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New approaches to DNA isolation methods from food and feed-derived products

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To my family and the friends made along the way.

This is also yours.

"Nothing is more powerful than an idea whose time has come".

Victor Hugo

Deutsche Kurzfassung

Die vorliegende Arbeit konzentriert sich auf die Entwicklung neuartiger DNA-Isolierungsmethoden aus Lebensmitteln und Futtermitteln unter Verwendung verschiedener Strategien.

In jüngster Zeit hat sich die Verwendung ionischer Flüssigkeiten (ILs) aufgrund ihrer hervorragenden Eigenschaften zum Auflösen von Biomasse und Biopolymeren als innovatives Lösungsmittel für die Biomasseverarbeitung herauskristallisiert. In der ersten Studie dieser Arbeit wurde eine neuartige, einfach anzuwendende und zeiteffiziente Methode zur direkten Extraktion von genomischer DNA (gDNA) aus gentechnisch verändertem Mais auf Basis wässriger IL-Lösungen entwickelt. Das einfache Protokoll beruht auf der Extraktion von Mais in einer 10 wt% Lösung von ILs in wässrigem Phosphatpuffer (Na_2HPO_4/NaH_2PO_4, 50 mM, pH = 8,5) für 5 Minuten bei Raumtemperatur (RT), gefolgt von einem Inkubationsschritt bei 95 °C für 10 min, um coextrahierte Proteine zu denaturieren. Anschließend erfolgt eine einfache Filtration über eine PTFE-Membran (0,2 µm) zur Entfernung von Polymerresten. Ein Set von 22 ILs wurde in einem Puffersystem getestet und 1-Ethyl-3-methylimidazoliumdimethylphosphat ($[C_2mim]$ Me_2PO_4) sowie das umweltfreundlichere Cholinformiat ([chol]fom) wurden als ideale Kandidaten identifiziert. Mit dieser Strategie konnte die Qualität der extrahierten gDNA signifikant verbessert und das Extraktionsprotokoll im Vergleich zu einer etablierten Methode stark vereinfacht werden. Nach bestem Wissen des Autors war diese Arbeit die erste veröffentlichte Studie, in der eine schnelle und effiziente Strategie vorgestellt wurde, die auf neuartigen IL-Technologien zur Extraktion von DNA direkt aus einer komplexen natürlichen Matrix basiert. Der Redakteur der Fachzeitschrift Analytical and Bioanalytical Chemistry wählte diese Publikation, wie von den Peer Reviewern betont, als Ausnahmeartikel für eine schnelle Veröffentlichung und damit als „Paper in Forefront" aus.

Die Vielseitigkeit der zuvor vorgeschlagenen IL-basierten Methode wurde auf eine weitere Studie zur Entwicklung eines Verfahrens zur Extraktion mitochondrialer DNA (mtDNA) aus Fleisch und Fleischprodukten ausgeweitet. Zu diesem Zweck wurde ein Set von 20 ILs untersucht, von denen das umweltfreundliche Cholinhexanoat in Kombination mit einem Phosphatpuffer die vielversprechendsten Ergebnisse lieferte. In diesem Fall wurden 100 mg IL in 900 µL Puffer gelöst und 200 mg Fleisch zugegeben. Es folgte ein optionaler Rührvorgang für 15 min bei RT, ein Denaturierungsvorgang für 10 min bei 95 ° C und eine anschließende Zentrifugation bei 13.000 U/min für 4 min. Der resultierende Überstand wurde dann zur weiteren Analyse bei -20 ℃ gehalten. Die Extraktion erfolgte in verschiedenen Fleischsorten

wie Rind, Schwein und Pferd in deutlich höheren Erträgen als im reinen Phosphatpuffer. Mit dieser IL extrahierte DNA zeigte eine hohe Spezifität, da bei der Durchführung einer Agarosegelelektrophorese keine Nebenprodukte nachgewiesen wurden, eine hohe Ausbeute aufwiesen und bei 20-tägiger Lagerung bei RT stabil waren. Darüber hinaus wurde der Einfluss der IL auf den Amplifikationsprozess während der qPCR untersucht, wobei ein inhibitorischer Effekt mit zunehmender Kettenlänge der IL und höheren Konzentrationen an ionischen Flüssigkeiten gezeigt wurde. Zusammenfassend ist das entwickelte Verfahren von großem Vorteil, da es nicht nur die Verwendung toxischer und flüchtiger organischer Lösungsmittel vermeidet, die in anderen Extraktionsmethoden und kommerziellen Kits zu finden sind, sondern auch einen zeit- und energiesparenden Prozess bietet.

Eine dritte Studie befasste sich mit der Entwicklung einer schnellen gDNA-Extraktionsmethode und ihrer Kombination mit einer helikaseabhängigen Amplifikation zum Nachweis von gentechnisch verändertem Mais. Das Verfahren basiert auf dem Einsatz eines wässrigen Puffersystems in Kombination mit einem Proteinase K-Verdau. Darauf folgt später eine helikaseabhängige Amplifikation als Alternative zur PCR zum Nachweis von transgenem Mais durch Screening auf den *P35S*-Promotor in den Maissorten NK603, Bt-11 und MON810. Die erhaltenen Daten entsprachen denen, die mit der komplexeren CTAB-Extraktionsmethode oder dem Promega Wizard DNA – Aufreinigungskit erzielt wurden. Die in dieser Studie vorgestellte gDNA-Isolierungsmethode kann vor Ort durchgeführt werden und ist kostengünstig, einfach und zeitsparend.

Die in dieser Arbeit vorgestellten entwickelten Methoden gelten als Ausgangspunkt für zukünftige Extraktionsverfahren der neuen Generation. Durch die Anwendung neuer Isolierungsstrategien wird der immer noch bestehende Engpass komplexer, zeitaufwändiger und laborabhängiger Isolierungsprotokolle behoben. Sie vermeiden Wasch- und Filtrationsschritte und reduzieren so die Abfallansammlung. Im Vergleich zu konventionellen Methoden und kommerziellen Kits führen sie zu einfachen, kostengünstigen und viel schnelleren Extraktionsverfahren und liefern zuverlässige Ergebnisse sowie einen geringen ökologischen Fußabdruck.

Abstract

The present work focuses on the development of novel isolation methods of DNA from food and feed derived products by using different strategies.

Recently, the use of ionic liquids (ILs) has emerged as innovative solvents for biomass processing, due to their excellent properties for dissolving biomass and biopolymers. In the first study of this thesis, a novel, easily applicable and time efficient method for the direct extraction of genomic DNA (gDNA) from genetically modified maize based on aqueous IL solutions was developed. The straightforward protocol relies on the extraction of maize in a 10 wt% solution of ILs in aqueous phosphate buffer (Na_2HPO_4/NaH_2PO_4, 50 mM, pH = 8.5) for 5 min at room temperature (RT), followed by an incubation step at 95 °C for 10 min for denaturing coextracted proteins. Afterwards, a simple filtration over a PTFE membrane (0.2 µm) to remove residual polymers is performed. A set of 22 ILs were tested in a buffer system and 1-ethyl-3-methylimidazolium dimethylphosphate ($[C_2mim]Me_2PO_4$), as well as the more environmentally benign choline formate ([chol]fom), were identified as ideal candidates. With this strategy, the quality of gDNA extracted was significantly improved and the extraction protocol was simplified to a large extent when compared to a well-established method. To the best of the author´s knowledge, this work was the first published study to present a fast and efficient strategy based on novel IL technologies for the extraction of DNA directly from a complex natural matrix. As emphasized by the peer reviewers, the editor from the journal Analytical and Bioanalytical Chemistry selected this publication as an exceptional paper for rapid publication and thus as a "Paper in Forefront".

The versatility of the previously proposed method based on ILs was expanded to another study for develop a procedure to extract mitochondrial DNA (mtDNA) from meat and meat derived products. A set of 20 ILs was investigated for this purpose, from which the environmentally benign choline hexanoate combined with a phosphate buffer gave the most promising results. In this case, 100 mg of IL were dissolved in 900 µL buffer and 200 mg of meat were added. This was followed by an optional stirring process for 15 min at RT, a denaturation process for 10 min at 95 °C and a subsequent centrifugation at 13,000 rpm for 4 min. The resulting supernatant was kept then at -20 °C for further analysis. The extraction was carried out in different types of meat such as beef, pork and horse in significantly higher yields compared to the pure phosphate buffer. DNA extracted with this IL showed high specificity as no by-products were detected when performing an agarose gel electrophoresis, had a high yield and was stable when stored at RT for 20 days. Furthermore, the influence of the IL on the

amplification process during qPCR was investigated, showing an inhibitory effect with increasing chain length of the IL and higher ionic liquid concentrations. As a summary, the developed method is highly advantageous, as it not only avoids the use of toxic and volatile organic solvents which can be found in other extraction methods and commercial kits, but it also provides a time and energy saving process.

A third study involved the development of a rapid gDNA extraction method and its combination with helicase dependent amplification to detect genetically modified maize. The method is based on the employment of an aqueous buffer system in combination with a proteinase K digestion. This is later followed by a helicase dependent amplification, as an alternative to PCR to detect transgenic maize by screening for the *P35S* promoter in the maize varieties NK603, Bt-11 and MON810. Data obtained were similar to those achieved with the more complex CTAB extraction method or the Promega Wizard DNA purification kit. The gDNA isolation method as presented in this study can be performed on-site, is inexpensive, simple and time saving.

The developed methods presented in this thesis are considered to be a starting point for future new generation extraction procedures. The application of new isolation strategies will remove the still existing bottleneck of complex, time consuming and laboratory dependent isolation protocols. They avoid washing and filtration steps and thus reduce accumulation of waste. When compared to conventional methods and commercial kits, they result in simple, inexpensive and much more faster extraction procedures, while providing reliable results and a low environmental footprint.

