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1 Introduction

Food is a necessity of life that contains nutrients, essential for the growth, repair and maintenance of body tissues and for the regulation of vital processes. Throughout the history people have used different ways of food production, transportation and safe storage. Hence, their food and food habits have been influenced by local natural resources, tradition and religion. Because of the globalization of food markets and food industry, the connection between food and territory has disappeared over the time. Nowadays, when buying food, consumers must trust product declaration, product logos and producer, retail chain, the country of production or the control system in the country. Thus, a number of food scandals involving false labeling left the consumers mistrusting food authenticity, but also the control system. For this reason, regulatory agencies and different research projects (e.g. Food Integrity - <https://secure.fera.defra.gov.uk/foodintegrity/>) have developed analytical techniques to identify potential food adulteration. Most commonly used techniques are high-performance liquid chromatography (HPLC), infrared spectroscopy (IR), gas chromatography (GC), isotope ratio mass spectrometry (IRMS) and polymerase chain reaction (PCR) [1, 2].

1.1 Food fraud

A literature research carried out by Moore *et al.*, 2012, showed an increased interest by consumers in the terms “food adulteration” and “food authentication” [3].

Food is adulterated if the identity or/and purity of the original product has been changed, by either replacing it with cheaper one or camouflaging the bad appearance or taste by addition of other ingredients. Furthermore, food declarations with false or misleading statements are also considered as adulteration [1, 4]. Altogether, food fraud is commonly driven by the motivation to increase financial gains, even at the cost of losing consumers’ trust.

According to the European Commission’s (EC) monthly summary of food fraud articles, in May 2017 cases about fish, meat, cheese, seafood, wine, honey and milk adulteration have been reported [5]. The analysis of scholarly report datasets carried out by Moore *et al.* in 2012 showed that olive oil, milk, honey, saffron, orange juice, coffee and

apple juice are the top seven vulnerable ingredients. Additionally, when categorized, 50% of the scholarly records showed oils, milk, jams, purees, preserves, spices, concentrates and fruit juices as target of fraudulent activities [3].

Berries like cranberry, blueberry, raspberry and blackberry are nutritional power houses and have become a healthy eating trend. These high-priced superfruits can be eaten raw or as jams, purees, juices, syrups, jellies and powders. To establish good quality and authenticity of these premium products, the Codex Alimentarius Commission proposed guidelines and standards regarding fruit contents and fruit processing [6, 7]. Hence, food adulteration is still a major problem. For instance, fruit juices can be adulterated by adding sugar, water, colorants, flavors, vitamins or cheaper fruits. Food processing but also the complex product matrix and the natural occurrence of fruit species and hybrids make the detection even harder. In most cases, more than one analytical technique and characteristic fruit parameters are required to provide sufficiently reliable results [8, 9].

1.2 Blueberry and bilberry

Blueberry (*Vaccinium corymbosum* L.) or “high blueberry”, “swamp blueberry”, or “blue huckleberry” is a part of the family Ericaceae, which consists of more than 4000 species and 128 genera [10]. Furthermore, it belongs to the subgenus *Vaccinium* and *Cyanococcus* section. Originally from North America, it is now cultivated all over the northern parts of Europe and Asia.

This tetraploid plant is a deciduous perennial shrub which grows in highly acidic and well-drained soils. Furthermore, the 12 mm wide fruit has dark blue color with green pulp inside [11].

Extensive production of blueberry jams, juices and other products has been recorded, due to their widely-known health benefits. So far, 15 anthocyanins have been identified in blueberries, which play an important role as antioxidants [12].

Bilberry (*Vaccinium myrtillus* L.) is also called European blueberry, “huckleberry” or “whortleberry” and it belongs to the section *Myrtillus*. It grows in dry forest heaths of northern Europe, northern Asia and Canada. Furthermore, it is self-fertile and it forms dark blue berries with a diameter of about 5-9 mm. The berries are smaller and darker than the blueberries and have purple inside [13].

Bilberries have been used for centuries in the folk medicine, mainly because of their antioxidant and anti-inflammatory effect. Just like in blueberries, anthocyanins are also the most abundant group of polyphenols in bilberries. Delphinidin-3-O-glucopyranoside, delphinidin-3-O-galactopyranoside and cyanidin-3-O-arabinopyranoside are the three dominant anthocyanins in bilberry, malvidin-3-O-arabinopyranoside and petunidin-3-O-galactopyranoside in blueberry [12]. These anthocyanins can be used to detect adulteration of bilberry products with blueberry. Typically, bilberries are wild and need to be harvested from forests, on the other hand blueberries are cultivated and commercially available [13].

1.3 Cranberry and lingonberry

American cranberry (*Vaccinium macrocarpon* Aiton) is also a member of the family Ericaceae, but it is classified in the subgenus *Oxycoccus*. Native to North America, in the past it was collected from August through fall, and used immediately, mixed with corn breads, cooked with fish or meat and dried and preserved for winter. Nowadays, the United States and Canada are still the biggest producers worldwide, but American cranberries are also cultivated in Europe, Central America, Central and South-East Africa and Asia.

Also known as “large cranberry”, *Vaccinium macrocarpon* A. is an evergreen shrub which thrives in nutrient poor acidic soils. Additionally, it is diploid, self-fertile and pollinated by bees. The fruit is a dark red berry with about 20 mm diameter [14].

The European Food Safety Authority (EFSA) acknowledged the beneficial effect of the American cranberry fruit on urinary tract infections (UTIs) [15]. This is due to the presence of different phenolic compounds, such as anthocyanins, phenolic acid, flavanols and tannins. The French Agency for Food, Environment and Occupational Health and Safety (ANSES) approved the claim, that *Vaccinium macrocarpon* A. prevents the urinary tract from the attachment of *Escherichia coli* [16].

The European cranberry *Vaccinium oxycoccos* Linnaeus, also known as “small cranberry”, “bog cranberry”, “mossberry” or “wild cranberry”, is classified just like the American cranberry. Primarily grown in the northern parts of Europe, North America and Asia, this berry grows on bogs and swamps by forming evergreen dwarf shrubs.

Furthermore, it is also self-fertile and pollinated by bees. The fruit is a dark red berry (6 mm in diameter) with juicy acidic taste, which gets sweeter after frost. Although smaller than the American cranberry, the European cranberry is very rich in nutrients and used as food and medicine [17].

Vaccinium vitis-idaea L. or lingonberry belongs to the same genus as the American and European cranberry, but to different subgenus (*Vaccinium*, sect. *Vitis-idaea*). Since they all have very similar physical characteristics, they get often mixed with each other. Another name for the lingonberry is “mountain cranberry”, “rock cranberry” or “cowberry” [18]. Its polyphenolic content and composition contributes to its high antioxidant capacity and anti-bacterial effect [18, 19]. Moreover, a lingonberry extract has shown antidiabetic properties *in vivo* [20].

As a matter of fact, there are a lot of studies which seem to reproduce the positive health effects of European cranberries and lingonberries, but still no verified health claims have been established. The three berry species have been used for the production of various juices, beverages, jams, purees, powders and dietary supplements. Producers took advantage of their similarity and used it for adulterating their products [21]. Product authenticity can be detected by application of different analytical techniques, like HPLC or GC [21, 22, 23]. Different parameters like anthocyanin levels, presence of melatonin, serotonin or ascorbic acid or metabolomic profiles can be used to distinguish between the berries [19]. For instance, cyanidin-3-O-arabinoside, peonidin-3-O-galactoside, cyanidin-3-O-galactoside and peonidin-3-O-arabinoside are four major anthocyanins detected in Finnish *V. oxycoccos* L. With 23.1% of the total anthocyanin amount, cyanidin-3-O-arabinoside can be used as characteristic anthocyanin for *V. oxycoccos* L. [23]. On the other hand, *V. macrocarpon* A. showed the same anthocyanin profile, however, with proportion of 38% peonidin-3-O-galactoside was found to be the main anthocyanin compound [24]. In contrast, the anthocyanin profile of *V. vitis-idaea* L. was rather simple, with cyanidin-3-O-galactoside being the main representative [25]. Nevertheless, the negative aspect is that anthocyanin profile may be influenced by temperature variations, weather changes, pollutants, pathogens and geographical location [26, 27, 28].

1.4 Pomegranate

Pomegranate (*Punica granatum L.*) is an ancient fruit tree, which originates from the Middle East, and has spread through the years to Asia, North Africa and the Mediterranean. Nowadays, most important growing countries are Iran, Israel, China, Turkey, Spain, India and USA. Furthermore, Spain is the largest European exporter of pomegranates, which are mainly grown in Alicante and Murcia [29].

Worldwide there are more than 1000 cultivars of pomegranates, differing in the characteristics and appearance of the fruit, flower and tree. A study by Melgarejo *et al.*, 2000, differentiated pomegranate varieties based on their acidity levels as “sweet” with 0.32% acidity, “sweet-sour” with 0.79% acidity and “sour” with 2.72% acidity [30]. Another study by Koppel *et al.*, 2010, has carried out a sensory evaluation of pomegranate juices, and came to the conclusion that pomegranate flavors can be sweet, sour, earthy, fruity aromatic and astringent [31].

Pomegranate fruit contains an outside peel (leathery exocarp or rind), an inside peel (leathery mesocarp or albedo), arils (pulp and seeds) and a membrane.

40% of the whole fruit consist of the peel, which is known for its antioxidant, antibacterial and anti-inflammatory properties. Accordingly, it is rich in polyphenolic compounds, such as punicalagin A, punicalagin B, ellagic acid, gallic acid, chlorogenic acid, caffeic acid, catechin, epicatechin, rutin, quercetin and galangal [32]. Due to their positive bioactive properties, peels are no more just agricultural waste, but used in cosmetic and food industry [33].

The arils are the edible berry fruit, which has high levels of sugar, vitamins, minerals, polyphenols and organic acids and is typically used to produce jams, juices and jellies. Seeds which make up 20% of the whole fruit, are also used in the food industry for the production of pomegranate seed oil. They are rich in polyunsaturated acids, tocopherols, fiber and lipids [29, 34].

In the last decade, pomegranate and pomegranate products have received an increased attention due to their superior antioxidant properties and prevention of various diseases. Indeed, their consumption may positively affect diabetes [35], may show chemopreventive and chemotherapeutic effects against prostate cancer [36, 37] and inhibit colon cancer proliferation [38].

Pomegranate juices can be either extracted from the whole fruit or only from the arils. In the first case, high levels of hydrolysable tannins (ellagitannin, punicalin and punicalagin) are present in the pomegranate juice, decreasing its sensory quality. Their binding to the salivary proteins results in dry, astringent and unpleasant mouthfeel, which is usually not enjoyed by most of the consumers. On the other hand, juice extracted from separated arils has lower levels of phenols and hydrolysable tannins, making it tastier [39]. In addition, industrial processes such as pasteurization may influence the aroma profile and juice coloring [40]. Therefore, optimization of food processing is crucial for achieving a juice of high quality.

This popular superfruit is on the one hand in high demand, but on the other it has short harvest season which leads to short supply and high price. This may motivate the producers to adulterate the original pomegranate juice by mixing it with other fruits or juices [40]. Another drawback is the characteristic astringent taste and pale brown color, leading to unattractive product. By addition of sugars or sweet juices, like peach juice or apple juice, the astringency of the tannins can be covered. Additionally, by mixing with chokeberry, raspberry or grape juices, a deep and intense coloring can be achieved. The adulteration is typically done with common and cheap fruits, which have same volatile profiles as the pomegranates. Often used fruits are apple, cranberry, bilberry, black mulberry and black chokeberry [41].

Adulterated pomegranate juice can be detected by employing different techniques for determining the concentration of polyphenols, sugars, organic acids, minerals and amino acids [41]. Variations in the defined range of these main quality characteristics can indicate the addition of other fruits. For instance, tartaric acid is an indicator for adulteration with grape juice, since it is not originally present in pomegranate juice [42]. Furthermore, six anthocyanins (delphinidin-3,5-di-O-glucoside, cyanidin-3,5-di-O-glucoside, delphinidin-3-O-glucoside, pelargonidin-3,5-di-O-glucoside, cyanidin-3-O-glucoside, pelargonidin-3-O-glucoside) are typical for pomegranate juice, and can be used as parameter to determine its authenticity [41]. Analysis is most commonly done by HPLC. One major disadvantage of detecting the chemical composition is that it is affected by season, climate, growing region and conditions, cultivar type, harvest time and storage

[43, 44]. However, this limitation can be overcome by employing DNA based techniques [45].

1.5 Detection of food fraud by PCR-HRM analysis and DNA barcodes

Analytical methods based on analyzing specific DNA sequences are already applied in routine analysis to detect genetically modified organisms, food adulterations, allergens or pathogens. The positive aspects of working with DNA are its stability upon 120°C and the possibility to distinguish between subspecies and populations.

The polymerase chain reaction plays a central role among analytical methods used to detect food fraud. Characterized by high efficiency, specificity and accuracy, PCR produces high quantities of double stranded DNA (dsDNA), which can help with e.g. detecting mutation, mapping chromosomes or screening gene-banks [46, 47].

High resolution melting (HRM) analysis is a powerful and fast technique that allows measurement of the dissociation rate of double stranded DNA to single stranded DNA while increasing the temperature. The dissociation can be monitored by using an intercalating fluorescence dye, which fluoresces only when bound to dsDNA [48, 49]. In the study from Ganopoulos *et al.*, 2011, the HRM analysis with microsatellites (Simple Sequence Repeats, SSRs) was used to genotype sweet cherry cultivars and detect adulteration in commercial sweet cherry products [50]. Mader *et al.*, 2011, developed two HRM assays for the authentication of *Helleborus niger*, one for detecting unknown adulterants and other for detecting *Veratrum nigrum*, a common admixture of *Helleborus niger* [51].

Another possibility is to analyze DNA barcodes by HRM. DNA barcoding is a molecular technique for rapid species identification based on a standardized sequence of the genome, called “DNA barcode”. The short DNA sequences usually consist of 400-800 bp and are appropriate fragments of the chloroplast (plants) or mitochondrial (animals) genome [52]. The international initiative “Barcode of Life” (<http://www.barcodeoflife.org/>) offers a database, in which DNA barcodes of all living species can be stored.

Most commonly, for the identification of animal species the 5' end part of the mitochondrial cytochrome c oxidase 1 gene (cox1 or COI) has been used. The advantage of COI is that it exhibits rapid evolution, shows high copy number and lacks introns [53]. With the help

of COI, in 2005 Ward *et al.* identified about 200 Australian fish species [54], while Lambert *et al.* identified extinct birds from New Zealand [55].

Because of the slow evolution of the plant mitochondrial DNA, COI is not suitable for plant species identification. Therefore, chloroplast and nuclear DNA regions have been utilized for differentiating a broad range of species of angiosperms, gymnosperms and cryptograms [56]. The Consortium for the Barcode of Life (CBOL) proposed the use of two plastid coding sequences as core-barcode regions [56]. The first gene, *maturase K* (*matK*), is probably the closest plant analogue to COI. The center of the gene consists of approx. 841 bp region, which is one of the most rapidly evolving coding regions of the plastid genome. The main drawback of *matK* may be the low amplification rate due to the lack of universal primers due to limited amount of available data [57]. The second gene, *rbcL*, consists of a 599 bp region at the 5' end of the gene, is easy to amplify and has modest discriminatory power [57, 58]. Besides *matK-rbcL*, the chloroplast intergenic spacer *trnH-psbA* is one of the most variable intergenic spacers in land plants. Furthermore, it is easy to amplify and results in an amplicon of approx. 450 bp [59]. The internal transcribed spacer (*ITS*) is among the most widely used supplementary loci from the nuclear ribosomal cistron (18S-5.8S-26S) region due to its easy amplification and high variability [57].

Due to its specificity and sensitivity, PCR-HRM analysis of DNA barcodes has already been applied to detect allergens but also adulterants in plants [48]. An overview of recently published studies using PCR-HRM barcoding is given in Table 1.

Allergenic compounds, such as nuts, can end up in food products, e.g. by unintended cross-contamination or false labeling, eliciting a variety of allergic symptoms in sensitized people. Madesis *et al.*, 2013, proposed using *trnL* primers for rapid detection of tree nut species in commercial food products. By analyzing the shape of the HRM melting curves, they were able to distinguish between tree nut species (almond, cashew nut, walnut, peanut, hazelnut, pecan, chestnut, pistachio Aigina, brazilnut and macadamia). Melting curves obtained for walnut, peanut and pecan showed three melting peaks, whereas for the other species, two melting peaks were obtained. Additionally, 0.01% of hazelnut traces were detected in biscuits [60].

In a study from Osathanunkul *et al.*, 2016, DNA barcoding has been used for distinguishing between three closely related *Uvaria* species, *Uvaria longpies*, *Uvaria wrayi* and *Uvaria siamensis*. Among the four regions (*matK*, *trnH-psbA*, *rbcL* and *trnL*) tested, *trnH-psbA* was found to be best suitable for distinguishing between these species [61]. Medicinal plants, in form of powders, tablets or teas have gained increased popularity as alternative medicines. However, they may be mixed with undesirable and unrelated species and thus have negative effect on consumers. Therefore, Osathanunkul *et al.*, 2016, applied six plastid markers (*matK*, *rbcLA*, *rbcLB*, *rbcLC*, *rpoC1* and *trnL*) on 25 Thai medicinal plant species and demonstrated, that DNA barcoding combined with PCR-HRM is a cost-effective and reliable method for species discrimination and detecting adulteration. In addition they showed, that no single marker could identify more than 58% of the species, and proposed a combination of four markers to be optimal for the plant species of interest [62].

According to Faria *et al.*, 2013, five commonly used fruits (orange, mango, peach, pear and pineapple) in Portuguese juices have been separated using universal primers for the *trnL* region. Furthermore, the difference plots allowed detection of admixtures, which may be useful in detecting adulteration with cheaper fruits [63].

Table 1: Overview of PCR-HRM coupled with DNA barcoding.

Aim	DNA barcode region	Application	Reference
Identification of allergenic tree nut species; Detection of tree nut species in food products	<i>trnL</i>	Eleven tree nut species and self-made biscuits	Madesis <i>et al.</i> (2013) [60]
Identification of <i>Uvaria</i> species	<i>matK</i> , <i>trnH-psbA</i> , <i>rbcL</i> and <i>trnL</i>	Three <i>Uvaria</i> species	Osathanunkul <i>et al.</i> (2016) [61]
Identification of medicinal plants; Discrimination, authentication and detection of adulteration of medicinal plants in herbal products	<i>matK</i> , <i>rbcL</i> , <i>rpoC1</i> and <i>trnL</i>	Twenty-five Thai medicinal plants	Osathanunkul <i>et al.</i> (2016) [62]
Authentication and detection of adulteration of fresh fruit juices	<i>trnL</i>	Five fruits	Faria <i>et al.</i> (2013) [63]
Identification and authentication of berry species and berry products	<i>ITS</i> , <i>rbcL</i> , <i>trnH-psbA</i> and <i>trnL</i>	Eight berry species and bilberry products	Jaakola <i>et al.</i> (2010) [45]
Identification of <i>Croton</i> species; Discrimination, authentication and detection of adulteration of <i>Croton</i> species in herbal products	<i>matK</i> , <i>rbcL</i> , <i>rpoC</i> , <i>trnL</i> and <i>ITS1</i>	Twelve <i>Croton</i> species	Osathanunkul <i>et al.</i> (2015) [64]
Identification of Mediterranean pines	<i>trnL</i>	Eight <i>Pinus</i> species and one hybrid	Ganopoulos <i>et al.</i> (2013) [65]

2 Aims of the master thesis

Food adulteration and mislabeling are widespread problems which affect not only the consumers but also the businesses and the economy. Consumers choose one product over another based on the product information provided, but also according to their lifestyle, religious or health concerns. Additionally, products containing only natural components are often more expensive than their synthetic equivalents. Food agencies have used different types of analytical techniques to verify product authenticity and detect adulterations. As previously shown, PCR-HRM DNA barcoding has been found to be very effective in not only detecting food fraud, but also in distinction between species.

The main objective of the present master thesis was to find suitable regions and design primer sets that can distinguish between different berries. Moreover, the primer sets should be applicable to raw and processed food.

Berries often belong to the same genus but different subgenus or section. In addition, they can derive from different cultivars or be a hybrid between two similar species. Therefore, the aim was to design appropriate primer sets enabling to distinguish between the berries. In the first place, it was important to distinguish between blueberry and bilberry, as well as between American cranberry, European cranberry and lingonberry.

From our cooperation partners from Macedonia, we have received eight pomegranate varieties from the same region. Thus, another aim of the master thesis was to investigate if these cultivars can be differentiated by PCR-HRM analysis.

Another aim was to check the accuracy of the labeling of different berry products. Hence, suitable primer sets and method optimization were required. In addition, DNA had to be extracted in sufficient amount and quality from highly processed products such as jams, juices, beverages, powders and caplets.

3 Theoretical background

3.1 Spectrophotometric measurements

The most commonly used technique for quantifying the amount of extracted DNA is the spectrophotometric estimation by a Nanodrop instrument (*ThermoFisher Scientific*). This spectrophotometer uses a unique sample retention technology, which eliminates the need of cuvettes. A simple drop of 0.5-2 μL sample on the pedestal is what makes this technique efficient and easy to maintain. Additionally, a broad range of DNA concentrations can be measured, because of the short path length. This allows measurement of non-diluted samples in less than a minute. With the help of Lambert-Beer law and the amount of absorbed UV light at 260 nm, the DNA concentration can be determined. By measuring the absorbance at 280 nm, the presence of proteins and polyphenolic compounds can be estimated. In order to determine the purity of the DNA, a ratio of absorbance at 260 nm and 280 nm is used and its value should be between 1.8 and 2.0. Lower values than 1.8 may indicate protein contamination. The main drawback of this technique is the lack of possibility to distinguish between DNA, RNA, proteins, polyphenolic compounds, salts and free nucleotide compounds. Furthermore, the low sensitivity may hamper the detection of low concentration samples [66, 67].

3.2 Fluorometric measurements

Fluorometric measurements were conducted using the Qubit (*ThermoFisher Scientific*) Fluorometer. With this instrument, DNA, RNA and proteins can be quantified, with the help of specific fluorescent dyes. The required volume of sample (DNA, RNA or protein) is 1-20 μL . The high sensitivity DNA kit (0.2-100 ng) uses DNA specific dyes, which have extremely low fluorescence when not bound. After incubation for 2 minutes, the dye will be bound to the DNA and an increase in fluorescence can be observed. With the help of two standards (S1 and S2), the DNA concentration can be determined. The advantages of Qubit are its high selectivity, sensitivity and molecule specificity [68].

3.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a fast and cost-effective technique used to copy (“amplify”) specific DNA sequences *in vitro*. This enzymatic assay requires small amounts

of DNA sample and allows researchers and clinicians to clone and sequence genes, e.g. to detect diseases. For this revolutionary discovery, Kary B. Mullis received the Nobel prize for chemistry in 1993.

Performed in a single tube, containing template DNA, primers, nucleotides and DNA polymerase, PCR doubles the amount of the target DNA with each cycle. Each cycle contains three phases, denaturation, annealing and elongation (Figure 1). After 30-50 cycles, more than one billion copies of the target DNA will be generated.

At the beginning, the initialization step takes place, where the reaction mixture is heated to 95°C for 5 minutes to activate the DNA polymerase. Then, the PCR cycle starts with the denaturation step by heating to 95°C. The heat breaks the hydrogen bonds of the double stranded DNA, leading to two single stranded DNAs. These single stranded DNAs are the template for the next step. Subsequently, the reaction is cooled to a lower primer specific temperature (50-65°C), allowing the primers to bind to the template. Primers (forward and reverse) always bind at the 3' end of the template DNA. In the last step, the so-called elongation, the temperature is increased to 72°C. The Taq polymerase extends the primers by adding nucleotides from 5' to 3' end and forming a new DNA strand. Finally, at the end of the cycle, two exact copies to the target DNA have been made [69].

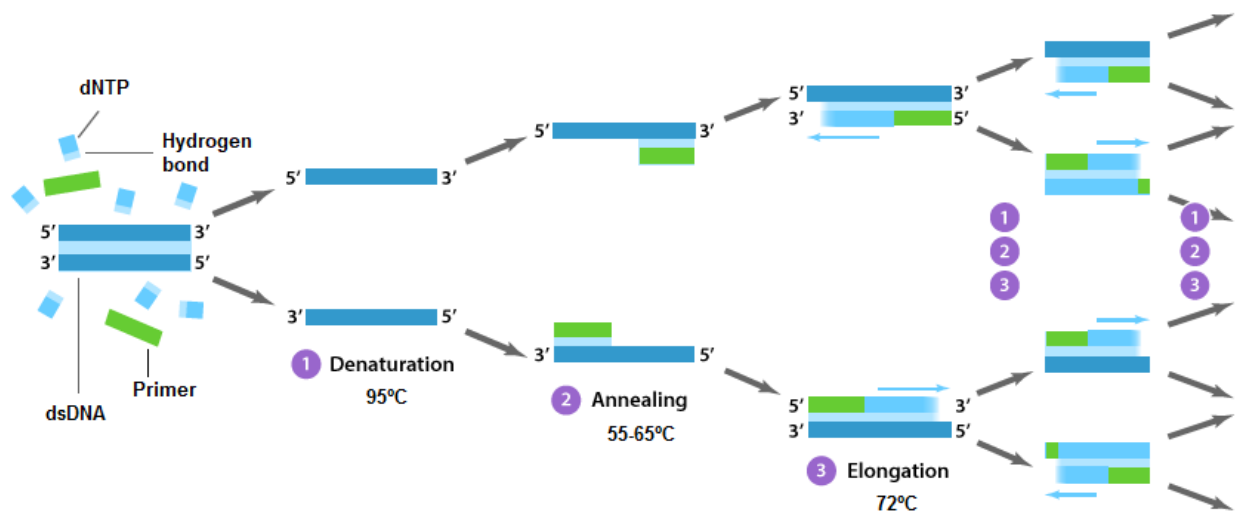


Figure 1: Schematic flow of PCR; from [70], modified.

