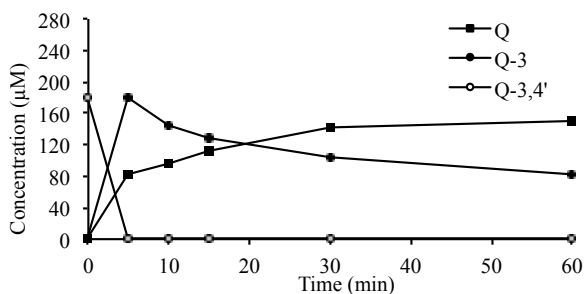


Figure 16. Catalysis by *TnBgl1A* with (a) Q3, (b) Q4' and (c) Q3,4'. 20 pmol of *TnBgl1A* and 200 nmol of substrate were used. Relative standard deviation (RSD) of the concentration values at the different sampling time points is ranging between 0.3 and 20.6% (n=3). Modified from **paper I**³.

By comparing Fig. 16a and 16b it is clear that the hydrolysis of glucose at 4'-position is faster than at 3-position. Fig. 16c also shows that in the case of Q3,4', it is glucose at 4'-position that is first hydrolysed, since only Q3 is detected after addition of enzyme. This finding is also supported by docking studies of the Q3,4' in the active site, which showed that in the diglucoside only the 4'-attached glucose could fit in the active site (unpublished data).

One way to improve the substrate affinity of an enzyme is to change the amino acid composition in the active site of the enzymes by mutation. In **paper II** mutants of the *TnBgl1A* were constructed. The amino acids of interest were F219, N221 and G222, since they are located close to the aglycone binding site in the active site. Four of the mutants and the wild type enzyme were used in a screening experiment to determine whether any of the mutations improve the conversion of Q3 to quercetin compared to the wild type enzyme (Fig. 17). The result was that one of the mutations, N221S/P342L, had higher conversion efficiency than the wild type and this enzyme variant was therefore used in **papers I, III and IV**.

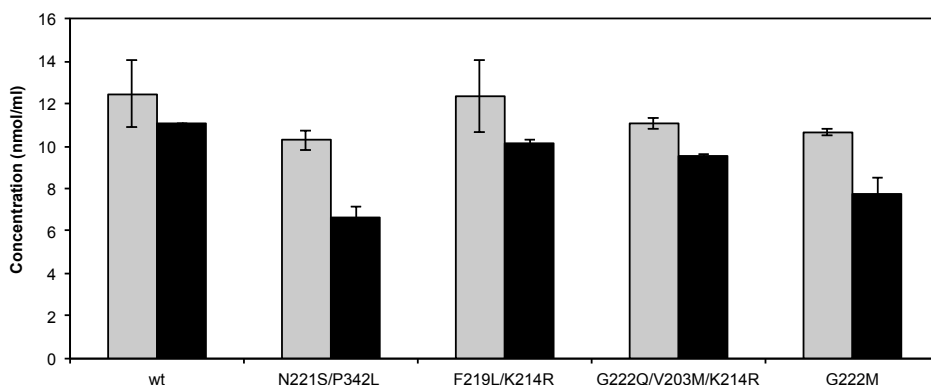


Figure 17. Analysis of quercetin-3-glucoside (Q3). Grey bars represent the Q3 concentration at time 0 min, and black bars the Q3 concentration remaining after 5 min incubation with the enzyme. Modified from **paper II** ¹¹²

In **paper I**, kinetics were measured for the hydrolysis of Q3 and Q4', using *TnBgl1A_N221S/P342L* (named *TnBgl1A_N221S* in **paper I**) and the wild type enzyme, *TnBgl1A*. The result was that the Michaelis Menten constant, K_m , was lower for Q4' than for Q3 for both enzymes and that the mutant showed lower K_m than the wild type, using either one of the two substrates. The conclusion is that the mutation improved the affinity of the enzyme for the quercetin glucosides, but

that the affinity for Q4' is still higher than for Q3. In **paper II** the reason why the affinity increased with the mutation of N221S was investigated by modelling of the substrate with the 3D structure of the enzyme. The model suggests that this is due to an additional hydrogen bonding to the hydroxyl group at 5-position compared to the wild type enzyme.