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Abbreviations

ILs	Ionic liquids
DNA	Deoxyribonucleic acid
gDNA	Genomic deoxyribonucleic acid
Na_2HPO_4	Sodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
RT	Room temperature
PTFE	Polytetrafluoroethylene
$[C_2mim]Me_2PO_4$	1-ethyl-3-methylimidazolium dimethylphosphate
[chol]fom	choline formate
mtDNA	Mitochondrial deoxyribonucleic acid
qPCR	Quantitative real-time polymerase chain reaction
CTAB	Cetyl trimethylammonium bromide
HACCP	Hazard Analysis and Critical Control Point
NASA	National Aeronautics and Space Administration
FMEA	Failure, Mode and Effect Analysis
FDA	Food and Drug Administration
FAO	Food and Agriculture Organization of the United Nations
WHO	World Health Organization
ISO	International Organization for Standardization
EC	European Commission
US	United States
FSMA	Food Safety Modernization Act
GMOs	Genetically modified organisms
GM	Genetically modified
GC-MS/MS	Gas chromatography tandem mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LC-TOF-MS	Liquid chromatography time of flight mass spectrometry
IRMS	Isotope ratio mass spectrometry
MC-ICP-MS	Inductively coupled plasma mass spectrometry
TIMS	Thermal ionization mass spectrometry
FTIR	Fourier transform infrared spectroscopy
FT-Raman	Fourier transform raman spectroscopy

Abbreviations

SyF	Synchronous fluorescence spectroscopy
SNIF-NMR	Site-specific natural isotope fractionation nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time of flight mass spectrometry
DART-MS	Direct analysis in real time mass spectrometry
ELISA	Enzyme-linked immune sorbent assay
EU	European Union
RTILs	Room temperature ionic liquids
B. amyloliquefaciens	*Bacillus amyloliquefaciens*
B. licheniformis	*Bacillus licheniformis*
^{31}P NMR	Phosphorous-31 nuclear magnetic resonance
EDTA	Ethylenediaminetetraacetic acid
HDA	Helicase dependent amplification
LAMP	Loop mediated isothermal amplification
RCA	Rolling circle amplification
SDA	Strand displacement amplification
NEAR	Nicking enzyme amplification reaction
RFLP	Restriction fragment length polymorphism
SSCP	Single strand displacement polymorphism

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1. Introduction

1.1 Aims and scope of this thesis

This thesis was conducted at the Molecular Diagnostics Group at the Center for Analytical Chemistry (IFA Tulln) under the supervision of University Professor Dr. Robert Mach and Dr. Kurt Brunner. The aim of this thesis was the development of new strategies for the fast extraction of genomic DNA (gDNA) with the purpose of detecting genetically modified maize as well as for food quality control.

The thesis consists of three chapters. The first chapter, the introduction, gives an overview of current legislation on food safety and its regulatory environment in the European Union and the United States of America, as well as current standards and quality control approaches implemented in the food industry. In addition, a short overview of genetically modified organisms and food authenticity is given. The following section within the chapter describes the use of ionic liquids for the isolation of biomolecules as well as their role in purifying and stabilizing DNA. The chapter continues by describing current approaches to DNA extraction together with the challenges posed when carrying out DNA extractions from different foods and its subsequent use in downstream applications. Afterwards, various DNA based techniques for the analysis of foods are presented. The second chapter continues with three Science Citation Index (SCI) publications selected for this thesis. Prior to each publication, a short summary is given. Finally, the conclusions draw attention to important achievements of the original works.

Different crop plants and raw food products must be tested for genetic modifications when entering the European Union or when processed to food and feed derived products to authenticate its origin. The standard method for testing a sample for this purpose is to extract the DNA and then perform either a qualitative or quantitative PCR to detect and quantify any artificial inserts into the genome or specific DNA sequences that differentiate species between each other. Unfortunately, PCR needs high-end equipment and skilled personnel. This drawback has been overcome by applying isothermal DNA amplification methods. Those simple procedures can be carried out at constant temperatures of 37 °C and 65 °C and PCR thermocyclers are not required anymore. However, for all those methods high quality DNA must be used as an input. Moreover, the isolation and purification of DNA is considered to be a bottleneck, as those methods are associated with extraction times ranging from 3 to 4 hours and multiple pipetting and centrifugation steps, which makes the entire procedure not only tedious and complicated, but also susceptible to sample cross-contamination. In addition, it

cannot be performed on-site as a centrifuge is always required. Among those methods, the most traditional ones employ surfactants and organic solvents, such as chloroform and isoamyl alcohol or ethanol for the dissolution of proteins, while keeping the DNA in the aqueous phase. Other extraction methods based on commercial kits are also available, but their price is very high, and the amount of sample usually extracted is very limited.

To face those drawbacks, this thesis proposes a first work that involves the development of a time efficient method for the direct extraction of gDNA with pure ionic liquids or their mixtures with aqueous buffer systems to finally detect genetically modified maize. The use of aqueous ionic liquid buffer systems was further expanded in a second work to develop an extraction method of mitochondrial DNA (mtDNA) from meat and meat derived products intended for food quality control. A third publication combines the development of a rapid gDNA extraction procedure with helicase dependent amplification to detect genetically modified maize. All of the performed experiments allow for quick, easy-to-perform and reliable methods of DNA extraction subsequently used for application.

1.2 Food Safety

1.2.1 Generals aspects and regulatory environment

Food safety refers to all the hazards, whether chronic or acute, that may make food harmful to the health of the consumer.[1] The incidence in which two or more persons experience a similar illness after the ingestion of a common food is known as a foodborne disease outbreak.[2] In this sense it is important to point out that food safety needs to be considered in the entire food chain, from production and market and later between market and consumer.

Foodborne disease outbreaks are considered to be important in the world, usually resulting in substantial morbidity, mortality and economic costs. [3] In humans, symptoms derived from foodborne pathogen infections range from milder complications like diarrhea, vomiting, abdominal pain, fever, headaches and muscle aches to more severe problems like enterotoxin poisoning, autoimmune complications, meningitis, septicemia, bloody diarrhea, hemorrhagic colitis and also miscarriage in pregnant women. [4] [5]

Norovirus is the most common cause of outbreaks registered in many countries, with the Center for Disease Control in the USA reporting this virus as being responsible for 50% of foodborne outbreaks. [5] Although norovirus is often spread through contact with persons, it can be transmitted through foods like lettuce, fresh fruits and shellfish. The infection with this virus

is characterized by vomiting and/or diarrhea, together with nausea and pain and cramps in the abdominal area. [6]

Another important major public health concern are foodborne outbreaks resulting from infections from *Salmonella* and *Campylobacter*, both Gram-negative bacteria. [7] [8] Recent statistics show that cases of campylobacteriosis and salmonellosis in the European Union have been relatively constant in the last decade. [9] Clinical manifestations of salmonellosis include enteric fever, gastroenteritis and bacteremia, [7] while campylobacteriosis is usually characterized by gastroenteritis, but can also lead in extreme cases to autoimmune diseases like the syndromes Guillain-Barré and Miller Fisher. [10] These pathogens are found in poultry, eggs and dairy products. [7]

Another microbiological contaminant is the bacterium *Clostridium botulinum*, which causes botulism by producing botulinum toxins. Foodborne botulism is caused by the ingestion of foods contaminated with the toxin. In this case, the bacterium will generate the toxin when specific conditions are present, like an anaerobic environment, a pH lower than 4.5, low salt and sugar content and a temperature of 4°C to 121°C. [11] Those conditions makes canned food the major source of contamination, which is why heat sterilization is always recommended. [12] The toxin produces skeletal muscle paralysis, double vision, dropping of eyelids, postural hypotension and difficulty with talking. [13]

Other important agents responsible of foodborne diseases include *Listeria monocytogenes*, *Escherichia coli*, *Trichinella spiralis*, *Shigella sonnei*, *Yersinia enterocolitica*, *Clostridium perfringens* and *Giardia*. [9] [14] [15]

To ensure the safety and quality of foodstuffs and to decrease the occurrence of the aforementioned foodborne diseases, the Hazard Analysis and Critical Control Point (HACCP) system was created. [16] The approach allows the production of safe products through a detailed analysis of production processes, identification of all hazards that are likely to occur in the production facility, the identification of critical points in the process at which these hazards may be introduced into the product and thus should be controlled, the creation and verification of critical limits for control at those points as well as the methods for monitoring the HACCP system by both the processing establishment and the regulatory authority. [17] The system was born out of two major developments. The first one relates to W.E. Deming, who was the developer of Total Quality Systems and it is widely recognized as a key person in improving the quality of Japanese products in the 1950s. The second development was associated with the HACCP application by the Pillsbury company, NASA and US Army laboratories; its initial concept was built on the proposal of "Failure, Mode and Effect Analysis" (FMEA), as used by

engineers in construction designs. [17] After its introduction in 1971, it has been widespread implemented in the food industry and included in the Codex Alimentarius Commission, European regulation EC 852/2004, FDA legislation and FAO/WHO guidelines. [16]

For its part, ISO 22000 (Food safety management systems – Requirements for any organization in the food chain) is a standard developed by the International Organization for Standardization, which is based within the framework of a structured management system and included in the overall management activities of the organization in order to establish, implement, monitor and update the most effective food safety systems. It also incorporates HACCP principles and application steps developed by the Codex Alimentarius Commission. [18]

The European Union is considered to have very strict standards in terms of food safety in the world. In this sense, regulation (EC) 178/2002 lays down the general principles and requirements of food law, establishing the European Food Safety Authority and also procedures to ensure food safety. [19] Additionally, this law forces all food and feed business operators to have adequate traceability systems in place, covering all stages from manufacturing, packaging and distribution, thus involving the declaration of ingredients, processes, test results, environment, transportation methods, etc. If any part of the supply chain has been compromised in terms of compliance, it also demands operators to immediately initiate procedures to withdraw the food or feed from the market and inform the authorities.