The detection of these PCR products is performed by using fluorescent dyes. Theoretically, after each PCR cycle, the number of amplified copies should be 2^n , where n represents the number of cycles. This would result in 100% PCR efficiency. However, in reality the amplification efficiency is commonly between 60 and 80%. By plotting the fluorescence signal against the number of cycles, the amplification can be observed. At first, the amount of newly synthesized dsDNA is quite low, compared to the amount of target dsDNA (phase 1, Figure 2). After a few cycles, an increase of the signal can be detected in the so-called exponential phase (phase 2, Figure 2). With every further cycle, the amount of product increases exponentially. At last, in the plateau phase, a saturation point is reached (phase 3, Figure 2). The polymerase efficiency is lowered due to several reasons, e.g. the limited availability of reagents and the loss of activity of the polymerase [71].

3.3.1 Reaction components

Each PCR requires target DNA, primers, DNA polymerase, nucleotides, water, PCR buffer and fluorescent dye. First, it is crucial, that the target DNA is of high quality and quantity. Second, forward and reverse primers are needed, so they can specify the region that needs to be amplified. By complementary base pairing the primers bind to the template DNA. Furthermore, in order to synthesize new DNA strand, an enzyme is required. Typically used is the Taq DNA polymerase, which has received its name after the heat-resistant bacterium from which it was isolated (*Thermus aquaticus*). Taq polymerase is active at around 70°C, but it remains intact during repeatedly heating at 95°C in the denaturation step. Indeed, the Taq polymerase requires deoxynucleosid triphosphates (dNTPs) to synthesize the new DNA strand. Four building blocks, deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP) in equimolar concentrations are being attached to the 3' end of the primer by the Taq polymerase. Besides that, the presence of Mg^{2+} is important, since Mg^{2+} works as a mediator between the 3'-OH group of the primer and the phosphate group of the dNTPs. Moreover, its concentration affects the specificity and efficiency of the reaction. Too low concentrations

of Mg^{2+} result in low amplicon yields, however, too high concentrations may lead to high amplicon yields and may cause formation of unspecific PCR products. Another important component is the PCR buffer, which provides monovalent salt environment and an optimal pH. Furthermore, the use of RNase free water in the reaction mixture provides ultrapure liquid medium for interaction [69]. Finally, to observe the amplification process in real-time, a fluorescent intercalating dye such as SYBR Green I is commonly used. The obtained amplification curve consists of three phases. The first phase, also known as initial lag phase, represents the area where no products can be observed (phase 1, Figure 2). Next, the exponential phase shows the intersection of the amplification curve with a threshold line (phase 2, Figure 2). This point is also known as threshold cycle C_t , and it indirectly correlates to the number of copies in the sample. High C_t values indicate low amounts of target DNA in the sample, and *vice versa*. The plateau phase is the end-point of the reaction (phase 3, Figure 2) [72].

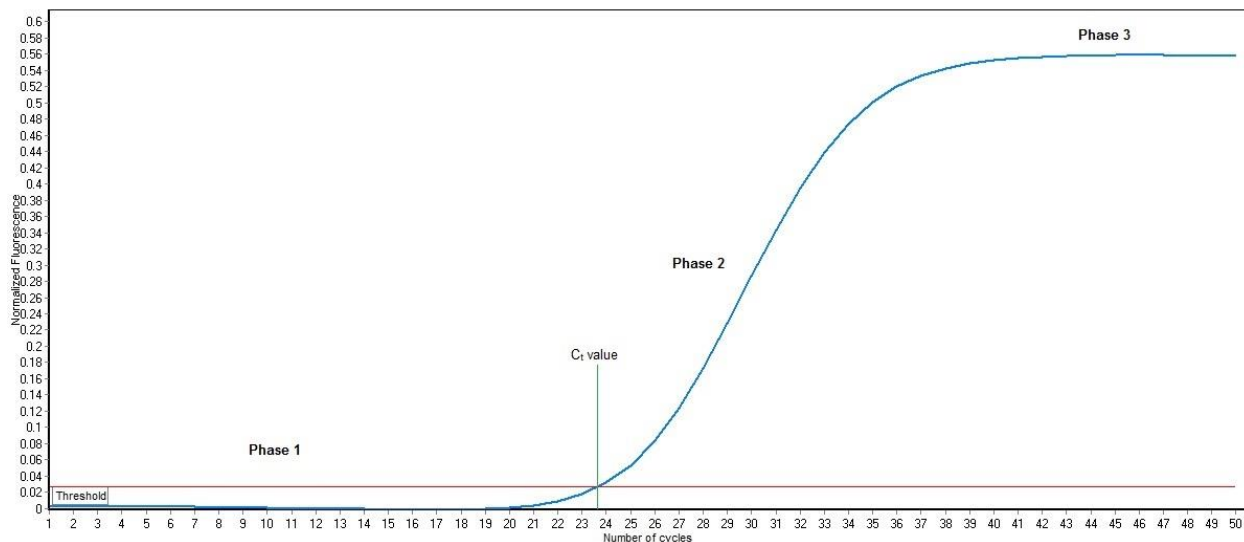


Figure 2: Example of the three phases of an amplification curve and C_t value.

3.4 High resolution melting (HRM) analysis

High resolution melting analysis is a quantitative technique which characterizes double stranded DNA based on its melting behavior. Main requirements for successful HRM

analysis are an adequate real-time PCR detection system and the use of intercalating DNA binding dyes. To precisely examine the melting behavior of dsDNA, HRM requires high concentrations of saturating DNA dyes. Furthermore, these dyes should neither inhibit the DNA polymerase, nor redistribute during melting. When bound to dsDNA, the dye exhibits high fluorescence. On the other hand, unbound saturating dye shows poor fluorescence. Typically used saturating dyes are EvaGreen®, SYT90® and ResoLight®. By gradually increasing the temperature from 65°C to 95°C with small increments (0.1°C-2.0°C/s), dsDNA is denatured. Subsequently the dye is slowly released and dsDNA dissociates into two single strands. A melting curve can be generated by plotting the fluorescence against the temperature (Figure 3). The temperature where 50% of the dsDNA is melted, is known as melting temperature (T_m). T_m is higher for guanine-cytosine (GC) rich PCR products, since more energy is required to break the three hydrogen bonds. On the contrary, two hydrogen bonds are formed between adenine (A) and thymine (T). Therefore, adenine-thymine (AT) rich PCR products melt at a lower T_m than GC rich amplicons. With the help of HRM software, pre- and post-melt regions of the melting curves can be defined. Additionally, the inflection point of the melting curve gives information about the T_m of the amplicon.

For better visualization, the first derivative of the melting curve is used. By normalizing the melting curve, differences in the background fluorescence can be eliminated. This provides better distinction between the melting curves (Figure 3).

When the differences between the melting curves are small, the use of difference plot can help. Therefore, normalized reference curve is subtracted from the sample curve [73, 74].

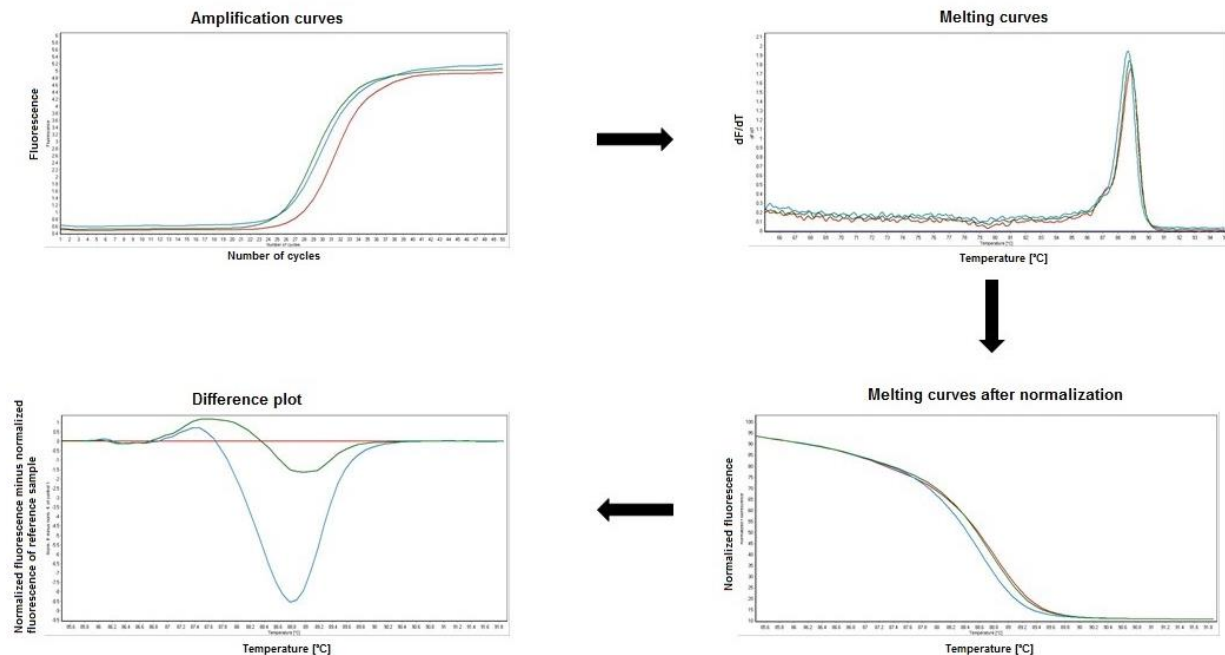


Figure 3: HRM analysis: examples of amplification curves, first derivative of the melting curves, melting curves after normalization and difference plot.

HRM analysis can be applied for detecting and categorizing genetic mutations, quantifying the proportion of methylated DNA or pathogen detection. Proper HRM assay design is crucial due to the high sensitivity of the analysis. This involves optimization of DNA quantity and quality, amplicon length, primer design and concentration, $MgCl_2$ concentration and dye selection. DNA samples with low-quality DNA may produce non-specific PCR products, which can lead to low sensitivity and incorrect reactions. Additionally, samples that amplify late or fail to reach high signal plateau, can result in low-resolution HRM data. To obtain DNA with good quality and quantity, firstly all samples should be extracted using the most suitable DNA kit. Secondly, extraction kits with lower buffer salt concentrations should be used, because salt carryover can lead to low reproducibility and low sensitivity. For instance, diluting salt contaminated samples can increase the data quality. The amplicon length is usually recommended to be between 80 bp and 300 bp. Shorter amplicons may produce lower fluorescence signals, whereas longer amplicons can lead to complex melting profiles. Another important factor is the design and concentration of forward and reverse primer. They should be optimized not only for robust performance, but also high specificity of the region of interest is required.

A variety of software tools are available to help check primer size, melting profile and secondary structure. The primer concentration will not have a significant influence on the final HRM result. By adjusting the optimal MgCl_2 concentration, non-specific amplification can be decreased, which leads to better distinction of the sequence variations. As mentioned above, the chosen dye has also an impact on the HRM analysis. There is the possibility to use non-saturating, saturating or release-on-demand dyes. In any case, the reaction settings have to be optimized to achieve high sensitivity [74].

All things considered, HRM analysis is a fast and non-destructive method, which requires no manual post-PCR processing and uses low cost reagents [74].

3.5 Primer design

Primer design is an important step which influences the whole PCR-HRM analysis. The following properties need to be considered in order to design suitable primers [69, 74]:

- Primers' length should be between 18 bp and 30 bp.
- GC content should be between 30 and 80%.
- T_m should be between 55°C and 65°C.
- The primers should be tested for secondary structures, which can lead to self-dimers, primer-dimers and cross-dimers.
- The 3' end should contain C or G, so that binding can be promoted. Furthermore, the 3' end must exactly match to the template DNA, since this end will be extended by the DNA polymerase.

3.6 Agarose gel electrophoresis

Agarose gel electrophoresis is an efficient and effective way of separating, purifying and visualizing DNA fragments with lengths between 100 bp and 25 kb. Agarose is a natural linear polymer extracted from seaweed. In solutions at high temperatures, agarose molecules have random coil structures. When cooled down, gelation occurs by physical cross-linking to a network of helical fiber bundles, which are connected by noncovalent hydrogen bonds. The size of the formed gel pores can affect DNA migration and depends on the agarose concentration. The solidified gel needs to be placed in an electrophoresis

cell, which contains positively and negatively charged electrodes. Afterwards, DNA samples or PCR-HRM products are loaded into the wells. When electric current of 100-150 V is applied, the negatively charged phosphate backbone of DNA will migrate to the positively charged anode. The migration speed is determined by the DNA size, thus smaller DNA fragments move faster through the gel than the longer ones. Visualizing of the DNA fragment is usually done with UV light and staining with ethidium bromide (EtBr). EtBr intercalates itself in the DNA, and when exposed to UV light, it fluoresces and DNA fragments can be visualized. By comparison of the DNA fragments with a standard DNA ladder, their size can be determined [75, 76].

4 Experimental part

4.1 Working with DNA

When working with DNA, different sources of contamination can influence the final result. To prevent contamination with other DNA samples or any air-born contaminants, three separated laboratories (A, B and C) were used. At laboratory A, DNA extraction took place. Laboratory B was used for PCR-HRM master mix preparation, laboratory C for adding the sample to the master mix. In the same manner, different freezers were used for storing kits, DNA extracts and PCR products.

Before starting, all working surfaces and pipettes were thoroughly cleaned with *DNA Exitus Plus IF* spray. Laminar flows, used at laboratories B and C, were additionally decontaminated with UV light for half an hour.

4.2 DNA extraction

Different types of samples (juices, jams, beverages, caplets, powders, etc.) were obtained from local supermarkets and pharmacies (Table 2). Fresh fruit samples were provided not only from Viennese supermarkets but also from our cooperation partners from Macedonia (Table 3). Furthermore, pressed plant voucher specimens were obtained from the Herbarium of the University of Vienna, Austria (Table 4). Also, five plants (blueberry, bilberry, American cranberry, European cranberry and lingonberry) were purchased from nursery garden Eggert (Vaale, Germany) (Table 3).

All samples were homogenized in a mortar, followed by grinding in a ball mill. DNA was extracted either using a commercial kit (*QIAamp® DNA Blood Mini Kit* or *DNeasy® Plant Mini Kit*) following the manufacturer's instruction or with modified CTAB protocol.

Table 2: Information about processed foods.

Product type	Product name and brand	Fruit	Declaration or information panel	Abbreviation used in this work
Dried fruit	Cashew cranberry-mix (Lorenz)	Cranberry	70% oil roasted cashew kernels, 30% dried sweetened cranberries, sugar, glycerol, citric acid, sunflower oil, palm oil	CCML
	Cranberry medley (Nature box)	Cranberry	pomegranate flavored dried cranberries (cranberries, sugar, sunflower oil), açai flavored dried cranberries (cranberries, sugar, sunflower oil, natural flavor)	CNB
	Craisins dried cranberries (Ocean spray)	Cranberry	cranberries, sugar, sunflower oil	COSD
	Cranberrys (S-Budget)	Cranberry	74% cranberries, sugar, sunflower oil	CSB
	Cranberries (Seeberger)	Cranberry	60% cranberries, sugar, sunflower oil	CSBE
	Superfrucht selection (Seeberger)	Cranberry, cherry, blueberry, chokeberry	16% chokeberry, cranberry, cherry, plums, sugar, glucose-fructose syrup, citric acid, sunflower oil, potassium sorbate	SSS
	Pflaumen entsteint (Seeberger)	Plum (prunes pitted)	pitted prunes, potassium sorbate	PPS2

Product type	Product name and brand	Fruit	Declaration or information panel	Abbreviation used in this work
Dried fruit	Cranberry nut mix (Spar)	Cranberry	33% peanuts, 25% sweetened cranberries (cranberries, sugar, sunflower oil), 20% raisins, 10% almonds, 9.6% cashew nuts, vegetable oils (sunflower, palm, corn, cotton, rapeseed)	CNS
	Cranberry Saft (DM)	Cranberry	100% north American cranberry juice (<i>Vaccinium macrocarpon</i>)	CSDM
Beverage	Cranberry Pur (Voelkel)	Cranberry	100% cranberry juice	CPV
	Cranberry (Pago)	Cranberry	26% cranberry juice from cranberry concentrate, water, cranberry aroma, sugar, lemon	CP
	Kombucha cranberry (Carpe diem)	Cranberry	5.1% cranberry juice, herbal tea, beet sugar, juice concentrate: chokeberry, apple, elderberry, lemon, acerola, kombucha cultures, lactobacillus	CCB
	Cranberrypulver (Biokia)	Cranberry	100% wild Cranberry (<i>Vaccinium oxycoccos</i>)	CBI
Powder	Preiselbeerpulver (Biokia)	Lingonberry	100% Finnish wild Lingonberry (<i>Vaccinium vitis-idaea</i>)	LBI

Product type	Product name and brand	Fruit	Declaration or information panel	Abbreviation used in this work
Jam	Hollerröster (Spar premium)	Black elderberry	85% elderberry, sugar, citric acid, pectin, cloves, cinnamon, vanilla extract	BES
	Waldheidelbeer (Hausensteiner)	Forest bilberry	60% bilberries, 40% sugar jelly, pectin, citric acid	FBH
	Schwarze Johannisbeere Cassis (St. Dalfour)	Black currant	45% black currants, 54.4% unsweetened fruit juice concentrate (grapes and dates), pectin	BCSD
	Cranberry und Heidelbeere (St. Dalfour)	Cranberry and blueberry	48.4% cranberry, 25.5% blueberry, unsweetened fruit juice concentrate (grapes and dates), pectin	CBSD
	Delikatess Powidl (Darbo)	Plum	plums, sugar, lemon juice concentrate	PPD
	Bio Ribisel (Ja!)	Red currant	55% red currant, sugar, lemon juice concentrate, pectin	RCJ
	Schwarze Ribisel (Spar)	Black currant	35% black currant, sugar, water, lemon juice concentrate, pectin	BCSJ
	Preiselbeere (Staud's)	Lingonberry	70% forest lingonberries, sugar, water, lemon juice concentrate, pectin	LS
	Zwetschken Röster (Efko)	Plum	90% plums, water, sugar, citric acid, cinnamon, cloves	PE

Product type	Product name and brand	Fruit	Declaration or information panel	Abbreviation used in this work
Caplet	Cranberry 1000 + vitamin C (Biomenta)	Cranberry	cranberry juice powder, ascorbic acid, hydroxypropylmethyl cellulose, magnesium salt, maltodextrin	CBM
	Cranberry (Finest Nutrition)	Cranberry	cranberry (<i>Vaccinium macrocarpon</i>), dicalcium phosphate, microcrystalline cellulose, hypromellose, magnesium hydroxide, croscarmellose sodium, silicon dioxide, magnesium stearate, stearic acid, polyethylene glycol, carmine color, dextrin, caramel color, dextrose, lecithin, sodium carboxymethyl cellulose, sodium citrate	CFN
	Cranberry + vitamin C (Vihado)	Cranberry	50% cranberry juice powder (<i>Vaccinium macrocarpon</i>), hydroxypropylmethyl cellulose, 16% acerola fruit extract, 17% L-ascorbic acid, silica powder, magnesium salt, iron oxide (E 172), titanium dioxide (E 171)	CVI
	Cranberry Kapseln (Well & Active)	Cranberry	cranberry juice powder, vitamin C	CWA

Product type	Product name and brand	Fruit	Declaration or information panel	Abbreviation used in this work
Syrup	Heidelbeer cassis sirup (Darbo)	Blueberry and black currant (cassis)	42% blueberry juice, 6% blueberry juice concentrate, 5% black currant juice concentrate, sugar, citric acid, natural aroma	BBD
	Cranberry Aronia sirup (Darbo)	Cranberry and chokeberry	42.9% cranberry, sugar, 6% chokeberry concentrate, acerola concentrate	CCDS
	Granatapfel sirup (Darbo)	Pomegranate	32% pomegranate juice, 19% pomegranate juice concentrate, citric acid, sugar	PDS
Jelly	Jellied cranberry sauce (Ocean spray)	Cranberry	cranberries, high fructose corn syrup, corn syrup, water	JCOS

Table 3: Information about fresh fruits and leaves.

Product type	Product brand	Fruit	Country of origin	Abbreviation used in this work (Cultivar)
Fresh fruit	Athos	Blueberry	Peru	BAP
	Spar	Plum Angeleno	Italy	PA
	Spar	Plum (orange)	-	PO
	Spar	Plum (purple)	-	PP
	Rubin garden	Pomegranate	-	9G (cultivar unknown)
	-	Pomegranate	Macedonia	1G (Bernarija)

Product type	Product brand	Fruit	Country of origin	Abbreviation used in this work (Cultivar)
Fresh fruit	-	Pomegranate	Macedonia	2G (Zumnarija)
	-	Pomegranate	Macedonia	3G (Ropkavac)
	-	Pomegranate	Macedonia	4G (Limfanka)
	-	Pomegranate	Macedonia	5G (Limfanka klon)
	-	Pomegranate	Macedonia	6G (Hidzas)
	-	Pomegranate	Macedonia	7G (Karamustafa)
	-	Pomegranate	Macedonia	8G (Valandovska kisela)
	-	Chokeberry	Macedonia	AmF
	-	Blackberry	Macedonia	RfF
	-	Bilberry	Macedonia	VmyF
	-	Blueberry	Macedonia	VcF
	Merkur	Bilberry	Germany	BM
	Spar	Cranberry	USA	CfS
	Hofer	White grape	Chile	WGC
	Hofer	Red grape	Chile	RGC
	-	Apple	-	APP (Pinova)
	Frutura	Apple	Austria	AGD (Golden delicious)
Fresh leaves	Nursery garden Eggert	Lingonberry	Germany	VviL
	Nursery garden Eggert	European cranberry	Germany	VoL
	Nursery garden Eggert	Bilberry	Germany	VmyL

Product type	Product brand	Fruit	Country of origin	Abbreviation used in this work (Cultivar)
Fresh leaves	Nursery garden Eggert	American cranberry	Germany	VmaL
	Nursery garden Eggert	Blueberry	Germany	VcL

Table 4: Information about herbarium specimens.

Species	Herbarium WU (Austria) ID number	Collection locality and date	Abbreviation used in this work
<i>Vaccinium vitis-idaea</i> L.	061340	Styria, Austria, 2007	1H
<i>Vaccinium vitis-idaea</i> L.	4823	Styria, Austria, 1997	2H
<i>Vaccinium vitis-idaea</i> L.	4854	North Siberian Lowland Russia, 2004	3H
<i>Vaccinium oxycoccos</i> L.	4883	Styria, Austria, 1987	4H
<i>Vaccinium oxycoccos</i> L.	004237	Upper Austria 1995	5H
<i>Vaccinium oxycoccos</i> L.	3114	Lower Austria, 1921	6H
<i>Vaccinium oxycoccos</i> L.	5824	Latvia, 2013	7H
<i>Vaccinium myrtillus</i> L.	061340	Austria, 2007	8H
<i>Vaccinium myrtillus</i> L.	045167/5114	Vorarlberg, Austria, 2008	9H
<i>Vaccinium myrtillus</i> L.	4883	Lower Austria, 1990	10H
<i>Vaccinium myrtillus</i> L.	5013	Poland, 2006	11H
<i>Vaccinium myrtillus</i> L.	004588	Russia, 2003	12H
<i>Vaccinium macrocarpon</i> A.	004680	Ontario, Canada, 1966	13H

Species	Herbarium WU (Austria) ID number	Collection locality and date	Abbreviation used in this work
<i>Vaccinium macrocarpon</i> A.	319	New Jersey, USA, 1948	14H
<i>Vaccinium macrocarpon</i> A.	249	Londonderry, UK, 1977	15H
<i>Vaccinium corymbosum</i> L.	023125/004418	North America, 1980	17H
<i>Vaccinium corymbosum</i> L.	023124/004418	North America, -	18H
<i>Vaccinium corymbosum</i> L.	004295	Virginia, USA, 1996	19H

4.2.1 DNA extraction with QIAamp® DNA Blood Mini Kit

At the beginning, 50 mg sample was weighed out in a 1.5 mL Eppendorf tube. Subsequently, 180 µL ATL Buffer and 20 µL Proteinase K were added, mixed by vortexing and incubated in a thermal block (*Thermal Mixer, ThermoFisher Scientific*) for 2 hours and 10 minutes at 56°C with 1400 rpm mixing frequency. The mixture was vortexed every 30 minutes to ensure efficient lysis. Afterwards, the tubes were briefly centrifuged and 20 µL Ribonuclease A (RNase A, c = 20-40 mg/mL) was added and incubated for 2 minutes at room temperature. The solution was mixed with 200 µL lysis buffer (AL Buffer) and incubated for 10 minutes at 70°C in the thermal block. Next, 200 µL of absolute ethanol were added and the solution was mixed by pulse-vortexing. Afterwards, the mixture was centrifuged for 1 minute at 8000 rpm. The solution was transferred to a *QIAamp Mini spin column* with a 2 mL collection tube and centrifuged for 1 minute at 8000 rpm. To provide pure DNA with no remaining contaminants, two washing steps were needed. First, 500 µL of wash buffer (Buffer AW1) were added to the *QIAamp Mini spin column* and centrifuged at 8000 rpm for 1 minute. Second, the *QIAamp Mini spin column* was once again transferred into a new 2 mL collection tube and washed with 500 µL Buffer AW2. After centrifugation for 3 minutes at 10000 rpm, the *QIAamp Mini spin column* was placed in a clean 1.5 mL Eppendorf tube, eluted with 200 µL of Buffer AE or RNase free water and incubated for 5 minutes at room temperature. By centrifugation for 1 minute at 8000 rpm, the DNA was eluted. In order to obtain higher DNA yields, the last step was repeated and the DNA extracts were stored at -20°C.