6.4 Immobilisation of the enzyme

Enzymes can be immobilised to allow their reuse and to simplify separation of the enzyme from the product in a biocatalytic process¹¹³⁻¹¹⁴. In **paper III**, the wild type enzyme, *TnBgl1A* and the mutant *TnBgl1A_N221S/P342L* were immobilised on two different support materials. When an enzyme is immobilised, the stability, activity and selectivity of the enzyme can be altered¹¹⁵. There are different immobilisation strategies but commonly used ones include support binding, entrapment, encapsulation and crosslinking¹¹³. Different kinds of materials can be used in immobilisation depending on the type of interaction between the support material and the enzyme. It is thus important that the surface properties of the enzyme and the support material match each other¹¹⁴. Other important factors in selecting support material are to select a material with high surface area and one that is chemically and mechanically stable¹¹⁴. In **paper III** enzymes were immobilised via support binding. In this method enzymes are linked to the support material via for example dispersive interactions, ionic bonds or covalent bonds¹¹³. If the enzymes are to be used in an aqueous system and/or at high temperatures, covalent bonding is a good alternative since then leaching of the enzyme from the support material is avoided^{113-114, 116}. In covalent binding the side chain of the amino acids can be utilised, for example amino, thiol, phenolic and imidazole¹¹⁷.

Common commercially available support material in covalent binding are for example Eupergit[®] C and Sepabeads[®]¹¹⁶, which both have epoxy groups on the surface that can form a covalent bond with the amino acid side chains mentioned above¹¹⁷. In **papers III-IV**, Eupergit[®] C, Eupergit[®] C 250 L and cryogel were used as support materials and they are all epoxy-activated materials. Eupergit[®] C and Eupergit[®] C 250 L are acrylamide-based beads¹¹⁸. The difference between Eupergit[®] C and Eupergit[®] C 250 L is pore size and number of oxirane groups per gram of material. The cryogel used was a acrylamide-based supermacroporous monolithic gel¹¹⁹.

One of the aims with immobilisation of *TnBgl1A_N221S/P342L* was to enable the use of the enzyme in a continuous flow system for on-line extraction and hydrolysis of quercetin glucosides from onion. Since the extraction of quercetin is performed at high temperatures, two different thermostability experiments were performed of Eupergit[®] C and cryogel in **paper III**. In one experiment the support

material (without enzyme) was incubated in buffer at 95 °C for 1 hour and scanning electron microscopy was done on heat treated and non-heat treated material, see Fig. 18.