Moreover, regulation (EC) 1169/2011 sets the rules on food labeling, presentation and advertising. It also establishes the need for nutrition labeling and specific information about the presence of allergens and ingredients. This regulation intends to protect local quality labels while ensuring the free movement of goods within the Community, the fairness of trade as well as protection of consumer´s health and interests. [20]

In the case of the United States, the Food and Drug Administration published the Food Code for the first time in 1993, which is updated every four years (latest version of 2017). The document provides very extensive and detailed guidelines for facility construction, waste handling, product labeling, safe food storage, handling and preparation, etc. [21] Additionally, the US Department of Agriculture guidelines cover both not only the design of the built environment, but also processing procedures to minimize transmission of pathogenic microorganisms. [22] More recently, the Food Safety Modernization Act (FSMA) was signed into law in 2011, changing the food safety focus from a reactive to a preventive approach. The law improves the capacity to prevent, detect and respond to food safety problems as well as improves the safety of imported food. [23]

A thorough analysis of the complex topics related to food safety and the regulations behind it go beyond the scope of this thesis. As a result, this work focuses on specific areas, such as the analysis of genetically modified organisms and authentication, where progress in the development of rapid on-site extraction and testing systems has been accomplished, together with the use of ionic liquids.

1.2.2 Genetically modified organisms

Genetically modified organisms (GMOs) are organisms whose hereditary disposition has been altered by following genetic engineering techniques that allow the insertion or deletion of specific sequences. [24] In the case of plants, for example, the modification can give a new characteristic to the organism, like tolerance to herbicides or resistance to insects. For the genes to be able to express the new characteristic a construct is required, formed by a promoter, a target gene and a terminator (table 1). [25] After this construct is designed, it should be inserted in the plant genome. Once this is done, each transformation results in a number of transformed cells that contains the same construct, but located in different parts of the genome. As a consequence, this region in the genome, called junction, can be identified by amplifying its sequence. This is known as event specific PCR. [24] The event numbers are often retained in the name of the product, such as Bt11, a genetically modified maize which carries after transformation event 11 two genes, a *pat* gene from *Streptomyces viridochromogenes* and a *cry1Ab* gene from *Bacillus thuringiensis*. [26]

Table 1. Common promoters and terminators. [24]

Element	Originating from organism
Promoter	
P-35S	Cauliflower mosaic virus (CaMV)
P-NOS	*Agrobacterium tumefaciens*
P-Ssu	*Arabidopsis thaliana*
P-FMV	Figworth mosaic virus
P-TA29	*Nicotiana tabacum*
Terminator	
T-NOS	*Agrobacterium tumefaciens*
T-35S	Cauliflower mosaic virus (CaMV)
T-E9	*Pisum sativum*
T-ocs	*Agrobacterium tumefaciens*
T-tml	*Agrobacterium tumefaciens*
Coding sequence (gene)	
Npt II	*Escherichia coli*
Pat	*Streptomyces viridochromogenes*
CP4EPSPS	*Agrobacterium tumefaciens*
Cry	*Bacillus thuringiensis*
GUS	*Escherichia coli*
Barnase	*Bacillus amyloquefaciens*
Barstar	*Bacillus amyloquefaciens*
Bla	*Escherichia coli*
Bla	*Streptomyces hygroscopicus*
Aad	*Escherichia coli*

China was the first country to commercialize a transgenic crop in the early 1990s, using an antibiotic resistant tobacco plant. [27] In 1994 the Food and Drug Administration in the USA approved marketing the tomato Flavr Savr™ (Flavor Saver) from the Californian company Calgene (later bought by Monsanto). The tomato contains the *pg* (sense or antisense) gene, which inhibits the production of polygalacturonase responsible for the breakdown of pectin molecules in the cell wall, which allows for the delayed ripening after the fruit is picked. Another gene inserted is *nptII* encoding the enzyme neomycin phosphotransferase II, to permit the metabolism of neomycin and kanamycin antibiotics during selection. [28] [29] Another desired

trait in GM plants include insect resistance due to the presence of gene variants of Cry proteins from *Bacillus thuringiensis* for the synthesis of Bt toxins. Another important feature is herbicide tolerance, which is conferred by the insertion of the EPSPS gene of *Agrobacterium tumefaciens*. The gene codes for the synthesis of the enzyme 3-enol-pyruvylshikimate-3-phosphate synthase that is not inhibited by the herbicide glyphosate and thus it makes it resistant to the Roundup Ready herbicide. [30] [31]

Biotech crops have increasingly expanded to include other plants, such as soybean, maize, cotton and canola. In 2016 farming areas for this kind of crops were globally increased from 179.7 to 185.1 million hectares. [32] According to the International Service for the Acquisition of Agri-Biotech Applications (ISAAA), countries like USA, Canada, Argentina, Brazil, China, India, Pakistan, Paraguay, South Africa and Uruguay were the top ten countries planting biotech crops in 2016. [33]

In spite of this continued growth and benefits, the position of the European Union is cautious, for the debate on the safety of GM crops for animal and human health is still ongoing. The introduction of numerous GMOs into feed and food products have occurred and thus Directive 2000/13/EC requires adequate labeling of GM products and their derivatives. [34] In this sense, the European Union introduced a threshold of 0.9 % for the mandatory labeling and traceability of GMOs, based on the Regulations (EC) 1829/2003 [35] and (EC) 1830/2003 [36]. GM contents with a higher percentage than the one specified, have to be labeled accordingly. In the case of unapproved biotech crops, zero tolerance is applied (0%), while a 0.5% threshold was defined for crops evaluated positively scientifically that have not received final approval through the European Commission.

To comply with those regulations, a great demand of robust and reliable analytical methods is needed, to allow for the detection, identification and quantification of DNA or proteins of a GM crop. However, there is a substantial amount of unresolved topics regarding the testing systems, such as sampling strategies, extraction methods, reference materials, multiplexing, sequencing, new targets, accreditation and the economic impact of testing. [37] The presence of unauthorized events is also playing an important role, as they have not received any regulatory approval in any country, so they could be considered unsafe and unknown. In this case, no or few event-specific methods are available to ensure the traceability in food and feed markets and therefore new approaches are needed. [38]

1.2.3 Food authenticity

The last two decades have seen the creation of more sophisticated and elaborated food safety control systems, dedicated institutions as well as public awareness of food safety [39]. In spite of this, incidents still occur and when issues arise, they are usually linked to one of the following categories: economic adulteration of high value foods, false description of the geographical, botanical or species origin, non-compliance with the established legislations as well as the implementation of non-acceptable process practices. [40]

The word authentic is usually defined as a synonym of reliability, trustworthy, of undisputed origin, genuine. [41] When applied to foods, this term involves the use of a wide array of chemical analytical methods to face the increasingly difficult challenge of detecting adulteration, misdescription and fraud. [42] Among these techniques stand out those based on genomics and proteomics (PCR, PCR-SSCP, RAPD, DNA fingerprinting), chromatography (GC-MS/MS, LC-MS/MS, LC-TOF-MS), isotopes (IRMS, MC-ICP-MS, TIMS), vibrational and fluorescence spectroscopy (FTIR, FT-Raman, SyF), magnetic resonance (SNIF-NMR, 1H-NMR), sensory analysis, non-chromatography mass spectrometry (MALDI-TOF-MS, DART-MS), immunology (ELISA) and chemometrics-bioinformatics. [43] [44]

One of the most well-known adulteration cases in recent times was the purposely contamination of milk powder with melamine in China in 2008. [45] [46] [47] The main reason behind the adulteration was to increase the protein content at less cost. This quality parameter is usually measured with methods like Kjeldahl and Duma, which take into account the nitrogen content in the matrix. The results obtained in those tests can be falsified by adding a nitrogen rich chemical like melamine, which also happens to be a white yellowish powder, covering up the appearance of powder milk. At that time the scandal involved several farmers and dairy manufacturers that were diluting the milk and infant formula, but were still meeting the high protein content requirements needed to pass the quality tests. The health impact of the crisis included renal failure and kidney stones in those persons that consumed the milk. Consumption of the infant formula also brought as a consequence the hospitalization of almost 51,900 children and the death of 6 children at the end of November 2008.

Another incidence of adulteration was the horse meat scandal in Europe in 2013. [48] [49] The crisis was caused by the substitution of beef with horse meat in the lasagna produced by Comigel and marketed by Findus, which lead to the checking of other meat products marketed in Europe. As a consequence, horse meat was identified in other products containing meat, which were initially declared to have meat from other animal species. [50] This incidence, however, did not represent a threat to human health, but involved a fraudulent labeling. The

fraudsters took advantage of the shortcomings of the supply chain and as a consequence both the image of the companies that did obey the law and the consumer interests were affected. After this incidence, the European food sector faced a consumer trust crisis where the consumption of red meat was reduced, products containing beef were boycotted and many consumers changed their dietary habits towards other types of meat or vegetarian diets. [48]

The identification of the origin of a food is certainly a complex task and includes several factors, such as the issue under investigation being dependent on legal requirements, standards or guidelines. In this case a correct interpretation of the results must consider the analytical uncertainty, the natural occurring variations and any tolerance allowed by the specifications that define the food in question. Additionally, a specific marker or markers that characterize a food, its ingredients, production and its geographical origin also needs to be taken into account. A third point deals with the availability of authentic samples for the development and assessment of the method. [44] [51] [52]

Labeling legislation also plays an important role in authenticating a food. In this case legal requirements force producers not only to declare the name given to the food, but also the ingredients used, the treatment and processing to which the food has be subjected as well as its geographic origin. In Europe, European regulation (EU) No 1169/2011 [20] demands that consumers should be appropriately informed about the food they consume. This is of great importance, so as to attain a high level of health protection and to guarantee their right to information, as well as to protect businesses of reliable producers from unscrupulous competition. Consumers´ choices can be influenced by health, economic, environmental, social and ethical considerations. The table 2 lists several ways in which food can be falsely described.

