Analysis of Acrylamide and Anthocyanins in Foods

Extraction Optimization for Challenging Analytes

ERIK V. PETERSSON
Dissertation presented at Uppsala University to be publicly examined in B22, BMC, Husargatan 3, Uppsala, Friday, December 4, 2009 at 13:15 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

In this thesis, the main concern has been to improve the reliability of different parts of the analytical workflow (Paper I, II, IV & V). Additionally, one of the resulting optimized methods was used in a real application (Paper III).

Paper I-II concerned the extraction of acrylamide (AA) from foods. In Paper I different parameters such as sample particle size, extraction solvent, extraction time and extraction temperature were optimized, leading to a method that showed good agreement with the assigned AA levels of several proficiency test samples. Later, after the publication of the paper, the method showed good performance in a collaborative trial validation, in terms of trueness, repeatability and reproducibility figures. It was labeled “undoubtedly fit for the purpose”.

In Paper II, it was shown that the ‘extra’ amounts of AA obtained during extraction of foods with an alkaline aqueous solution was not due to improved extractability of AA from the food matrix. Strongly alkaline conditions seemed to rather induce net formation of AA from water-soluble precursors formed during thermolysis. This phenomenon should therefore be regarded as an extraction artifact.

Paper III was an application of the optimized method from Paper I, where it was used to study the reduction of AA in potato chips (crisps) by using pre-treatments and frying at reduced pressure. There were significant reductions in AA, down to below the limit of quantification (5 µg/kg) for the method.

Paper IV-V concerned analysis of anthocyanins (AC) in red onion. In Paper IV, a new separation method using capillary electrophoresis was developed, and its rapidness combined with an acidic background electrolyte helped in preventing AC degradation. Furthermore, its alternative separation mechanism is a complement to that of the more commonly used liquid chromatography technique. In Paper V, simultaneous extraction and degradation of anthocyanins from red onion was studied in a static batch reactor at 110°C. The extraction and degradation kinetics were successfully separated, and an ideal theoretical extraction curve was constructed by compensating mathematically for degradation effects, showing that more anthocyanins, 21 to 36% depending on different species, could be extracted if no degradation occurred. The results give important information about the different kinetics competing during an extraction procedure, and also show that quantitative extraction is not to recommend in the batch system used in the study.

Keywords: Analysis, Extraction, Optimization, Acrylamide, Anthocyanins, Foods, Challenges

Erik V. Petersson, Department of Physical and Analytical Chemistry, Analytical Chemistry, Box 577, Uppsala University, SE-75123 Uppsala, Sweden

© Erik V. Petersson 2009

ISSN 1651-6214
urn:nbn:se:uu:diva-109752 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-109752)
“Violence is Italian art!”
Lucio Fulci
List of Papers

The thesis is based on the following five papers, which are referred to in the text by their Roman numerals:

I. Critical factors and pitfalls affecting the extraction of acrylamide from foods: An optimisation study

II. Impact of extraction conditions on the content of acrylamide in model systems and food

III. Acrylamide-free potato chips

IV. Analysis of anthocyanins in red onion using capillary electrophoresis-time of flight-mass spectrometry (CE-TOF-MS)

V. Pressurized hot water extraction of anthocyanins from red onion: A study on extraction and degradation kinetics

Reprints were made with kind permission from the publishers.
Author contribution:

Paper I: Took part in planning and made all experimental work. Took care of most of the evaluation. Wrote the manuscript.

Paper II: Took part in planning, experimental work, evaluation and writing in the part of the paper concerning measurement of acrylamide in food matrices.

Paper III: Supervised the implementation of the method for analysis of acrylamide, evaluated, and wrote the related parts of the manuscript.

Paper IV: Planned the project together with Angel Puerta, took part in the experimental work, evaluated all MS-data, and wrote the major parts of the manuscript.

Paper V: Planned the project together with Charlotta Turner, made the experimental work and evaluations together with Jiayin Liu, wrote the manuscript.

Papers not included in the thesis:

Effect of extraction pH on acrylamide content in fresh and stored rye crisp bread
Contents

Introduction .......................................................................................................................... 11

Chemical risks in foods ................................................................................................ 13
  Acrylamide .................................................................................................................. 13
    Background ............................................................................................................... 13
    Properties .................................................................................................................. 13
    Toxicity ..................................................................................................................... 14

Beneficial compounds in foods ...................................................................................... 15
  Anthocyanins ............................................................................................................... 15

Dietary intake ................................................................................................................... 17
  Acrylamide .................................................................................................................. 17
    Risk characterization of acrylamide intake ......................................................... 18
  Anthocyanins ............................................................................................................... 18
    Health benefits of anthocyanin intake ................................................................. 19

Formation and degradation of compounds in foods ....................................................... 20
  Acrylamide .................................................................................................................. 20
  Anthocyanins ............................................................................................................... 22

The analytical workflow .................................................................................................. 24

Extraction ......................................................................................................................... 25
  Conventional extraction techniques ........................................................................... 26
  Improved extraction techniques .................................................................................. 27
    Soxhlet extraction ...................................................................................................... 27
    Ultrasonic extraction ................................................................................................. 28
    Pressurized fluid extraction ...................................................................................... 29

Methods of analysis ........................................................................................................ 30
  Acrylamide .................................................................................................................. 30
  Anthocyanins ............................................................................................................... 33

Challenges in the analysis of acrylamide and anthocyanins in foods ......................... 36
  Acrylamide .................................................................................................................. 36
  Anthocyanins ............................................................................................................... 39
Abbreviations

Chemicals
3-APA 3-aminopropionamide
3-MCPD 3-monochloropropane-1,2-diol
AA Acrylamide
AC Anthocyanins
DNA Deoxyribonucleic acid
HCA Heterocyclic amines
HMF Hydroxymethylfurfural
PAH Polycyclic aromatic hydrocarbons

Organizations
BfR German Federal Institute for Risk Assessment
CSL Central Science Laboratory
IARC International Agency for Research on Cancer
JECFA Joint FAO/WHO Expert Committee on Food Additives
EC DG-JRC European Commission’s Directorate General Joint Research Centre
NMKL Nordic Committee on Food Analysis
SNFA Swedish National Food Administration

Techniques
ASE® Accelerated solvent extraction
CE Capillary electrophoresis
ESI Electrospray ionization
GC-MS Gas chromatography mass spectrometry
IT-MS Ion trap mass spectrometry
LC-MS/MS Liquid chromatography tandem mass spectrometry
LC Liquid chromatography
MS Mass spectrometry
MS\(^2\) Mass spectrometry with fragmentation
NMR Nuclear magnetic resonance
PDA Photodiode array
PFE Pressurized fluid extraction
PHWE Pressurized hot water extraction
SPE Solid phase extraction
TOF-MS Time of flight mass spectrometry
UE Ultrasonic extraction
UV-VIS Ultraviolet-visible spectrophotometry
Introduction

Food is an absolute necessity for human life. Generally, foods consist of macronutrients - carbohydrates, fats and proteins - and/or micronutrients like minerals and vitamins. However, the definition of foods can be wider than that - in Sweden, water and even the tobacco product snuff are classified as foods.

Macro- and micronutrients are essential for our body. All nutrients are absorbed by our body and made available to the cells, mainly for energy production or as building blocks. Similarly to the micronutrients, other compounds are also present in small quantities in foods. These can be important compounds for our well-being, like flavor and aroma compounds, or health-promoting like antioxidants, but there might also be unwanted toxic compounds present.

The purpose of eating foods is to sustain life, but in extreme cases this action might have somewhat opposite effect. Food related risks can be divided into nutritional, microbial and chemical ones. There is a nutritional risk taken when eating foods with unhealthy composition of nutrients, i.e. unnecessarily high in energy-rich fat and sugar, and/or low in vitamins and minerals. Furthermore, there is a microbial risk when foods contain pathogenic (disease or illness causing) microorganisms or mould. Finally, there is a chemical risk when foods contain toxic (e.g. cytotoxic, neurotoxic, mutagenic, carcinogenic or teratogenic) compounds.

On the other hand, eating foods with non-essential, but still beneficial chemical compounds, have been shown to give health-promoting effects, such as reduced risk of cancer, degenerative diseases and heart diseases. Beneficial chemical compounds are for example antioxidants.

In either case, whether it is a beneficial or toxic compound, it is of interest to know how much of it we are eating. In order to know this, chemical analysis is needed followed by intake calculations. For the analyst’s part, there are always doubts - can we trust our analysis? Are we really measuring the compound we are interested in? If so, are we measuring the correct amount? Or are we under/overestimating the content? These are the analytical challenges we are constantly facing. Thus, the main concern of this thesis has been to
improve the reliability of different parts of the analytical workflow, especially the extraction step, in order to reach a little bit further towards the ultimate goal for quantitative analysis – measuring the true value.
Chemical risks in foods

We are exposed to a variety of chemical risks from foods, for example naturally occurring toxic compounds (toxins), environmental contaminants, compounds added during food cultivation, compounds added during food production, and compounds released from packaging materials.

In this thesis, **Paper I-III** concerns acrylamide (AA), a process-induced contaminant. The process-induced contaminants differs somewhat from the earlier mentioned groups, since the chemicals are not coming from some source outside the foods. Instead, they are formed during processing of the foods, which is a necessary step in order to kill microbes and to give the food its desired properties like texture and taste.