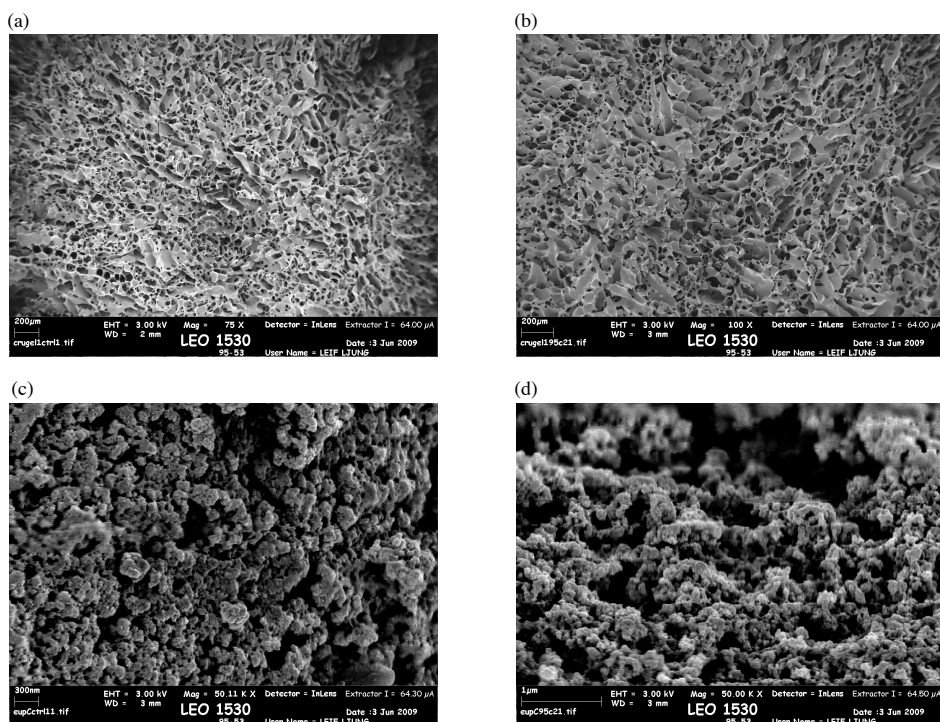


Figure 18. Scanning electron microscope images of (a) non-heat treated cryogel, (b) heat treated cryogel (1 h at 95 °C), (c) non-heat treated Eupergit® C and (d) heat treated Eupergit® C (1 h at 95 °C). From **paper III**.

As seen in Fig.18, there were no obvious changes in the conformation of the support material. The other thermostability experiment carried out with Eupergit® C and cryogel in **paper III** was thermogravimetric analysis (TGA). The result of the TGA was that the material started to decompose around 250 °C. The two experiments show that the material would be stable for use in a continuous flow system for on-line extraction and hydrolysis.

In the immobilisation of enzyme in **paper III**, the effect of bonding the enzyme directly to the epoxy group or via a spacer arm (glutaraldehyde) was studied, as well as the effect of addition of glucose and bovine serum albumin on the enzyme activity. In **paper III** it was shown that the enzyme immobilised on cryogel had

lower specific activity than the enzyme immobilised on Eupergit® C (250 L). One reason why the enzyme activity was lower using cryogel might be the diffusion limitation into the cryogel in the activity measurement. In **paper IV** both Eupergit® C 250L and cryogel were used in the continuous flow system and diffusion limitation was not a problem in this system.

In **paper I**, as already mentioned in section 3.2 *Life Cycle Assessment*, the use of enzyme is environmentally more favourable than HCl since less energy is needed with the former (Fig. 5), and also since HCl is a corrosive solvent. However, when the enzyme is immobilised, the energy performance changes since then you also have to include the support material in the assessment. Kim *et al.*¹²⁰ performed an LCA of three different enzymes immobilised on Sepabeads®. The result was that the immobilisation step had an increased effect on the different environmental impact categories studied.

In **paper IV** a heat stability test was carried out at 84 °C for 144 hours and there was no significant decrease in activity during the first 48 hours of activity measurement. The question is whether the inactivation of the immobilised enzyme is reversible or irreversible. If it is irreversible, then it might not be worth it from an environmental perspective to immobilise the enzyme, and instead enzyme in solution should be used. Of course more studies are needed before this can be concluded and in section 8 *Conclusion and future perspectives* this will be further discussed.

7 Hyphenation

Hyphenation within analytical chemistry is the coupling of different analytical techniques together. It can for example be combining of an extraction technique with a chromatographic separation technique¹²¹, the combination of chromatographic separation together with several detection techniques¹²² or combining chromatographic separation techniques¹²³. In the steps listed for the analysis of the sample in section 3.1 *Green Analytical Chemistry*, the most time-consuming steps are sample collection and sample preparation; they can account for 60-80% of the time of the analysis chain^{8, 124}. One way to decrease the analysis time per sample is to combine different sample preparation steps, separation and/or detection of the sample. Apart from saving time, combining analytical techniques brings other advantages such as reduced risk of contamination, sample loss and degradation of analytes⁸. From an environmental perspective the coupling of different techniques is also an advantage since less solvent per sample is usually used.