4.2.2 DNA extraction with DNeasy® Plant Mini Kit

For the extraction with *DNeasy® Plant Mini kit*, 100 mg wet or 20 mg dry sample was weighed out in a 1.5 mL Eppendorf tube. Then, 400 µL of Buffer AP1 and 4 µL RNase A (c= 20-40 mg/mL) were added, briefly vortexed and incubated in a thermal block (*Thermal Mixer, ThermoFisher Scientific*) for 2 hours and 10 minutes at 65°C (mixing frequency 1400 rpm). To ensure efficient lysis, the mixture was vortexed every 30 minutes. To precipitate proteins and polysaccharides, 130 µL of Buffer P3 was added, mixed and incubated on ice for 5 minutes. The lysate was centrifuged for 5 minutes at 14000 rpm

and pipetted into a *QIAshredder spin column* placed in a 2 mL collection tube. After centrifugation for 2 minutes at 14000 rpm most precipitates and cell debris should be removed. The flow-through was transferred into a new 1.5 mL Eppendorf tube and washed by adding 1.5 volumes of Buffer AW1. 650 μ L of the solution was pipetted to a *DNeasy Mini spin column* placed in a 2 mL collection tube and centrifuged for a minute at 8000 rpm. The flow-through was removed and the step was repeated with the remaining sample. Afterwards, the *DNeasy Mini spin column* was placed in a new 2 mL collection tube, washed by adding 500 μ L of Buffer AW1 and centrifuged at 8000 rpm for a minute. Since ethanol residues may interfere with extraction, drying the membrane was of high importance. To achieve this, another 500 μ L of washing buffer (Buffer AW2) were added and centrifuged for 2 minutes at 14000 rpm. After carefully removing the spin column and transferring it to a clean 1.5 mL Eppendorf tube, 100 μ L of elution buffer (Buffer AE) was added, incubated for 5 minutes at room temperature and centrifuged for 1 minute at 8000 rpm. The last step was repeated and the DNA extracts were stored at -20°C.

4.2.3 DNA extraction with CTAB protocol

At the beginning, 100 mg of sample was weighed out and mixed with 500 μ L CTAB extraction solution and vortexed. After incubation for half an hour at 65°C in a thermal block (Thermal Mixer, *ThermoFisher Scientific*) with stirring frequency of 1400 rpm, 15 μ L of Proteinase K was added and incubated for further 2 hours and 10 minutes at 50°C (1400 rpm). Then 20 μ L of RNase A (c = 20-40 mg/mL) was added and the mixture was incubated at room temperature for 2 minutes and then centrifuged for 10 minutes at 13000 rpm. The upper phase was transferred into new 1.5 mL Eppendorf tube and 200 μ L of chloroform were added, followed by vortexing for 1 minute and centrifugation for 10 minutes at 13000 rpm. Afterwards, the aqueous phase was transferred into a new 1.5 mL Eppendorf tube, mixed with two parts by volume of CTAB precipitation solution (5 g/L CTAB and 0.04 M NaCl) and incubated in the thermal block for 2 hours and 10 minutes. After centrifugation (10 minutes, 13000 rpm), the supernatant was transferred into a new 1.5 mL Eppendorf tube without destroying the pellet, dissolved in 350 μ L of

1.2 M NaCl solution and extracted with 350 μ L of chloroform. Then, the mixture was vortexed for 1 minute and centrifuged for 10 minutes at 13000 rpm. The upper aqueous phase was transferred into a new 1.5 mL Eppendorf tube and then the DNA was precipitated with 0.6 parts by volume isopropanol and centrifuged (10 minutes, 13000 rpm). Without destroying the pellet, the supernatant was discarded. Then the pellet was washed with 500 μ L ice cold 70% (v/v) ethanol, vortexed for 1 minute and centrifuged for 10 minutes at 13000 rpm. The upper phase was once again discarded and the pellet was dried long enough to remove the alcohol (ca. 20 minutes) at 60°C in the thermal block. Finally, the pellet was dissolved by adding 100 μ L RNase free water and warming in the thermal block (20 minutes, 56°C, 500 rpm). The extracted DNA was stored at -20°C for further use.

4.3 Spectrophotometric and fluorometric measurements

Yield and purity of the DNA extracts were measured using Nanodrop 2000c Spectrophotometer (*ThermoFisher Scientific*) and Qubit Fluorometer (*ThermoFisher Scientific*).

At the beginning of the spectrophotometric measurement a zero adjustment with 1.0 μ L elution solution (Buffer AE or RNase free water, respectively) was done. After that, 1.0 μ L of DNA extract was loaded on the pedestal and the absorbance in the wavelength range 220-350 nm was measured.

For the fluorometric measurements, a high sensitivity kit (0.2-100 ng) was used. First, two standards, S1 and S2, were prepared by mixing 10 μ L standard solution (S1 or S2) with 190 μ L Qubit working solution. Second, 2 μ L DNA sample were added to 198 μ L Qubit working solution for the first measurement, whereas for the other two measurements 20 μ L DNA and 180 μ L Qubit working solution were used. The two standard solutions (S1 and S2) and the DNA sample solutions were briefly vortexed, incubated for 2 minutes and measured.

Adjustment of the DNA concentration of the extracts to 5000 ng/mL was done based on the concentration values obtained with the Nanodrop instrument.

4.4 PCR

4.4.1 Primer design

At the beginning, primers based on the sequences of internal transcribed spacer (*ITS*) and plastid loci regions (*rpl36-rpl38* and *trnL-F*) designed by Jaakola *et al.*, 2010, were applied. All three primer sets had been designed based on bilberry sequences submitted to GenBank (GU361894 for *ITS*, GU361915 for *rpl36-rpl38* and GU361900 for *trnL-F*). In addition, primers for *ITS* and *matK* were designed within this work. Primer design was performed with *PyroMark Assay Design 2.0* according to the settings mentioned in chapter 3.5. The primer sets *matK_1* and *matK_2* were designed based on the *matK* sequences for plum (HQ235147) and bilberry (AF382810), respectively. For the design of the *ITS_2* and *ITS_3* primers, the *ITS* sequence for pomegranate (JQ740192.1) was used.

The melting temperatures were calculated with the webserver *Oligo Calc* [77]. Furthermore, the formation of secondary structures was checked with the webserver *RNA fold* [78] and the formation of primer dimers with *Oligo Analyzer 3.1* [79]. Table 5 summarizes the primer sequences used in the present work.

Table 5: Primer sequence, GenBank accession number and amplicon length.

Gene	Primer	Primer sequence 5'-3'	Species and GenBank Accession number	Amplicon length [bp]
<i>ITS</i>	<i>ITSVm1f</i>	ATTGTCGAAAACCTGCCA	Bilberry (GU361894)	244-291
	<i>ITSVm1r</i>	GAGATATCCGTTGCCGAG		
<i>rpl36-rps8</i>	<i>rp1f</i>	TTGAAAAATCGCCTAATTCTCC	Bilberry (GU361915)	188-359
	<i>rp1r</i>	GATCCCACACGAGGACGTAT		
<i>trnL-F</i>	<i>trnLVm1f</i>	TTAGCCGTTCCAAATTCCTT	Bilberry (GU361900)	242-312
	<i>trnLVm1r</i>	GGGTCTATGTCAATTAAAAGAACGA		
<i>matK</i>	<i>matK_1f</i>	TTTTTCAAAAAGTAATCCACGATT	Plum (HQ235147)	77-218
	<i>matK_1r</i>	ATGTATTGCTCAAAAAAGATCCC		
<i>matK</i>	<i>matK_2f</i>	TTCGTTTTTCTTCGCAATCAATCT	Bilberry (AF382810)	675-1019
	<i>matK_2r</i>	TAATCGTACGCTTGAAAGATAGC		
<i>ITS</i>	<i>ITS_2f</i>	ACAGTGTAACCCCGGC	Pomegranate (JQ740192.1)	289-408
	<i>ITSVm1r</i>	GAGATATCCGTTGCCGAG		
<i>ITS</i>	<i>ITS_3f</i>	TCGCCCCAAAACCTCCAC	Pomegranate (JQ740192.1)	552-688
	<i>ITS_3r</i>	GTCGCTCCCGTGCTCCTTT		

4.4.2 Primer ordering

The primers were synthesized by *Sigma Aldrich*. To achieve a concentration of 100 μM , the lyophilized primers were diluted with RNase free water following the manufacturer's instruction and stored in 32 μL aliquots at -20°C .

4.4.3 PCR-HRM preparation

PCR amplification followed by HRM analysis was performed on *Rotor-Gene Q Series* from *Qiagen*. Either the *Epitect HRM PCR Kit (Qiagen)* or the *Type-it HRM PCR Kit (Qiagen)* was used. Both Kits were used following the manufacturer's instructions. The reaction mixture consisted of:

- HRM Master mix, 2x
- Primer solution (forward and reverse) (10 μM)
- RNase free water
- DNA solution
- MgCl_2 solution (40 mM) *

*optional

Prior pipetting, all reagents were thawed and the working place was cleaned with *DNA Exitus Plus IF* spray to avoid contamination. At working station B, 18 μL of master mix were pipetted in 200 μL strip tubes placed in a cooled aluminum block (Table 6).

Table 6: Pipetting scheme for master mix, depending on the MgCl_2 concentration.

Component	Volume [μL]	Volume [μL]	Volume [μL]
HRM Master Mix	10	10	10
Forward primer	0.5	0.5	0.5
Reverse primer	0.5	0.5	0.5
RNase free water	7	6	5
MgCl_2 solution	0	1	2

2 µL of DNA solution were added to the master mix at working station C. For the *no template control* (NTC) instead of DNA, RNase free water was used.

4.4.4 PCR-HRM settings

Real-time PCR was conducted following the general settings shown in Table 7, unless otherwise noted.

Table 7: General PCR and HRM settings.

		Temperature [°C]	Time
Initial denaturation step		95	5 min
3 step cycling (50 cycles)	Denaturation	95	10 s
	Annealing	57→51*	30 s
	Extension	72	10 s
Denaturation		95	1 min
Hybridization		40	1 min
HRM		65-95	0.1°C per 2 s

*Touchdown: 1°C for the first seven cycles

Subsequently, the PCR amplicons were denatured for HRM at 95°C for 1 min followed by cooling at 40°C for 1 min to form random DNA duplexes. The temperature was then raised from 65°C to 95°C with an increment of 0.1°C every 2 s with fluorescence acquisitions. The fluorescence data was further processed to generate melting curves for the PCR products.

4.4.5 PCR-HRM optimization

In order to achieve efficient amplification and amplification curves reaching plateau as well as low threshold cycles (C_t), several PCR conditions had to be optimized. Firstly, all DNA extracts were diluted with RNase free water to a concentration of 5000 ng/mL. DNA

extracts with a DNA concentration below 5000 ng/mL were used undiluted. Furthermore, the annealing temperature (57°C-68°C), elongation time (10s or 30s) and the MgCl₂ concentration (0 µL, 1 µL or 2 µL, c = 40 mM) were varied. Also, an extension step for 7 min at 72°C was included in some experiments.

4.5 Agarose gel electrophoresis

4.5.1 Sample preparation

For the agarose gel electrophoresis, a *Mini Sub CellnGT*, *BioRad* coupled with power supplier *Power Pac HV* was used. The DNA ladder (*25 bp DNA Ladder*, *Invitrogen*TM) was provided from *ThermoFisher Scientific*, the 5x loading dye (5x Nucleic Acid Sample Loading Dye) from *BioRad*. 10 µL of the PCR-HRM product were mixed with 2 µL 5x loading dye. In the case of the DNA ladder solution, 2 µL DNA ladder were mixed with 8 µL RNase free water and 2 µL 5x loading dye.

4.5.2 Procedure

At first, by mixing of 20 mL 50x tris-acetate-EDTA (TAE) buffer with 1000 mL distilled water, the 1xTAE buffer solution was prepared. Then, 2 g agarose were weighed out, dissolved in 100 mL 1xTAE buffer solution and boiled until a clear solution was obtained. 10 µL of GelRedTM were added to the agarose solution and the mixture was slowly poured into a gel tray with a well comb and let solidify for 30 minutes at room temperature. Then, the agarose gel was transferred into the electrophoresis cell and covered with 1xTAE buffer solution. The well comb was removed and the wells were loaded with PCR-HRM products (5 µL or 10 µL) or DNA ladder (1 µL or 2 µL). After closing the electrophoresis cell, electric current of 120 V was applied for approx. 1 hour. The agarose gel was visualized with the help of a UV transilluminator (*Herolab*).

5 Results and discussion

The aim of the present master thesis was to develop and optimize different primer sets which can be used for authentication of various red fruits and their products. A previous study by Jaakola *et al.*, 2010, was taken as a starting point. Their goal was to develop a method, that can distinguish bilberry (*V. myrtillus* L.) from other same-colored commercially important berries. Therefore, they have designed primers based on several standard DNA regions, and used them for PCR-HRM analysis. They successfully distinguished bilberry from other berry species in raw but also in dried berry products. At the beginning of my work, I used three primers pairs (*ITSVm1*, *rp1* and *trnLVm1*) designed by Jaakola *et al.*, 2010, and applied them to different berry species (fresh berries and berry products). One objective was to differentiate berry species from the same family but different subgenus. However, we were also interested in detecting potential adulteration with other fruits. In order to achieve these objectives, I designed various primer sets (*matK_1*, *matK_2*, *ITS_2*, *ITS_3*) with the help of *PyroMark Assay Design 2.0* software according to the rules mentioned in Chapter 3.5.

5.1 Primer sets

5.1.1 *ITSVm1*

Figure 4 shows a part of the *ITS* region for *V. myrtillus* L. The accession number of the sequence is GU361894 (Jaakola *et al.*, 2010). The binding positions of forward and reverse primer are marked in blue and green, respectively. Characteristics of the primers are summarized in Table 8.

```
GGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAAAACCTGCCAAGCAGA
AAACCCGCGAACTCGTCTATACTCTCGGGGAACGATGCGGGGGTTCGTGGCCCGGTTGCCTCGCCTTCCAT
TTTTTCCCCTTGCGAGCAGATGCGCACGGAACCTTCGGGCAACGTGTTTCGTCTGCTTGTCAAACAACGAA
CCCCGGCGCAAAACGCGCCAAGGAAAAATCGAACAAAGAGCGCGCTCCCTGCCCGTTCTCGGGCGGTGT
TGGCGTCTGCAATCTTTCTTGTAACCTGAACGACTCTCGGCAACGGATATCTCGGCTCTTGCATCGATGAA
GAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAG
TTGCGCCTGAAGCCATTAGGTTGAAGGCACGTCTGCCTGGGCGTCACGCATTGCGTCACCCACTCCCCC
GTGCCCCAAGCGGGCACGTGCGAGCGTGGGCGGATATTGGCCCCCGTTTCGCATCCGTGCGCGGTGCGCC
TAAAAACGGGTCCCCAATGACGGACATCACGACAAGTGGTGGTTGCTAAACCGTCGCGTCACGTCGTGC
ATGCCATCGTTTGTGCGGGTTGGCCATTTGACCCTGAAGTGCCGTTTAACTGCGGCGCCTCAACTGCG
ACCCAGGTGAGGCGGGATTACCC
```

Figure 4: Sequence of the *ITS* region for bilberry (GU361894); forward primer: blue, binding site of the reverse primer: green.

Table 8: Characteristics of the *ITSVm1* primers.

	Forward primer	Reverse primer
Sequence 5'→3'	ATTGTCGAAAACCTGCCA	GAGATATCCGTTGCCGAG
Length [nt]	18	18
T_m [°C] according to Sigma Aldrich	61.7	60.8
T_m [°C] according to Jaakola et al., 2010	56.0	56.0

5.1.2 *rp1*

A part of the *rpl36-rps38* region for *V. myrtillus* L. with accession number GU361915 is shown in Figure 5 (Jaakola et al., 2010). The binding positions of forward and reverse primer are marked in blue and green, respectively. Characteristics of the primers are summarized in Table 9.

```

TTTCTTACCGTAATTTTGATCTACTTCTTGGAAGAAAAAAGTTTAGTGAAATTTTGAATCTTGAATTGT
ATTCTTACCAAAGGAATGTTAGGATTGAAAAATCGCCTAATTCTCCGAAGCCTTGTTGGGCTTCTTCGAA
GCCTTGTTGGGCTTCTCCGAAGCCTTGTCGGGCTTTTCTGAAGCCTTGTTGGGATTCTCCGAAGCCTTGT
CGGGCTTCTCTGAAGCCTTGTTGGGCTTCTCTGAAGCCTTGTTGGGATTCTCCGAAGCCTTGTCTGGGCTT
GTCGGGCTTCTCTGAAGCCTTGTTGAGATTCTCCGAAGACTTCTTCGAAGGCTTCTTAACGGGGAGTCGA
TAAATTATACGTCCTCGTGTGGGATCATATTGACTTACTTCAATTTGGACTCTATCTCCGGGAATATCC
GTATCAAACGACGTCTGTATATTTCTGAAATATAACCTAGAATAATATCTGTATTATGGTTATCCACTTT
ATCGTCGTTATCTAAAAGAACC CGGAACCTACCATCGGGAAATGAATCGGTAACCTAAACCTTCATAAATT
CTTTTTTGTTCTTTTTTACTCATTTTTTGAAGATCCTCCTGTAATTCAAAAAATTGATTAATCTCCTCT
TCTTCTTTTTTTTTTGAAGAAAAAAGTTTCGGATAGAATTCGGAT

```

Figure 5: Sequence of the *rpl36-rps38* region for bilberry (GU361915); forward primer: blue, binding site of the reverse primer: green.**Table 9:** Characteristics of the *rp1* primers.

	Forward primer	Reverse primer
Sequence 5'→3'	TTGAAAAATCGCCTAATT CTCC	GATCCACACGAGGAC GTAT
Length [nt]	22	19
T_m [°C] according to Sigma Aldrich	62.9	63.8
T_m [°C] according to Jaakola et al., 2010	56.0	56.0

5.1.3 *trnLVm1*

Forward and reverse primer for the *trnL-F* region were designed based on the bilberry sequence (GU361900) shown in Figure 6 (blue and green). Additional information about the primers length and melting temperature is given in Table 10.

```
TCTATCCCCAAAAGCCTGTTTGACTCCTTAACAATACTATTAAGTATAGTATTTATCCGATCCTTTTTTA
GTTAGCCGTTCCAAATTCCTTATCTTTCTCATTCACTCGATTCTTTCACAAAATAAACGGATCARAGCAR
AAATACTTTACTCATATCACAAACAAGTCTTAGGACATGTGATAGACGTACAAATGAATATCTTTGAGCA
AGAAATCCCATTGGAATGATTGATGATCTATATCATTATTCGGGCTGAAATTCAAAAAGTTTTCTTTTT
TTAATCCAAGAAATTACAGGGCGAGGGTAAGACTTTGTAATACCCTTTCGTTCTTTTAATTGACATAGAC
CCAAATCATCTAGTAAAATTGARAATGATGGATCGGGAATGGTCAGGATAGCTCAGCTGGTARAGCARAG
GACTGAAAATTCTCGTGTACC
```

Figure 6: Sequence of the *trnL-F* region for bilberry (GU361900); forward primer: blue, binding site of the reverse primer: green.

Table 10: Characteristics of the *trnLVm1* primers.

	Forward primer	Reverse primer
Sequence 5'→3'	TTAGCCGTTCCAAATTCC	GGGTCTATGTCAATTAAAA
	TT	GAACGA
Length [nt]	20	25
T _m [°C] according to Sigma Aldrich	62.3	63.0
T _m [°C] according to Jaakola et al., 2010	56.0	56.0

5.1.4 *matK_1*

A sequence of the *mat K* region for the most common plum species (*Prunus domestica*) is displayed in Figure 7. Additionally, the binding positions of forward and reverse primer are marked in blue and green, respectively.

CTTCTTTGCATTTATTACGACTCTTTCTTCACGAGTATTATAAATTTGAATAGTCCTATTACTTCAAATAA
TTTTTTTCTTTTCAAAAAGTAATCCACGATTATTCTTACTCCTATATAATTCTCATGTATGTGAATACGAA
TTCATCTTACTTTTTCTTCGTAATCAATCTTCTCATTACAAATTAACCTCTTCTGGGATCTTTTTTGAGC
GAATACATTTCTATGAAAAAATAAAATATCCTGTAGAAGAAGTCTTTGCTAATGATTTTCCGGCCTCCAT
CTTATGGTTCTTCAAGGATCCTTTTATGCATTATGTTAGATATCAAGGAAAATCAATTCTGGCTTCGAAG
GATACCCCTCTTCTGATGAATAAGTGGAAATATTATCTTGTCAATTTATGGCAATGTCATTCTTATGTGT
GGTCTCAACCGGGAAGGATTTATATAAATAAATTATCCAAGCATTCCCTTGATTTTTTGGGTTATTTTTTC
AAGTATCCGACCAAACCTTTTCAGTGGTACGGAGTCAAATGCTAGAAAATTCATTTATAACGGATAATGCT
ATGAAGAAGCTCGATACATTAGTTCCAATTATTCGGTTGATTGGATCATTGGCTAAAGTGAAATTTTGTA
ACGCATTAGGGCATCCTATTAGTAAGTCCACCTGGGCGGATTATCGGATTTTGATATTATCGACCGATT
TCTGCGTATATGCAGAAATATTTCTCATTATTACAGCGGATCCTCAAGAAAAAAGAGTTTGTATCGAATA
AAATATAT

Figure 7: Sequence of the *matK* region for plum (HQ235147); forward primer: blue, binding site of the reverse primer: green.

Table 11 summarizes the characteristics of the *matK_1* primers, including melting temperatures and predicted secondary structures. None of the primers showed a tendency to form secondary structures.

Table 11: Characteristics of the *matK_1* primers.

	Forward primer	Reverse primer
Sequence 5'→3'	TTTTTCAAAAAGTAATC CACGATT	ATGTATTCGCTCAAAAA AGATCCC
Length [nt]	24	24
Secondary structures		
T_m [°C] according to Sigma Aldrich	61.4	64.9
T_m [°C] calculated with Oligo Calc [82]	55.0	60.3

5.1.5 *matK_2*

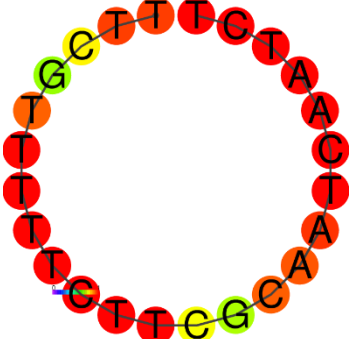
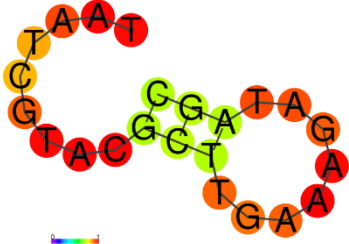
The design of the second *matK* primer set, *matK_2*, was based on the bilberry sequence (AF382810). Figure 8 shows the binding sites of the forward and reverse primer (blue and green, respectively).

```
TGGAGGAATTCAAAAGATATTTANAAC TAGATAGATCTCNGCAACATGACTTCATNTATCCACTTATTTT
TCAGGAGTATATTNATGCACTTGCTCATGATCGTGGGTAAATAGATCTATTGTTTTTCAAAATGCAGAT
TATGACAATAAATCTAGCTTACTAATTATGAAACGTTTAATCACTCATTTAATTACTCAAATGTATCAAC
AGAATCATTTTTCTTTTTTATACTAATGATTTTAATCCAAATAAATTTTTGGGGTACAATACATATTTGTA
TTCTCAAATGATATTTGAAGGATTTGCGGTGCTTGTGGAAATTCATTTTTATCTACAATTACTATCTTTT
CTAGAAGGGAAAAGAAAGAGTCAAATCTCATAATTTACGATCACTTCATTCAATATTTCCGTTTTTAGAGG
ACAAATTTTCCCATTTAAATTATGTGTTAGATATACTAATACCTCATCCCGTCCATCTGGAAATCTTGGT
TCAAACCTCTTCGCTACTGGGTAAAAGATCCCTCTTCTTTGCATTATTACGATTTTTTCTCCACGAGTAT
CCTAATCGGAATAGTCTTATTACTCCAAAGAAATATAGTTTTTCTTTTTCAAAAAGAAATCAAAAATTCT
TCTTGTTCCATATATAAATTTTCATGTATGTGAATACGAATCTATCTTCGTTTTTCTTCGCAATCAATCTTC
TCATTTATGCTCAATATCTTTTGAAACCTTTCTAGAACGAATCTTGTTCTATAAAAAAATAGAACTAGAA
GTCTTTGTTAAGGATTTTAAGGGCATTCTATGGGTTTTCAAAGACCCTTTCCTGCATTATGTTAGATATC
GAGGAAAAATCAATTTTGGCTTCAAACGTTTCGTCTCTTTTGATGAATAAATGGAAATATTACCTTGTCAA
TTTTCTGGGAATGTTATTTTTCCATATGGGCTCAACCAAGAAAGGATCCATATAAAACCAATTATCAAATAAT
TCCCTCGACTTTCTGGTCTATCTTTCAAGCGTACGATTAAACCTTCAATGGTACGGAGTCAAATGATAG
AAAATTCATTTCTAATAGAGAATGCTAGTAAGAAGTTCGATACTCTAGTGCCAATTACTCCAATGATTGC
ATCATTGTCTAAAGCGAAATTTTGTAACGTGTTAGGACATCCCATGAGTAAGCCGGTCTGGAGCGGTTTA
TCAGATTCTGATATTATTGAACGATTGGGGCGTATATATAGAAATCTTTCTCATTATTATAGCGGATCAT
TAAAAAAAATGAGTTTGTATCGAATAAAGTATATACTTCGACTTTCTTGTGCTAGAACTTTGGCACGTAA
ACACAAAAGTACGGTACGTTCTTTTTTGAAAAGATTAGGAGTGGGATTATTAGAAGAATTTTTTACGGAG
GAAGAGCAGTTTTTTTATTTGACCTTTCCAAAAGCTTCTTCGACTTCAAGGAAGTTATATCAAAGGCGGA
TTTGGNATTTGGATATTTTTTGNGTAAATGATACNGCAAATCATGAATGA
```

Figure 8: Sequence of the *matK* region for bilberry (AF382810); forward primer: blue, binding site of the reverse primer: green.