*Table 2*. Examples of false description of foods. [44]

Substitution of one ingredient by a similar but a cheaper one	- - -	Using whiting or pollack in place of cod Substituting bonito for tuna or sea trout for salmon Labelling cheaper varieties of potatoes as "King Edward" variety
Extending or adulterating food with a cheaper or base material	- - - - - - -	Adding water to increase weight of chicken breasts Mixing long-grain rise with Basmati rice Mixing cow´s milk with buffalo milk before production or buffalo mozzarella cheese Adding common wheat to durum wheat pasta labeled 100% durum wheat Extracting soluble coffee from beans mixed with skins and husks Adding cheaper vegetable oils to named higher value vegetable oils Adding water, sugar, acids and coloring to fruit juices
Presence of undeclared ingredients	- - -	Offal in processed meat products Donkey or horse meat in salami Mechanically separated meat in processed meat products
Extending or adulterating food to increase value	- -	Adding mandarin or tangerine juice to orange juice to improve color of juice Adding glycerol to wine to improve body
Non-declaration of false declaration of processes	- - -	Labeling poultry as fresh even though it has been previously frozen Failure to declare that food has been irradiated Failure to declare that juice has been prepared from concentrate
Over-declaration of a quantitative ingredient	-	Including hydrolyzed protein as part of the meat content
False claims regarding geographical or production origin	- - - -	Labeling South American beef as British beef Declaring farmed fish as "wild" Labeling conventionally produced food as organic Claiming that extra virgin olive oil is from a particular geographical region

1.3   Ionic liquids

1.3.1   Fundamentals

Ionic liquids (ILs) are a broad class of salts with considerable liquid ranges that melt at or below 100° C. Those ILs that are liquid at room temperature (~25°C) are known as room temperature ionic liquids (RTILs). In most common cases, RTILs have an asymmetrically substituted nitrogen containing cation (e.g. imidazolium, pyrrolidininium, pyridinium) and a halogen-based anion (e.g. Cl-, Br-, I⁻, $[BF_4]^-$, $[AlCl_4]^-$, $[PF_6]^-$). Examples of organic anions can be trifluoromethylsulfonate $[CF_3SO_3]^-$, bis[(trifluoromethyl)sulfonyl]imide $[(CF_3SO_2)_2N]^-$ (*i.e.*, NTf₂) and trifluoroethanoate $[CF_3CO_2]^-$. [53] The following picture (figure 1) shows the structures of the most common cations and anions present in ILs:

Common cations:
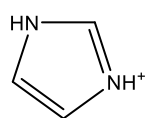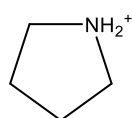
imidazolium   pyrrolidinium   pyridinium   tetraalkylammonium
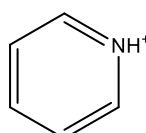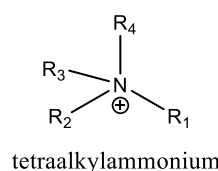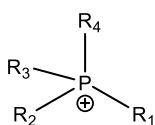
tetraalkylphosphonium

Common anions:
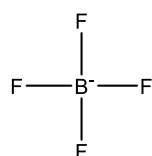
tetrafluoroborate   hexafluorophosphate   chloride   trifluoroacetate
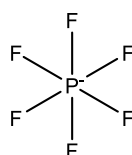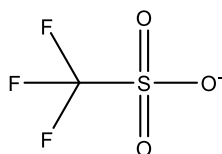
trifluoromethanesulfonate   bis((trifluoromethyl)sulfonyl)imide

Figure 1. Chemical structures of common cations and anions used in ionic liquids.

The first IL described in literature [54], ethylammonium nitrate with a melting point of 12°C, dates back from 1888. This same IL was studied in a more systematic and thorough way by

Paul Walden some years later, in 1914. [55] However, the discovery was ignored for a long time, most probably due to the prevalent opinion at that moment that insufficient purity had resulted in a liquid instead of the expected solid. [56]

Ever since, hundreds of ILs have been reported in literature. In this sense, an almost limitless number of ILs can be achieved, due to the variable combination of cations and anions which gives them outstanding properties. Besides their low melting point, IL have many other exceptional physicochemical properties, such as broad liquid ranges, low vapor pressures, good thermal stabilities and good extractabilities for various organic compounds and metal ions. [57] [58] By changing the combination of cation and anion in an IL, their miscibility with water and organic solvents, as well as their viscosities, can be adjusted. For this reason, they are also known as designable solvents. [59]

Those properties can be adjusted by varying the length and branching of the alkyl groups incorporated into the cation. In this case Seddon et al. [60] showed how the replacement of the $[PF_6]^-$ anion with $[BF_4]^-$ in 1-alkyl-3-methylimidazolium hexafluorophosphate $[C_nmim][PF^6]$ considerably increased the solubility of the IL in water. On the other hand, the replacement with the $[Tf_2N^-]$ anion decreased water solubility. In addition, the change in length of the 1-alkyl chain from 1 to 9 carbons on the same IL could turned the liquid from being soluble in water to very immiscible.

In terms of pricing, the substitution of common solvents with ILs should be called into question and needs to be justified, as their non-volatility can turn their recovery and purification process into expensive and complex processes. Additionally, ILs themselves are high-priced. In spite of their good commercial availability nowadays, they are con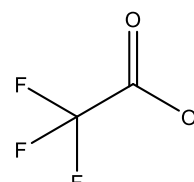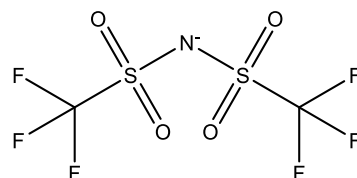sidered to be 5 to 20 times more expensive than the organic solvents they intended to substitute. [61] [62] Nonetheless, Chen et al. [63] managed to synthesize [HSO4]- based solvents, such as triethylammonium hydrogen sulfate $[HNEt_3][HSO_4]$ and 1-methylimidazoliumhydrogen sulfate $[C_1Him][HSO_4]$, via acid-base neutralization at a relatively inexpensive cost. It is also important to point out that the aforementioned financial drawbacks should be also weighed according to their significantly outstanding property.

For the extraction of DNA in different matrices, more than 50 ILs containing numerous cations and anions were produced in this thesis. The general method taken into account for the synthesis of ILs is shown in figure 2, including synthesis via metathesis and acid-base reactions. [64] The first step (step I) involves the formation of the desired cation, which can be synthesized by the protonation of the amine by an acid or by means of quaternization reactions (nucleophilic alkylations) of the amine with a haloalkane and by heating the mixture. A second

step involves either anion exchange reactions (step IIa), which can be carried out by treating halide salts with Lewis acids to form acid-base liquids or by anion metathesis (step IIb).
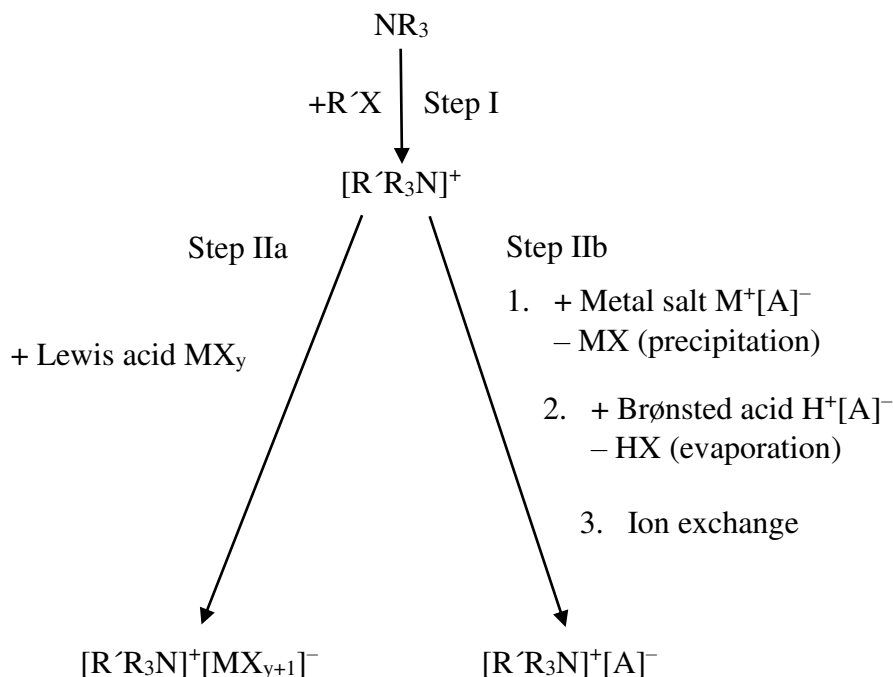
Figure 2. Synthesis pathways for the preparation of ionic liquids represented for an ammonium salt. [12]

The most studied and utilized ILs based on Lewis acids are salts based on $AlCl_3$.[65] Their preparation takes into account a simple mixing of the Lewis acid and the halide salt, forming as a result more than one anionic species depending on the ratio of quaternary halide salt Q^+X^- and Lewis acid MX_n.