7.1 On-line hydrolysis

The immobilisation of *TnBgl1A_N221S/P342L* described in **paper III** makes it possible to use the enzyme in a continuous flow system. The advantages of combining the extraction and hydrolysis of flavonoid glucosides in a continuous flow system are decreased time and less sample handling, and elimination of the step to separate the enzyme from the onion extract. In **paper III**, a test was carried out where a mixture of onion extract spiked with Q3,4' and diluted with buffer was pumped through a cell with *TnBgl1A_N221S/P342L* immobilised on Eupergit C[®] 250 L for approximately 10 min. The result of the test was that almost all quercetin glucosides pumped through the cell, with immobilised enzyme, was hydrolysed to quercetin.

In the work described in **paper IV**, it was decided to first optimise the hydrolysis conditions regarding temperature, pH and concentration of ethanol in the extraction solvent, using a surface response design. The optimal conditions were found to be: temperature 84 °C, pH 5.4 (cryogel) or pH 5.6 (Eupergit C[®] 250L) and 5% of ethanol. In the optimisation of the hydrolysis conditions, the extraction was not included, since the extraction kinetics of quercetin from onion is also

affected by the tested conditions. Instead buffer with a known concentration of Q3,4' was used.

Even though a flow rate of 3 ml/min at the optimal hydrolysis conditions gave the highest extraction yield, as mentioned in section 5.7 *Extraction of quercetin from onion*, in combined extraction and hydrolysis of quercetin glucosides from yellow, red and shallot onion a flow rate of 0.75 ml/min was used. This lower flow rate was used because at higher flow rates quercetin glucosides were detected in the extract collected. Total extraction and hydrolysis time, 2 h, was longer compared to **paper I**, where extraction and hydrolysis was carried out in two steps that together lasted approximately 30-40 minutes. The main reason for the on-line method taking longer time than off-line methods is the slow extraction of quercetin derivatives under the conditions used. However, with further optimisation of the method and another design of the instrumental set-up in **paper IV**, it would be possible to improve the efficiency of the on-line system. This will be further discussed in section 8 *Conclusions and Future perspective* below.

7.2 Molecularly Imprinted Polymers (MIP)

Once the extraction and hydrolysis have been optimised, the next step could be to isolate quercetin from the onion/plant extract, if the aim of the process is for example to obtain pure quercetin as the end product. However, for some applications it can be sufficient to dry the extract, and in this case the end product would be the dry plant extract powder. Water extracts can be dried using hot- or freeze-drying; however, an alternative technique for this purpose is water extraction and particle formation on-line, WEPO¹²⁵⁻¹²⁶. The isolation of an analyte from an extract can be done before separation and detection if the extract is too complex to analyse, or in an isolation step if one is interested in obtaining a pure compound. Solid phase extraction (SPE) is one technique used to isolate flavonoids from plant extracts⁴. Different kinds of material have been used for SPE of flavonoids from plant extracts, and a common material to use is silica with C18 bonded groups^{4, 46}. An alternative material is molecularly imprinted polymers (MIP). In **paper V**, an attempt was made to produce MIPs that could be used as a solid phase material in SPE for selective extraction of quercetin from onion extract.

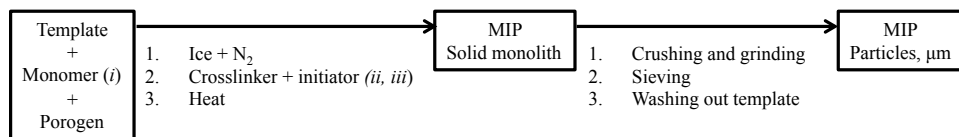
In a review published in 2006, Alexander *et al.*¹²⁷ defined molecular imprinting as follows: “*The construction of ligand selective recognition sites in synthetic polymers where a template (atom, ion, molecule, complex or a molecular, ionic or macromolecular assembly, including micro-organisms) is employed in order to facilitate recognition site formation during the covalent assembly of the bulk phase by a polymerization or polycondensation process, with subsequent removal of*

some or all of the template being necessary for recognition to occur in the spaces vacated by the templating species". In the construction of MIP one needs a template molecule, a monomer, a cross-linker, an initiator and a porogen (solvent)¹²⁷⁻¹²⁹. In the imprinting process (Fig. 19a), the template is first dissolved in the porogen and the monomer is added. The template and monomer then form interactions between each other. Next, a cross-linker and initiator are added and the polymerisation can be initiated using for example heat or light, depending on which initiator has been selected¹²⁹.