Table 12 displays the properties of the primer set. The criteria for primer design mentioned in chapter 3.5 were not fulfilled. The reverse primer showed a low tendency for secondary structures, which may lead to poor annealing of the primer. In addition, the melting temperatures given by the distributor *Sigma Aldrich* and by the webserver *Oligo Calc* deviated. Therefore, two different annealing temperatures (58°C and 60°C) were tested. Additionally, in one measurement the extension time was prolonged from 10 s to 30 s, to investigate if more time is required for sufficient synthesis of the complementary DNA strand.

Table 12: Characteristics of the *matK_2* primers.

	Forward primer	Reverse primer
Sequence 5'→3'	TTCGTTTTTCTTCGCAA TCAATCT	TAATCGTACGCTTGAA AGATAGC
Length [nt]	24	24
Secondary structures		
T_m [°C] according to Sigma Aldrich	66.5	61.1
T_m [°C] calculated with Oligo Calc [82]	58.3	59.2

5.1.6 ITS_2

Alignment of the *ITS* sequences for plum and bilberry indicated that the reverse primer for the *ITS* region from Jaakola *et al.*, 2010, should bind to the DNA of both species, whereas the binding site of the forward primer differed in four bases. Thus, I designed a novel forward primer for *ITS* (Figure 9).

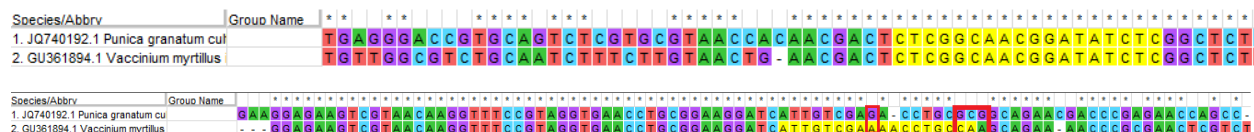


Figure 9: Partial sequence of the *ITS* region for pomegranate (*Punica granatum*) and bilberry (*Vaccinium myrtillus*); constant sites are marked with *, variable sites are marked with different colors. In the first alignment, binding site of the reverse primer is marked yellow, in the second alignment, the forward primer is marked yellow; the red boxes indicate the differences between the two species.

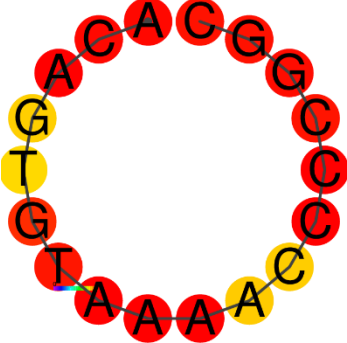
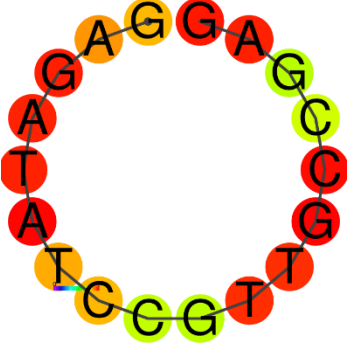
Figure 10 shows the sequence of the *ITS* region for pomegranate with accession number JQ740192.1. The binding positions of forward and reverse primer are marked in blue and green, respectively.

TTGTACACACCGCCCGTCGCTCCTACCGATTGAATGGTCCGGTGAAGTGTTTCGGATCGCGGCGACGTGGG
TGCTTCGTGCGCGACGACGTGCGGAGAAGTCCACTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAAC
AAGGTTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTGAGACCTGCGCGGCAGAACGACCCGAGAACCA
GCCGCTCCAAATGGGCCGGGGTCCCCCGGCCCGGGATGGCGGGGGCGCGCTTCGTGCGCTTTCCCGTC
CCGGTCGTACAGTGTAACACCCGGCGCGGAAGGCGCCAAGGATCAGAAACAGGAGAGGCACGCCGCCG
CCTGAGGGACCGTGCAGTCTCGTGCGTAACCACAACGACTCTCGGCAACGGATATCTCGGCTCTGCATC
GATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCCTTGAT
CGCAAGTTGCGCCCGAAGCCATCCGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCAA
AACCTCCACGCCCTCGTCATCCATCCCTCGGGGGGGATGAGGGGGGGGTGCGTGGCCGCTATGGGCGCGG
AAGTTGGCCTCCCGTGGCACACCGCTGCGGCTGGCCAAAAAGGAGCACGGGAGCGACGCGCTCCGCGGC
GCGCGGTGGCGGCAGTACATGGCCCCTCGGGCATCCGTCCGGAGCCGTCCCTTCCGCGTTGCTCGGTGGC
CTTCCATCCAAATAACGCGACCCAGGTGAGGCGGGGCCACCCGCTGAGTTTAAGCATATCAATAAGCGG
AGGAAAAGAAACT

Figure 10: Sequence of the *ITS* region for pomegranate (JQ740192.1); forward primer: blue, binding site of the reverse primer: green.

Table 13 summarizes the characteristics of the *ITS_2* primers, including melting temperatures and predicted secondary structures. None of the primers showed a tendency to form secondary structures.

Table 13: Characteristics of the *ITS_2* primers.

	Forward primer	Reverse primer
Sequence 5'→3'	ACAGTGTAACCC CCGGC	GAGATATCCGTTG CCGAG
Length [nt]	18	18
Secondary structures		
T_m [°C] according to Sigma Aldrich	62.4	60.8
T_m [°C] calculated with Oligo Calc [82]	56.3	56.3

5.1.7 ITS_3

Figure 11 shows a sequence of the *ITS* region for pomegranates amplified with a newly designed primer set. The binding positions of forward and reverse primer are marked in blue and green, respectively.

```
TTGTACACACCGCCCGTCGCTCCTACCGATTGAATGGTCCGGTGAAGTGTTCCGGATCGCGGCGACGTGGG
TGCTTCGTCGCGGACGACGTGCGGAGAAGTCCACTGAACCTTATCATTAGAGGAAGGAGAAGTCGTAAC
AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCGCGGCAGAACGACCCGAGAACCA
GCCGCTCCAAATGGGCCGGGGTCCCCCGGCCCGCGGGATGGCGGGGGCGCGCTTCGTGCGCTTTCCCGTC
CCGGTCGTACAGTGTAACCCCGCGCGGAAGGCGCCAAGGATCAGAAACAGGAGAGGCACGCCGCCCG
CCTGAGGGACCGTGCACTCTCGTGCGTAACCACAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATC
GATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCCTTGAT
CGCAAGTTGCGCCCGAAGCCATCCGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTTCGCCCCAA
AACCTCCACGCCCTCGTCATCCATCCCTCGGGGGGGATGAGGGGGGGGTGCGTGGCCGCTATGGGCGCGG
AAGTTGGCCTCCCGTGGCACACCGCTGCGGCTGGCCCAAAGGAGCACGGGAGCGACGCGCTCCGCGGC
GCGCGGTGGCGGCAGTACATGGCCCCTCGGGCATCCGTCCGGAGCCGTCCCTTCCGCGTTGCTCGGTGGC
CTTCCATCCAAATAACGCGACCCCAAGTCAGGCGGGGCCACCCGCTGAGTTTAAGCATATCAATAAGCGG
AGGAAAAGAACT
```

Figure 11: Sequence of the *ITS* region for pomegranate (JQ740192.1); forward primer: blue, binding site of the reverse primer: green.

Further details about this primer set are summarized in Table 14. As can be seen, the reverse primer shows a low tendency to form secondary structures. This can result in poor annealing of the primer.

Table 14: Characteristics of the *ITS_3* primers.

	Forward primer	Reverse primer
Sequence 5'→3'	TCGCCCCAAAACC TCCAC	GTCGCTCCCGTGCT CCTTT
Length [nt]	18	19
Secondary structures		
T_m [°C] according to Sigma Aldrich	68.4	68.9
T_m [°C] calculated with Oligo Calc [82]	58.4	61.6

5.2 Differentiation between blueberry, bilberry, (American and European) cranberry and lingonberry

We investigated if PCR-HRM is applicable to differentiate between berry species. Two primer sets (*ITSVm1* and *rp1*) were taken from the literature (Jaakola *et al.*, 2010).

5.2.1 *ITSVm1*

The main purpose of the primer set *ITSVm1* was to differentiate between blueberry and bilberry. Therefore, DNA extracts from blueberries were prepared from fresh leaves (VcL) and two herbarium samples (17H and 18H), DNA extracts from bilberries from fresh leaves (VmyL) and four herbarium samples (9H, 10H, 11H and 12H). The herbarium samples were collected in various years, starting from 1990 until 2008 in different regions in the world. On the other hand, the fresh leaf samples were collected shortly before the DNA extraction took place. Details on the samples are given in Chapter 4.2, Table 3 and 4. DNA was extracted with *QIAamp® DNA Blood Mini Kit* as described in Chapter 4.2.1. From the herbarium samples DNA was eluted with Buffer AE, from fresh leaf samples with RNase free water.

Afterwards, with Nanodrop and Qubit instruments the concentration and purity were determined. Table 15 shows that DNA concentrations determined with the Nanodrop instrument were much higher compared to the concentrations obtained with the Qubit instrument. The main drawback of Nanodrop is its low selectivity, thus the presence of polyphenols and polysaccharides leads to inaccurate results. Results obtained by fluorometric measurements show that DNA extracts from fresh leaves (VmyL and VcL) were higher concentrated than extracts from herbarium samples (9H-12H, 17H and 18H). $A_{260/280}$ values obtained for samples 9H-12H, VmyL and VcL were in the range from 1.1 to 1.4, while blueberry samples (17H and 18H) showed a value of 1.6. However, all samples showed values below the expected range of 1.8-2.0. According to literature, extracting DNA with good quality from herbarium specimen is rather challenging. The amount of DNA in dried herbarium leaves is usually very low and can be influenced by the preservation method, fumigation method and herbarium treatment [80].

Table 15: DNA concentration and purity of the extracts determined by spectrophotometric analysis and DNA concentration determined by fluorometric analysis.

Sample	Nanodrop [ng/mL]	$A_{260/280}$	Qubit [ng/mL]
Bilberry 9H	8000	1.4	496
Bilberry 10H	11000	1.1	205
Bilberry 11H	11400	1.3	201
Bilberry 12H	8800	1.2	252
Blueberry 17H	4500	1.6	65
Blueberry 18H	4300	1.6	48
Bilberry VmyL	8000	1.4	1180
Blueberry VcL	23900	1.3	3020

PCR-HRM was carried out as described in Chapter 4.4.4. DNA extracts were analyzed undiluted in duplicates. For sample VcL, only one of the duplicates led to an increase of the fluorescence signal within 50 cycles. C_t values obtained for fresh leaves were in the range from 12 to 16, those for herbarium samples from 17 to 31.

Regarding the melting behavior, the primer set *ITSVm1* divided the bilberry samples into two groups. The first group consisted of samples 10H, 11H and 12H, the second group of 9H and VmyL. The peak maximum was at 87.0°C and 87.3°C, respectively. Melting

curves obtained for blueberry samples also showed a peak maximum of 87.0°C (Figure 12).

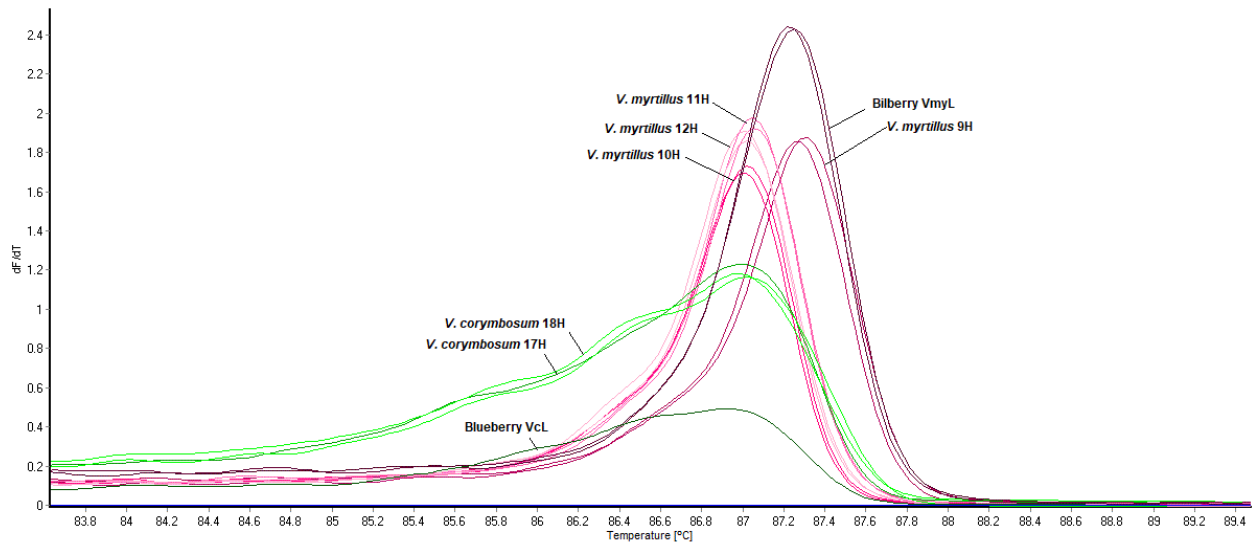


Figure 12: Melting curves of PCR products obtained for blueberry (green) and bilberry (pink) with primer set *ITSVm1*. For sample denomination see Chapter 4.2, Table 3 and 4.

Even though blueberry and bilberry samples resulted in similar melting temperatures, the different shapes of their melting curves help in the distinction between these two berry species. The normalized melting curves can also be used to differentiate between blueberry and bilberry (Figure 13).

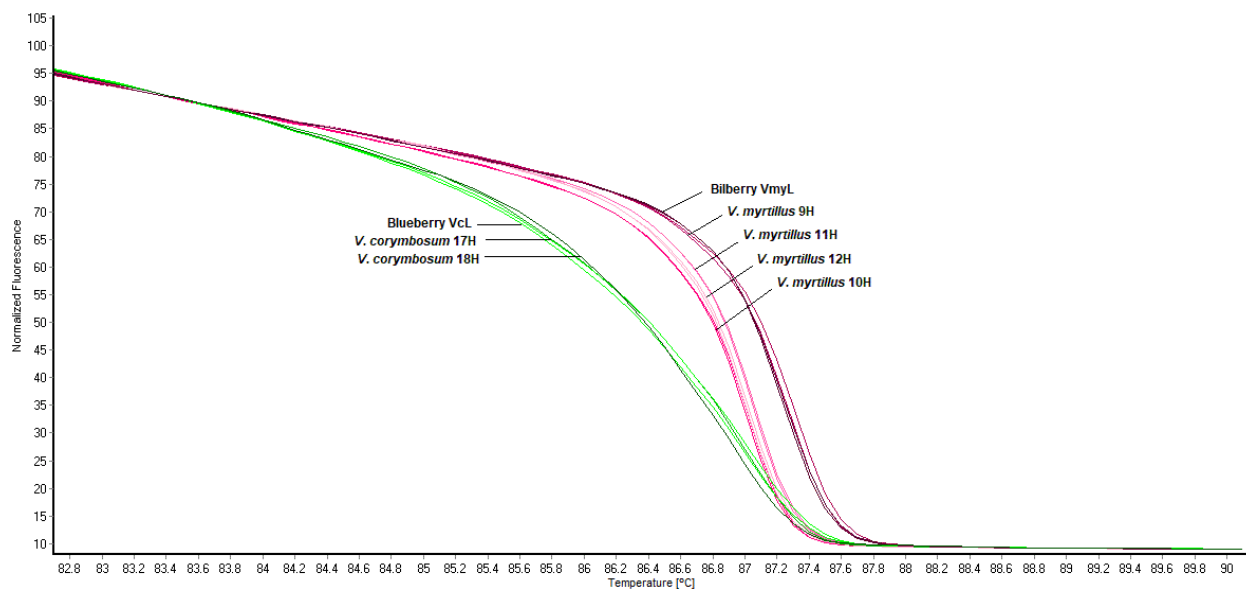


Figure 13: Normalized melting curves of PCR products obtained for blueberry (green) and bilberry (pink) with primer set *ITSVm1*. For sample denomination see Chapter 4.2, Table 3 and 4.

With sample 17H as reference, the difference plot in Figure 14 presents once again that blueberry and bilberry are readily distinguishable with the primer set *ITSVm1*.

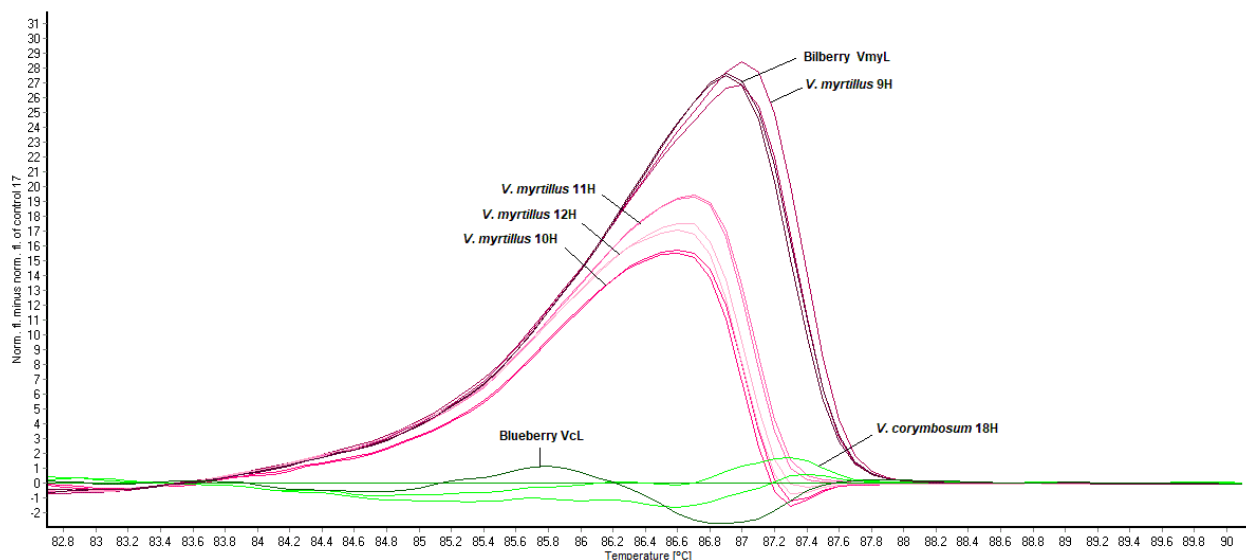


Figure 14: Difference plot of PCR products obtained for blueberry (green) and bilberry (pink) with primer set *ITSVm1*. Reference: *V. corymbosum* 17H. For sample denomination see Chapter 4.2, Table 3 and 4.

Another purpose of the primer set *ITSVm1* was to distinguish blueberries and bilberries from cranberries (American and European) and lingonberries. Therefore, DNA was extracted from fresh leaves (VcL, VmyL, VoL, VmaL and VviL) and fresh fruits (BAP, BM and CfS) using *QIAamp® DNA Blood Mini Kit* as described in Chapter 4.2.1. DNA from fresh fruits was eluted using Buffer AE, from fresh leaves with RNase free water. Details on the samples are given in Chapter 4.2, Table 3. DNA extracts were analyzed in duplicates.

Subsequently, with Nanodrop and Qubit instruments the concentration and purity were determined. Table 16 shows that DNA concentrations determined with the Nanodrop instrument for fresh leaf samples were much higher compared to the concentrations obtained with the Qubit instrument. DNA concentration for fresh fruit samples was determined only with Nanodrop instrument. $A_{260/280}$ values obtained for all samples were in the range from 1.2 to 1.6, which is below the expected range of 1.8-2.0

Table 16: DNA concentration and purity of the extracts determined by spectrophotometric analysis and DNA concentration determined by fluorometric analysis.

Sample	Nanodrop [ng/mL]	$A_{260/280}$	Qubit [ng/mL]
Blueberry BAP	8300	1.6	-
Bilberry BM	8500	1.2	-
American cranberry CfS	1600	1.6	-
American cranberry VmaL	10100	1.4	527
European cranberry VoL	6500	1.6	159
Lingonberry VviL	9800	1.2	374

Before subjecting the DNA extracts (BAP, BM, VcL, VmyL, VmaL, VoL and VviL) to PCR-HRM analysis, they were diluted with RNase free water so that a final concentration of 5000 ng/mL was achieved. DNA extract from sample CfS was subjected undiluted. The amplification was successful for all DNA extracts and the C_t values ranged from 15 to 28 for samples from fresh leaves and from 13 to 20 for fresh fruit samples.

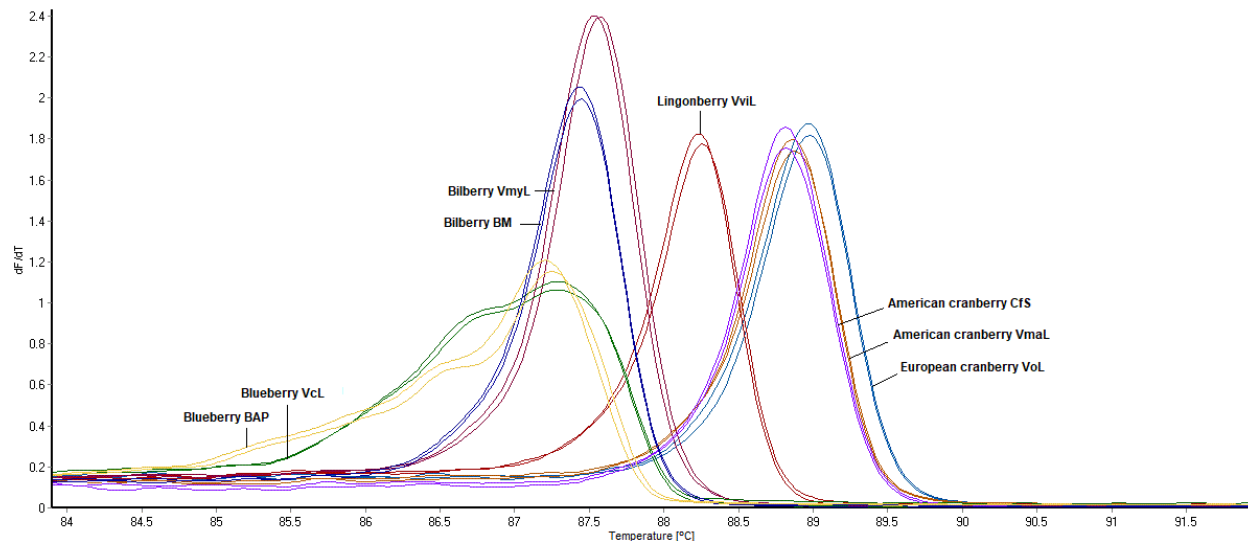


Figure 15: Melting curves of PCR products obtained for blueberry (BAP: yellow, VcL: green), bilberry (BM: dark blue, VmyL: dark pink), lingonberry (VviL: red), American cranberry (CfS: purple, VmaL: orange) and European cranberry (VoL: light blue) with primer set *ITSvm1*. For sample denomination see Chapter 4.2, Table 3.

The blueberry samples (VcL and BAP) resulted in a peak maximum at 87.2°C, while the bilberry samples (VmyL and BM) led to a peak maximum at 87.5°C. The peak maximum for the lingonberry sample (VviL) was at 88.2°C. Melting curves obtained for American cranberry (VmaL and CfS) and European cranberry (VoL) showed a peak maximum at 88.8°C and 88.9°C, respectively.

Both, melting curves and the difference plot show a clear distinction between blueberry, bilberry, cranberry (American and European) and lingonberry. However, this primer set failed in the separation between American and European cranberry.

5.2.2 *rp1*

In order to better investigate the ability of the primer set *rp1* to distinguish between the different berry species, firstly it was applied on the positive controls. As positive controls were used DNA extracts from herbarium samples and fresh leaf samples. Thus, herbarium samples 1H-3H represented the lingonberries, 4H-7H the European cranberries, 9H-12H the bilberries, 13H-15H the American cranberries and samples 17H and 18H the blueberries. Also, for each berry species fresh leaf samples (VviL, VoL, VmyL, VmaL, VcL) were tested. DNA was extracted with *QIAamp® DNA Blood Mini Kit*

as described in Chapter 4.2.1. DNA from herbarium samples was eluted with Buffer AE, from fresh leaf samples with RNase free water. DNA extracts were analyzed undiluted in duplicates. Detailed information about the samples as well as the PCR-HRM settings are summarized in Chapter 4.2 and Chapter 4.4.4, respectively.