When preparing water and air stable ILs based on 1,3-dialkylimidazolium cations, anion metathesis is the most appropriate reaction for this purpose. This method comprises the treatment of the halide with the silver/sodium/potassium salts of NO_2^-, NO_3^-, BF_4^-, SO_2^- and $CH_2CH_3^-$ or with the free acid of the appropriate anion. [65]

1.3.2 Use of ionic liquids for the isolation of biomolecules

Due to their exceptional dissolution properties, ILs are considered to be innovative solvents for the isolation of biomolecules. Different biopolymers, such as cellulose and wood composites like lignin and chitin have been dissolved and treated in ILs. [66] Other examples of dissolution and efficient separation of biomolecules from different matrices include antibodies, [67] proteins, [68] antibiotics, [69] as well as lipids and related compounds like essential oils, fatty acid methyl esters, and tocopherols. [70]

Usually, both cations and anions of ILs are considered to be important in stabilizing the proteins. [71] The Hofmeister ion series influences the interaction of cations and anions of ILs

with proteins. [72] [73] These interactions are based on the kosmotropicity and chaotropicity of the ions composing the ILs. Kosmotropes increase the stability of proteins in solution. On the contrary, chaotropes decrease the stability of proteins in aqueous medium. A mixture of both chaotropic (weakly hydrated) and kosmotropic (strongly hydrated) is the best way to understand enzyme – IL interactions. [74] The effect of cations and anions on protein stability is shown in figure 3:

Order of protein stabilization

⟵

Anions: F^- > PO_4^{3-} > SO_4^{2-} > CH_3COO > Cl^- > Br^- > I^- > SCN^-

Cations: $(CH_3)_4N^+$ > $(CH_3)_2NH_4^+$ > NH_4^+ > K^+ > Na^+ > Cs^+ > Li^+ > Mg^{2+} > Ca^+ > Ba^+

Order of protein destabilization

⟶
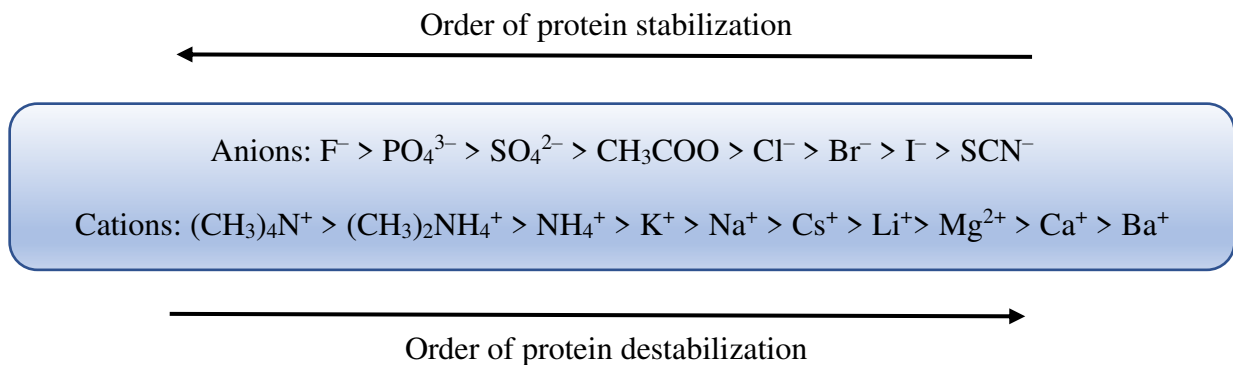
Figure 3. The Hofmeister series as an order of the ion effect on protein stability. [75] [76] [77]

Fujita et al. [78] observed that hydrated [chol][H_2PO_4] was able to maintain the structural activity of cytochrome C in dissolution, being this IL comprised of a chaotropic cation and a kosmotropic anion. Similar results were obtained by Zhao et al., [79] where it was shown that kosmotropic anions, such as CF_3COO^- and CH_3COO^-, and chaotropic cations like [BuPy]$^+$ and [emim]$^+$ could stabilize the enzyme amano protease P6. On the other hand, a destabilization occurred in solution with chaotropic anions like tosylate and BF_4^- and kosmotropic cations like [bmim]$^+$.

Other studies showed that anions are highly polarizable and more strongly hydrated than cations. [80] [81] As a consequence, anions have a more dominating effect and are more efficient than cations. In this case, when a protein is dissolved in an aqueous solution, it has many charged groups on its surface that are responsible for the interactions with the ions in solution. [82] These charged groups were found to have a strong interaction with the chaotropic anions (e.g. SCN^-) and kosmotropic cations (e.g. Na^+, K^+).

Anion kosmotropicity is, however, not the only factor responsible for enzyme performance in ILs, as enzymes in an aqueous solution of ILs may differ in behavior when compared to that in pure IL or ILs containing traces of water. [83] In this case, it is not clear how IL hydrophobicity influences the kosmotropicity. For example, the salt of PF_6^- (chaotropic anion) in combination with Na^+ denatures enzymes in aqueous solution. On the other hand, ILs containing PF_6^- are

known to be hydrophobic and enzyme stabilizing solvents. Thus, the Hofmeister effect (anion kosmotropicity) may be inadequate to explain the enzyme behavior in hydrophobic ILs or their mixtures with water. [84]

It has also been observed that ILs with long alkyl chains exert a destabilizing effect on enzymes. [85] In this case it was reported that when [Bmim][Cl] and [Hmim][Cl] have a long alkyl chain, the enzymatic stability and activity of α-amylase from *B. amyloliquefaciens* and *B. licheniformis* were reduced. [86] Likewise, imidazolium and phosphonium cation based hydrophobic ILs, like [Bmim][Cl], [Bmim][BF$_4$] and TBPBr, were considered to be weak stabilizers for α-chymotrypsin when having longer alkyl chains. On the contrary, small chain ILs had the opposite effect. [87]

1.3.3   Separation and stabilization of DNA in ionic liquids

Extraction of DNA from biological samples is typically achieved by protocols involving chemical solvents, like phenol and chloroform followed by ethanol or isopropanol precipitation. Those methods based on organic solvents are time consuming and include many steps. As a consequence, more environmentally friendly techniques with a high throughput have been developed. Finding an organic medium where the DNA solubilizes and is kept stable at the same time is considered to be a bottleneck in molecular biology. In recent times various studies have reported the use of ILs for the separation and extraction of DNA. [88] [89] [90]

In this sense Li et al. [91] reported that the optimization of the IL properties can enhance the extraction efficiency of DNA. The authors investigated the effect of IL substituent alkyl chain length in the presence of hydroxyl groups, obtaining the best extraction efficiencies of DNA with C$_{16}$POHIM–Br and [(C$_{10}$)$_2$NMDG-Br].

Another study [90] also involved the extraction of double stranded DNA with [C$_4$mim]PF$_6$ in aqueous solution. In this case the high extraction efficiency obtained was explained by the hydrophobic interactions between the cationic imidazolium groups and the phosphate groups in DNA. Proteins and metal species did not interfere either with the separation and purification process.

Preservation of DNA over time under ambient conditions is a major challenge. Slow hydrolytic reactions like depurination and deamination can cause an important damage to its structure. The role of ILs in the stability of DNA was first published in 2010 by the group of MacFarlane [92], which studied the long-term stability of DNA in various choline-based IL – water solutions. When DNA from salmon was analyzed by circular dichroism spectra it was seen that the double helix structure of the DNA dissolved in choline lactate ([chol][lac]) was

still present after storing the samples for 6 months at room temperature and even in the presence of nucleases with strong hydrolytic activity towards DNA. In contrast, when dissolved in water, the structure had been lost after 1 month. Those choline-based ILs were found to stabilize the secondary structure of DNA through interactions with the minor groove of the double helix. The same effect was confirmed later by Chandran et al. [93]

Jumbri et al. [94] studied the interactions between calf thymus DNA and alkylimidazolium-based ILs ([C$_n$bim]Br, where n = 2, 4 and 6) via circular dichroism and fluorescence quenching constant. Results obtained showed strong $\pi - \pi$ electrostatic interactions between the polar imidazolium ring cation and the negative charge of DNA phosphate groups, as well as the retention of the duplex B – conformation of the DNA. Those strong electrostatic interactions also prevented the intermolecular interactions between DNA strands, which could change the structure from B- to A – DNA conformation. Moreover, the addition of methylene in the linear chain of the IL cations contributed to the increase in thermal stability of DNA as observed from UV-vis spectra. The partial dehydration of DNA by the ILs studied also prevented the hydrolytic reactions that could denature DNA by decreasing water diffusion through the hydrophobic interactions.

Another study [95] carried out measurements on different analytical techniques to further investigate the dissolution process of DNA from salmon testes and bio-based ILs like choline-glycolate (Cho-Gly) and choline-pyruvate (Cho-Pyr). FTIR spectra showed shifts of the symmetric and anti-symmetric stretching vibrations of the PO_2^- groups of DNA dissolved in Cho-Gly, from 1073 cm^{-1} to 1086 cm^{-1} and 1223 cm^{-1} to 1241 cm^{-1}, respectively. Those shifts reported were due to electrostatic interaction between the choline cation of the IL and the phosphate group of DNA, which facilitated the dissolution process. This interaction was also confirmed by ^{31}P NMR. As with the study from Jumbri et al. [94], circular dichroism spectra confirmed the B – conformation of the DNA studied, together with $\pi - \pi$ base stacking and the molecule´s helicity. Additionally, further measurements with isothermal titration calorimetry revealed excessive H-bonding interactions between the hydroxyl groups of the Cho-Gly and the DNA base pair, being the binding of this IL to the DNA an endothermic process.