Other examples of process-induced contaminants are furan, heterocyclic amines (HCA), polyaromatic hydrocarbons (PAH), 3-monochloropropane-1,2-diol (3-MCPD), and hydroxymethylfurfural (HMF).

**Acrylamide**

**Background**

The German chemist Moureu was the first to synthesize AA (1893). During the 1950’s it became more widely available when the Hercules company started commercial production. Since then, AA has mainly been used to produce polyacrylamides. Polymers of AA have many applications, for example in laboratory electrophoresis, in water and waste-water treatment, as a cosmetic additive, and in various industrial processes (oil, paper, mineral, concrete, textile) [1].

**Properties**

Acrylamide (2-propenamide; MW 71.08 g/mol) is a white, odorless, crystalline solid with low volatility (melting point 84.5°C, boiling point/polymerization 136°C) [2,3,4]. The small size of the molecule combined with the properties of the amide group and the carbon-carbon double
bond makes AA a polar and fairly reactive molecule. The structure of AA is found in Figure 1.

![Structure of Acrylamide and Glycidamide](image)

*Figure 1.* Acrylamide (left) and its metabolite glycidamide (right).

**Toxicity**

Acrylamide is neurotoxic to animal and man [5] and causes carcinogenic, genotoxic and reproductive toxicity effects in experimental animals [6]. Acrylamide is classified as “probably carcinogenic to humans” (IARC group 2A) by the International Agency for Research on Cancer (IARC) [7]. The IARC classifies chemicals into five groups depending on the scientific evidence for carcinogenicity, with “group 1” being the highest (carcinogenic to humans) and “group 2A” the second highest [8].

Acrylamide is biotransformed in vivo by cytochrome P450 2E1 to a reactive epoxide, glycidamide [9,10], which has been reported to be 100-1000 times more reactive towards DNA than AA [11]. It has been concluded that the mutagenicity of AA in human and mouse cells is based on the capacity of its metabolite, glycidamide, to form promutagenic DNA adducts [12]. Figure 1 shows the structure of glycidamide.
Beneficial compounds in foods

Foods consists of beneficial chemical compounds, some are essential and exists in large quantities, like macronutrients such as carbohydrates, fats and proteins. Some are essential and exist in minor quantities, like micronutrients such as minerals and vitamins. There are also non-essential compounds in small quantities, that are health-promoting. Examples of these are phytochemicals and antioxidants (some antioxidants fall into several categories – they can also be vitamins and/or phytochemicals).

Oxidation reactions can produce free radicals, which start chain reactions that damage or kill cells, so called oxidative stress. This kind of stress might be an important cause of many human diseases, like neurodegenerative diseases, cancer and coronary heart disease. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions, since the AC are oxidized primarily. An example of the antioxidant class of compounds in foods is anthocyanins (AC), which are the analytes in Paper IV-V.

Anthocyanins

Anthocyanins are a class of natural pigments found in flowers, fruits and berries. They consist of an aromatic ring bonded to a heterocyclic ring that contains oxygen, which in turn is bonded by a carbon-carbon bond to a third aromatic ring (Figure 2). Anthocyanins belong to the polyphenolic class of compounds and usually occur in the plant as glycosides and acylglycosides of the anthocyanidin aglycones (see example in Figure 3). In this form, anthocyanidins are called anthocyanins. Several different aglycones exist, with varying configurations of hydrogen-, hydroxy-, and methoxy-groups, as can be seen in Figure 2. Cyanidin, Delphinidin, Pelargonidin, Peonidin, Petunidin and Malvidin are the most common aglycone bases in fruits and vegetables [13], and the most common glycosides are: 3-monosides, 3-biosides, 3,5-diglycosides and 3,7-diglycosides [13].

Anthocyanins can be useful as non-toxic colorants for foods (giving varieties of orange, red, violet and blue colors), or for human health, as they are antioxidants and free radical scavengers. Potential therapeutic effects include
preventive action against cancer [14], improvement of visual functions [15], improve cardiovascular health [16], and showing preventive action against Alzheimer’s and Parkinson’s diseases [17]. These factors have lead to increased interest in using AC in food and pharmaceutical industries as a substitute to synthetic colorants and antioxidants. However, anthocyanins are particularly unstable – they are especially sensitive to light, heat [18,19] and pH above the optimal range of pH 1-3 [20]. Around pH 1 there is a reddish color (due to the flavlylium cation form of the aglycone), between pH 2 and 4 the quinoidal blue species are predominant, between pH 4-6 four structural forms of the AC coexist: flavlylium cation, anhydrous quinoidal base, colourless carbinol base and pale yellow chalcone [21]. At pH values higher than 7, the AC are starting to degrade, though at different rates depending on their substituent groups [21]. Anyhow, the color continues to turn more intensely towards blue with higher pH, and some stability is actually shown for some AC at pH 8-9 (although less than at the optimal range pH 1-3) [20].

Figure 2. General structure of the anthocyanidin aglycones.

Figure 3. Structure of the conjugated anthocyanin Cyanidin 3-O-glucoside.
Dietary intake

Acrylamide

As earlier mentioned, AA has many uses in the industry and therefore work related exposure has been known for a long time. Tobacco smoke has also been known as a source of human exposure to AA [22], but the finding of high levels of AA in foods was completely unexpected.

It all started when workers in the Hallandsås tunnel (in the south-west part of Sweden) were exposed to a grouting agent containing monomeric AA and N-methylolacrylamide. To investigate the degree of exposure, the blood of exposed workers (and also the blood of cattle exposed through contaminated water) was investigated for AA hemoglobin adducts (reaction products between AA and the blood protein hemoglobin). When investigating the control groups consisting of unexposed humans and cattle, it was found that unexposed humans had an unexpectedly high background level of such hemoglobin adducts. Since humans eat cooked foods (and animals usually do not), and it was known that AA is formed during heating (e.g. pyrolysis of tobacco), the possibility of dietary AA was investigated by feeding laboratory animals with fried food. The results strongly indicated that AA could be formed by the heating of foods [23]. The work continued and unexpectedly high levels of AA were found in several different foodstuffs, as was announced in a press conference organised by Stockholm University and the Swedish National Food Administration, 24th of April 2002.

In early 2005, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated certain food contaminants, amongst them AA. JECFA estimated AA intakes at national levels ranging from 0.3 to 2.0 μg/kg body weight per day for the average in the general population. For high percentiles consumers (90th to 97.5th) intake estimates ranged from 0.6 to 3.5 μg/kg body weight per day, and up to 5.1 μg/kg body weight per day for the 99th percentile consumer [24]. This corresponds to a margin of exposure (MOE; calculated by dividing the dose in animals that cause a 10% increased cancer rate by the estimated human exposure) from 75 for high consumers of AA to 300 for average consumers of AA. The major contributing foods to total exposure for most countries were: potato chips and French fries (16-30%); potato crisps and chips (6-46%); coffee (13-39%); pastry, sweet biscuits and cook-
ies (10-20%); bread and rolls/toasts (10-30%). Other food items contributed with less than 10% of the total exposure [24].

In Paper III efforts were successfully made to lower the AA amounts in potato chips (crisps), one of the foods with the highest AA levels, by applying pre-treatment steps before frying, and then using an alternative frying technique at reduced pressure.

Risk characterization of acrylamide intake
In the JECFA evaluation of AA in 2005, the Committee considered the human exposure of AA to be high “…for a compound that is genotoxic and carcinogenic…” and that this “…may indicate a human health concern. Therefore, appropriate efforts to reduce acrylamide concentrations in foodstuffs should continue.” [24]. The Committee recommended that the risk characterization of AA should “…be re-evaluated when results of ongoing carcinogenicity and long-term neurotoxicity studies become available.” [24].

There are epidemiological studies that have not detected any relationship between dietary AA and cancer [25,26,27,28]. One explanation could be that the statistical power of standard epidemiological studies can be far too low to detect an increased risk for cancer due to background exposure of AA [29,30]. Other epidemiological studies have found an increased cancer risk related to dietary AA. One study suggested a higher risk of breast cancer in women who frequently consumed french fries at pre-school age [31]. Another study found a significantly increased risk of ovarian cancer among postmenopausal women and a significantly increased risk of endometrial cancer among never smoking women in association with dietary exposure to AA [32]. A third study suggested an increased risk of renal cell cancer among men and women in association with dietary AA exposure [33].

Anthocyanins
Anthocyanins have been a part of our diet for a long time – especially in countries with berry eating traditions. For example, in Finland, where people eat a lot of berries, dietary intake of anthocyanins in adults has been estimated to 47 mg/day – and almost 90% of the intake in the study came from berry related foods [34]. In USA, on the other hand, the anthocyanin intake was lower, and estimated to 12.5 mg/day [35].
Health benefits of anthocyanin intake

In a German study, it was shown that individuals who consumed an anthocyanin/polyphenolic rich fruit juice had reduced oxidative DNA damage, as well as a significant increase in reduced glutathione levels (meaning better protection against free radical induced organ injury) when compared to controls [36]. In another investigation, concerning patients with Barrett’s esophagus (a condition in which the normal tissue in the swallowing tube has been replaced by an abnormal precancerous tissue), the oral administration of 45 or 32 g (males and females, respectively) of lyophilized black raspberry powder (containing about 5–7% anthocyanins) in a water slurry daily for six months reduced levels of 8-epi-prostaglandin F2α (8-Iso-PGF2) and 8-OHdG (markers of oxidative stress) in urine [37].