There are several different kinds of molecular imprinting approaches, for example covalent-, non-covalent- and metal-ion imprinting¹²⁷⁻¹²⁸. In **paper V** the non-covalent approach was used, and four different MIPs were constructed using quercetin as the template molecule. There are different kinds of polymerisation methods, for example bulk polymerisation and precipitation polymerisation¹²⁷. In **paper V** bulk polymerisation was used. In bulk polymerisation, the MIP produced is a solid monolith that is usually crushed, ground and sieved before use. The next step is the removal of the template, which is necessary before the application of the MIP. For this step, different washing strategies are used depending on which approach is applied to break the interaction between the template and the monomer/polymer. MIPs and NIPs in **paper V** were washed in four steps using methanol:acetic acid 9:1 (v/v), methanol, methanol:triethylamine 9:1 (v/v) and methanol, in that order, to remove quercetin, but an alternative could be to use PFE. Batlokwa *et al.*¹³⁰ found that when using PHWE, quercetin was removed from MIPs within 70 minutes, compared to Soxhlet extraction with methanol:acetic acid, which took more than 10 h. In addition to producing the MIP, non-imprinted polymers (NIP) should also be prepared, which are produced in the same way as MIPs, but with the template excluded. The NIP is used to compare the difference between the capacity of NIP and MIP to bind the analyte so as to be able to distinguish between specific and non-specific binding. Binding of analyte to NIP is only non-specific since no imprint of the template is made in the polymerisation.

In **paper V**, 4-vinylpyridine was used as monomer, ethylene glycol dimethacrylate as cross-linker and 1,1'-azobis(cyclohexanecarbonitrile) as initiator, see Fig. 19 for structures and the scheme of the MIP production.

(a)



(b)

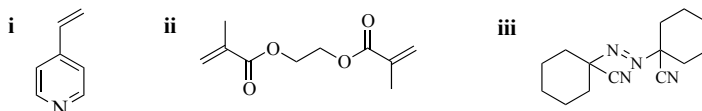


Figure 19. (a) steps in the MIP production and (b) structures of (i) 4-vinylpyridine, (ii) ethylene glycol dimethacrylate and (iii) 1,1'-azobis(cyclohexanecarbonitrile).

In the production of quercetin MIPs, different solvents have been used, for example acetone¹³¹⁻¹³², acetonitrile¹³² and THF¹³²⁻¹³³. In **paper V** the following porogens were tested: methanol, THF, a mixture of methanol and water or a mixture of methanol, THF and water. As mention above in section 3 *Sustainable Chemistry*, THF is one of the solvents that has a significant environmental impact, and an alternative method for producing MIP would be to use scCO₂ technology¹³⁴, see further discussion in the section 8 *Conclusion and future perspectives*. 4-vinylpyridine has been used previously as a monomer in quercetin MIP^{131, 135} and other monomers that have also been used are acrylamide¹³²⁻¹³³ and methacrylic acid¹³⁵⁻¹³⁶.

In **paper V** the adsorption of quercetin to the different MIPs and NIPs was studied as a function of time 25 °C and 84 °C. The adsorption studies, and other binding studies, were performed at 84 °C because we were interested in how the MIP would function at the temperature used for on-line extraction and hydrolysis in the studies described in **paper IV**. Fig. 20 shows the adsorption as a function of time for one of the MIPs (M2, see **paper V**) and its corresponding NIP (N2, see **paper V**) at 25 °C and at 84 °C.