Before subjecting the DNA extracts to PCR-HRM analysis, their concentration and purity were determined. Concentrations obtained with the Nanodrop instrument were much higher than those determined with the Qubit instrument. Results obtained with the Qubit instrument indicate that the extracts from herbarium samples and fresh samples for the lingonberries were in the same range. On the contrary, the amount of DNA in the extracts from American cranberry (VmaL), blueberry (VcL) and bilberry (VmyL) was higher compared to the amount extracted from fresh leaves (Table 15 and 16). Also, the determined DNA concentration of extracts from two herbarium samples of the European cranberry (5H and 6H) was as twice as low as the extract concentration from fresh leaf sample (VoL). With the Nanodrop instrument also the purity of the samples was detected and it was in the range from 1.1 to 1.8 (Table 17).

Table 17: DNA concentration and purity of the extracts determined by spectrophotometric analysis and DNA concentration determined by fluorometric analysis.

Sample	Nanodrop [ng/mL]	A _{260/280}	Qubit [ng/mL]
Lingonberry 1H	12700	1.1	410
Lingonberry 2H	11800	1.1	281
Lingonberry 3H	7300	1.0	119
European cranberry 4H	3500	1.8	121
European cranberry 5H	6100	1.4	62
European cranberry 6H	5900	1.5	61
European cranberry 7H	7100	1.6	136
American cranberry 13H	4900	1.4	18
American cranberry 14H	4400	1.6	11
American cranberry 15H	7900	1.3	14

The C_t values obtained for dried lingonberry samples (1H-3H) was in the range from 18 to 20, while for fresh lingonberry leaves (VviL) it was 16. The dried European cranberry samples showed C_t values in the range from 18 to 23, being slightly higher compared to the C_t value obtained for the fresh leaf sample (C_t = 17). C_t values obtained for fresh

American cranberry samples were in the range from 26 to 31, while fresh American cranberry leaves showed a value of 14. C_t values obtained for dried bilberry samples were in the range from 21 to 26, those for dried blueberry samples were in the range from 31 to 32. Fresh blueberry sample showed a C_t value of 24, fresh bilberry sample of 15. Altogether, the fresh samples showed lower C_t values compared to the dried samples. Regarding the melting behavior, the primer set *rp1* divided the five berry species into two groups. Group one included the lingonberries and cranberries (American and European), while group two consisted of blueberries and bilberries. The PCR products of the first group showed peak maxima between 78.2°C and 82.2°C, the second group between 83.6°C and 84.5°C (Figure 16).

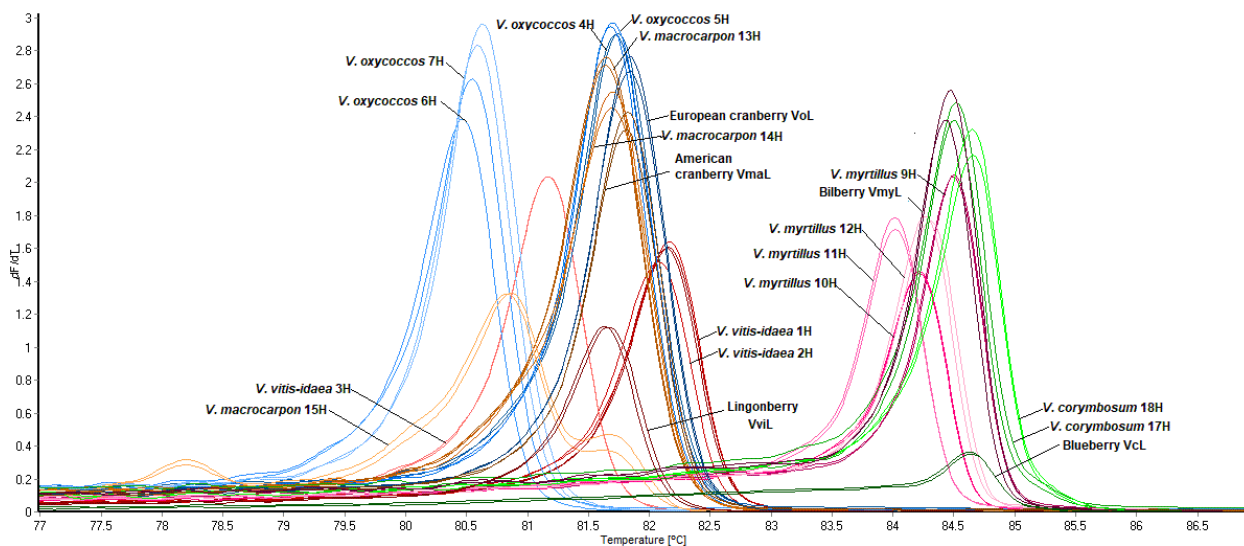


Figure 16: Melting curves of PCR products obtained for blueberry (green), bilberry (pink), lingonberry (red) and cranberries (American and European, orange and blue) with primer set *rp1*. For sample denomination see Chapter 4.2, Table 3 and 4.

A difference plot with bilberry species (9H) as a reference shows that the primer set *rp1* separated the five berry species into two groups. The first group included the lingonberries and the cranberries (American and European), while the second one consisted of bilberry and blueberry (Figure 17). The DNA extract from fresh blueberry leaves (VcL) resulted in melting curves with a very small peak compared to the other samples (Figure 16). In addition, it showed a rather characteristic curve shape in the

difference plot. Most probably, the sample is derived from another cultivar than the other blueberry samples.

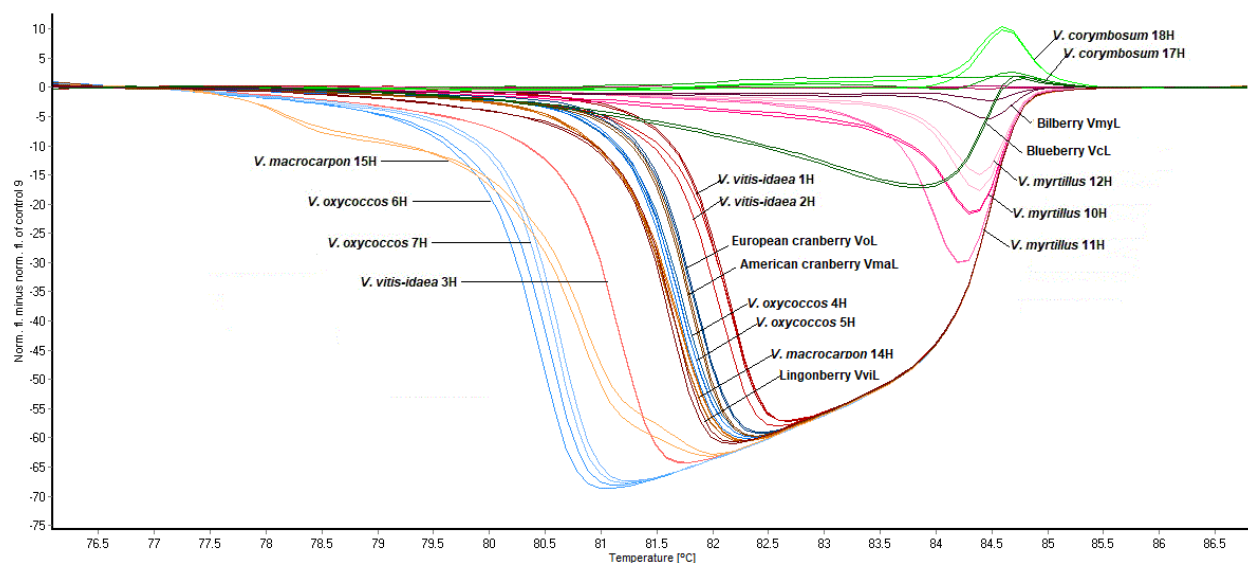


Figure 17: Difference plot of PCR products obtained for blueberry (green), bilberry (pink), lingonberry (red) and cranberries (American and European, orange and blue) with primer set *rp1*. Reference: *V. myrtillus* 9H. For sample denomination see Chapter 4.2, Table 3 and 4.

5.3 Differentiation between different pomegranate cultivars

We investigated if different pomegranate cultivars can be distinguished by PCR-HRM analysis. Two primer sets (*ITSVm1* and *rp1*) taken from the literature and one primer set (*ITS_3*) designed in-house were tested for their applicability.

5.3.1 *ITSVm1*

Although the primer set *ITSVm1* was designed for bilberries, we investigated if it can be used to distinguish between different pomegranate cultivars. Samples 1G-8G (Bernarija, Zumnarija, Ropkavac, Limfanka, Limfanka klon, Hidzas, Karamustafa and Valandovska kisela, respectively) were obtained from the cooperation partners from Macedonia, while sample 9G was purchased from a Viennese supermarket. DNA was extracted with *QIAamp® DNA Blood Mini Kit* and eluted with Buffer AE as described in Chapter 4.2.1. Afterwards, with Nanodrop and Qubit instruments the concentration and purity were determined. Table 18 shows that DNA concentrations determined with the Qubit

instrument were much lower compared to the concentrations obtained with the Nanodrop instrument. The obtained $A_{260/280}$ values with the Nanodrop instrument for all samples were in the range from 0.7 to 1.0. The major disadvantage of the Nanodrop instrument is the lack of selectivity, which can be influenced by the presence of polyphenols and polysaccharides and leads to inaccurate results (Table 18).

Table 18: DNA concentration and purity of the extracts determined by spectrophotometric analysis and DNA concentration determined by fluorometric analysis.

Sample	Nanodrop [ng/mL]	$A_{260/280}$	Qubit [ng/mL]
Pomegranate 1G (Bernarija)	12800	0.7	1860
Pomegranate 2G (Zumnarija)	4600	0.9	1260
Pomegranate 3G (Ropkavac)	17100	0.7	670
Pomegranate 4G (Limfanka)	12900	0.7	1960
Pomegranate 5G (Limfanka klon)	5800	0.9	3020
Pomegranate 6G (Hidzas)	9000	0.8	940
Pomegranate 7G (Karamustafa)	5300	0.8	773
Pomegranate 8G (Valandovska kisela)	18400	0.7	2570
Pomegranate 9G (cultivar unknown)	7100	1.0	2890

Before subjecting the DNA extracts to PCR-HRM analysis they were diluted with RNase free water so that a final concentration of 5000 ng/mL was achieved. The DNA extract from sample 2G (Zumnarija) was used undiluted. PCR-HRM was carried out as described in Chapter 4.4.4. DNA extracts were analyzed in duplicates.

C_t values for pomegranate samples ranged from 24 to 31. Melting curves obtained for eight pomegranate samples (1G (Bernarija), 3G (Ropkavac), 4G (Limfanka), 5G (Limfanka klon), 6G (Hidzas), 7G (Karamustafa), 8G (Valandovska kisela) and 9G (cultivar unknown)) had a peak maximum at 87.5°C, while sample 2G (Zumnarija) resulted in a peak maximum at 87.2°C (Figure 18).

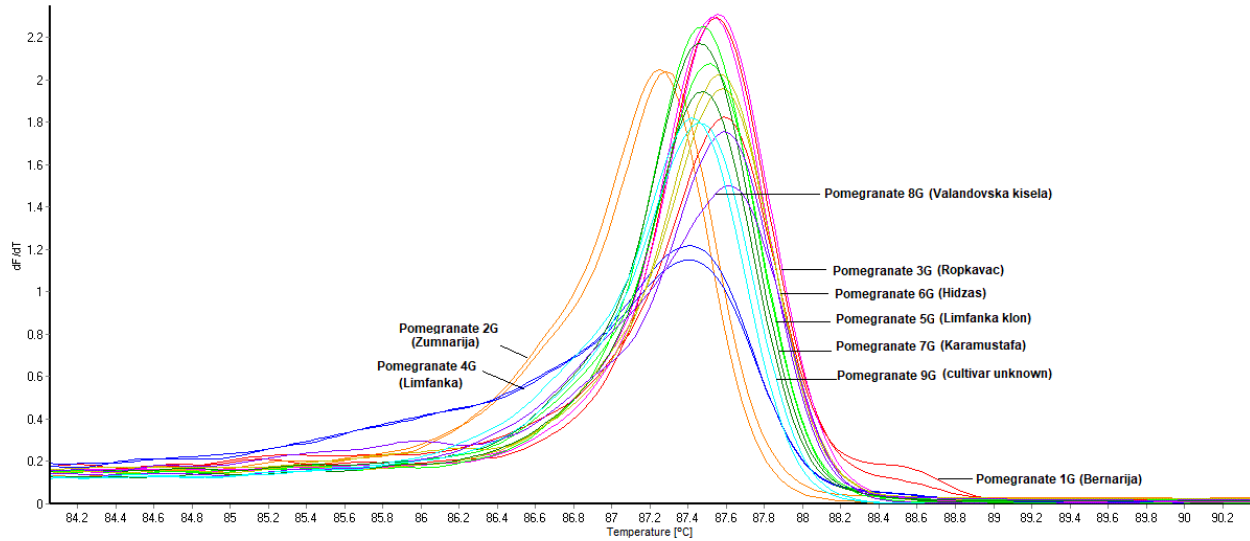


Figure 18: Melting curves of PCR products obtained for pomegranate cultivars (1G (Bernarija): red, 2G (Zumnarija): orange, 3G (Ropkavac): pink, 4G (Limfanka): dark blue, 5G (Limfanka klon): light green, 6G (Hidzas): yellow, 7G (Karamustafa): dark green, 8G (Valandovska kisela): purple and 9G (cultivar unknown): light blue) with primer set *ITSVm1*. For sample denomination see Chapter 4.2, Table 3.

Figure 19 shows the difference plot with pomegranate cultivar 1G (Bernarija) as a reference. It demonstrates that the primer set *ITSVm1* is not suitable for distinguishing between all the pomegranate cultivars tested, due to the small differences in the curve shapes.

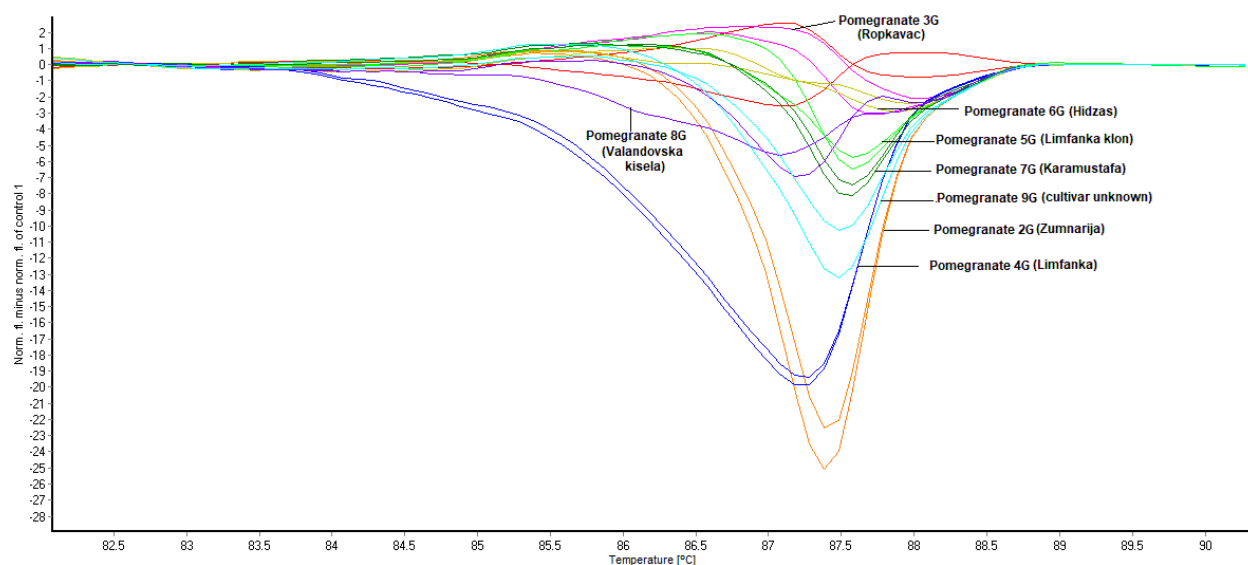


Figure 19: Difference plot of PCR products obtained for pomegranate cultivars (1G (Bernarija): red, 2G (Zumnarija): orange, 3G (Ropkavac): pink, 4G (Limfanka): dark blue, 5G (Limfanka klon): light green, 6G (Hidzas): yellow, 7G (Karamustafa): dark green, 8G (Valandovska kisela): purple and 9G (cultivar unknown): light blue) with primer set *ITSvm1*. Reference: pomegranate 1G (Bernarija). For sample denomination see Chapter 4.2, Table 3.

5.3.2 *rp1*

The primer set *rp1* was tested for its suitability of differentiating between different pomegranate cultivars, although for primer design the bilberry sequence had been used. DNA was extracted from the nine pomegranate cultivars (1G (Bernarija), 2G (Zumnarija), 3G (Ropkavac), 4G (Limfanka), 5G (Limfanka klon), 6G (Hidzas), 7G (Karamustafa), 8G (Valandovska kisela) and 9G (cultivar unknown)) as described in Chapter 5.3.1. Detailed information about the samples as well as the PCR-HRM settings are summarized in Chapter 4.2 and Chapter 4.4.4, respectively. DNA concentration of the extracts was examined with Nanodrop and Qubit instrument. Also, the purity of the DNA extracts was detected with the Nanodrop instrument (see Chapter 5.3.1, Table 18).

DNA extracts were analyzed in duplicates. For sample 9G only one of the duplicates led to an increase of the fluorescence signal within 50 cycles.

C_t values obtained for the nine pomegranate cultivars ranged from 31 to 38. Regarding the melting behavior, the primer set *rp1* divided the pomegranate cultivars into three groups. The first group consisted of samples 2G (Zumnarija) and 8G (Valandovska kisela) and resulted in a peak maximum at 82.0°C. The second group included samples 4G

(Limfanka), 7G (Karamustafa) and 9G (cultivar unknown) and led to a peak maximum at 85.0°C. Melting curves obtained for samples 1G (Bernarija), 3G (Ropkavac), 5G (Limfanka klon) and 6G (Hidzas) showed two peak maxima, at 82.0°C and 84.9°C (Figure 20).

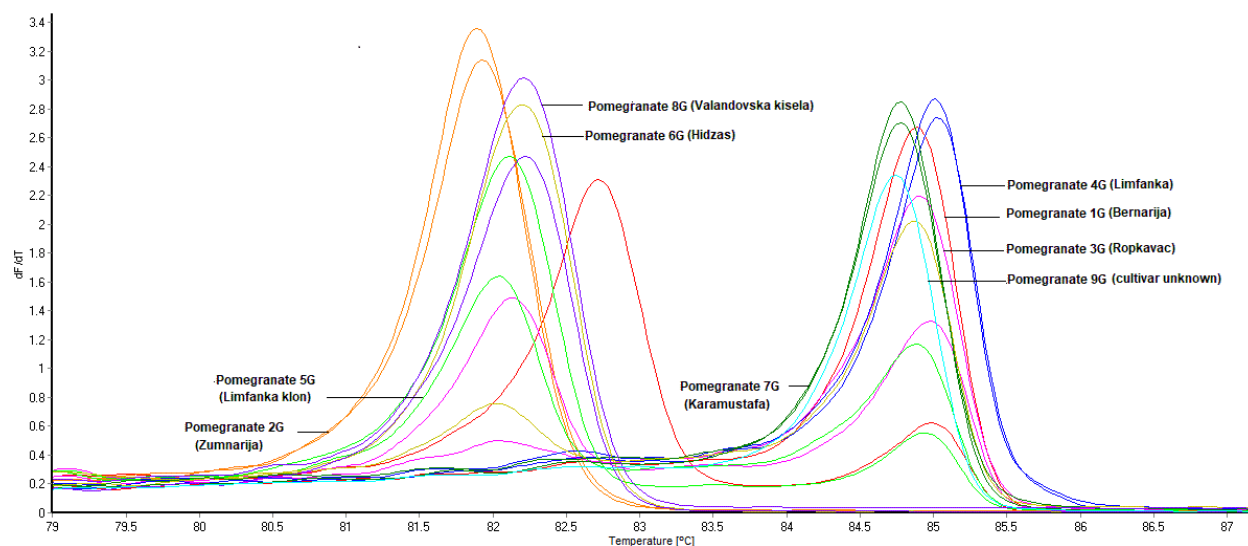


Figure 20: Melting curves of PCR products obtained for pomegranate cultivars (1G (Bernarija): red, 2G (Zumnarija): orange, 3G (Ropkavac): pink, 4G (Limfanka): dark blue, 5G (Limfanka klon): light green, 6G (Hidzas): yellow, 7G (Karamustafa): dark green, 8G (Valandovska kisela): purple and 9G (cultivar unknown): light blue) with primer set *rp1*. For sample denomination see Chapter 4.2, Table 3.

In the difference plot in Figure 21, sample 2G (Zumnarija) was used as a reference. Melting curves obtained by analyzing samples 8G (Valandovska kisela), 1G (Bernarija) and 5G (Limfanka klon) in duplicates showed quite different shapes.

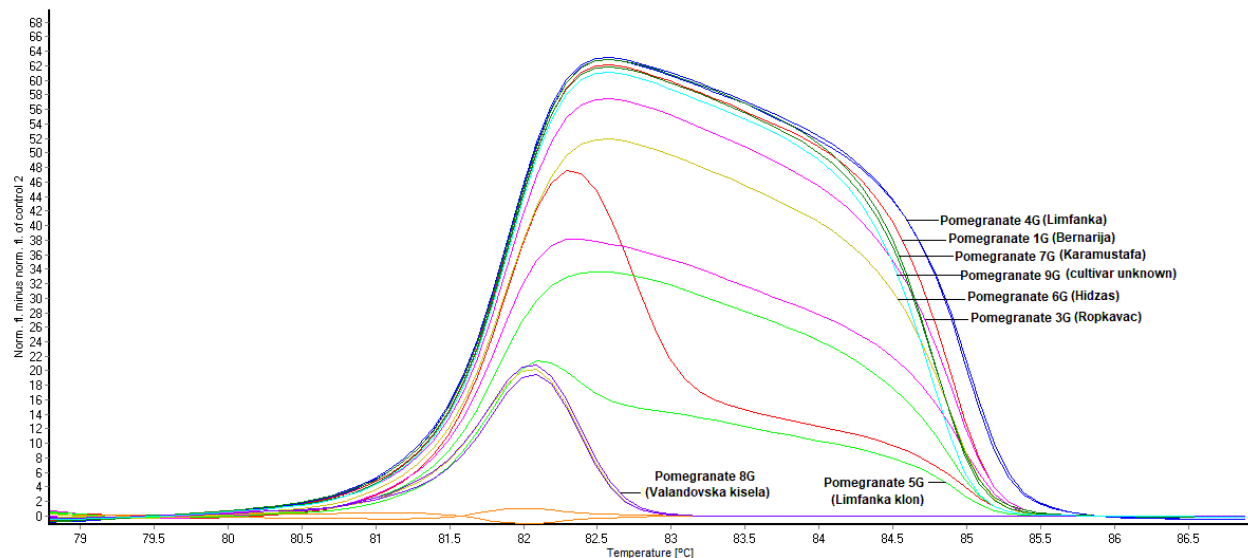


Figure 21: Difference plot of PCR products obtained for pomegranate cultivars (1G (Bernarija): red, 2G (Zumnarija): orange, 3G (Ropkavac): pink, 4G (Limfanka): dark blue, 5G (Limfanka klon): light green, 6G (Hidzas): yellow, 7G (Karamustafa): dark green, 8G (Valandovska kisela): purple and 9G (cultivar unknown): light blue) with primer set *rp1*. Reference: pomegranate 2G (Zumnarija). For sample denomination see Chapter 4.2, Table 3.

Figure 22 shows the results obtained after loading the PCR-HRM products amplified with the primer set *ITSVm1* or *rp1* onto an agarose gel. As described above, melting curves of PCR products obtained with primer set *ITSVm1* had only one peak maximum. Agarose gel electrophoresis confirmed that only the specific amplicon was formed during PCR amplification. However, for PCR products obtained with primer set *rp1*, more than one band was seen on the agarose gel, indicating that unspecific amplicons were formed in addition to the specific PCR product.



Figure 22: Results obtained by subjecting PCR products from nine pomegranate cultivars (1: 1G (Bernarija), 2: 2G (Zumnarija), 3: 3G (Ropkavac), 4: 4G (Limfanka), 5: 5G (Limfanka klon), 6: 6G (Hidzas), 7: 7G (Karamustafa), 8: 8G (Valandovska kisela) and 9: 9G (cultivar unknown)) to agarose gel electrophoresis; M represents the marker, green and blue numbers refer to PCR products obtained with primer sets *ITSvm1* and *rp1*, respectively.

5.3.3 *ITS_3*

The primer set *ITS_3* was designed to be used for the differentiation of pomegranate cultivars. Before the method was applied, the annealing temperature and the $MgCl_2$ were optimized. Optimization was carried out with DNA extracts from two pomegranate cultivars (1G Bernarija and 2G Zumnarija). In order to optimize $MgCl_2$ concentration, either 0 μL or 1 μL of a 40 mM $MgCl_2$ solution were added to the reaction mixture. The highest amplification efficiency was obtained without further adding $MgCl_2$. For optimization of the annealing temperature, DNA extracts from the same two cultivars were used. The annealing temperature was either 66°C or 68°C. The annealing temperature did not have a strong influence on the amplification. In further experiments, an annealing temperature of 68°C was applied.

C_t values obtained for the pomegranate samples were in the range from 22 to 27. Figure 23 shows that the melting curves of PCR products obtained for pomegranate samples (1G (Bernarija), 3G (Ropkavac), 4G (Limfanka), 5G (Limfanka klon), 6G (Hidzas), 7G (Karamustafa), 8G (Valandovska kisela)) and 9G (cultivar unknown)) showed a peak maximum at 88.8°C. Only the peak maximum obtained for sample 2G (Zumnarija) was slightly different (88.6°C).