![Figure 4. A selection of different berries. Picture from USDA public domain.](image-url)
Formation and degradation of compounds in foods

Acrylamide

Acrylamide is formed in foods mainly in a Maillard type of reaction between the amino acid asparagine and different reducing sugars (e.g. glucose, fructose, or other α-carbonyl compounds) [38,39]. The AA formation pathway is not fully clear, but is believed to consist of several steps [40,41,42,43], as can be seen in Figure 5. As a first step, the nucleophilic amino group of asparagine may react with the carbonyl group of the sugar to form an N-substituted glycosylamine. The glycosylamine may form a Schiff base in the next step (via condensation). In the following step, the Schiff base can decarboxylate into an intermediary azomethine ylide, which after tautomerization leads to a decarboxylated Amadori compound. The decarboxylated Amadori compound (or perhaps a tautomer) can then release AA directly by a β-elimination reaction and cleavage of the carbon-nitrogen covalent bond; or via hydrolyzation to form 3-aminopropionamide, which then deaminates to release AA. In Paper II it was shown that some of the water-soluble precursors of AA are labile to alkaline pH, and that artefactual formation (and thereby faulty results) is induced when employing alkaline extraction methods, such as the one published by Eriksson and Karlsson [44]. Maillard type reactions are generally known to be favored by low moisture and high temperatures, but products can also be formed with higher moisture levels, which was seen in the formation experiments performed in Paper II. The formation also at high moisture levels explains the extraction artifact discovered in Paper I, where AA was formed during overnight water extraction of a chocolate sample at 60ºC.
Other, less important routes of AA formation include reaction paths through 3-aminopropionamide [42,43], acrolein [45], acrylic acid [46] and pyruvic acid [47].

When cooking foods, the amount of AA formed can be minimized in several ways, based on the knowledge about the formation mechanisms. For example, in potato products, reductions of AA can be achieved by: lowering the frying temperature [48], removing precursors with blanching (washing the raw material with hot water) [48], and by reducing the reactions by lowering the pH [49]. These measures were applied in Paper III, where AA in potato chips (crisps) was minimized firstly by two pre-treatment steps. The first of those, blanching, was used to reduce the content of the precursors asparagine and sugar. As the second pre-treatment step, a citric acid bath was used, to remove more of the precursors, and also to lower pH to reduce the AA formation by protonating the R-amino group of asparagine, which hinders the formation of the N-substituted glycosylamine [49], the first step in the AA formation (Figure 5). After the pre-treatments, the frying step was altered. As an alternative to conventional frying at atmospheric pressure, frying at reduced pressure was used in Paper III. This technique shortens the frying time by lowering the boiling temperature of water. As a consequence of shorter frying time, there was less AA formation.
Anthocyanins

Anthocyanins belong to a large group of secondary plant metabolites collectively known as flavonoids. Secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of the organism. Unlike primary metabolites, absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of the organism's survivability, fecundity, or aesthetics, or in no significant change at all. Secondary metabolites are usually a narrow set of species within a phylogenetic group. From a chemical point of view, AC are glycosides of 2-phenylbenzopyrylium (flavylium) salts derivatives. Flavonoids, like AC, are formed in both the shikimate and phenyl propanoid pathways, producing aminophenylalanine and malonyl-coenzyme A, respectively, then followed by forming a tetrahydroxy chalcone (1,3-diphenylpropanone) [50]. A further five or more enzymatically catalyzed reaction stages produce the final pigments [51].

Concerning degradation at pH 1, deglycosylation reactions represent the initial steps of AC degradation yielding the respective aglycones. The latter can then be cleaved into terminal products phenolic acid and a phenolic aldehyde [52] as seen in Figure 6. At pH 3.5, typical in fruit juices, the scheme looks a little different as seen in Figure 6, where a ring opening first takes place and a chalcone glycoside is formed. Then follows deglycosylation, and finally ring opening, yielding the same end products, phenolic acid and a phenolic aldehyde, as at pH 1 [53]. Degradation of AC in foods follows first order reaction kinetics [52,53], which means that the reaction has a rate proportional to the concentration of one of the reactants. The first-order degradation curves that were obtained in Paper V agree well with this.
Figure 6. Degradation of anthocyanins at pH 1 adapted from [52], and at pH 3.5 adapted from [53].
To be able to perform a correct risk or benefit assessment of a toxic or beneficial food compound, it is essential to properly identify it and to securely quantify the amount we are ingesting. A chemical analysis procedure is a complex matter, comprising several steps which all have a potential risk of failure. An analysis of an organic compound present in foods can consist of the following steps:

- **Sampling** – a representative and sufficient amount of material is collected and stored properly.
- **Homogenization** – the sample is treated mechanically to become sufficiently fine-divided and homogenous. Special attention was put on the impact of particle size in Paper I.
- **Weighing** – an appropriate amount of sample is carefully weighed in.
- **Extraction** – the sample is penetrated by a solvent (for a certain time, temperature, pressure and vigorousness) in order to release the analyte into the extraction solution. Paper I, II and V focus on investigating the extraction step.
- **Clean-up** – the extract is treated physically (e.g. by centrifugation) or chemically (e.g. by precipitation) in order to separate the analyte from disturbing matrix components. The importance of defatting was investigated in Paper I.
- **Separation** – the cleaned extract is chromatographically separated in a column or a capillary in order to separate the analyte from interfering compounds. Paper IV focus on development of the separation step, and partly Paper V as well.
- **Detection** – the analyte is determined in the separated sample plug with emphasis on structure and/or concentration. Paper IV focuses on the development of the detection step, and together with Paper V on structure elucidation.
Extraction

Since this thesis mainly focuses on extraction, this process will be discussed more in-depth than the other parts of the analytical workflow. An extraction could be considered as the process of bringing a desired compound that is “trapped” somewhere – for example in a solid – into solutions. A well known example of an extraction is coffee-making. Compounds soluble in hot water are extracted from the solid powder, then the solid residues are removed by filtration, and the result is the beverage in the cup.

Important factors to consider when choosing a suitable solvent are: the solubility of the analytes (i.e. polarity), their diffusivity, and the matrix characteristics. Important factors affecting these characteristics are the nature of the matrix (water and organic/inorganic content), and its physical characteristics such as homogeneity, porosity and particle size. Thus, it is important to understand the mass transfer mechanisms across chemical/physical interfaces, since this is generally the limiting factor when working with analytical applications [54].

Going back to the “trapped” molecule - in a solid matrix, during an extraction, the target molecule could be found in several places (modified from [54]):

- Adsorbed at the surface of the matrix
- Dissolved in the pore solvent and/or adsorbed at the pore surface
- Dissolved/adsorbed in a micro/nano pore
- Chemically bonded to the matrix
- Physically trapped in the matrix
- Dissolved in the bulk solution
Figure 7. Schematic of a household waste particle and a model of the extraction process, adapted from [54]. Graphics by Andreas Dahlin.

The extraction process could consist of the following stages (modified from [54]), as depicted in Figure 7:
1. Diffusion of the solvent into the matrix
2. Desorption of the analyte from the matrix (including breaking of chemical bonds)
3. Solvation of the analyte into the extraction solvent
4. Diffusion of the analyte out from the matrix
5. Diffusion of the analyte through the stagnant solvent layer and into the bulk solvent

When extracting solid samples, as in Paper I-V the choice of a solvent is crucial for the extraction efficiency. Quite commonly, the “best” solvent for extraction could be the worst from an environmental point of view. Especially for non-polar compounds, the best solvents are often toxic, like hexane or halomethanes. For the polar analytes that Paper I-V concerns, environmentally friendly water or water/alcohol solutions were found optimal for extraction.

Conventional extraction techniques

Conventional solid-liquid extraction can, for example, be performed by shaking or stirring the solid-liquid mixture. Generally, this requires a relatively long extraction time in order to get a good extraction yield – but on the
other hand, it is a gentle treatment of the sample, and could be performed at ambient temperature, or lower, for sensitive samples. A more vigorous way of mixing the solid-liquid mixture is to use an ULTRA-TURRAX® mixer. It works in a similar way as a hand-held household blender, grinding and propelling the sample at high speed, which can increase the extraction speed. However, its drawbacks are that only one sample at a time can be extracted, it is non-automated, needs cleaning between each sample, and there is a risk for evaporation from the open extraction vessel, which also allows for light and oxygen to enter. Conventional extraction of acrylamide from foods by ULTRA-TURRAX® was successfully replaced by an automated horizontal shaker in Paper I, since the throughput could be increased by shaking of 10-folds of samples at the same time, and the time spent per sample was greatly reduced. This optimized method was also applied in Papers II-III. In literature, conventional extraction techniques are commonly used for extracting anthocyanins, as for example by Revilla et al. [55].