1.4   Methods of extraction of DNA from foods

The success of DNA detection and quantification methods depends on efficient DNA extraction protocols, which should provide a high quality and quantity of DNA. Food products are complex matrices that might contain a substantial amount of PCR inhibitors like polysaccharides, polyphenols and proteins. [96] Moreover, they have been frequently subjected

to one or more steps of processing, such as mechanical, thermal, chemical, or enzymatic treatment, affecting the integrity of DNA. As a result, the isolation of DNA from foods is sometimes considered to be a challenge in food analysis. In this sense, heat exposure can cause fragmentation of high molecular weight DNA. In addition, physical and chemical treatments will result in random breaks in DNA strands, thus decreasing the average DNA fragment size. DNA is also very sensitive to acid and alkaline agents due to the mechanism of hydrolytic degradation of DNA. As such, an acid environment will depurinate the nucleic acid backbone due to the cleavage of N-glycosidic bonds between deoxyribose residues and bases. Resultantly, adjacent 3´, 5´- phosphodiester linkages are hydrolyzed, leading to the shortening of DNA strands. [97] [98] Furthermore, condensation of carbonyl groups from reducing sugars with primary amines, known as the Maillard reaction, occurs due to the high temperatures that result from industrial processing. As a result, extensive cross-linking of proteins and nucleic acids are formed, which makes it difficult for DNA to be extracted and afterwards amplified. [99] [100]

1.4.1   Approaches to DNA extraction

DNA extraction methods involves three steps: lysis, purification and DNA recovery, where lysis is considered to be the most critical step. [101] DNA can be extracted by conventional methods or by using commercial kits. Conventional methods are usually dependent on chemicals that require preparation before the extraction takes place. These methods are considered to be tedious and time consuming and may introduce inhibitors due to the use of solvents, like chloroform and isopropanol. On the other hand, DNA extraction with kits are rapid and easier, but expensive.

1.4.2   Challenges during extraction and downstream applications

Choosing the suitable method for DNA extraction involves certain factors, such as time, cost and toxicity of the chemicals employed. In this case, a conventional method of extracting DNA from animal species generally requires the addition of phenol and chloroform, which increases the risks of contaminating the DNA and health hazards. [102] In addition, the method is also time consuming.

A key factor in nucleic acid amplification is the purity of the extracted DNA, more than it is the yield. [103] With this in mind, it is important to decrease as much as possible the presence of inhibitors or contaminants originating from foods. In 1996 [104] it was reported that protein in milk diminishes the solubility of pelleted cells from which DNA is extracted. Additionally,

lipids and phenolic compounds may also contaminate the DNA. [105] Moreover, some chemicals used during the extraction have been identified as PCR inhibitors. As such, the chelating agent EDTA (ethylenediaminetetraacetic acid) can form a complex with magnesium, thus affecting the concentration of this ion needed for PCR. To prevent this from happening, a higher concentration of magnesium is needed to sustain amplification. [106]

Other chemicals like phenol can cause the polymerase to denature. The usage of alcohols like ethanol and isopropanol in DNA precipitation steps can affect the quality of DNA obtained if they are not completely evaporated from the final pellet. [107] These inhibitors can interfere with downstream applications, as they can completely inhibit the polymerase activity during the amplification. [108]

Other factors, such as primer specificity, amplicon size and gene copy number should be considered for an efficient amplification, hence the addition of an endogenous control in downstream application. This additional step may verify potential amplification variations due to changes in the amount and quality of the DNA extracted from the sample. [109]

1.4.3 DNA extraction from different foods

High amounts of polyphenolic and polysaccharide compounds are present in vegetables, which may restrict the enzymatic activity of the DNA polymerase during PCR and thus they must be removed during the extraction process. To achieve this, silica based commercial kits such as the one from Promega (Promega Wizard Magnetic® DNA Purification) have been reported to effectively extract DNA from vegetable foods, as they can effectively remove fat content, polyphenolics and polysaccharides by using a mobile solid phase. [108] Conversely, kits like the DNeasy® Tissue Kit included a silica column-based system, which improves the recovery of DNA and eliminates inhibitors from processed foods and difficult to extract matrices. The high sensitivity of these methods has been demonstrated by accomplishing PCR amplification of target genes, with detection limits from 0.05 ng/µL to 2.5ng/ µL.

Additionally, the use of silica binding columns were shown to be a more effective method than the classical laboratory method based on the CTAB surfactant (cetyltrimethylammonium bromide) for extracting DNA from wheat (*Triticum aestivum*) grains, following charring at 200°C for 1h. [110] The authors of the study demonstrated that a sample incubation with N-phenacylthiazolium bromide (PTB) in a binding buffer before transferring the sample to the silica binding column together with extended washing of bound DNA with washing buffer can eliminate inhibitors in the charred wheat.

Nevertheless, the CTAB method has been successfully used for extracting DNA from fish and seafood. In this case it is important to consider the concentration of CTAB, as the chemical can complex the DNA. [111] As a result, it is recommended to pretreat the samples for fat removal before continuing with the extraction. [112]

Extraction of DNA from canned tuna (*Thunnus albacares*) can be difficult, due to the presence of filling media. In a study where four different types of filling media were assessed (oil, brine, tomato sauce and vinegar), tuna canned in oil showed the highest DNA concentration, independent of the DNA extraction method used. In addition, the CTAB method proved to be very efficient, as high quality of DNA was obtained with the ratio of A_{260}/A_{280} having a result closed to two. The Wizard kit from Promega also showed DNA preservation in oil, as demonstrated by the amplification of 300 base pairs against the sample extract. [113]

Other processes to which fish and seafood are subjected include smoking, curing and cooking, which can degrade the DNA. In this case it is known that phenolic compounds resulting from the smoking process can contribute to a failed PCR. In order to guarantee the amplification of DNA, the replacement of NaOH with KOH in the lysis buffer during the extraction generates KCl after neutralization with Tris-HCl. This new salt formed has been identified to enhance the activity of the Taq polymerase. [114]

Different techniques for extracting DNA from meat samples have been reported by Yalçınkaya et al. [115] The authors reported a modified salt method to the one proposed by Cawthorn et al. [113] as the best method of the ones studied, which included extraction procedures based on chemicals like CTAB, urea, guanidinium thiocyanate as well as commercial kits from Promega, Qiagen, Zymogen and Eurofins. In this case, the method included an incubation of the sample in lysis buffer together with SDS and proteinase K, after which a 6M solution of NaCl was added. The mixture was centrifuged and an equal volume of isopropanol was given, which was followed by an incubation at -20°C for 10 min. After centrifugation, the supernatant was removed, the pellet was dried and dissolved in TE buffer. Results showed very high concentrations of DNA (49.6 ± 2.6 ng/μL) as well as A260/A280 and A260/A230 ratios of 1.98 ± 0.02 and 1.91 ± 0.02, respectively. Early Cq values of 12.2 ± 0.5 indicated a high yield and quality of the DNA isolated, together with a very low amount of inhibitors coextracted.

As for dairy products, it is known that the DNA extracted from this type of matrix may be influenced by its content in proteins, lipids and calcium. In this case, a comparative study showed the CTAB method giving the best results and therefore the method of choice when eliminating those inhibitors. With this method, the highest concentrations of DNA were

obtained, which was detected by agarose gel and early cycle detection in qPCR. Other methods evaluated included a Tween and SDS based methods, as well as commercial kits like the Nucleo-Spin® Food, Wizard® Resin, Charge Switch® Forensic DNA Purification Kit and QIAamp DNA Stool® Mini Kit. [116]

Quigley et al. [117] performed a study where commercial kits (modified QIAamp DNA Stool® Mini Kit, Chemagen Food Basic kit, Wizard® Magnetic DNA isolation kit, Milk Bacterial DNA isolation kit and PowerFood™ Microbial DNA Isolation Kit) and laboratory developed methods (lytic method and guanidine thiocyanate) were compared in order to extract bacterial DNA from raw milk and cheese. One of the kits studied, the PowerFood™ Microbial DNA Isolation Kit, was found to most consistently extract highly concentrated and pure DNA for subsequent use in PCR downstream applications. The kit also efficiently extracted DNA from spiked *Listeria monocytogenes* EGDe and *Salmonella enterica* serovar Typhimurium LT2.

1.5 DNA based methods for the analysis of foods

1.5.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was developed by Kary Mullis in 1983, which earned him the Nobel Prize in chemistry in 1993. This method allows the amplification of DNA fragments for the subsequent analysis via electrophoretic or fluorometric techniques. [118] Currently, PCR is a standard analytical tool of significant relevance for different kind of analysis with organisms as targets. The method considers frequent changes of temperature, which are known as cycles, where a single DNA molecule can be exponentially amplified. One cycle usually involves three steps: denaturation, annealing and extension. Before the first cycle, an initialization step is induced, which is only needed for DNA polymerases that require heat activation. The rest of the cycles start with the denaturation step in which the double stranded DNA (dsDNA) is separated into two single stranded DNA (ssDNA) strands by heating up the reaction to 95°C. This is followed by the annealing step, where temperatures are reduced to 50°C – 70°C. This phase allows short oligonucleotides, known as primers, to bind to one of the separated strands indicating the starting point for the strand extension. The optimal annealing temperature is calculated by the base composition and length of the primers and can be determined as follows:

$$T_m[°C] = 2°C \; x \; (A + T) + 4°C \; x \; (G + C)$$

Where T_m indicates the melting temperature and A, T, G and C the number of the single nucleotides in the DNA sequence: adenosine, thymine, guanine and cytosine, respectively.