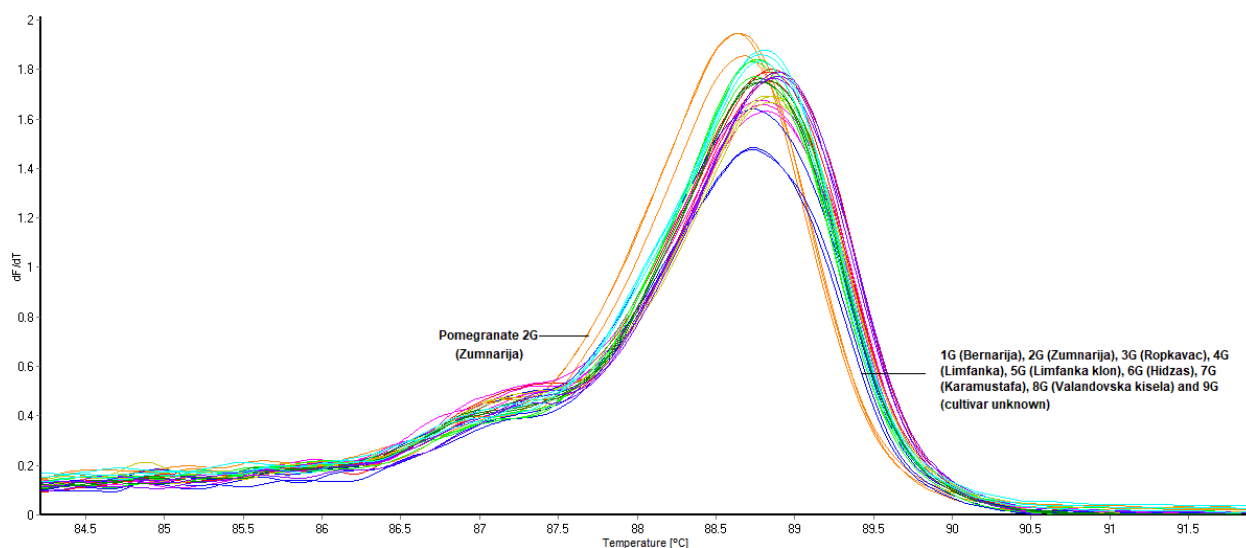


Figure 23: Melting curves of PCR products obtained for pomegranate cultivars (1G (Bernarija): red, 2G (Zumnarija): orange, 3G (Ropkavac): pink, 4G (Limfanka): dark blue, 5G (Limfanka klon): light green, 6G (Hidzas): yellow, 7G (Karamustafa): dark green, 8G (Valandovska kisela): purple and 9G (cultivar unknown): light blue) with primer set *ITS_3*. For sample denomination see Chapter 4.2, Table 3.

The difference plot in Figure 24 with pomegranate sample 1G (Bernarija) as a reference indicates some differences between the pomegranate cultivars. However, the curves obtained for pomegranate samples 5G (Limfanka klon) and 9G (cultivar unknown) showed similarity. In addition, cultivars Hidzas (6G) and Bernarija (1G) resulted in very similar melting curves.

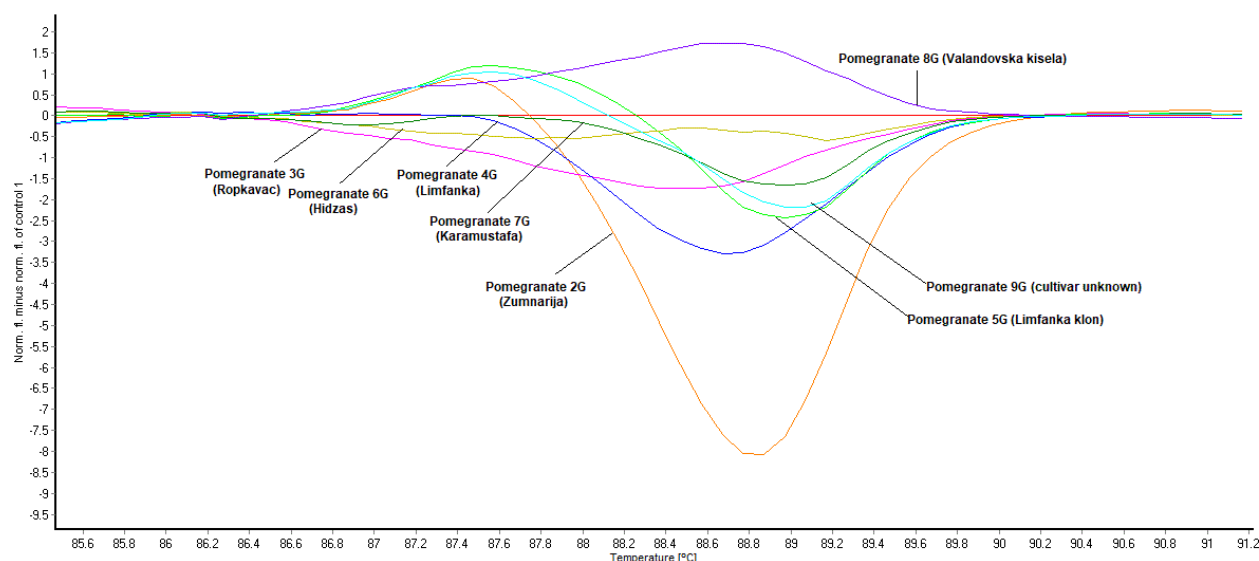


Figure 24: Difference plot of PCR products obtained for pomegranate cultivars (1G (Bernarija): red, 2G (Zumnarija): orange, 3G (Ropkavac): pink, 4G (Limfanka): dark blue, 5G (Limfanka klon): light green, 6G (Hidzas): yellow, 7G (Karamustafa): dark green, 8G (Valandovska kisela): purple and 9G (cultivar unknown): light blue) with primer set *ITS_3*. Reference: pomegranate 1G (Bernarija); replicate view. For sample denomination see Chapter 4.2, Table 3.

In order to investigate in more detail, if primer set *ITS_3* can be used to differentiate pomegranate cultivars, more representatives from one and the same cultivar had to be analyzed. However, since the harvest season of pomegranates has ended in September, additional samples were only available for the cultivars 1G (Bernarija), 4G (Limfanka) and 7G (Karamustafa). These samples were named “B” and “C”.

Details on the samples are given in Chapter 4.2, Table 3. DNA was extracted with *QIAamp® DNA Blood Mini Kit* and eluted with Buffer AE as described in Chapter 4.2.1. DNA concentration and purity of the DNA extracts are given in Table 18 and 19.

Table 19: DNA concentration and purity of the extracts determined by spectrophotometric analysis and DNA concentration determined by fluorometric analysis.

Sample	Nanodrop [ng/mL]	A _{260/280}	Qubit [ng/mL]
Pomegranate 1G B (Bernarija)	10500	0.8	614
Pomegranate 1G C (Bernarija)	22500	0.8	6000
Pomegranate 4G B (Limfanka)	4500	0.8	796
Pomegranate 4G C (Limfanka)	4300	1.0	1330
Pomegranate 7G B (Karamustafa)	8700	0.8	1230
Pomegranate 7G C (Karamustafa)	5800	0.9	1060

Before subjecting the DNA extracts to PCR-HRM analysis they were diluted with RNase free water so that a final concentration of 5000 ng/mL was achieved. DNA extracts from samples 4G B (Limfanka) and 4G C (Limfanka) were used undiluted. PCR-HRM was carried out as described in Chapter 4.4.4. DNA extracts were analyzed in duplicates. The nine pomegranate samples resulted in C_t values in the range from 22 to 24. The resulting melting curves showed a peak maximum at approx. 88.8°C (Figure 25).

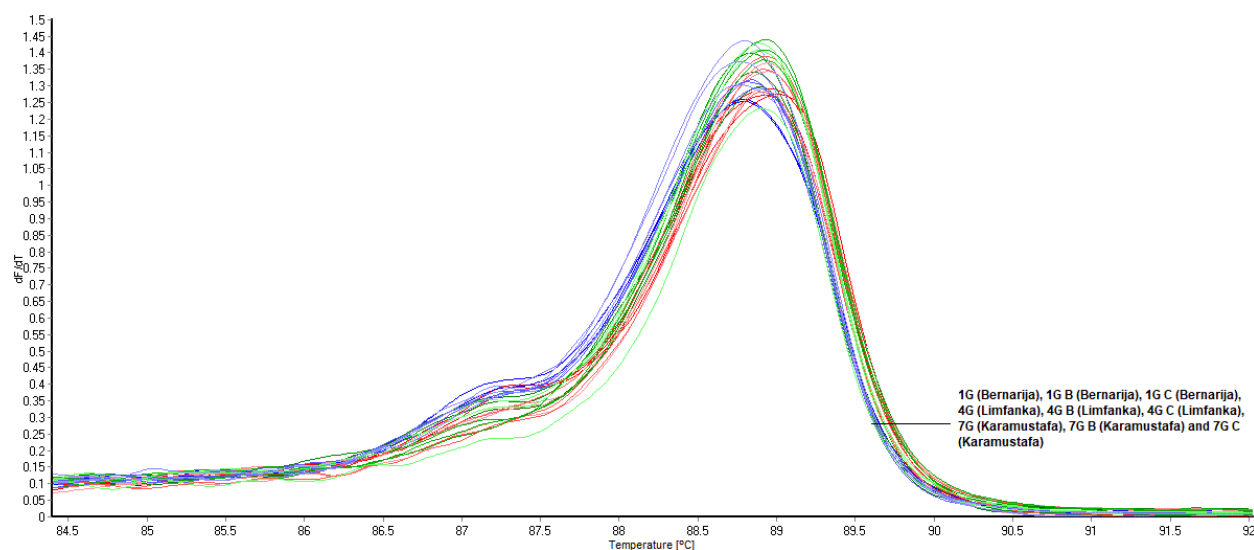


Figure 25: Melting curves of PCR products obtained for pomegranate cultivars (1G (Bernarija): dark red, 1G B (Bernarija): light red, 1G C (Bernarija): very light red, 4G (Limfanka): dark blue, 4G B (Limfanka): light blue, 4G C (Limfanka): very light blue, 7G (Karamustafa): dark green, 7G B (Karamustafa): light green and 7G C (Karamustafa): very light green) with primer set *ITS_3*. For sample denomination see Chapter 4.2, Table 3.

With sample 1G (Bernarija) as a reference, the difference plot shows that the three samples from pomegranate cultivar 4G (Limfanka) can be grouped together (Figure 26). Also, samples 7G (Karamustafa) and 7G C (Karamustafa) resulted in similar curves. Surprisingly, the melting curve obtained for sample 7G B (Karamustafa) was similar to the curves obtained for samples 1G (Bernarija) and 1G B (Bernarija). We assume that the sample was either falsely declared or mixed with samples from cultivar 1G (Bernarija). Altogether, more samples from each cultivar need to be tested to obtain more accurate results.

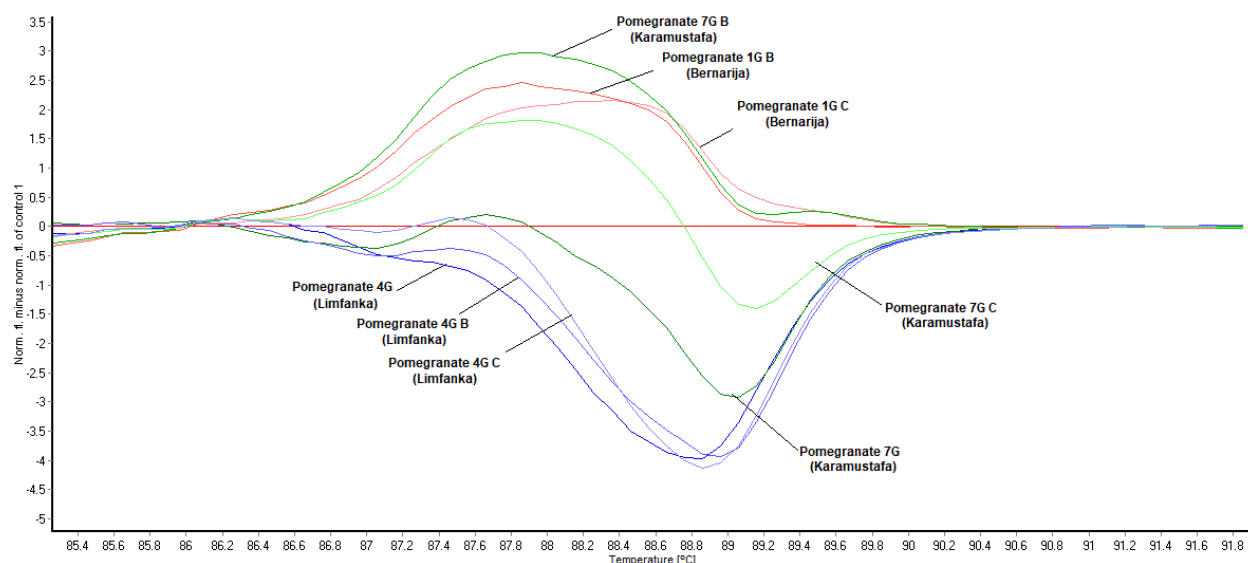


Figure 26: Difference plot of PCR products obtained for pomegranate cultivars (1G (Bernarija): dark red, 1G B (Bernarija): light red, 1G C (Bernarija): very light red, 4G (Limfanka): dark blue, 4G B (Limfanka): light blue, 4G C (Limfanka): very light blue, 7G (Karamustafa): dark green, 7G B (Karamustafa): light green and 7G C (Karamustafa): very light green) with primer set *ITS_3*. Reference: pomegranate 1G (Bernarija); replicate view. For sample denomination see Chapter 4.2, Table 3.

5.4 Detection of adulteration

In order to detect the admixture of undeclared fruits in highly processed products such as jams, juices, beverages, powders and caplets and confirm their authenticity, various primer sets were used. Three primer sets (*ITSVm1*, *rp1* and *trnLVm1*) were taken from the literature and three primer sets were designed in-house (*matK_1*, *matK_2* and *ITS_2*).

5.4.1 *ITSVm1*

The primer set *ITSVm1* was used to confirm product authenticity and detect possible adulteration in caplets. Therefore, DNA was extracted with *QIAamp® DNA Blood Mini Kit* and eluted with RNase free water from four caplet samples declared to contain cranberry. Cranberry extracts from fresh leaves (VoL and VmaL) were used as positive controls. Details on the samples are given in Chapter 4.2, Table 2 and 3.

DNA concentrations determined with the Nanodrop instrument were highest for sample CBM, which was declared to contain cranberry juice powder (Table 16 and 20). On the other hand, the lowest DNA concentration was detected in the DNA extract from sample CVI, declared to contain 50% cranberry juice powder. DNA extracts from samples CVI and CWA were only analyzed with the Nanodrop instrument. The concentration of DNA

extracts from samples CBM and CFN was below the limit of detection (LOD) of the instrument. $A_{260/280}$ values, indicating the purity of the extracts, ranged from 1.4 to 1.6, with the exception of the extract from sample CFN ($A_{260/280}$: 0.4, Table 20). Caplets contain different amounts of excipients, disintegrants, sweeteners, pigments and polymer coatiers which can lead to impure DNA extracts and may lower amplification efficiency.

Table 20: DNA concentration and purity of the extracts determined by spectrophotometric analysis and DNA concentration determined by fluorometric analysis.

Sample	Nanodrop [ng/mL]	$A_{260/280}$	Qubit [ng/mL]
Cranberry caplet CBM	34000	1.5	< LOD
Cranberry caplet CFN	7400	0.4	< LOD
Cranberry caplet CVI	1400	1.5	-
Cranberry caplet CWA	18300	1.6	-

However, DNA extracts from samples CBM, CFN, CWA, VoL and VmaL were diluted with RNase free water so that a final concentration of 5000 ng/mL was achieved. DNA extract from sample CVI was used undiluted. Then, the DNA extracts were subjected to PCR-HRM analysis. DNA extracts were analyzed in duplicates. For samples CBM and CVI, only one of the duplicates led to an increase of the fluorescence signal within 50 cycles. For sample CWA, no increase of the fluorescence signal within 50 cycles was observed. C_t values for samples VoL, VmaL, CFN, CVI and CBM were 25, 28, 33, 40 and 41, respectively.

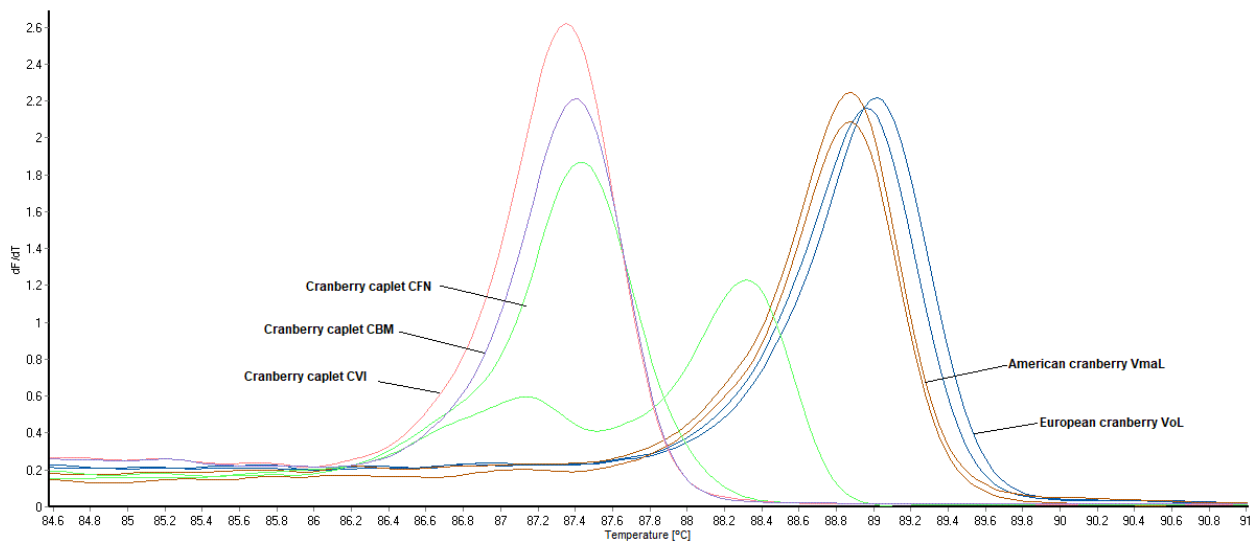


Figure 27: Melting curves of PCR products obtained for American cranberry (brown), European cranberry (blue) and cranberry caplets (CFN: green, CBM: purple and CVI: orange) with primer set *ITSVm1*. For sample denomination see Chapter 4.2, Table 2 and 3.

Melting curves for samples CVI and CBM resulted in a peak maximum at 87.4°C. One duplicate of sample CFN led to a peak maximum at 87.4°C, whereas the melting curve obtained for the other duplicate showed two peak maxima (87.4°C and 88.3°C). The extract from American cranberry led to a peak maximum at 88.8°C, that from European cranberry to a peak maximum at 88.9°C (Figure 27). The difference in the melting temperatures obtained for positive controls and test samples may be due to the strong influence from other compounds contained in caplets or due to the use of cranberry hybrids.

5.4.2 *rp1*

We also investigated if the PCR-HRM method based on primer set *rp1* allows the detection of fruits that are typically used for adulteration. Thus, DNA was extracted from fresh chokeberry (AmF), blackberry (RfF), plum (PA), American cranberry (CfS), blueberry (BAP), bilberry (BM) and from dried chokeberry (SSS). As positive controls were used DNA extracts from fresh blueberry (VcF) and bilberry (VmyF) fruits. DNA was extracted with *QIAamp® DNA Blood Mini Kit* and eluted with Buffer AE as described in Chapter 4.2.1. Additionally, two self-made DNA extract mixtures were tested. Therefore, 1 µL bilberry or blueberry DNA extract and 1 µL chokeberry DNA extract were added to

the master mix prior PCR-HRM analysis (VmyF+AmF and VcF+AmF, respectively). Details on the samples are given in Chapter 4.2, Table 2 and 3.

Next, DNA concentration and purity of the extracts were determined with the Nanodrop instrument. The DNA concentration was found to be in the range from 3700-17000 ng/mL, the $A_{260/280}$ values from 1.2 to 1.6 (Table 16 and 21).

Table 21: DNA concentration and purity of the extracts determined by spectrophotometric analysis.

Sample	Nanodrop [ng/mL]	$A_{260/280}$
Chokeberry AmF	5200	1.4
Blackberry RfF	14000	1.4
Plum PA	17000	1.3
Dried chokeberry SSS	5400	1.2
Blueberry VcF	7300	1.3
Bilberry VmyF	5900	1.2

Before subjecting the DNA extracts (AmF, RfF, PA, BAP, BM, SSS, VcF and VmyF) to PCR-HRM analysis, they were diluted with RNase free water so that a final concentration of 5000 ng/mL was achieved. DNA extract from sample CfS was subjected undiluted. DNA extracts were analyzed in duplicates. C_t values obtained for American cranberry, blueberry and bilberry samples ranged from 21 to 24, those for chokeberries, blackberry and plum ranged from 33 to 38. The mixtures VmyF+AmF and VcF+AmF resulted in a C_t value of 22 and 25, respectively.

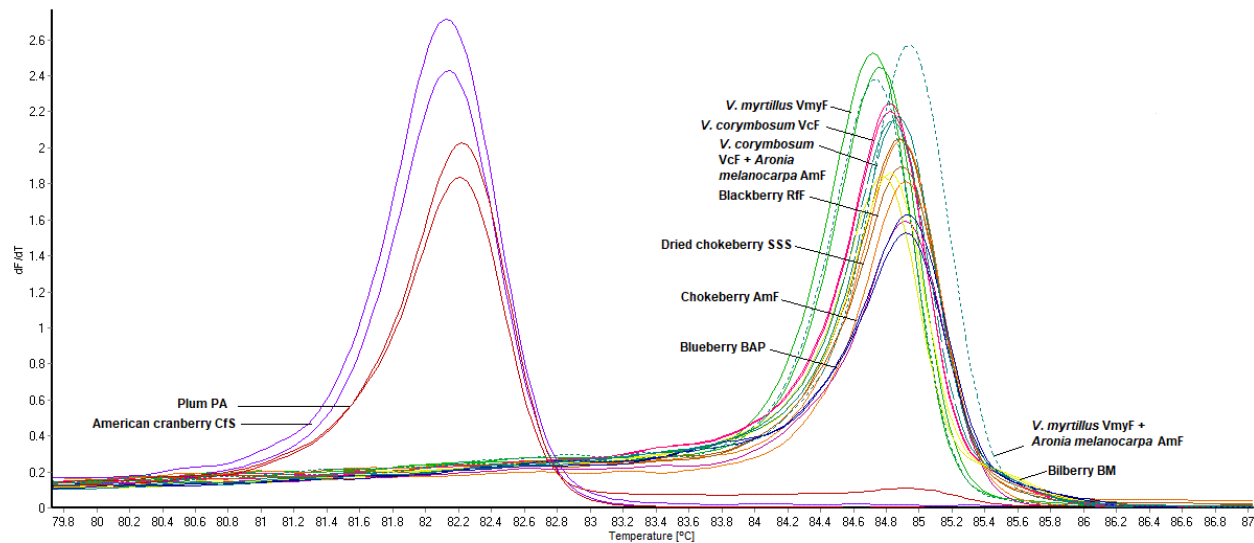


Figure 28: Melting curves of PCR products obtained for chokeberry (light orange), blackberry (dark pink), plum (red), American cranberry (purple), blueberry (VcF: light pink and BAP: dark blue), bilberry (VmyF: green and BM: yellow), dried chokeberry (dark orange), bilberry and chokeberry mixture (VmyF+AmF: dashed light blue) and blueberry and chokeberry mixture (VcF+AmF: dotted light blue) with primer set *rp1*. For sample denomination see Chapter 4.2, Table 2 and 3.

Both, melting curves and the difference plot show that only samples for American cranberry (CfS) and plum (PA) can be distinguished from the other tested fruit samples (Figure 28 and 29). These two species resulted in a peak maximum at 82.2°C. The two positive controls as well as the rest of the samples showed a peak maximum at 84.8°C. Melting curves obtained for mixtures VmyF+AmF and VcF+AmF had a peak maximum at approx. 84.8°C (Figure 28).

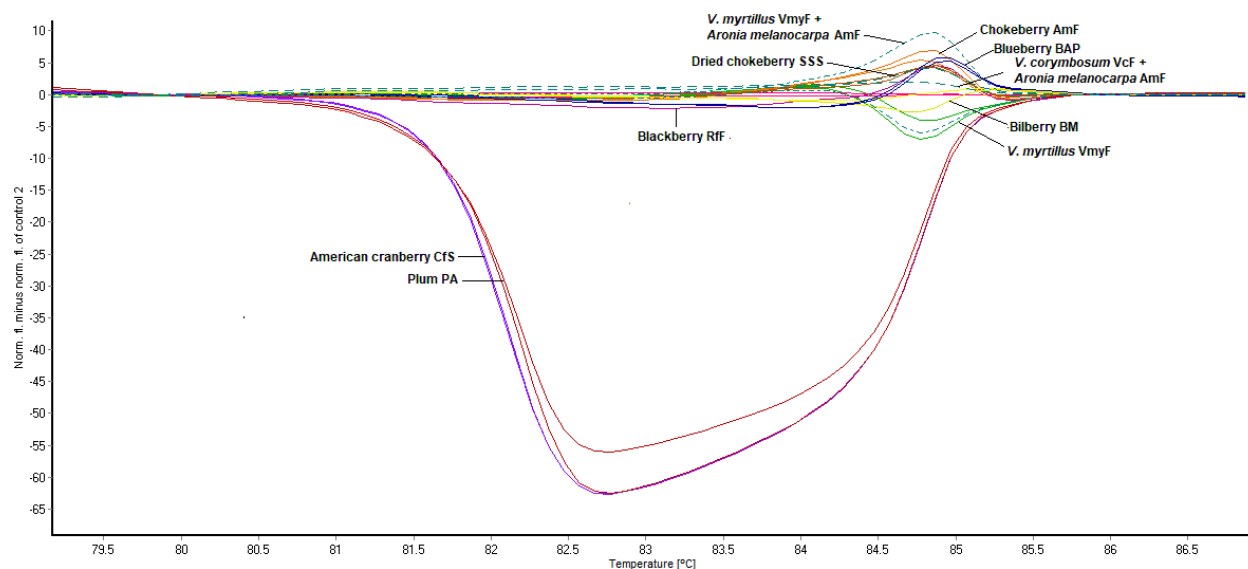


Figure 29: Difference plot of PCR products obtained for chokeberry (light orange), blackberry (dark pink), plum (red), American cranberry (purple), blueberry (VcF: light pink and BAP: dark blue), bilberry (VmyF: green and BM: yellow), dried chokeberry (dark orange), bilberry and chokeberry mixture (VmyF+AmF: dashed light blue) and blueberry and chokeberry mixture (VcF+AmF: dotted light blue) with primer set *rp1*. Reference: blueberry VcF. For sample denomination see Chapter 4.2, Table 2 and 3.