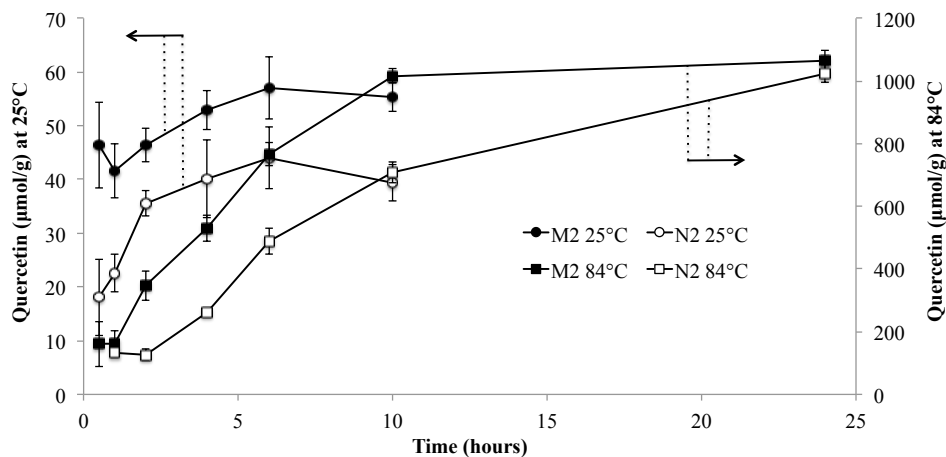


Figure 20. Effect of contact time on binding capacity of polymers M2 and N2 at 25 °C (left y-axis) and 84 °C (right y-axis). pH was 5.5 and solvent was MeOH/H₂O, 7:3, (v/v). M2=MIP and N2=NIP, see **paper V** for further description. Modified from **paper V**.

As the figure shows, the adsorption of quercetin to both M2 and N2 was much higher at 84 °C, compared to at 25 °C. A reason for the higher adsorption at a higher temperature can be due to higher mass transfer and faster adsorption kinetics and maybe also due to weakening of the hydrogen bonding, i.e. less competition of the binding site with water and methanol. An increase of temperature also results in a decrease of the solvents viscosity and surface tension, which can result in faster penetration of the MIP and therefore higher adsorption of quercetin at higher temperature. At 25 °C a difference in quercetin adsorption could be seen between the M2 and N2. There was no difference at 84 °C between the M2 and N2 after 24 h, since all quercetin present in solutions had been adsorbed to the polymers. The M2-MIP was also added to an onion extract sample, and the onion extract was analysed by HPLC-UV before and after the addition of M2, see Fig. 21a and 21b. The M2 was first washed with methanol and then with methanol:acetic acid 9:1 (v/v) and the wash solutions were analysed using HPLC-UV, see Fig. 21c and 21d, respectively.