Improved extraction techniques

Different extraction techniques exist that increase the speed or yield of the extractions. This means, for example, that it is possible to choose a more environmentally friendly solvent, and still have better performance than its conventional extraction counterpart. Examples of such techniques can be seen below.

Soxhlet extraction

The Soxhlet extractor was invented in 1879 by Franz von Soxhlet. The equipment can be seen schematically in Figure 8. The extraction solvent is located in a heated flask, on top of that is the main chamber, containing the solid material (usually placed in a filter paper). Solvent vapor travels up a distillation arm, and condenses into the sample chamber, which is filled with warm solvent. The extraction temperature cannot be changed, and is a few degrees lower than the boiling point of the solvent. Some of the desired compound will dissolve, and when the chamber is filled with solvent, a siphon side arm will automatically empty the extract down to the heated flask again. The process restarts, and the desired compound is by this process concentrated at the bottom flask. The cycle can be repeated many times, over hours or days. After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. Because of the elevated temperatures used, in combination with long extraction times, Soxhlet extraction may not be suitable for thermolabile compounds. Soxhlet extraction has been used for extraction of acrylamide from foods [56].
Ultrasonic extraction

One of the techniques that has potential for speeding up and simplifying sample treatment is ultrasonic extraction (UE). Ultrasonic energy, when imposed into solutions, causes acoustic cavitation – which means bubble formation and subsequent implosion. The collapse of bubbles created by the sonication of solutions results in the generation of extremely high local temperature and pressure gradients, referred to as localized “hot spots”. These hot spots contains effective temperatures of around 5000 K, pressures of about 1000 atm, and heating and cooling rates above $10^{10}$ K/sec [57]. The result is highly effective solid-liquid extractions, when analytes are “burst out” from the solid matrix. The temperature of the bulk solvent is by default well below its boiling point. Ultrasonic extraction has been used for extracting acrylamide [58] as well as anthocyanins [59] from foods.
Pressurized fluid extraction

Pressurized Fluid Extraction (PFE), sometimes referred to as Pressurized Liquid Extraction (PLE®), Pressurized Solvent Extraction (PSE®) and Accelerated Solvent Extraction (ASE®), is an innovative technique that has already been used for extraction of anthocyanins from various plants [60,61,62], and acrylamide from foods [63]. Pressurized Hot Water Extraction (PHWE) is a variety of PFE, where the solvent is mainly water, and was utilized in Paper IV-V. Similar equipment to the one used in the papers can be seen in Figure 9.

A comprehensive review on the PHWE technique was written by Kronholm et al. [64]. Pressurized fluid extraction in general means that the temperature of the extraction solvent is raised above its atmospheric boiling point, and pressure is applied to maintain the solvent in liquid state. Under such circumstances, in the case of PHWE, the fast movement of the water molecules weakens the intermolecular hydrogen bonding, which lowers the dielectric constant and gives water more non-polar solvent characteristics [64]. Moreover, the viscosity and surface tension of the solvent decreases, and the solubility and diffusion rate of the target compounds increase. The penetration of the solvent into the matrix and the transportation of the compounds out from the matrix is faster than in a similar extraction process performed at room temperature [64]. Hence, compared to conventional extraction methods, the PHWE technique attains more rapid and efficient extraction and reduces the need/consumption of organic solvent. However, since elevated temperatures are used, one must be aware of potential degradation problems [64].
Methods of analysis

Acrylamide

Since the discovery of AA in foods, there have been numerous publications on analytical methods for its determination, and these have been reviewed several times [65,66,67]. Generally, there are two major analytical techniques used: analysis of brominated AA with Gas Chromatography Mass Spectrometry (GC-MS) or direct analysis of AA with Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS). The latter technique was used in Papers I-III. The distinctions between different methods based on these two techniques are usually found in the extraction and clean-up procedures [65,66,67]. Typical GC-MS and LC-MS/MS methods for analysis of AA in foods are presented below.

Analysis using Gas Chromatography-Mass Spectrometry

The first published GC-MS method for analysis of AA in foods has improved over the years [68,69,70,71] and this is its current shape: Isotope-labeled AA is dissolved in water and added to the sample. A mixture of ethyl acetate and cyclohexane is added, and the mixture is shaken to extract the fat from the sample. Then, additional water is added and the solution is shaken for 15 minutes or longer. After centrifugation, an aliquot of the aqueous phase is transferred to a glass vial and derivatized by adding a mixture of potassium bromide, hydrobromic acid and saturated bromine water. Bromination takes place in the dark at 4°C within 1 hour. Excessive derivatization reagent is destroyed with a few drops of an aqueous sodium thiosulphate solution. The resulting 2,3-dibromopropanamide is extracted with ethyl acetate and dried over anhydrous, granular sodium sulphate. The extract is then transferred into another clean vial where the volume is reduced below 0.5 mL (e.g. under a stream of nitrogen). To avoid uncontrolled dehydrobromination in the hot injector of the GC, 50 μL of triethylamine is added in order to completely dehydrobrominate the analyte. The formed product, 2-bromopropenamide (Figure 10), is analyzed by GC–MS on a mid-polar GC column, e.g. containing 50% phenyl–50% polymethylsilicone as stationary phase. The mass spectrometer is run in selected ion monitoring mode. The monitored mass to charge ratios are 106, 133, 135, 149 and 151 for 2-bromopropenamide, and 152 and 154 for the $^{13}$C$_3$-labeled internal standard.
Analysis using Liquid Chromatography-Mass Spectrometry
The first published LC-MS/MS method for analysis of AA in foods has improved over the years [72,73,74,75,76]. Its latest revision is the one developed in Paper I, and used in Paper I-III. Isotope-labeled AA is added to the sample, which is then extracted with water (e.g. shaking for 45 minutes). After centrifugation, an aliquot of the extract is passed through a preconditioned Isolute Multimode® solid phase extraction (SPE) cartridge. The stationary phase of the Multimode® retains ionic and non-polar matrix components. The resulting eluate is collected and loaded on an Isolute ENV+® SPE cartridge, which retains AA. Matrix components are washed out, and then the AA-extract is eluted with a methanol solution and reduced to a final volume of about 500 μL. A quantity of 10 μL is injected on a Hypercarb® column, which is run isocratically with 0.1% acetic acid in water at a flow rate of 400 μL min⁻¹. The LC is connected to a triple quadrupole mass spectrometer, where the transitions m/z 72→55, 72→54 and 72→44 are recorded for AA, whereas the transition 75→58 is used for the isotope-labeled internal standard (see Figure 11).
A collaborative trial validation study of the two methods above was organized by the European Commission’s Directorate General Joint Research Centre (EC DG-JRC), the Swedish National Food Administration (SNFA), the Central Science Laboratory (CSL) and the Nordic Committee on Food Analysis (NMKL) in 2006. The collaborative trial was designed as a blind duplicate study of 11 different samples, and included potato crisps and bakery products with AA contents from 20 to about 9000 μg/kg. The results showed that the LC–MS/MS method (the optimized method developed in Paper I) was superior to the GC–MS method in terms of precision parameters and interlaboratory variation [77].

Sixteen laboratories from Europe and elsewhere reported results using the LC-MS/MS method. Relative repeatability figures of the method were 3-9% and the corresponding reproducibility values were 5-13%. For evaluation of the method trueness, the study included a spiked potato powder (500 μg/kg) and a candidate reference material consisting of crisp bread (980±90 μg/kg). The mean results for these were 485 μg/kg (Coefficient of Variation, CV=9%) and 950 μg/kg (CV=5%), respectively, indicating that the method bias was low or non-existent. Finally, it was observed that the precision of
the results for all samples obtained by the LC–MS/MS method was even better than predicted by the Horwitz equation (which gives an estimation of the expected “normal performance”) [78]. In summary, it was concluded that the LC-MS/MS method from Paper I is undoubtedly fit for the purpose [77].

Anthocyanins

Analysis of anthocyanins in foods has been ongoing for a long time and many methods are available. The levels found in foods are usually higher than those of acrylamide, therefore a wider range of (also less sensitive) techniques can be used. A comprehensive review on analysis of AC was written by Castaneda-Ovando and colleagues [21]. The review describes that in general, the methods of analysis starts with AC being extracted with weakly acidified methanol or ethanol, of which methanol is the most efficient. However, in the food industry, ethanol is preferred due to lower toxicity. After the extraction step, sometimes solid phase extraction (SPE) is applied as a clean-up step, to remove co-extracted compounds such as sugars and organic acids. Regarding the separation step, Castaneda-Ovando et al. describe that Liquid Chromatography (LC) is the most common technique, and such a method was developed in Paper V. Several methods using Capillary Electrophoresis (CE) are also available, for example the one developed in Paper IV, or the one used by Bednar et al. [79]. For detection, Castaneda-Ovando et al. conclude that UV-VIS or Photo Diode Array (PDA) detectors are commonly used, as in Paper V, as well as mass spectrometer (MS) detectors, as in Paper IV-V. Electrospray ionization (ESI) is the most common technique for ionizing the molecules prior to entering the MS detector, as in Paper IV-V. Regarding the type of MS detector, various techniques are used. Single MS can be used, like the time-of flight (TOF) MS that was utilized in Paper IV. Another alternative is tandem MS that for example Wu et al. utilized [80]. The photometric and mass spectrometric methods can be coupled together, for extra powerful identification and confirmation, and this was utilized in Paper V.