In a further experiment, various lingonberry and cranberry products were tested to confirm product authenticity. Hence, DNA was extracted from cranberry powder (CBI), dried cranberries (CSBE) and two cranberry juices (CPV and CSDM) using the *QIAamp® DNA Blood Mini Kit*. To find out if the DNA extraction kit has an influence on the results, DNA from lingonberry powder (LBI) and cranberry powder (CBI) was extracted using the *QIAamp® DNA Blood Mini Kit* (LBI M and CBI M) as well as the *DNeasy® Plant Mini Kit* (LBI P and CBI P). In both cases, Buffer AE was used for elution. Fresh American cranberries (CfS) and lingonberry jam (LS) were used as positive controls. Details on the samples are given in Chapter 4.1, Table 2. DNA was extracted as described in Chapter 4.2.1 and 4.2.2. A further aim was to extract DNA from all samples using the CTAB Protocol (Chapter 4.2.3). The DNA concentration of the extracts was below the limit of detection of the Nanodrop instrument. Thus, the extracts were not subjected to PCR-HRM analysis.

DNA concentration and $A_{260/280}$ values indicating the purity are summarized in Table 22. The concentration was in the range from 2400 ng/mL to 16300 ng/mL, the $A_{260/280}$ values were in the range from 1.2 to 1.9 (Table 16 and 22).

For samples CfS and CSBE, only one of the duplicates led to an increase of the fluorescence signal within 50 cycles.

Table 22: DNA concentration and purity of the extracts determined by spectrophotometric analysis.

Sample	Nanodrop [ng/mL]	A_{260/280}
Cranberry powder CBI M	12500	1.6
Cranberry powder CBI P	7600	1.4
Lingonberry powder LBI M	16300	1.8
Lingonberry powder LBI P	4800	1.5
Dried cranberry CSBE	6700	1.2
Cranberry juice CPV	5300	1.4
Cranberry juice CSDM	2400	1.9
Lingonberry jam LS	3600	1.3

Before subjecting the DNA extracts (CBI M, CBI P, LBI M, CSBE AND CPV) to PCR-HRM analysis they were diluted with RNase free water so that a final concentration of 5000 ng/mL was achieved. DNA extracts from samples LBI P, CSDM, CfS and LS were used undiluted. DNA extracts were analyzed in duplicates. C_t values obtained for cranberry samples ranged from 24 to 38, those for lingonberry samples were 27 and 31. The positive control for the cranberry samples (CfS) showed a C_t value of 21, whereas that for the lingonberry samples (LS) showed a C_t value of 35.

The melting curves of the lingonberry samples had a peak maximum at 82.2°C, those for cranberries (American and European) showed a peak maximum at 82.7°C (Figure 30).

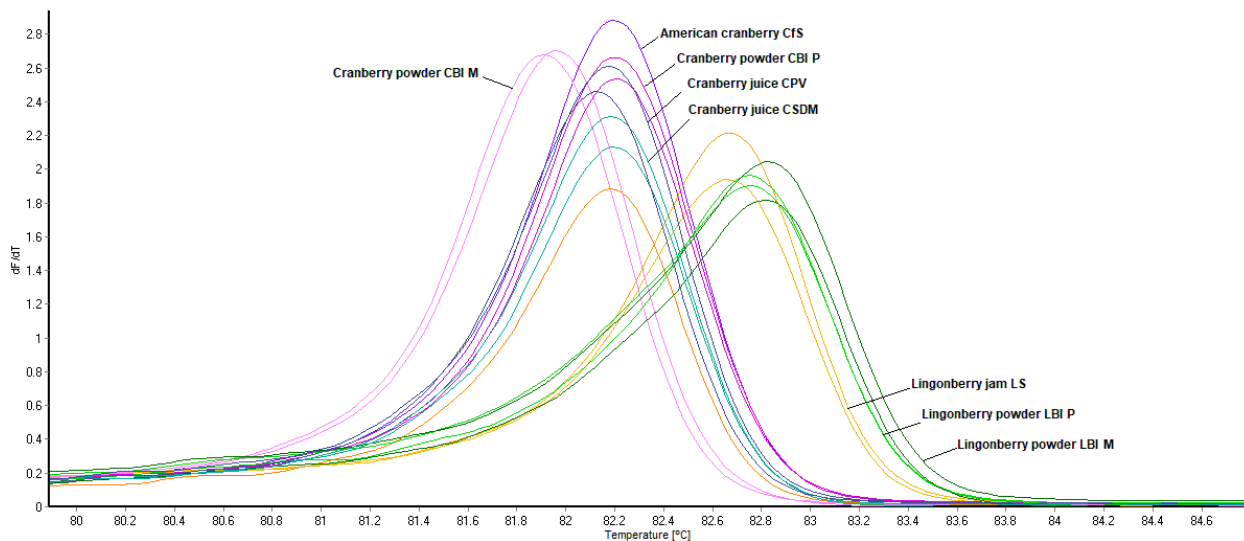


Figure 30: Melting curves of PCR products obtained for lingonberry (LBI M: dark green, LBI P: light green and LS: yellow) and cranberry (CBI M: light pink, CBI P: dark pink, CSDM: light blue, CPV: dark blue and CfS: purple) products with primer set *rp1*. For sample denomination see Chapter 4.2, Table 2.

Moreover, as can be seen in Figure 30, the kit type did not influence the curve shape obtained for lingonberry powder samples (LBI M and LBI P) or cranberry powder samples (CBI M and CBI P). In addition, both lingonberry samples resulted in very similar peak maximum, LBI M at approx. 82.8°C and LBI P at approx. 82.7°C. On the other hand, cranberry samples CBI M and CBI P resulted in peak maxima at 81.9°C and 82.2°C, respectively.

The primer set *rp1* was applied on more products declared to contain cranberry, such as juices, beverages, jelly and dried fruits. Thus, DNA was extracted from cranberry juices (CP and CSDM), cranberry jelly (JCOS), dried cranberries (CNB, CNS, CCML, COSD, CSB and CSBE) and cranberry beverage (CCB). As positive controls were used DNA extracts from American and European cranberry leaves (VmaL and VoL). DNA was extracted with *QIAamp® DNA Blood Mini Kit* as described in Chapter 4.2.1. Details on the samples are given in Chapter 4.2, Table 2 and 4. For samples CSB, CSBE and COSD, DNA was eluted either with Buffer AE or with RNase free water to find out if the elution solution influences the purity of the DNA extract. These samples were named “A” and

“R”. DNA from the other samples (CNB, CNS, CCML and CCB) was eluted with Buffer AE, that from the positive controls (VmaL and VoL) with RNase free water.

Afterwards, with the Nanodrop instrument the concentration and purity were determined. For samples CP, JCOS, CCB, VoL and VmaL the concentration was additionally determined with the Qubit instrument (Table 23). The DNA concentration for these samples obtained with the Nanodrop instrument were much higher compared to the concentrations obtained with the Qubit instrument. Concentrations determined for the extracts from samples CSB A and CSBE A were higher than those determined for the extracts from samples CSB R and CSBE R, which may indicate higher amount of DNA but also high amount of other substances like sugars and polyphenols. The purity of the extracts from samples CSB and CSBE was not affected by the type of elution solution. For all DNA extracts, $A_{260/280}$ values ranged from 1.2 to 1.9 (Table 16, 22 and 23).

Table 23: DNA concentration and purity of the extracts determined by spectrophotometric analysis and DNA concentration determined by fluorometric analysis.

Sample	Nanodrop [ng/mL]	$A_{260/280}$	Qubit [ng/mL]
Cranberry juice CP	2800	1.8	15
Cranberry jelly JCOS	4200	1.3	4
Cranberry dried CNB	9900	1.4	-
Cranberry dried CNS	8800	1.3	-
Cranberry dried CCML	18700	1.3	-
Cranberry dried COSD A	20100	1.3	-
Cranberry dried COSD R	9600	1.3	-
Cranberry dried CSB A	28200	1.3	-
Cranberry dried CSB R	28500	1.3	-
Cranberry dried CSBE A	17900	1.2	-
Cranberry beverage CCB	2900	1.8	29

Extracts with a DNA concentration > 5000 ng/mL were diluted to a concentration of 5000 ng/mL, extracts with lower concentrations were used undiluted.

PCR-HRM was carried out as described in Chapter 4.4.4. DNA extracts were analyzed in duplicates with the exception of VoL and VmaL (which had already been analyzed repeatedly in previous experiments). Within 50 cycles, only one of the duplicates carried out for sample CP led to an increase of the fluorescence signal, while for sample CCB no increase of the fluorescence signal was observed.

C_t values obtained for the positive controls VmaL and VoL were 16 and 19, respectively. The two juice samples (CP and CSDM) and the jelly sample (JCOS) resulted in C_t values in the range from 34 to 37. On the other hand, dried cranberry samples led to C_t values in the range from 24 to 33.

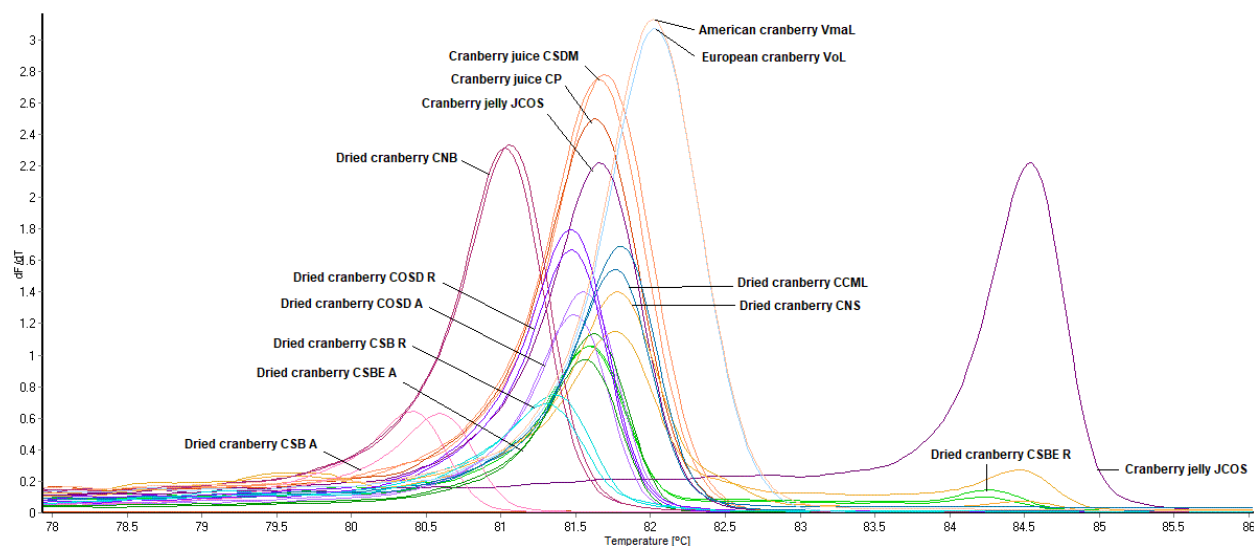


Figure 31: Melting curves of PCR products obtained for dried cranberry (CNB: dark red, CNS: yellow, CCML: dark blue, COSD A: light purple, COSD R: dark purple, CSB A: light pink, CSB R: light blue, CSBE A: dark green and CSBE R: light green), cranberry jelly (JCOS: dark purple), cranberry juice (CSDM: light orange and CP: dark orange) products with primer set *rp1*. For sample denomination see Chapter 4.2, Table 2 and 4.

The two positive controls VoL and VmaL resulted in melting curves with a peak maximum at 82°C. Samples CSB A and CNB resulted in peak maxima at 80°C and 81°C, respectively. The PCR products of the samples COSD R, COSD A, CSB R, CSBE A, CCML, CP and CSDM showed a peak maximum in the range from 81°C and 82°C. The cranberry jelly sample JCOS resulted in melting curves with peak maxima at different temperatures (82°C and 85°C). Samples CSBE R and CNS resulted in melting curves with two peak maxima, at approx. 82°C and the other at 85°C. Only these three samples showed an additional peak at approx. 85 (Figure 31).

In a further experiment, DNA was extracted from three syrup samples using the DNA QIAamp® DNA Blood Mini Kit and eluted with Buffer AE as described in Chapter 4.2.1.

One syrup was declared to contain blueberry and black currant (BBD), another was declared to contain cranberry and chokeberry (CCDS). As positive controls were used DNA extracts from fresh leaves from blueberry (VcL), American cranberry (VmaL), European cranberry (VoL) and one black currant jam (BCSJ). DNA extracts for the positive controls were also extracted with the *QIAamp® DNA Blood Mini Kit*. DNA from leaf samples was eluted with RNase free water, from jam sample with Buffer AE as described in Chapter 4.2.1. Details on the samples are given in Chapter 4.2, Table 2 and 4.

Concentrations determined for the two syrup samples with the Nanodrop instrument were much higher compared to the concentrations obtained with the Qubit instrument. The purity of the DNA extracts was found to be in the range from 2.0 to 2.2 (Table 15, 16 and 24).

Table 24: DNA concentration and purity of the extracts determined by spectrophotometric analysis and DNA concentration determined by fluorometric analysis.

Sample	Nanodrop [ng/mL]	A _{260/280}	Qubit [ng/mL]
Blueberry and black currant syrup BBD	3000	2.2	6
Cranberry and chokeberry syrup CCDS	2400	2.0	14
Black currant jam BCSJ	6200	1.6	-

Afterwards, the DNA extracts were subjected to PCR-HRM analysis. With the exception of VoL and VmaL (which had already been analyzed repeatedly in previous experiments), DNA extracts were analyzed undiluted in duplicates. The positive controls VoL and VmaL were diluted with RNase free water so that a final concentration of 5000 ng/mL was achieved. For samples BBD and CCDS only one of the duplicates led to an increase of the fluorescence signal within 50 cycles. C_t values obtained for samples BBD and CCDS were 36 and 37, respectively. The positive controls VcL, VoL and VmaL showed C_t values in the range from 15 to 19.

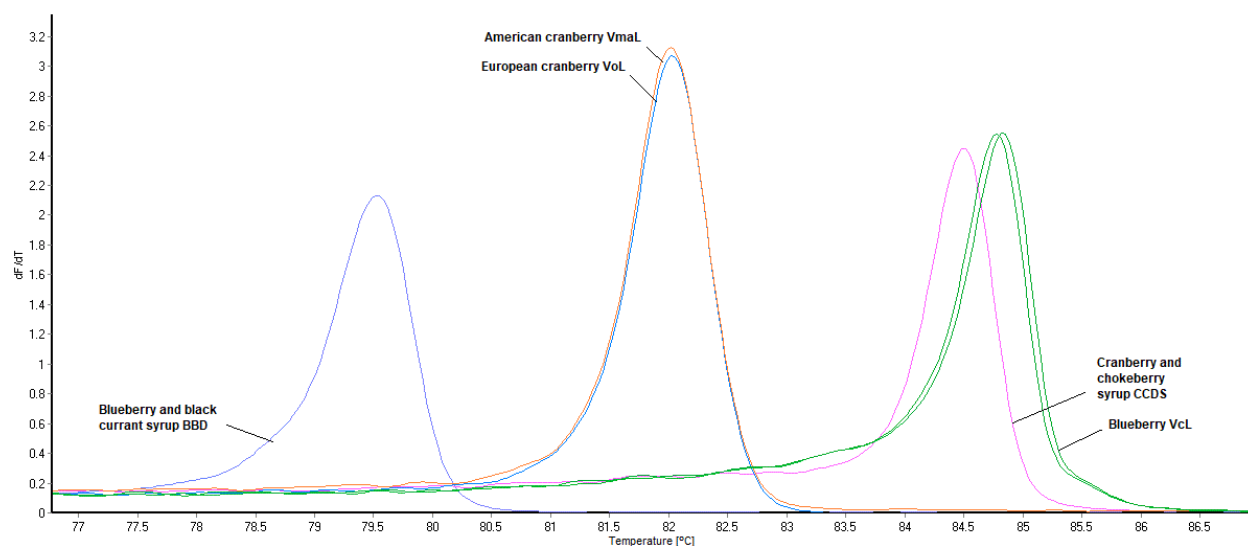


Figure 32: Melting curves of PCR products obtained for blueberry and black currant syrup (purple), cranberry and chokeberry syrup (pink), blueberry (green), American cranberry (orange) and European cranberry (blue) with primer set *rp1*. For sample denomination see Chapter 4.2, Table 2 and 4.

The melting curves obtained for sample CCDS showed a peak maximum at 84.5°C, whereas the positive controls VoL and VmaL resulted in a peak maximum at 82.0°C (Figure 32). Even though the syrup was declared to contain 42% blueberry juice and only 5% black currant juice, melting curves obtained for the positive control for blueberry (VcL) and the syrup sample (BBD) showed peak maxima at different temperatures (84.8°C and 79.5°C). Also the PCR product obtained for the other syrup, declared to contain 42.9% cranberry and 6% chokeberry, differed in its melting behavior from that of the two positive controls (VoL and VmaL). The chokeberry sequence for the *rp136-rp138* region is more similar with the bilberry sequence than with the cranberry sequence. This could lead to better binding of the primer set to the chokeberry sample and explain the peak maxima at 84.5°C. Syrups are concentrated fruit juices, which are processed and usually contain substances like sugar, citric acid and different aromas. These substances could lower the amplification efficiency.

5.4.3 *trnLVm1*

DNA extracts from various fruits were subjected to PCR-HRM analysis to investigate if the primer set *trnLVm1* allows the detection of admixture of undeclared fruits in blueberry products. Therefore, a DNA extract from fresh blueberry sample (VcF) was used as

positive control and DNA extracts from fresh chokeberry (AmF), American cranberry (CfS), pomegranate (9G (cultivar unknown)), plum (PO and PA) were the test samples. Additionally, two jams, one declared to contain 70% forest lingonberries (LS) and another declared to contain 85% black elderberry (BES), were analyzed. Details on the samples are given in Chapter 4.2, Table 2 and 3. DNA was extracted with *QIAamp® DNA Blood Mini Kit* and eluted with Buffer AE as described in Chapter 4.2.1.

DNA concentration and purity of the extracts was determined with the Nanodrop instrument (Table 16, 18, 21, 22 and 25). $A_{260/280}$ values in the range from 1.0 to 1.7 indicate the presence of proteins and polysaccharides.

Table 25: DNA concentration and purity of the extracts determined by spectrophotometric analysis.

Sample	Nanodrop [ng/mL]	$A_{260/280}$
Plum PO	13000	1.2
Black elderberry jam BES	2200	1.7

Before subjecting the DNA extracts (VcF, AmF, 9G, PO and PA) to PCR-HRM analysis they were diluted with RNase free water so that a final concentration of 5000 ng/mL was achieved. DNA extracts from samples CfS, LS and BES were used undiluted.

DNA extracts were analyzed in duplicates. For samples PO, LS and BES, only one of the duplicates led to an increase of the fluorescence signal within 50 cycles. Indeed, the jams were processed, which may lead to degraded DNA and low amount of DNA. Also, they contain other substances like sugars or citric acid which can inhibit the PCR amplification. C_t values obtained for samples VcF and CfS were 19 and 21, respectively. For extracts from the other samples, C_t values in the range from 19 to 42 were observed. Melting curves of the analyzed PCR products showed one peak maximum, ranging from 77.2°C to 77.5°C (Figure 33). These results indicate that PCR-HRM based on the primer set *trnLVm1* is not applicable for the detection of chokeberry, American cranberry, pomegranate, plum, lingonberry and black elderberry in blueberry products.

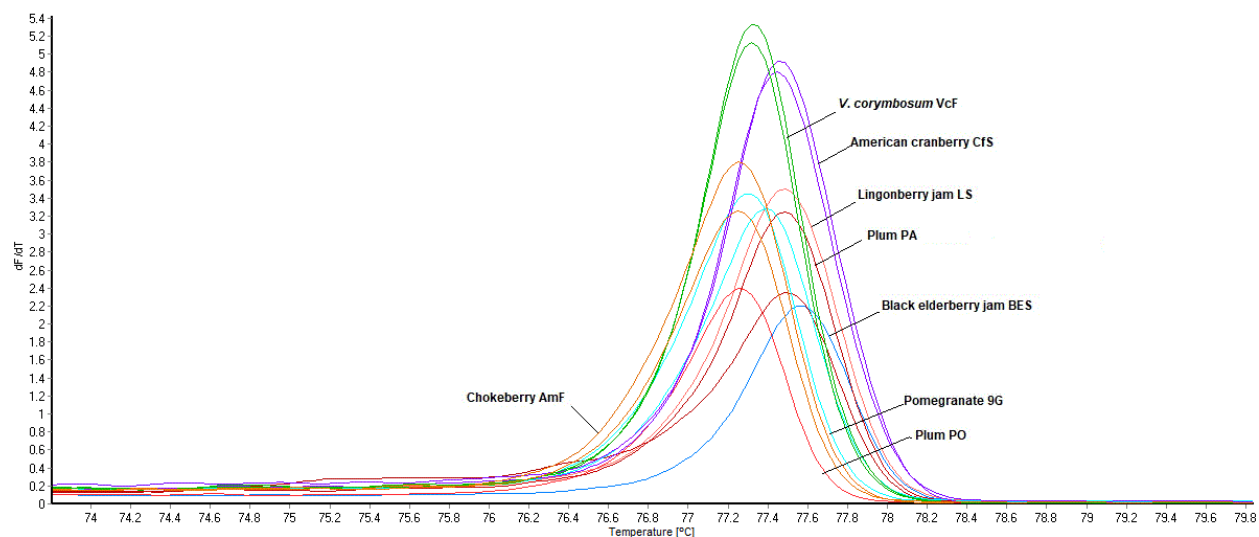


Figure 33: Melting curves of PCR products obtained for blueberry (green), chokeberry (orange), American cranberry (purple), plum (PA: dark red, PO: light red), lingonberry (light orange) and elderberry (blue) with primer set *trnLVm1*. For sample denomination see Chapter 4.2, Table 2 and 3.

5.4.4 *matK_1*

The primer set *matK_1*, designed based on the *matK* sequence for plum, was investigated for its suitability to detect admixtures of typical fruit species in blueberry products. For this aim, for the positive control DNA was extracted with *QIAamp® DNA Blood Mini Kit* from fresh blueberries (VcF) and for the possible adulterants from fresh plums (PA), dried plums (PPS2), plum jam (PE), fresh American cranberries (CfS) and fresh chokeberries (AmF). DNA was eluted with Buffer AE as described in Chapter 4.2.1. DNA concentrations determined with the Nanodrop instrument were in the range from 1600 ng/mL to 17000 ng/mL, while the $A_{260/280}$ values were in the range from 1.3 to 1.6 (Table 16, 21 and 26).

Table 26: DNA concentration and purity of the extracts determined by spectrophotometric analysis.

Sample	Nanodrop [ng/mL]	$A_{260/280}$
Dried plum PPS2	4400	1.4
Plum jam PE	6400	1.4

After adjusting the DNA concentrations to 5000 ng/mL, the extracts were subjected to PCR-HRM. Extracts with lower concentrations were used undiluted. DNA extracts were analyzed in duplicates. For sample PPS2 only one of the duplicates led to an increase of the fluorescence signal within 50 cycles.

C_t values obtained for the analyzed samples could be separated into three groups. The first group consisted of samples PA and AmF and showed C_t values of 27 and 30, respectively. The second group with a C_t value of 37 included the samples PE and VcF, while the third group consisted of samples CfS and PPS2 (C_t value of 44).