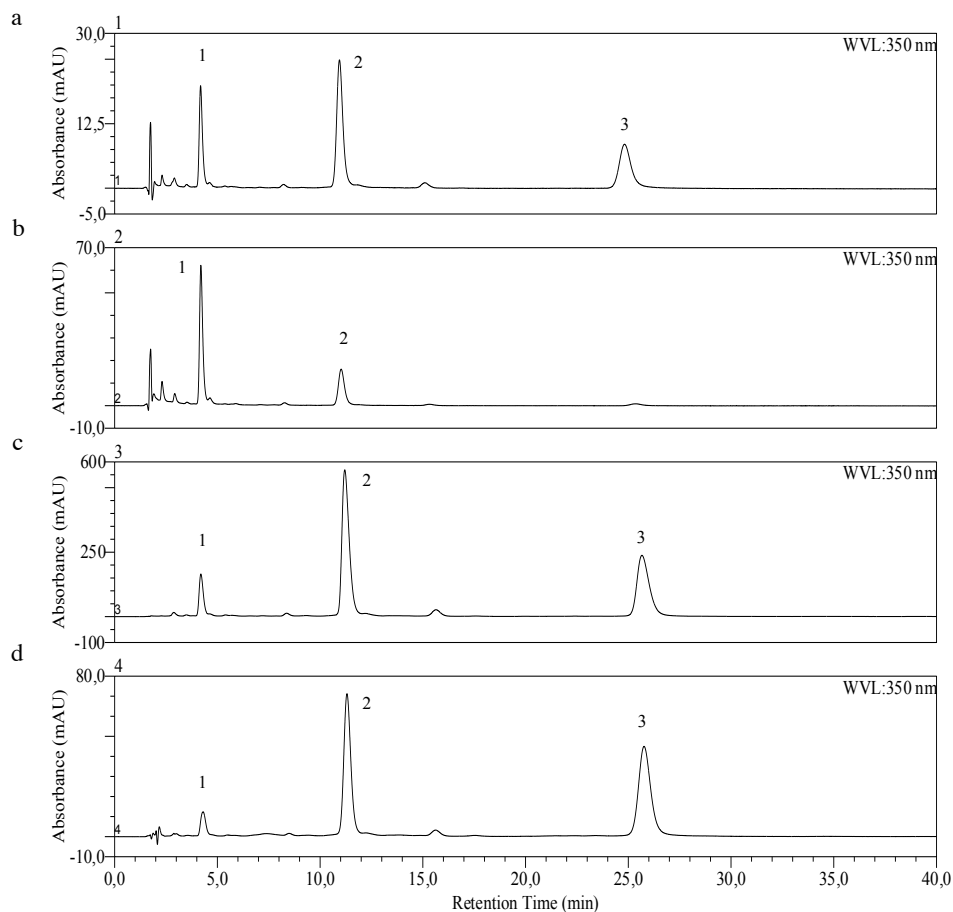


Figure 21. HPLC chromatograms of (a) onion extract before addition of M2 MIP, (b) onion extract after addition of M2 MIP, (c) collected MeOH washing solution and (d) collected MeOH/acetic acid washing solution. 1 = Q3,4', 2 = Q4', 3 = quercetin. From **paper V**.

When Fig. 21a and 21b above are compared, it can be seen that almost all quercetin within the onion extract was adsorbed to the M2, and also Q4'. Both Q3,4', Q4' and quercetin were washed out from the M2 using methanol (Fig. 21c) and methanol:acetic acid (Fig. 21d). These results show that the M2 is probably more selective for quercetin than for its glucosides, but this has to be studied further. However, since the aim is to hydrolyse extracted quercetin glucosides to quercetin before use of MIP, there will not be any glucosides present in the extract in future application. As the reader may have noticed, there are more experiments

left to do regarding **paper V** and this will be further discussed in section 8 *Conclusion and future perspectives*.

8 Conclusions and future perspectives

When I started my PhD in May 2007 my knowledge of PFE was almost zero, but at least I had worked with enzyme-catalysed reactions during my undergraduate studies. Now, however, almost 5 years later, I have learnt a great deal about the research around PFE, enzymes, quercetin and the other techniques and methods I have worked with in **paper I-V**.

Although **papers I** and **II** present many interesting results I will concentrate here on certain of them. The test using Q3, Q4' and Q3,4' standards showed that the enzymatic hydrolysis of glucose at the 4'-position of quercetin is faster than that at the 3-position, but the affinity of glucose at the 3-position was improved by mutating a specific residue in the enzyme. On the other hand, the mutation lead to a slight decreased thermostability compared to the wild type enzyme. PHWE with enzyme catalysed hydrolysis has less environmental impact compared to the conventional method. In one of the papers not included in this thesis, Khan *et al.*¹³⁷, other mutants have been used to study whether the affinity towards glucose can be further improved at the 3-position of different flavonoids. Another suggestion might be to screen for other thermostable enzymes that might have higher affinity for glucose bound at the 3-position of the flavonoid backbone.

In **paper III**, the major conclusions are that the support materials selected are stable at the high temperatures necessary for hot water extraction of quercetin, that the immobilisation improved the thermostability of the enzyme and that the activity of the enzyme was higher when enzymes were immobilised on acrylic beads compared to monolithic gel. Regarding immobilisation, other support materials that could be tested are for example glass beads or polystyrene beads. A third alternative material could be electrospun fibers. The diameter of electrospun fibers can be as small as nanometers, which results in high surface area for immobilisation of the enzyme. I tested electrospinning of polystyrene together with *TnBgl1A* during a short visit at Rhodes University in South Africa. Even though the enzyme was exposed to a high electric field, it was still active towards hydrolysis of quercetin glucosides (unpublished data).