Analysis using Capillary Electrophoresis-Mass Spectrometry

The method developed in Paper IV used an accelerated solvent extractor for the extractions, and the extraction program was a modified version of one published earlier [60]. The extraction solvent was ethanol/formic acid 99:1 v/v, and the total extraction time was seven minutes (five minutes pre-heating time plus two minutes extraction time) at 99ºC. The ethanol extract was evaporated to dryness under a gentle stream of nitrogen, and then the volume was made-up with water/formic acid 99:1 v/v to a final volume of 0.4 mL. The chromatographic capillaries (50 μm inner diameter, 365 μm
outer diameter, 57 cm total length) were coated with poly-LA 313 according to a procedure previously described [81]. Separation of the anthocyanin extract was performed with a negative CE voltage of –210.5 V/cm applied, and the background electrolyte was 15.0 mM ionic strength formic acid with pH 1.9. The separation capillary was coupled to an orthogonal acceleration time-of-flight mass spectrometer with a sheath liquid, methanol/water/acetic acid 80:19.9:0.1 v/v/v flowing at 2 μL/min (without nebulization). The molecular weights of the anthocyanins extracted from the mass spectrum compared well with those found in red onion in literature [80]. A typical electropherogram can be seen in Figure 12.

![Figure 12. An electropherogram from the analysis of anthocyanins in red onion with the CE-TOF-MS method used in Paper IV. Peaks shown are: EOF = electro-osmotic flow; 1 = either cyanidin 3-(malonoyl)-glucoside-5-glucoside or cyanidin 3-(6”-malonoyl-laminaribioside); 3 = either cyanidin 3-(3”malonoyl-glucoside) or cyanidin 3-(6”-malonoylglucoside); 5 = cyanidin 3-glucoside.](image)

**Analysis using Liquid Chromatography-Photo Diode Array Detection-Mass Spectrometry**

In Paper V, a pressurized and temperature controlled batch reactor was used for the extractions. The extraction liquid was water/ethanol/formic acid 94:5:1 v/v/v, and the temperature was set to 110°C. The pre-heating time from room temperature to target temperature was 8 minutes, and the extract peak level of different anthocyanin species was reached after approximately 5-17 min additional extraction time. The sample extracts were then separated with Liquid Chromatography (LC) on a Synergi Max-RP 2x100mm 2.5μm LC column (Phenomenex, Torrance, CA, USA). The chromatographic elu-
tion was performed using mobile phase A (1 vol% formic acid in acetonitrile) and mobile phase B (1 vol% formic acid in water). The flow rate was 200 µL/min and the optimal elution profile was a multistep linear solvent gradient: 0-2 min 0% A, 2-62 min 0-15% A, 62-70 min 15% A, 70-75 min 15-0% A, and 75-85 min 0% A. Detection was performed with a Photo Diode Array (PDA) detector (main wavelength monitored was 520 nm) coupled to an Ion-Trap Mass Spectrometer operated in positive mode ESI and Auto MS$^2$ mode. Peaks that had typical AC absorption at 520nm were screened for MS$^2$ transitions that corresponded to known AC molecular weights and fragmentation patterns. A typical chromatogram with PDA signals can be seen in Figure 13.

Figure 13. A chromatogram from the analysis of anthocyanins in red onion with the LC-PDA-IT-MS method used in Paper V. The peaks represent the absorbance at 520 nm. Major peaks that were evaluated: 1 = cyanidin 3-O-glucoside; 2 = cyanidin 3-laminaribioside; 3 = cyanidin 3-(6"-malonoylglucoside); 4 = cyanidin 3-(6"-malonoyl-laminaribioside).
Challenges in the analysis of acrylamide and anthocyanins in foods

Acrylamide

In a review of analytical methods for determination of AA in foods 2003, special attention was given to sample preparation, which represented the greatest differences between the methods [65]. It was concluded that several steps of the extraction procedure had not been thoroughly investigated, including the influence of extractant composition, extraction temperature/time, defatting of the sample and mechanical treatment during extraction. Another review from 2005 also requested optimization of extractive conditions, such as: grinding under dry or wet conditions and variable temperatures, extraction with various solvents (water, with or without organic solvents), swelling (e.g. of starch containing materials), extraction temperature, extraction time, number of extraction cycles and special needs such as defatting of the sample prior to the extraction [67].

An evaluation of the results from a proficiency test organized by the German Federal Institute for Risk Assessment (BfR) in 2002 showed large inter-laboratory differences, supposedly related to different extraction procedures [82]. In particular, there was a significant difference between aqueous and nonaqueous extraction of AA from cocoa powder. Also, in another proficiency test, jointly organized by BfR and EC DG-JRC, a cocoa sample presented large variations, which made it impossible to establish an assigned value [83]. In 2004, an evaluation of an inter-laboratory comparison study arranged by EC DG-JRC showed that a large number of the measured AA levels were outside the acceptable range, especially for crisp bread samples [84]. The choice of analysis technique (LC-MS/MS or GC-MS) had a significant influence on the results; GC-MS without derivatization was found to sometimes lead to an overestimation of the AA content [84]. Furthermore, the composition of the extraction solvent had a significant influence on the results, especially for crisp bread samples. It was concluded that additional work is necessary in order to identify problematic steps in the analytical procedures [84].
Certain pitfalls affecting the extraction of AA have been proposed. Three authors suggested *in situ* formation of AA during Soxhlet extraction of potato chips at 60°C in methanol [85,86,87], which gave faulty results in a method published by Pedersen and Olsson [56]. Another group, who used pressurized fluid extraction of food samples with ethyl acetate at elevated temperature and pressure, found incomplete extraction of AA from cacao and milk powder compared to the use of water as extraction solvent. Under the same extraction conditions, formation of AA in raw sugar samples was suspected, but not in the raw mashed potatoes blank [63]. These results indicated both incomplete extraction and formation as possible pitfalls. Furthermore, formation has also been suggested in the injection port during direct GC-MS analysis (if AA precursors are present) [66]. Other possible pitfalls include contamination of AA from lab-ware such as syringe- and ultra-filters [74] and thermal degradation of AA.

As a response to the above needs, a thorough investigation of common extraction factors and their resulting AA yield was performed in *Paper I*, in order to both optimize the extraction and to identify possible sources of errors and pitfalls. The tested samples were foods relevant for the daily intake of AA [24], *i.e.* coffee, crisp bread, potato crisps, milk chocolate and a mashed potatoes blank. Experimental designs were used to investigate the following extraction factors: sample particle size (fine or coarse), defatting (yes or no), extraction solvent (water or methanol/water 80:20 v/v), homogenization by ULTRA-TURRAX® (yes or no), extraction temperature (25 or 60°C), and extraction time (5 min to 17 h).

From the obtained results in *Paper I*, an optimized extraction procedure was devised that should be suitable for a wide range of food matrices. The method consisted of the use of disintegrated samples (particle size <1mm), water as extraction solvent with horizontal shaking of the sample (100 rpm) at 25°C for 45 min. This is a gentle and simple method without using organic solvents. It allows for a high sample throughput since multiple samples can be extracted in simultaneously. Both the defatting step and the ULTRA-TURRAX® homogenization were found to be superfluous.

In general, the results from the optimization experiments in *Paper I* indicated that incomplete extraction is the most likely cause of erroneous results in the extraction of AA. This might occur when the food is not divided finely enough, or when using organic solvent extraction, short extraction time, or low extraction temperature - in particular when using these factors in combination. Other possible error sources might be the destruction or formation of AA during the extraction procedure. Formation was not seen under any of the experimental conditions employed when a fructose-enriched blank potato sample was extracted. However, increased AA levels were noted at pro-
longed extraction times when a chocolate sample was extracted at 60°C for up to 17 h. Based on recovery experiments, it was concluded that the destruction of AA during extraction and work-up was small or absent for the investigated foods.

The new extraction procedure from Paper I was employed within an established in-house LC-MS/MS method using an effective two-stage solid phase extraction (SPE) clean-up of the extracts. The analytical results correlated well with those obtained by the original [73], more labor-intensive, extraction procedure and showed good agreement with the assigned AA levels of several proficiency test samples analyzed for evaluation. Furthermore, when the method later was evaluated in a collaborative trial validation study with sixteen participating laboratories, the performance of the method in terms of trueness, repeatability and reproducibility figures showed that it was “undoubtedly fit for the purpose” [77].

In 2006, there was a report on the impact of high pH on the extraction of AA from different food matrices. The results showed up to about four times higher amounts of AA extracted from certain foods at strongly alkaline conditions (pH 12) compared to extraction at normal conditions (pH 6). The authors proposed that under such alkaline conditions “…the structure of the matrix can be changed and made to facilitate free AA to get into solutions.” [44]. If this claim is true, all current analytical methods and exposure calculations are seriously underestimating the dietary AA intake.
As a response to the finding above, an extensive study was performed in Paper II, testing an alternative hypothesis - that the conversion of precursors under harsh extraction conditions furnishes AA. Acrylamide was heat-generated in a food environment containing an AA isotopomer (13C1- or 2H3-labeled), and the ratio of AA and the AA-isotopomer was compared after extraction at pH 7 (given), 9 and 12. The food matrices tested, both raw and fried, were potatoes and rye cakes. After extraction and centrifugation, the sample extracts were divided into a water-soluble part and a solid matrix pellet. Experiments were also performed without a food matrix, in chemical model systems consisting of pyrolysates of asparagine and fructose.