Extension takes then place at temperatures ranging from 70°C to 80°C. During this step the thermostable DNA polymerase binds to the primer – template complex and starts synthesizing a new complimentary strand by adding desoxyribunocleotides in a 5´ to 3´ direction. Typically, 25 to 45 cycles are carried out. The principle is shown in the following picture.
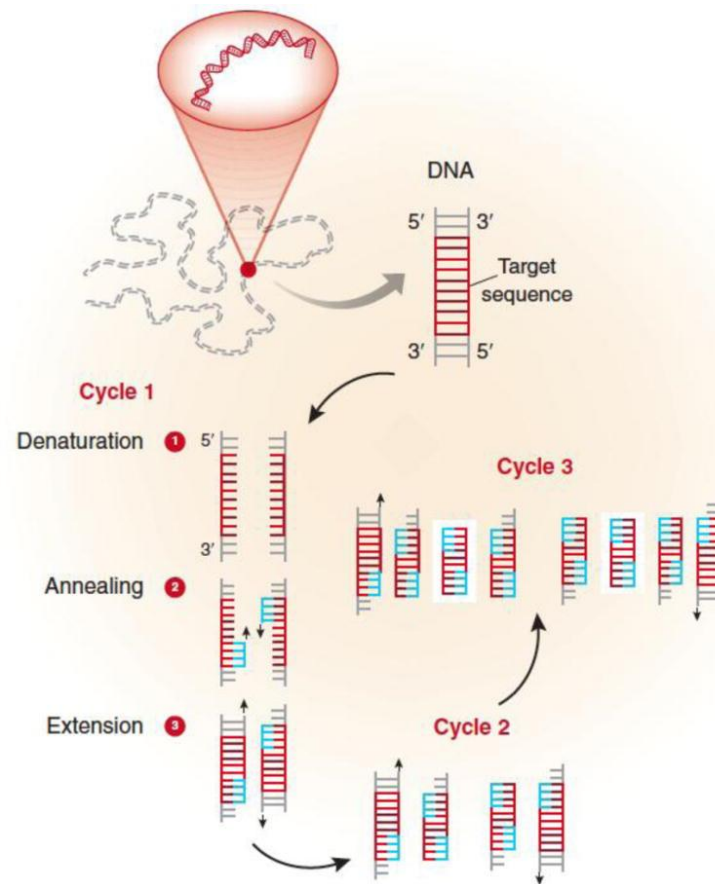
Figure 4. Polymerase chain reaction [119]

During each cycle, the concentration of the DNA target is doubled. To confirm the generation of the desired amplicon, the products formed can be separated according to their sizes by agarose gel electrophoresis and compared with a molecular weight marker. [120] [121]

An advanced approach to the classical PCR is the quantitative real time PCR (qPCR). This type of PCR combines amplification of the target sequence together with the detection of the generated amplicon. The process can be monitored in real time due to a fluorescence signal that is detected with a sensor during each cycle. [122] It typically makes use of two detection chemistries. The first one is a DNA binding dye, most commonly SYBR green, which is an inexpensive alternative. However, there is a major disadvantage to its usage, such as the lack of specificity to bind double stranded DNA. The second one is a hydrolysis probe approach

that generally uses TaqMan probes to increase the specificity of the assays. This last chemistry approach requires the effective binding of a specific probe in addition to the binding of the PCR primers to achieve a positive identification. [123] [124]

Another development in this field with a significant potential in the DNA analysis of foods is digital PCR (dPCR). The method uses limiting dilutions, PCR and Poisson statistics to quantify the absolute number of targets that are present in the sample. [125] In this sense, the PCR mix is distributed across a large number of partitions containing zero, one or more copies of the target nucleic acid. A PCR is then carried out in each of these partitions and is positively or negatively measured depending on whether the PCR product is present or not. [126] The use of binomial Poisson statistics allows the determination of the number of target nucleic acid molecules that are contained in the original sample while taking into account the ratio of positive to total partitions. [127] The use of dPCR has found that the quantitative analysis of GMOs shows comparable sensitivity and an improved repeatability at low range concentrations together with a greater tolerance to inhibitors when compared to qPCR. [126] Moreover, this technique enables the accurate determination of DNA copies without the need for a reference calibrator and is not dependent on amplification efficiency. [128]

1.5.2 Isothermal amplification techniques

Recent advances in molecular biology of DNA synthesis *in vivo* permit the possibility of amplifying DNA in isothermal conditions without the need of a thermocycler. DNA polymerase replicates DNA with the help of various accessory proteins. As a result, this has enabled the development of new in vitro isothermal amplification methods, imitating these *in vivo* mechanisms. [129] This is considered an advantage over PCR, as the reaction can be performed on a heating block or in water bath, which eliminates the need for sophisticated thermocyclers. [130] Additionally, they are considered to be highly tolerant to inhibitory material that affect the PCR efficiency. [131] However, some isothermal amplification techniques have complicated reactions mechanisms and experimental design and results are not quantifiable, as there is no controllable sequence of cycles. In this case, only the initial target copy number can be estimated and so a qualitative analysis can be provided. [129]

The following techniques are considered the most important isothermal amplification methods:

- Helicase dependent amplification (HDA): This technique was used in this study and therefore it is described in more detail in this section. The method was first patented in 2002 [132] and it uses the unwinding activity of the helicase to separate two complimentary DNA

strands into each single stranded DNA during replication. [133] During this process, heat denaturation followed by thermocycling are not necessary, as the helicase is also able to displace DNA strands alongside the exponential amplification reaction. As a result, the entire HAD process can be carried out at one temperature, which makes it a very useful tool to amplify DNA under isothermal conditions. In addition, it is highly sensitive, has a relatively simple primer design as well as the possibility of multiplexing. [134] The following figure (figure 5) shows a schematic diagram of this method.
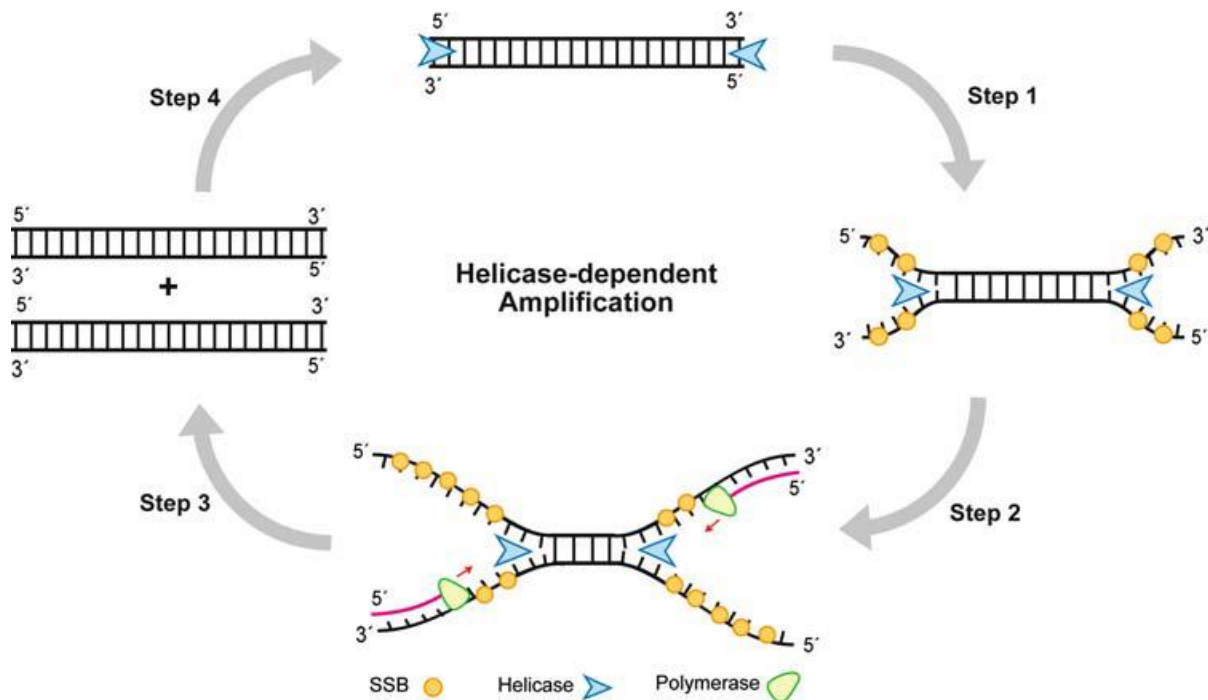
Figure 5. Schematic diagram of the helicase dependent amplification. Helicase unwind dsDNA and SSB proteins bind to exposed ssDNA. Subsequently, DNA polymerases start synthetizing the complimentary strand from the bound primers, and the cycles repeat continuously. [133]

Helicase dependent amplification has been used to successfully detect a wide range of pathogens causing foodborne outbreaks. Recently, Du et al. [135] developed this method combined with a lateral flow assay to detect *Salmonella* in cultured bacteria and also in contaminated chicken and milk, as well as infant formula. Moreover, Chen et al. [136] were able to use this technique to detect *Staphylococcus aureus* in contaminated milk powder and pork samples. The authors added SYBR Green I to the amplicons, which allow them to observe the fluorescence intensity by using a UV lamp or a fluorescence spectrophotometer.