In **paper IV**, extraction and hydrolysis was combined in an on-line continuous flow system. Extraction profiles showed that an increase in pH, concentration of ethanol and flow rate increased the total quercetin extraction yield from yellow onion. Due to the design of the instrumental set-up, a lower flow rate was used in the combined extraction and hydrolysis, which resulted in a lower quercetin yield. A higher flow rate can be used, if the design of the extraction cell is changed. For example a longer cell can be used, which would increase the residence time of the extract within the cell, thus allowing a higher flow rate to be used.

In **paper IV**, the process was optimised concerning the hydrolysis but not the extraction. It would also be interesting to study the effect on the extraction and hydrolysis reactions, using water with and without ethanol. This variation would allow us to see whether the ionic strength of the buffer has any effect on the extraction. The pH of a water-onion extract is around 4-5 and the question is how the hydrolysis rate is affected if no buffer is present. The re-use and stability of the immobilised enzyme in the on-line continuous flow system is another aspect that would be interesting to study. Other questions of interest include for how many extractions the enzyme can be re-used, why is the enzyme inactivated and is the inactivation reversible or irreversible. Once support material, stability of the immobilised enzyme and the questions above have been settled, it would be interesting to carry out an LCA to investigate whether the immobilisation step is still favourable compared to using enzyme in-solution.

In **paper V**, an interesting result was that the binding of quercetin to both MIP and NIP increased when the temperature was increased. The method for studying the adsorption of quercetin with time needs to be improved so that not all quercetin present in the solution is adsorbed to the MIP or NIP. This can be accomplished by for example increasing the ratio between the amount of quercetin and the MIP or NIP in the solution. The adsorption of quercetin to the MIP at even higher temperatures than 84 °C is another interesting experiment to perform, as well as the fundamentals around why the higher binding effect is seen.

Once the fundamental studies of the MIP are completed, it would be interesting to include the MIP in the on-line extraction and hydrolysis of quercetin from onion. Would the residence time of quercetin within the cell packed with MIP be long enough to ensure full adsorption? Would there be any change in the binding capacity of the MIP with number of usage?

In the production of the MIP, THF was used in the porogen mixture. Since THF is one of the most environmentally burdensome solvents, it would be of great interest to use techniques and methods with less of environmental impact, for example scCO₂-technology. The problem with such approach would be the very low solubility of quercetin in scCO₂, and possibly also a low solubility of the monomer

and crosslinker. However, an organic modifier such as methanol could be added to increase the solubility.

So far, we have only worked with one enzyme, *TnBgl1A*. Glucose is one of the most commonly substituted groups on flavonoids, but another abundant sugar group is rhamnose, which *TnBgl1A* cannot hydrolyse. Quercetin rhamnosides are for example found in apples, another industrial by-product in southern Sweden. That the *TnBgl1A* is not active towards a broad range of sugars limits its use toward other plants. However, it might be difficult to find one enzyme that is active towards all different sugars bound to flavonoids and then a mixture of different enzymes could be used instead. If a mixture of enzymes is used, with different glycoside specificity, the same instrumental set-up could be used for different plant by-products or for a mixture of plant by-products.

Another challenge that I have been thinking about throughout the projects is whether it is possible to scale-up the different steps from an analytical scale to an industrial process. Would it be preferable to use a continuous flow on-line extraction, hydrolysis and isolation of flavonoids from plant based by-products, or would such set-up be too complex? The complexity can be due to the fact that the extraction, hydrolysis and isolation have different optimal conditions. So to get a high yield of the high-value compounds without too many different steps, it might be easier to do it in a batch-wise approach, i.e. first extraction, then hydrolysis and finally isolation of high-value compounds from the plant extract in three separate containers.

During the projects there has been a great deal of focus on how we as analytical chemists can decrease the impact of different techniques on the environment. I hope that this thesis can help out making other chemists aware of that there are alternative techniques to use.

In the last, almost, five years I have learned that it is important to collaborate with people in other research area such as biotechnology, environmental and energy studies and economics. By collaboration it is possible to develop new methods and techniques that can help improve the sustainability of chemistry. Though it is hard to understand details of other research areas I have tried to learn as much as possible from my collaborators and I have also taken courses in other fields such as life cycle assessment and enzyme technology.

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