The results in Paper II showed a clear difference in AA levels between the aqueous sub samples extracted at pH 7 and 12, whereas no significant difference was observed between the samples extracted at pH 7 and 9. The same pattern was observed in the solid subsamples, although these contained only minor AA amounts (probably from residual aqueous AA). The results support the hypothesis that the AA released at pH 12 is furnished from water-soluble components of the matrix, and is not released physically from the solid matrix. The recovery of isotope-labeled AA added before roasting of potato or rye cakes was not influenced by pH. This shows that the incremental AA that was released at pH 12 in these food models was not due to improved extraction of AA that had been physically or chemically ‘bound’ to food components after formation, as the same behavior would be applicable to the isotope-labeled analogue. Instead, the final results of Paper II indicate that AA can be formed by unknown reaction pathways, probably involving water-soluble precursors, in high pH water extracts of heat-treated foods. Since no AA was detected in alkaline extracts of the raw foods, the precursors must have been formed during the roasting process.

It was suspected that the compounds responsible for the chemical formation of AA at alkaline pH would not have much relevance in terms of contributing to the bioavailability of AA, given the pH of the human gastrointestinal tract [88] and foods as such. Not surprisingly, the suspicions were confirmed later in a bioavailability study in mice [23].

Anthocyanins

As mentioned earlier, AC are sensitive to high temperature, light, and pH above the optimal range of pH 1-3 [18,19,20] and this of course creates challenges all through the analytical workflow. The longer time the AC stays in one of the above conditions, the worse. Therefore, analytical methods should be designed to avoid these conditions, or the corresponding holding time should be minimized. One simple way to avoid degradation by light is of
course to protect the sample from light at all times, which should be rather easy in most laboratories. The other two problems, temperature and pH, might be harder to avoid, depending on the analytical methods and facilities the analyst has access to. For example, regarding pH, some extraction equipment such as PSE® from Applied Separation; PLE® from Fluid Management Systems; and the older models of ASE® (models 100 and 200 – see Figure 15) from Dionex Corporation cannot withstand low pH (which is optimal for AC stability). Regarding temperature, sometimes the extraction equipment or premises used does not have cooling systems that allow the analyst to extract at a “safe” temperature (for the desired extraction time).

Figure 15. The Dionex ASE® 200 system.

Optimization of separation and detection of anthocyanins

As mentioned above, time is of the essence when analyzing anthocyanins, in order to avoid degradation. For the reason of speed, capillary electrophoresis (CE) is a promising separation technique, since it is in general more rapid than the more commonly used Liquid Chromatography (LC) technique [89]. Furthermore, CE can be considered an environmentally friendly technique since it uses only minor amounts of solvents. However, newer, improved and miniaturized LC techniques such as nano-LC could compete with CE in the respects above. The advantages of nano-LC over traditional LC include better separation efficiencies, shorter analysis time, and reduced flow rates [90]. Anyhow, no nano-LC method for analysis of AC could be found in literature - and it is important to have alternative separation techniques, such as CE, using a complementary separation mechanism to that of LC, to be able to solve the separation problems that LC perhaps cannot.

There have been attempts to analyze AC using CE-UV-VIS. Bridle and co-workers achieved separation of AC using CE with a pH 8 borate buffer [93,94]. However, since AC are not stable under alkaline conditions, the applicability of the method is limited. The use of acidic buffers has been tried by da Costa et al. [92] and Bednar et al. [79]. Da Costa et al. used an
The use of Mass Spectrometry (MS) for detection offers more structural information, and sometimes also higher sensitivity, than common ultraviolet-visible (UV-VIS) detection. Several varieties of MS exist. The one used in **Paper IV**, Time-Of-Flight (TOF) MS, has the potential to provide low mass errors, and thereby enables the identification of empirical formulas of molecules with higher accuracy as compared to the perhaps more common Triple Quadrupole-MS (tandem-MS) or Ion-Trap MS (IT-MS) techniques. Moreover, since TOF-MS does not use a more time-consuming scan mode to collect the full m/z range, as the two other techniques, it is very well suited for the detection of the narrow peaks that CE provides. A schematic of a TOF-MS can be seen in **Figure 16**.

Coupling CE with MS is an attractive opportunity; however, certain specific problems arise when doing so. Normally, non-volatile borate or phosphate buffers are used in CE in order to get highly efficient separations, but these buffers have poor compatibility with MS [91]. Furthermore, borate buffers usually have a slightly alkaline pH around 8-9, and could thereby cause problems with degradation of the anthocyanin analytes [92]. Non-coated capillaries are sometimes used for separation of AC in CE [79,92,93,94,95], although a risk of band-broadening due to interactions between negatively charged silica surface and positively charged AC cannot be excluded. Instead, the use of coated capillaries might be a better approach to avoid band-broadening effects [96]. All in all, a suitable approach for AC analysis with CE-MS seems to be the use of a volatile, acidic buffer, together with a coated capillary. This was tested in **Paper IV**.

A recent review reported that the use of CE-MS for analysis of natural antioxidants is a very rare combination [97]. In one of the few reports on this topic, Bednar et al. coupled CE to an IT-MS, and utilized both acidic and alkaline buffers in non-coated capillaries [79]. Detection limits were 0.8-1.5 mg/L with acidic buffer, and 4-10 mg/L with alkaline buffer. The problem when applying this method to real samples was that the preferred (more selective) buffer was the alkaline borate-ammonium (pH 9), once again with questionable applicability to pH-labile anthocyanins.

In **Paper IV**, a capillary electrophoresis-time of flight-mass spectrometry (CE-TOF-MS) analysis method for detecting anthocyanins was developed and used together with red onion samples for the first time. The method was environmentally friendly, rapid and sensitive. No organic solvents were needed in the final method, and very small amounts of samples were con-
sumed in the CE analysis. The run time for each analysis was relatively rapid, around 20 minutes including conditioning, which together with an acidic running buffer (pH 1.9) helped in preventing anthocyanins from degradation during the analysis. Since the running buffer was volatile, it was MS-compatible. The use of a coated capillary helped in preventing band-broadening effects (which could occur due to silica interactions with the positively charged anthocyanins). The limit of detection for the final method in Paper IV was estimated to 1.4 mg/L for cyanidin 3-glucoside; fully inline with the CE-IT-MS technique [79]. By using high resolution TOF-MS (Agilent LC/MSD TOF) with pre-run tuning of masses, low mass errors (12.5-29.0 ppm) were achieved in the determination of the conjugated AC. A simultaneous up-front fragmentation showed the presence of masses corresponding to the aglycone backbones of the AC, which increased the reliability of the identification. Most AC (at least seven out of ten) known in red onion from the literature were tentatively identified, as well as one new for this matrix.

Figure 16. Schematic of a Time of Flight Mass Spectrometry detection unit. Graphics by Andreas Dahlin.
For the experiments in Paper V, an alternative method of analysis was developed. An LC instrument with PDA-detector was used for routine analysis and quantification (at 520 nm), and an optional IT-MS detector was connected when more structural information was needed. For the routine analysis with the PDA-detector, a full separation of the AC species was required, since the quantification wavelength (520 nm) would otherwise overlap between peaks. Optimization of the LC gradient led to the method described in an earlier chapter, starting with 100% mobile phase B (1 vol% formic acid in water) for 2 minutes, and then an increase of the eluent, mobile phase A (1 vol% formic acid in acetonitrile), with 0.25% per min, up to 15% total acetonitrile content of the mobile phase. A faster increase of the acetonitrile content than 0.25% per min resulted in poor separation of some of the AC species.

The peaks with characteristic AC absorbance at 520 nm were investigated with an MS - Bruker Daltonics HCT ESI Ion Trap. The MS was set to operate with positive ionization and the Auto MS² mode, which runs a continuous full MS scan followed by fragmentation of the two largest peaks found during the cycle. The molecular ions of the two largest peaks are fragmented, and the m/z of the fragmented species is recorded. In the case of Paper V, the glycosylated molecular ion is fragmented into several ions, including the aglycone backbone of the AC. Thereby information is given about which of the AC aglycones, as described earlier in Figure 2, the molecule is based on. Furthermore, information is obtained about how many and which kind of sugar/acyl groups the molecule contained, since the weight of the sugar/acyl loss can be calculated as the weight difference between the molecular ion and the aglycone. With this method of analysis, ten AC were found totally, out of which seven were tentatively identified as AC found in red onion in the literature, and three could be found in other matrices [80].

More structural certainty for unknown compounds is obtained with the IT-MS running in Auto MS² mode, in Paper V, compared to using the TOF-MS in Paper IV. Although there was up-front fragmentation in the TOF-MS method, giving signals with molecular weights corresponding to both the aglycone backbone and the molecular ion, one can not be 100% certain that they are connected in the meaning that they come from the same molecule - even though they have the same migration time. This is because all molecules sprayed in the interface at a certain time can be potential sources of the recorded fragments during the up-front fragmentation. On the other hand, in Paper V, the fragmentation is controlled, since the MS isolates the selected molecular ion, and fragments it. Thereby all fragments formed should belong to this particular molecule – and the certainty of identification increases.