• Loop mediated isothermal amplification (LAMP): In the LAMP reaction, gene amplification proceeds through repetition of two types of elongation reactions that occur via the loop regions, like template self-elongation from the stem loop structure formed at the 3´ -

terminal and subsequent binding and elongation of new primers to the loop region. In this reaction, pairs or inner and outer primers are used. Each of the primers possesses a sequence complimentary to one chain of the amplification region at the 3´-terminal and identical to the inner region of the same chain at the 5´-terminal. The elongation reactions are sequentially repeated by DNA polymerase-mediated strand displacement synthesis using the previously mentioned loop regions as a stage. The method is based on the principle of the production of a large quantity of DNA amplification products with a mutually complementary sequence and an alternating, repeated structure. [136] The efficiency of this technique is strongly influenced by the guanine/cytosine content, the distance between the primer binding regions and the melting temperature of the oligonucleotides. [138] Additionally, a highly sensitive and specific assay depends on the primer design [139] and the length of the target sequence, as a LAMP amplicon usually consists of around 140 to 220 base pairs. Shorter targets can increase the complexity derived from the primer design and thus the assay. [139]

- Rolling circle amplification (RCA): The method was first developed by Fire and Xu [140] and later modified by Lizardi et al. [141] It is an enzymatic isothermal process where a short DNA sequence is amplified by means of a circular DNA template and a highly processive DNA polymerase (bacteriophage Φ29, Bst and Vent exo-DNA polymerase) to create a long ssDNA containing tens of hundreds of tandem repeats that are complimentary to the circular template. On the other hand, a linear DNA template can also be first ligated to form the circular DNA template assisted by double strand formation with the target. RCA is performed at a constant temperature (30°C-65°C) in solution, on a solid support or in a complex biological environment. However, its reaction set-up is arduous, as it has to be divided into two steps, one to perform the ligation and the second one for the amplification. Additionally, an initial denaturation step prior to ligation is required to successfully perform hybridization of the open circle probe to the target. Non-specific background amplification can occur though, for which a restriction digestion to remove any non-ligated probes in recommended. [142]

- Strand displacement amplification (SDA): The method was first published by Walker et al. in 1992. [143] [144] SDA uses bifunctional primers combining a target recognition sequence and a restriction endonuclease recognition sequence. In this method, an initial heat denaturation step separates a dsDNA target into two single strands upon attachment of the primer to the target strand with a 5´ overhang which contains a specific sequence that the HincII restriction enzyme recognizes. As thiol modified deoxyadenosine triphosphate is used, the newly synthesized DNA strand contains a triophosphate modification which is inactive to HincII and

so the original primer is cleaved by HincII, but not the synthesized DNA strand. The method heavily depends on polymerases with the ability with strong strand-displacing power to extend the 3´ end and displace the downstream DNA strand and the restriction enzyme to nick the unprotected primer strand at the recognition site. Because the polymerase lacks the 5´– 3´ exonuclease activity, it extends the 3´ end at the nick place and displaces the downstream strand in the temperature range of 30°C – 55°C. This generates a new nickable region and so exponential amplification is accomplished, as a result of coupling sense and antisense SDA in which strands displaced serve as secondary targets.

- Nicking enzyme amplification reaction (NEAR): This technique can be identified by the use of nicking endonucleases that allow the hydrolyzation of one oligonucleotide strand. Therefore, modified nucleotides are no longer required. This technique only uses the inner primer pairs, with their recognition site for the restriction endonuclease being replaced by the corresponding site for a nicking enzyme. The reaction is performed at 54°C-60°C with Bst DNA polymerase. [145] When employing a polymerase together with restriction endonucleases, non-specific amplification can occur, which can affect the sensitivity of the assay. [146] [147]

1.5.3 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) relies on the use of restriction endonuclease enzymes to digest DNA into fragments of various lengths that may be species-specific. The DNA to be digested is usually an amplicon of, for example, combined ITS and D1/D2 regions to ensure an adequate DNA concentration for separation by agarose on gel electrophoresis and subsequent detection by Southern blot hybridization to a labelled DNA probe that identifies the locus under investigation. [148] Labeling of the probe can be carried out by either radioactive isotopes or alternatively by non-radioactive stains, like digoxigenin or fluorescein. Probes are generated through the construction of gDNA or cDNA libraries and so may be composed of a specific sequence of unknown identity or a sequence of a functional gene. The basis of length polymorphism is formed by the DNA sequence variation, which has a direct effect on the absence of presence of recognition sites of restriction enzymes, as well as insertions and deletions within two adjacent restriction sites. [149] This technique has been used for the identification of fraud and adulteration in food, like industrial meat products, [150] [151] and fish species. [152] Additionally, it has also been used for the identification of meat in animal feedstuff. [153]

1.5.4 Single strand conformation polymorphism

Single strand conformation polymorphism (SSCP) is a technique used for detecting mutations and genotyping. Its principle relies on the fact that ssDNA has a defined conformation; its alteration due to a single base change in the sequence can cause ssDNA to migrate differently under nondenaturing electrophoresis conditions. As a consequence, wild type and mutant DNA samples display different band patterns. The technique takes into account four steps: 1.) polymerase chain reaction amplification of target DNA; 2.) denaturation of double stranded PCR products; 3.) cooling of the denatured DNA (single-stranded) to maximize self-annealing and 4.) detection of mobility difference of the single stranded DNAs by electrophoresis under non-denaturing conditions. In order to visualize the SSCP motility shifts, different methods like radioisotope labeling, fluorescent dye-labeled PCR primers, silver staining and capillary based electrophoresis have been developed. [154] SSCP has been used for the authentication of different fish species, [155] [156] analysis of microbial populations in wines [157] and milk. [158]

2. Scientific publications

Publication I

Publication II

Publication III

A rapid genomic DNA extraction method and its combination with helicase dependent amplification for the detection of genetically modified maize

Eric Gonzalez Garcia, Andreas H. Farntleitner, Robert L. Mach, Rudolf Krska and Kurt Brunner; *Analytical Methods*, 8, 2016, 136-141

DOI: 10.1039/C5AY02628H

3.   Conclusions and outlook

Several contributions to improve the DNA isolation process have been made in the course of this thesis. The herein developed methods allow for the extraction to be carried out on-site. Furthermore, these procedures are significantly less expensive and simpler to be performed, they fully avoid the use of toxic and volatile organic solvents as well as allow for a time and energy saving process. The developed protocols also avoid repetitive washing and filtration steps as well as reduce accumulation of waste.

The first study carried out in this thesis involved the use of a set of different ionic liquids to extract gDNA from genetically modified maize. For the first time, to the best of the author´s knowledge, gDNA was directly extracted from a complex natural matrix by using IL technologies. While the use of pure ILs for gDNA extraction was not appropriate as reproducibility problems arose, it was found that the combined IL – aqueous buffer systems could notably increase the amount of extracted DNA compared to conventional buffers systems. Important changes in starch morphology were seen after incubating the maize with IL – aqueous buffer systems, thereby emphasizing the influence of ILs in biomass treatment, even when using them in aqueous solutions. Among all systems tested, the IL [C_2mim]Me$_2$PO$_4$ in combination with sodium phosphate buffer (50 mM, pH = 8.5) was found to be the most efficient extraction system. The gDNA fraction extracted with this IL had the highest quality and was stable when stored at RT for up to 10 days. The optimization of extraction parameters resulted in a simplified and time-efficient procedure in comparison to the conventional CTAB method for the direct extraction of gDNA from genetically modified maize powder; a time saving of approximately 3h was achieved when extracting 10 samples in parallel with both methods.

In a following step, the developed extraction method for DNA was expanded to another food matrix. In this case, mtDNA was extracted from different types of meat and meat derived products. From all of the systems tested, choline hexanoate at a concentration of 10 wt% in sodium phosphate buffer (5m mM, pH = 8.5) was found to be most efficient. mtDNA extracted with this IL showed high specificity as no by-products were detected when performing an agarose gel electrophoresis, had a high yield and was stable when stored at RT for 20 days. Further investigations showed a strong influence of the alkyl chain length and the concentration of ILs on the extraction of DNA from meat and on the amplification process. When extracting with choline carboxylates with chain lengths of less than 8 carbon atoms, ILs concentrations lower than 11 ng/µL did not affect the amplification of DNA in the qPCR. These results were

in accordance with the observed concentration dependency in the extraction experiments, where a higher concentration of IL in buffer influenced DNA amplification and resulted in higher Cq values.

In this study, the optimized extraction procedure based on the biodegradable IL choline hexanoate in aqueous buffer systems provides general advantages, as the solvent is environmentally friendly and saves both energy and time. This easily applicable protocol avoids washing and filtration steps and reduced therefore accumulation of waste. When compared to conventional methods, such as the extraction kits from Promega, r-Biopharm and Amani et al. 2011, the here proposed protocol resulted in a simple, inexpensive and five times faster extraction procedure, while providing reliable results and a low environmental footprint.

Additionally, these two methods developed here can be considered to be the foundation of a new generation of isolation procedures, which are not limited to the analysis of genetically modified organisms or for food authenticity, but that can also be applied to the extraction of DNA from any biological material. DNA analysis became mobile during the last decade by developing portable PCR cyclers and simple amplification systems like isothermal procedures. The application of ILs as an extraction facilitator will remove the still existing bottleneck of complex, time consuming and laboratory dependent DNA isolation protocols.

The isolation of gDNA from genetically modified maize was further investigated by developing another method based on an aqueous buffer system in combination with a proteinase K digestion and later followed by a filtration over a polypropylene membrane. Detection was carried out by helicase dependent amplification (HDA), as an alternative to PCR for the detection of the *ADH1* gene and the promoter *P35S*. The method was simple, inexpensive and fast. Optimization of the parameters led to a simplified protocol where a high yield of gDNA could be achieved and the amount of coextracted inhibitors was kept low. The protocol developed showed comparable results to the conventional CTAB method and the commercial kit from Promega, while saving 3.5 h when comparing the CTAB method and 5.5 h when comparing to the Promega kit while extracting 10 samples in parallel with these methods. Its combination with HDA for the detection of the *P35S* promoter in genetically modified maize makes it suitable for reliable on-site detections of genetically modified sequences in maize containing this promoter with concentrations as low as 0.5 %. Moreover, as HDA can be performed using an inexpensive heating block instead of an expensive and difficult to operate thermal cycler, the costs of the assays could be remarkably decreased.

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