It should be noted that the identification of the AC in Paper IV-V is only tentative. For higher certainty, the identity of all compounds would have to
be confirmed with standards (though not all are available), and possibly also the use of Nuclear Magnetic Resonance (NMR) Spectroscopy, that probably could give the highest structural certainty.

**Optimization of extraction of anthocyanins**

In order to avoid degradation of AC during extraction, most extraction methods employ extraction liquids with low pH [21]. Regarding temperature, some research groups perform their extractions at very low temperature (down to -25°C), and then a long extraction time of up to four days is necessary [55]. As earlier mentioned, PHWE is an attractive technique, since it gives more rapid and efficient extraction, and minimizes the need for organic solvent, compared to conventional extraction methods. However, due to the AC instability, there is a potential problem in using PHWE - an increasing temperature will certainly achieve higher AC yield in a shorter period, but at the same time also lead to degradation of these thermo-labile compounds.

If anthocyanins are exposed to high temperature, non-acidic pH, or light during an extraction, the extraction curve might look as the blue curve in Figure 17. The blue curve, which is the actual extraction curve - what we can measure - is a combination of both extraction and degradation, due to the presence of degradation (red curve). If, however, we could avoid the degradation, we would have the ideal theoretical extraction curve (green) which would remain constant after a certain time of extraction, when all AC have been extracted.

![Figure 17. Calculation of an ideal theoretical extraction curve from experimentally obtained data on extraction and degradation. Graphics by Jiayin Liu.](image)

At high temperature over 100°C there is a lack of reliable data concerning degradation kinetics of AC [98]. Furthermore, most studies so far have been done using fruit juice or lab-produced food products that contain already...
extracted AC, therefore these studies are focused on the stability of AC in food products rather than the degradation during an extraction procedure. To make PHWE a more advantageous and applicable technique for AC extraction, the study of extraction and degradation kinetics from a natural vegetable matrix at high temperature is of vital importance. Therefore, extraction and degradation kinetics when extracting AC from red onion samples using PHWE at 110ºC were carefully investigated in Paper V. The working hypothesis was that the ideal theoretical extraction curve can be obtained by fitting together the actual extraction/degradation curve and degradation curve. The actual extraction/degradation curve and degradation curve can be measured separately from experiments, then the degradation effect can be compensated for by calculations, and the ideal theoretical extraction curve can be constructed. The overall goal was to develop a strategy, especially for thermolabile compounds, to distinguish between extraction kinetics from a sample and degradation/reaction speed in the extraction solvent.

The experimental procedure consisted of putting samples in the batch reactor in a solvent of water:ethanol:formic acid (94:5:1 v/v/v), and the temperature was set to 110ºC. The pre-heating time was about 8 min to heat up the 80-90 ml solvent that was used. Samples were taken out before heating, after pre-heating, and then every 10 min during the following 100 min. The actual extraction/degradation curve was obtained by extraction of a red onion sample (solid blue curve in Figure 18). The degradation curve was obtained by putting a red onion extract, free from solids (removed by filtering), in the reactor, and then follow the degradation (dashed blue curve in Figure 18).

The results from Paper V showed that extraction and degradation kinetics of AC could be successfully separated based on the experimental data as obtained above. It was observed that extraction and degradation effects compete already from the start of the extraction process. Firstly, the extraction effect dominated, but after a certain time, a maximum level of AC was reached, then degradation effects overcame the extraction effects, and the level decreased. A maximum (apparent) peak level of AC was reached after approximately 13-25 min extraction (out of which 8 min is pre-heating time) in the reactor, then the degradation effects took over and the AC levels started to decrease (see Figure 18). The apparent peak level is probably sometimes regarded as the “optimized” extraction point, and at the same time mistaken for complete extraction of the analyte.

An ideal theoretical extraction curve (describing extraction under ideal conditions without degradation present) was established using two different methods (Method A = red and Method B = green curve in Figure 18). In method A, the rate of degradation was calculated from the experimental degradation curve, while for method B it was calculated from the very last part
of the experimental extraction/degradation curve. The two methods gave similar results, i.e. theoretically, 21 to 36% more peak yield of AC (depending on species) could be obtained from red onions if there would be no degradation problems. This would require an extraction time of around 20-40 min (plus 8 min pre-heating time). The results in Paper V give important information about the different kinetics competing during an extraction procedure, and are to some extent probably applicable to lower temperatures as well (but then with slower rates).

Figure 18. Derived ideal theoretical extraction curves established with two different methods (Method A = red and Method B = green) for Cyanidin-3-(6’malonylglucoside). The Figure also shows the combined extraction/degradation data points as circles (triplicates) with the model values (calculated for each minute) as an adjacent solid blue curve. The degradation data points are shown as crosses (triplicates) and the corresponding model as an adjacent dashed blue curve.
Conclusions and future aspects

In this thesis, the main concern has been to improve the reliability of different parts of the analytical workflow (Paper I, II, IV & V). Additionally, one of the resulting optimized methods was used in a real application (Paper III).

The investigations concerning extraction of acrylamide (AA) from foods in Paper I resulted in an optimized extraction procedure that showed good performance when evaluated; and the method was applied successfully in a reduction study of AA in potato chips in Paper III. In Paper II, the same method was used to investigate a rather sensational extraction effect that had been proposed, and the conclusion was that this effect was actually a formation artifact.

The research concerning analysis of anthocyanins (AC) in red onion resulted in a new separation method using capillary electrophoresis in Paper IV. Its rapidness together with acidic background electrolyte helped in preventing degradation of AC. In Paper V, AC were extracted from red onion using pressurized hot water extraction at 110ºC, and the simultaneous extraction and degradation kinetics were successfully separated, and an ideal theoretical extraction curve was constructed by compensating mathematically for the degradation effects.

In summary, the investigations performed in this thesis takes us a little closer to measuring the true content of our analytes, by avoiding error sources such as: formation, degradation, and incomplete extraction. But this is just a small step forward – there are numerous challenges left in this enormous research area.

Regarding future work for acrylamide, the optimized extraction procedure should be combined with optimized clean-up and chromatography/detection steps. For anthocyanins, the CE method should be coupled to MS techniques that give even more structural information. Regarding the pressurized hot water extraction of anthocyanins, the quantitative potential of dynamic instead of static extraction should be investigated, to see if the yield could be increased.
Introduktion


Syftet med att äta är att hålla kroppen vid liv, men i extrema fall kan denna handling ge delvis motsatt effekt. Livsmedelsrelaterade risker kan delas in i nutritionella, mikrobiella och kemiska. Man utsätts för en nutritionell risk när man äter livsmedel med en ohälsosam sammansättning av näringsämnen, med en hög andel av energirika kolhydrater och fett och en låg andel vitaminer och mineraler. En mikrobiell risk kan vara när livsmedlet innehåller sjukdomsorsakande bakterier eller mögel. Sist men inte minst finns det kemiska risker när livsmedlet innehåller giftiga kemiska ämnen. I denna avhandling har det giftiga ämnet akrylamid studerats, vilket förekommer i en del värmebehandlade livsmedel.

Att äta livsmedel med icke-essentiella, men ändå nyttiga kemiska ämnen, har visat sig ge hälsofrämjande effekter, som t.ex. minskad risk för cancer, åldersrelaterade sjukdomar och hjärtssjukdomar. Exempel på sådana nyttiga ämnen är antioxidanter, varav en grupp ämnen som kallas antocyaniner har studerats i denna avhandling. Dessa förekommer som de röda och blå färgämnen som vi ser i bland annat bär, frukt, grönsaker och blommor.

Denna avhandling bygger på fem vetenskapliga artiklar, varav Artikel I–III behandlar analys av det giftiga ämnet akrylamid i värmebehandlade livsmedel och Artikel IV–V behandlar analys av antioxidanterna antocyaniner i rödlök.

**Den analytisk-kemiska arbetsgången**

För att kunna utföra en korrekt risk- eller nyttoberäkning av ett giftigt eller nyttigt ämne i livsmedel så är det viktigt att på ett korrekt sätt identifiera och kvantifiera det. En kemisk analys är en komplex procedur bestående av flera steg, som alla har en potentiell risk att misslyckas. En kemisk analys av ett livsmedel kan bestå av följande steg:

- **Provtagning** - en representativ och tillräcklig mängd material samlas upp och förvaras så att inte nedbrytning sker.
- **Homogenisering** - provet sönderdelas mekaniskt för att bli tillräckligt finmalet och blandat. Partikelstorleks betydelse undersöktes i Artikel I.
- **Vägning** - en väl avpassad mängd prov, tillräcklig för att ge en representativ bild, vägs noggrant in.
- **Extraktion** - en extraktionsvätska strömmar genom det invägda provet (under en viss tid, temperatur, tryck) för att få allt det önskade ämnet i lösning. Extraktionssteget undersöktes särskilt i Artikel I, II och V.
- **Upprening** - extraktet behandlas fysiskt (t.ex. centrifugeras) eller kemiskt (t.ex. genom utfällning) för att separera det önskade ämnet från andra ämnen i lösningen som kan störa analysen. En sorts upprenning som undersöktes i Artikel I var avfettning.
- **Separation** - det upprenade extraktet separeras kromatografiskt på en kolonn eller kapillär (olika ämnen vandrar olika snabbt genom dessa) för att ytterligare separera det önskade ämnet från störningar. I Artikel IV utvecklades och förbättrades separationssteget, likaså en del i Artikel V.
• Detektion - det önskade ämnet detekteras med avseende på identitet och mängd efter separationssteget. I Artikel IV fokuserades det på utveckling av detektionssteget, och i denna artikel samt i Artikel V studerades strukturbestämningssteget noggrant.

Resultat och diskussion

Denna avhandling har speciellt handlat om att förbättra extraktionssteget i den analytisk-kemiska arbetsgången. Några av de problem som kan uppstå under detta steg är bland annat: ineffektiv extraktion, ofrivillig bildning av ämnet man vill mäta, samt ofrivillig nedbrytning av ämnet man vill mäta.

Undersökningarna rörande extraktion av akrylamid från livsmedel i Artikel I resulterade i en optimerad extraktionsmetod som uppvisade utmärkt prestanda när den utvärderades i en extern avprövning. Metoden applicerades även framgångsrikt i Artikel III för att mäta minskningen av akrylamid i potatischips. Denna minskning uppnåddes genom att använda förbättrade förbehandlingar och friteringsmetoder. Även i Artikel II användes extraktionsmetoden, i detta fall för att undersöka en sensationell extraktionseffekt som gick ut på att extrahera akrylamid med mycket högt (basiskt) pH. Det visade sig att denna effekt var en extraktionsartefakt som gav ofrivillig bildning av akrylamid, och därmed felaktigt högre halter.


Slutsatser

Sammanfattningsvis har studierna som genomförts i denna avhandling tagit oss ett litet steg närmare på vägen mot att försöka mäta det sanna innehållet av olika ämnen i livsmedel. Detta genom att hitta sätt att undvika vanliga felkällor. Men detta är givetvis bara ett litet steg på vägen - antalet kvarvarande utmaningar är stort i detta enorma forskningsområde.
För fortsatta studier kring akrylamidanalyse vore det önskvärt att kombinera den framtagna, optimerade extraktionsmetoden med förbättrade upprenings- och separations-/detektionssteg. Framtida studier vad gäller antocyaniner skulle kunna vara att undersöka andra detektionsmöjligheter att koppla den framtagna separationsmetoden till, för att få ännu mer strukturell information. Vad gäller extraktion av antocyaniner med trycksatt hett vatten så borde det testas om ett dynamiskt flöde, i stället för statisk extraktion, kan ge bättre extraktionsutbyte. Detta på grund av bättre möjlighet till snabb nedkylning av de värmeäktsliga antocyaninerna efter avklarad extraktion.
Acknowledgements

Supervisors:
Charlotta Turner and Rolf Danielsson (Uppsala University), Johan Rosén and Karl-Erik Hellenäs (National Food Administration)

Collaborators:
Richard Stadler, Till Goldmann, Adrienne Perisset and Marie-Claire Bertholet (Nestlé), Lilia Masson, Conrado Camilo, Cristian Encina, Jose Muñoz, Luis Hernandez and Nalda Romero (University of Chile), Afaf Kamal-Eldin, Roger Andersson and Per Åman (Swedish University of Agricultural Sciences), Angel Puerta, Jiayin Liu, Arwa Mustafa and Jonas Bergquist (Uppsala University).

Proof readers:
Lars Petersson, Christina Classon, Jakob Axén, Ingrid Axén, Arwa Mustafa, Irene Rodriguez, Jiayin Liu, Michelle Co, Sofia Lindahl, Oskar Werner, Mikael Fridén.

Colleagues at Uppsala University and the National Food Administration.

Room-mates:
Ganna Shevchenko, Heidi De Brabandere; and especially Jörg Hanrieder for inspiring scientific discussions and friendship.

“Power Meet” accompanies:
Jakob Axén and David Malmström for friendship and cultural activities.

Exchanges:
Jana Hajslova for the stay in Prague, Lilia Masson for the stay in Santiago, Annalisa Romani for the stay in Florence.

Min familj:
Lars, Karin, Jonas och Thore Petersson, Maria Eklöf, Björn Petersson med familj, Lars Westerberg med familj; och alla andra släktingar. Jan och Marita Classon med släkt. Speciellt tack till min sambo Christina Classon!

Everyone that I forgot.
References


tion of black carrot [Daucus carota ssp sativus var. atrorubens Alef.] anthocy-
ianins. *European Food Research and Technology, 226*, 363-370.

62 Ju Z. Y., & Howard L. R. (2003). Effects of solvent and temperature on pressur-
ized liquid extraction of anthocyanins and total phenolics from dried red grape

63 Hoenicke, K., Gatermann, R., Harder, W. & Hartig, L. (2004). Analysis of
acrylamide in different foodstuffs using liquid chromatography – tandem mass
spectrometry and gas chromatography – tandem mass spectrometry. *Analytica

with water at elevated temperatures and pressures. *Trends in Analytical Chemis-
try, 26*, 396-412.

the determination of acrylamide in food products: a review. *Food Additives and
Contaminants, 20*, 885-902.

66 Castle, L. & Eriksson, S. (2005). Analytical methods used to measure acryla-

of acrylamide in heat-treated foods. Review and recent developments. *Journal of

monomer in hydroponically grown tomato fruit by capillary gas chromatogra-
phy mass spectrometry. *Journal of the Science of Food and Agriculture, 54*,
549-555.

69 Castle, L. (1993). Determination of acrylamide monomer in mushrooms grown on

(2002). Verifications of the findings of acrylamide in heated foods. *Food Addi-
tives and Contaminants, 19*, 1116-1124.

71 Nemoto, S., Takatsuki, S., Sasaki, K., & Maitani, T. (2002). Determination of
acrylamide in foods by GC/MS using 13C-labeled acrylamide as an internal

72 Rosén, J. & Hellenäs, K.-E. (2002). Analysis of acrylamide in cooked foods by

73 Fredriksson, H., Tallving, J., Rosén, J. & Åman, P. (2004). Fermentation re-
duces free asparagine in dough and acrylamide content in bread. *Cereal Chemis-
try, 81*, 650-653.

The acrylamide intake via some common baby food for children in Sweden dur-
ing their first year of life – an improved method for analysis of acrylamide.
*Food and Chemical Toxicology, 43*, 951-959.

Critical factors and pitfalls affecting the extraction of acrylamide from foods:

76 Rosén, J., Nyman, A. & Hellenäs, K.-E. (2007). Retention studies of acrylamide
for the design of a robust liquid chromatography-tandem mass spectrometry
77 Wenzl, T., Karasek, L., Rosén, J., Hellenäs, K.-E., Crews, C., Castle, L. & Ank-  

lam, E. (2006). Collaborative trial validation study of two methods, one based  
on high performance liquid chromatography–tandem mass spectrometry and on  
gas chromatography–mass spectrometry for the determination of acrylamide in  
78 Horwitz, W. & Albert, R. (1996). Reliability of the determinations of poly-  
chlorinated contaminants (biphenyls, dioxins, furans). *Journal of AOAC Inter-  
national*, **79**, 589-621.  
79 Bednar, P., Papouskova, B., Müller, L., Bartak, P., Stavek, J., Pavlousek, P. &  
(CE/MSn) for the study of anthocyanin dyes. *Journal of Separation Science*, **28**,  
1291–1299.  
cyanins by high-performance liquid chromatography-electrospray ionization-  
tandem mass spectrometry in common foods in the United States: Vegetables,  
polyamine coating for enhanced capillary electrophoresis of basic proteins and  
82 Webpage of Federal Institute for Risk Assessment, Berlin, Germany. Acryla-  
mide interlaboratory study.  
(Accessed 090909)  
83 Wenzl, T. (2005). Detailed report on the third European inter-laboratory com-  
parison study on the determination of acrylamide in food. Joint Research Centre  
– Institute for Reference Materials and Measurements, Geel, Belgium.  
84 Wenzl, T., de la Calle, B., Gatermann, R., Hoenicke, K., Ulberth, F. & Anklam,  
E. (2004). Evaluation of the results from an inter-laboratory comparison study  
of the determination of acrylamide in crispbreads and butter cookies. *Analytical  
85 Grob, K., Biedermann, M., Hoenicke, K. & Gatermann, R. (2004), ‘Comment  
on "Soxhlet extraction of acrylamide from potato chips" by J. R. Pedersen and J.  
86 DeVries, J. W. & Post, B. E. (2004). ‘Comment on "Soxhlet extraction of  
acrylamide from potato chips" by J. R. Pedersen and J. O. Olsson, Analyst,  
on "Soxhlet extraction of acrylamide from potato chips" by J. R. Pedersen and J.  
*Danish Medical Bulletin*, **46**, 183–196.  
cations in nanoliquid chromatography. *Journal of Separation Sciences*, **30**,  
1589–1610.  
ing separation solutions in capillary electrophoresis mass spectrometry. *Journal  
of Chromatography A*, **817**, 49-57.


A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology”.)