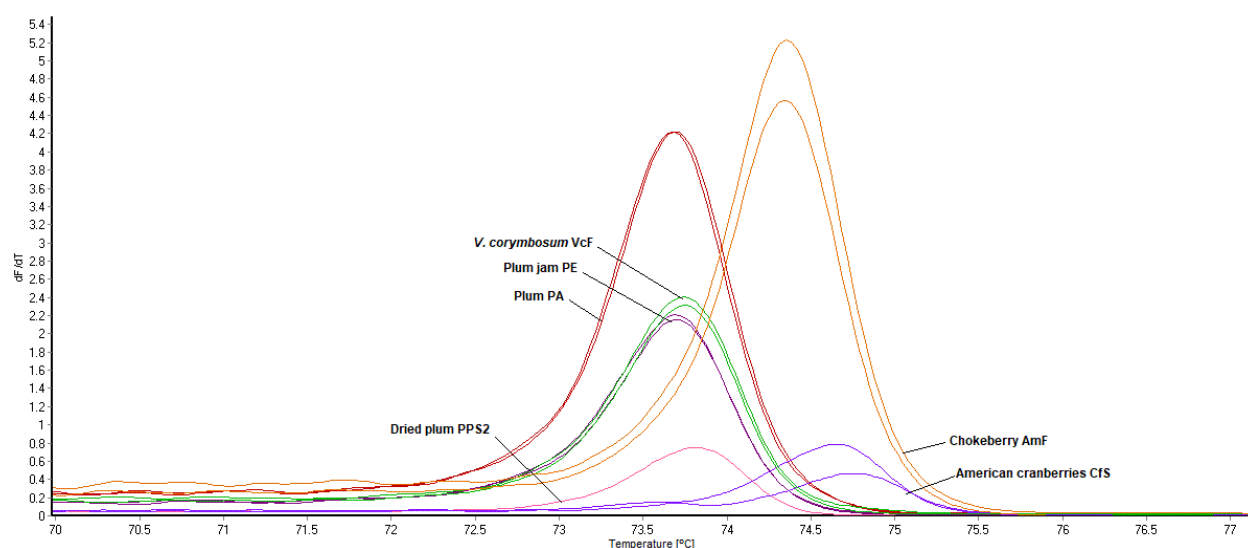


Figure 34: Melting curves of PCR products obtained for blueberry (green), plum (PA: red, PPS2: light pink and PE: dark pink), American cranberry (purple) and chokeberry (orange) with primer set *matK_1*. For sample denomination see Chapter 4.2, Table 2 and 3.

The resulting melting curves divided the samples into two groups, one for the blueberry and three plum samples with peak maximum at 73.7°C, another for chokeberry and American cranberry with a peak maximum at 73.4°C (Figure 34). Both, melting curves and difference plot show that the primer set *matK_1* cannot distinguish blueberry and plum species (Figure 34 and 35). However, it could be used for detecting the admixture of American cranberry and chokeberry in blueberry products.

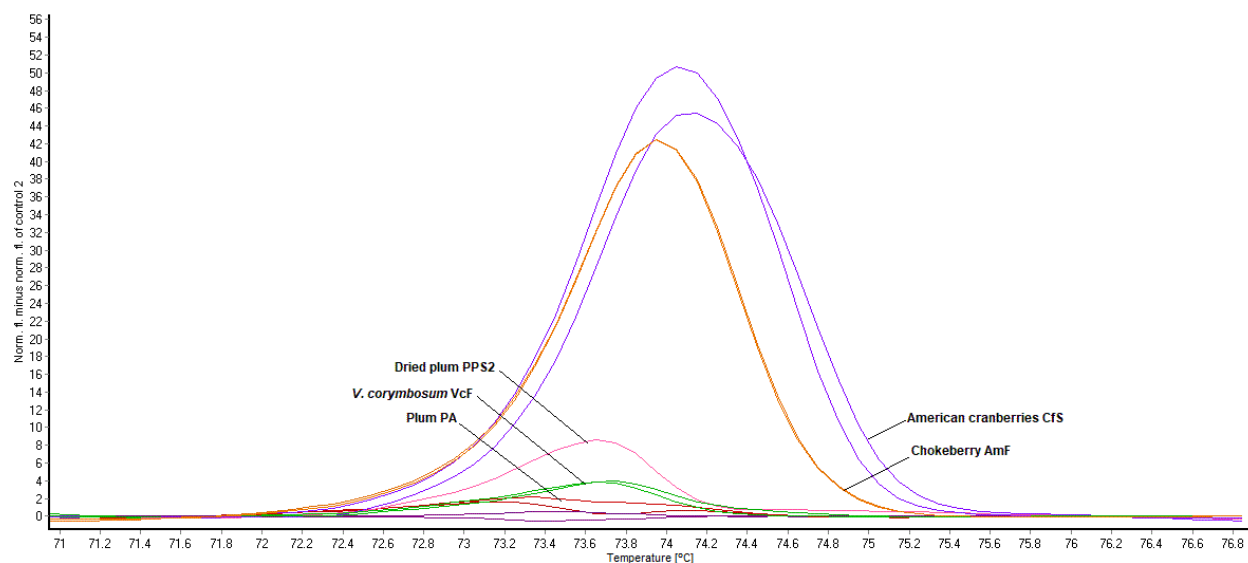


Figure 35: Difference plot of PCR products obtained for blueberry (green), plum (PA: red, PPS2: light pink and PE: dark pink), American cranberry (purple) and chokeberry (orange) with primer set *matK_1*. Reference: plum jam PE. For sample denomination see Chapter 4.2, Table 2 and 3.

5.4.5 *matK_2*

Another primer set for *matK*, *matK_2*, was designed, based on the *matK* sequence of blueberry. At the beginning, DNA was extracted with *QIAamp® DNA Blood Mini Kit* and eluted with Buffer AE from blueberry (VcF), bilberry (VmyF), chokeberry (AmF), plum (PA, PP and PO), plum jams (PPD and PE) and dried plums (PPS2). DNA extracts were subjected in duplicates to PCR-HRM. Detailed information about the samples as well as the PCR-HRM settings are summarized in Chapter 4.2 and Chapter 4.4.4, respectively. Before subjecting the DNA extracts to PCR-HRM analysis they were diluted to a final concentration of 5000 ng/mL. Since the concentration of the extract from sample PPS2 was < 5000 ng/mL, it was subjected undiluted.

In order to investigate if the DNA extracts contain polymerase inhibiting compounds, they were tested undiluted and 1:50 diluted with RNase free water. With the exception of the plum sample PA, for none of the plum samples an increase of the fluorescence signal within 50 cycles was observed.

DNA concentrations determined with the Nanodrop instrument were in the range from 4400 ng/mL to 17000 ng/mL, while $A_{260/280}$ values were in the range from 1.2 to 1.4 (Table 21, 25, 26 and 27).

Table 27: DNA concentration and purity of the extracts determined by spectrophotometric analysis.

Sample	Nanodrop [ng/mL]	A _{260/280}
Plum PP	12000	1.2
Plum jam PPD	6800	1.4

To find the best PCR-HRM conditions, two different annealing temperatures (58°C and 60°C), as well as two different elongation times (10 s and 30 s) were tested. C_t values obtained for blueberry (VcF), bilberry (VmyF), plum (PA) and chokeberry (AmF) samples at 58°C and 60°C were 22, 24, 36 and 37, respectively. The increase of the temperature did not lead to lower C_t values for plum and chokeberry samples. Therefore, a temperature of 58°C was used in the following experiments. In order to ensure full-length replication of complementary strand upon synthesis, a prolonged extension time (10 s → 30 s) was tested. However, prolonging the extension time did not have an influence on the results.

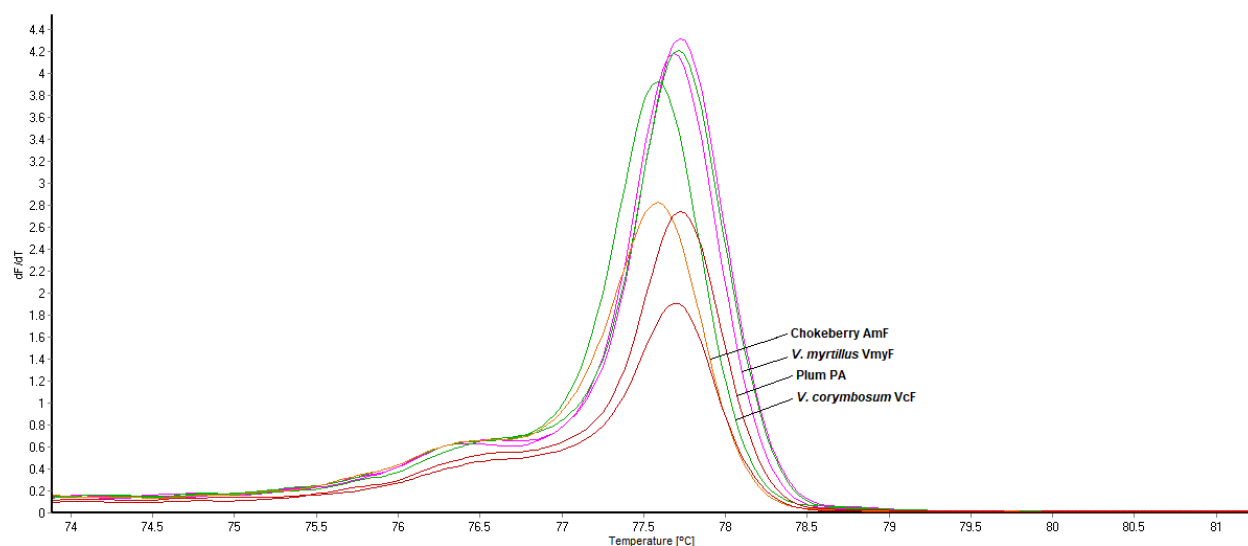


Figure 36: Melting curves of PCR products obtained for blueberry (green), bilberry (pink), chokeberry (orange) and plum (red) with primer set *matK_2*; annealing temperature 58°C, elongation time 30 s. For sample denomination see Chapter 4.2, Table 3.

PCR products obtained for blueberry, bilberry, chokeberry and plum did not differ in the peak maximum. Our results indicate that the PCR-HRM method based on the primer set *matK_2* is not suitable for detecting the admixture of plums and chokeberries in blueberry products.

5.4.6 ITS_2

The primer set *ITS_2* was designed in course of this master thesis. For primer design, the *ITS* sequence for pomegranate was used. We tested if PCR-HRM based on this primer set allows the detection of fruits which are typically used to adulterate pomegranate juices.

Before the method was applied, the annealing temperature and the $MgCl_2$ were optimized. Optimization was carried out with DNA extracts from two pomegranate cultivars (1G Bernarija and 2G Zumnarija). In order to optimize $MgCl_2$ concentration, either 0 μ L, 1 μ L or 2 μ L of a 40 mM $MgCl_2$ solution were added to the reaction mixture. The highest amplification efficiency was obtained when 1 μ L of $MgCl_2$ was added. For optimization of the annealing temperature, DNA extracts from the same two cultivars were used. The annealing temperature was either 57°C, 59°C, 61°C or 63°C. The annealing temperature did not have a strong influence on the amplification. In this experiment, an annealing temperature of 61°C was applied.

DNA was extracted with *QIAamp® DNA Blood Mini Kit* from two fresh pomegranate varieties (1G Bernarija and 2G Zumnarija), two apples (APP and AGD), white grapes (WGC), red grapes (RGC), American cranberry (VmaL and CfS), European cranberry (VoL), red currant jam (RCJ), black currant jam (BCSJ) and black elderberry jam (BES). DNA from sample VoL was eluted with RNase free water, from the other samples with Buffer AE. DNA concentrations determined with the Nanodrop instrument were much higher compared to the concentrations obtained with the Qubit instrument (Table 16, 18, 24, 25 and 28). In accordance with results described above, concentration in the extract from American cranberry (CfS) was below the limit of detection of the Qubit instrument. On the contrary, DNA was successfully extracted from red currant jam (RCJ), although it was highly processed and pasteurized. For samples BCSJ and BES the concentrations was measured only with the Nanodrop instrument.

Table 28: DNA concentration and purity of the extracts determined by spectrophotometric and fluorometric analysis.

Sample	Nanodrop [ng/mL]	A _{260/280}	Qubit [ng/mL]
Apple APP	3400	1.9	27
Apple AGD	3600	1.9	93
White grapes WGC	2300	2.5	68
Red grapes RGC	3600	2.0	91
Red currant jam RCJ	6600	1.6	46

Before subjecting the DNA extracts (1G (Bernarija), VmaL, VoL, RCJ and BCSD) to PCR-HRM analysis they were diluted with RNase free water so that a final concentration of 5000 ng/mL was achieved. DNA extracts from samples 2G (Zumnarija), APP, AGD, WGC, RGC, CfS and BES were subjected undiluted. PCR-HRM was carried out as described in Chapter 4.4.4 but with annealing temperature of 61°C and an additional extension step for 7 min at 72°C. DNA extracts were analyzed in duplicates. For samples 1G (Bernarija), VoL and CfS only one of the duplicates led to an increase of the fluorescence signal within 50 cycles.

Two pomegranate cultivars (1G Bernarija and 2G Zumnarija) led to a C_t value of 24. All other fresh fruit samples and pasteurized samples resulted in a C_t value in the range from 36 to 43.

The melting curves in Figure 37 show that the American cranberry (VmaL) and European cranberry (VoL) resulted in two peak maxima, at 85.0°C and 86.5°C. The only sample with three peak maxima was the black elderberry (BES). They were at 81.2°C, 83.2°C and 86.5°C. The rest of the samples had one peak maximum at 86.5°C. The additional peaks obtained for American and European cranberry and black elderberry can be used to detect them in adulterated pomegranate juices.

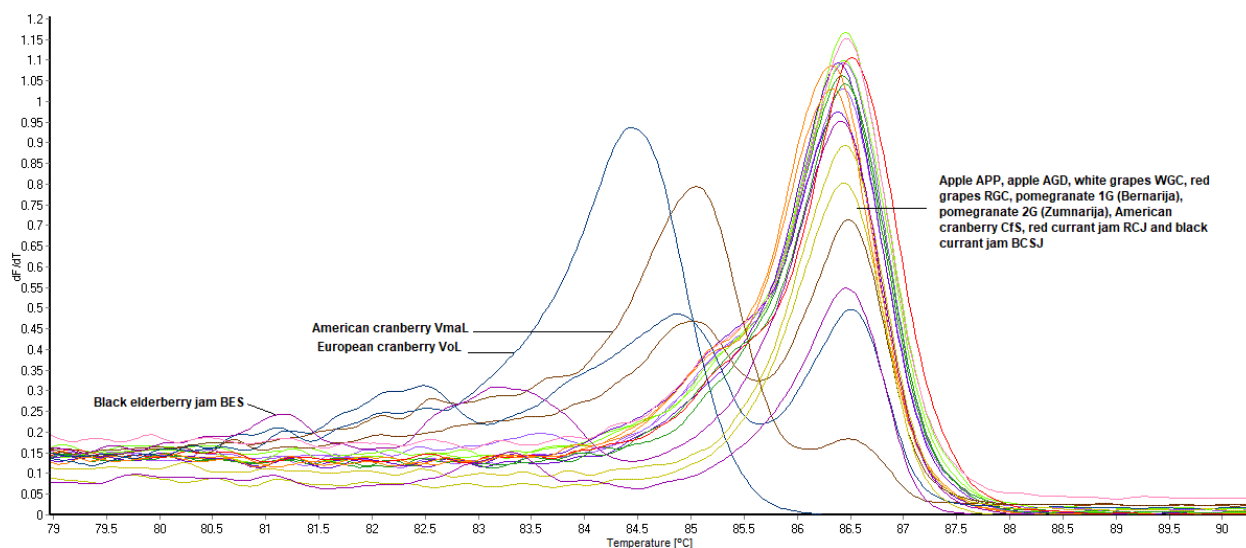


Figure 37: Melting curves of PCR products obtained for pomegranate (1G Bernarija: red and 2G Zumnarija: orange), apple (APP: dark purple and AGD: light purple), grape (WGC: light green and RGC: dark green), cranberry (VmaL: brown and VoL: dark blue), currant (RCJ: light green and BCSD: light blue) and elderberry (BES: dark pink) with primer set *ITS_2*. For sample denomination see Chapter 4.2, Table 2, 3 and 4.

With sample 1G (Bernarija) as a reference, the difference plot in Figure 38 shows that the duplicates did not result in the same melting curves, most probably due to low amplification efficiency. With the primer set *ITS_2*, only black elderberry and cranberries could be differentiated from pomegranates. In addition, the primer set *ITS_2* failed to differentiate between different cultivars. As can be seen, pomegranate 1G (Bernarija) and 2G (Zumnarija) could not be distinguished.

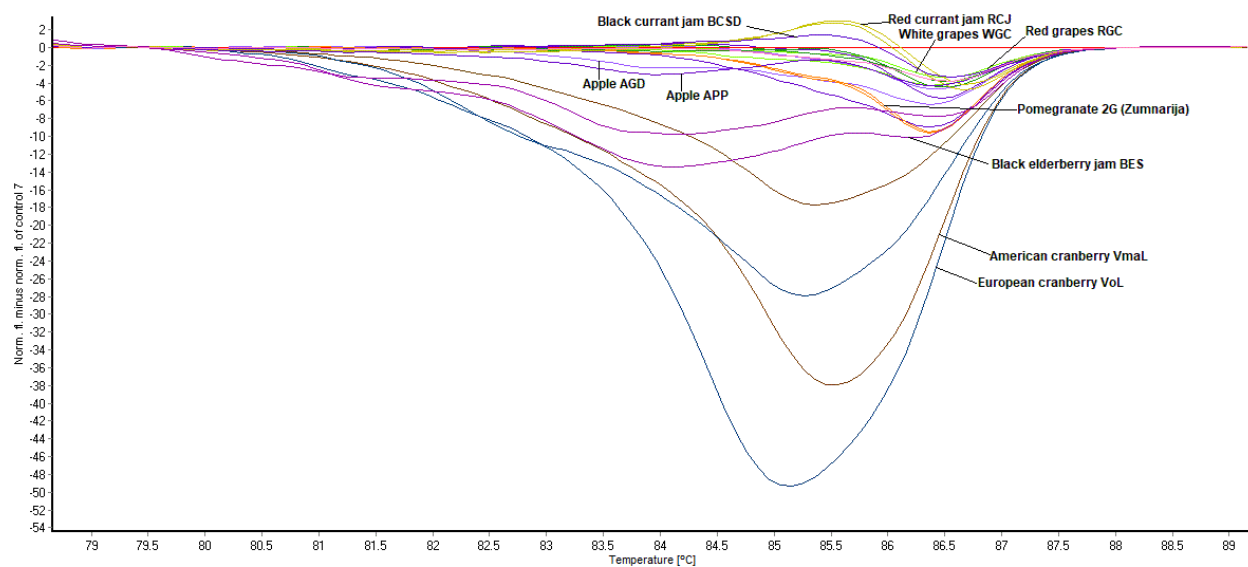


Figure 38: Difference plot of PCR products obtained for pomegranate (1G Bernarija: red and 2G Zumnarija: orange), apple (APP: dark purple and AGD: light purple), grape (WGC: light green and RGC: dark green), cranberry (VmaL: brown and VoL: dark blue), currant (RCJ: light green and BCSD: light blue) and elderberry (BES: dark pink) with primer set *ITS_2*. Reference: pomegranate 1G (Bernarija). For sample denomination see Chapter 4.2, Table 2, 3 and 4.

6 Conclusion

The aim of the master thesis was to investigate the suitability of high resolution melting (HRM) analysis for differentiation of berry species and cultivars. Among the primer sets tested, *ITSVm1* and *rp1*, targeting the *ITS* and *rpl36-rps38* region, respectively, were found to be suitable for distinguishing between blueberry and bilberry. However, in future more blueberry cultivars have to be analyzed in order to demonstrate the applicability of the HRM methods. In addition, with these primer sets, blueberry and bilberry could be separated from lingonberry and cranberry. However, both primer sets failed in the distinction between American and European cranberry.

Primer set *ITS_3* enabled the differentiation between different pomegranate cultivars. However, more samples from one and the same cultivar have to be analyzed in order to assess the intra-cultivar variability of pomegranates in this region.

Various primer pairs were tested for their applicability to detect adulteration of commercial berry products, including jams, juices and caplets. DNA could be isolated from all products. However, the purity of some extracts was very low and thus the DNA could not be amplified by PCR. Our results indicate that primer set *rp1* can be used to detect substitution of cranberry products by lingonberry and *vice versa*.

7 Appendix

7.1 Abstract

Food adulteration and mislabeling are widespread problems which affect not only the consumers but also businesses and the economy. Consumers choose one product over another according to their lifestyle, religious or health concerns, but also based on the product information provided. Studies have shown that the polymerase chain reaction (PCR) coupled with high resolution melting (HRM) analysis is a fast and accurate tool for species identification and product authentication.

The aim of the master thesis was to investigate the suitability of HRM analysis for differentiation of berry species and cultivars. Two primer sets, one targeting the *ITS* and the other one targeting the *rpl36-rps38* region, were found to be suitable for distinguishing between the cultivated blueberry (*Vaccinium corymbosum*) and the wild form (bilberry, *Vaccinium myrtillus*). However, more blueberry cultivars have to be analyzed to investigate the selectivity of the HRM assays in detail. In addition, with these primer sets, blueberry and bilberry could be separated from lingonberry (*Vaccinium vitis-idaea*) and cranberry. However, both primer sets failed in the distinction between American (*Vaccinium macrocarpon*) and European (*Vaccinium oxycoccos*) cranberry.

Another primer set targeting the *ITS* region seems to enable the differentiation between different pomegranate cultivars. However, the intra-cultivar variability of pomegranates in the investigated part of the *ITS* region has to be determined in more detail by analyzing a higher number of samples from one and the same cultivar.

In addition, various primer sets were tested for their applicability to detect adulteration of commercial berry products, e.g. jams, juices and caplets. DNA could be isolated from all products. However, the purity of some extracts was very low and thus the DNA could not be amplified by PCR. Our results indicate that the primer set targeting the *rpl36-rps38* region is applicable to detect substitution of cranberry products by lingonberry and *vice versa*.

7.2 Zusammenfassung

Verfälschung und fehlerhafte Kennzeichnung von Lebensmitteln sind weitverbreitete Probleme, welche nicht nur die Verbraucher, sondern auch Unternehmen und die Wirtschaft betreffen. Verbraucher entscheiden sich für ein bestimmtes Produkt aufgrund ihres Lebensstils, ihrer Religion oder aufgrund gesundheitlicher Aspekte, aber auch aufgrund der zur Verfügung gestellten Produktinformationen. Studien haben gezeigt, dass die Polymerasekettenreaktion (PCR), gekoppelt mit der Hochauflösenden Schmelzkurvenanalyse (HRM), eine schnelle und genaue Methode zur Speziesidentifizierung und Produktauthentifizierung ist.

In der vorliegenden Masterarbeit sollte die Eignung der HRM Analyse zur Differenzierung von Beerenspezies und Beerensorten untersucht werden. Zwei Primersets, eines für die *ITS* und das zweite für die *rpl36-rps38* Region, erwiesen sich als geeignet für die Unterscheidung von kultivierter Heidelbeere (*Vaccinium corymbosum*) und wilder Heidelbeere (*Vaccinium myrtillus*). Zur Überprüfung der Selektivität dieser HRM Assays müssen jedoch noch mehrere Heidelbeersorten analysiert werden. Mit den beiden Primersets konnten außerdem die kultivierte und die wilde Heidelbeere von der Preiselbeere (*Vaccinium vitis-idaea*) und der Cranberry unterschieden werden. Die beiden Primersets sind jedoch nicht geeignet, um zwischen der Amerikanischen (*Vaccinium macrocarpon*) und der Europäischen (*Vaccinium oxycoccos*) Cranberry zu differenzieren.

Ein anderes Primerset für die *ITS* Region scheint die Unterscheidung von verschiedenen Granatapfelsorten zu ermöglichen. Weitere Proben von den einzelnen Granatapfelsorten müssen jedoch noch analysiert werden, um die Intravariabilität der Sorten in der untersuchten *ITS* Region abschätzen zu können.

Weiters wurden verschiedene Primersets auf ihre Eignung zur Detektion der Verfälschung von kommerziellen Beerenprodukten, wie z.B. Konfitüren, Säften und Kapseln untersucht. DNA konnte zwar aus allen Produkten isoliert werden, die Reinheit einiger Extrakte war aber so gering, so dass die DNA nicht mittels PCR amplifiziert werden konnte. Unsere Ergebnisse lassen darauf schließen, dass mit dem Primerset für die *rpl36-rps38* Region eine Verfälschung von Cranberry Produkten mit Preiselbeere und umgekehrt nachgewiesen werden kann.

7.3 List of utensils

7.3.1 Chemicals and kits

<i>25 bp DNA Ladder Invitrogen™</i>	<i>ThermoFisher Scientific</i>
5x Nucleic Acid Sample Loading Dye	<i>BioRad</i>
Acetic acid	<i>Sigma Aldrich</i>
Agarose	<i>Sigma Aldrich</i>
Cetrimonium bromide (CTAB)	<i>Sigma Aldrich</i>
Chloroform	<i>VWR</i>
<i>DNA Exitus Plus IF spray</i>	<i>AppliChem</i>
<i>DNeasy® Plant Mini Kit</i>	<i>Qiagen</i>
<i>Epitect HRM PCR kit</i>	<i>Qiagen</i>
Ethanol absolute (EtOH)	<i>VWR</i>
Ethylendiaminetetraacetic acid (EDTA)	<i>Merck</i>
GelRed™	<i>VWR</i>
Hydrogen chloride (HCl)	<i>VWR</i>
Isopropanol	<i>Merck</i>
Magnesium chloride hexahydrate	<i>Sigma Aldrich</i>
Primers	<i>Sigma Aldrich</i>
Proteinase K	<i>Qiagen</i>
<i>QIAamp® DNA Blood Mini Kit</i>	<i>Qiagen</i>
<i>Qubit high sensitivity Kit</i>	<i>ThermoFisher Scientific</i>
Ribonuclease A solution from bovine pancreas	<i>Sigma Aldrich</i>
RNase free water (ultra-filtered and autoclaved)	<i>Sigma Aldrich</i>
Tris(hydroxymethyl)aminomethane (Tris)	<i>Sigma Aldrich</i>
<i>Type-it HRM PCR kit</i>	<i>Qiagen</i>

7.3.2 Consumables

Diverse adjustable pipettes (10-1000 µL)	<i>Eppendorf</i>
Filter pipette tips	<i>VWR</i>
PCR tubes 200 µL	<i>VWR</i>
Reaction tubes 1.5 mL	<i>VWR</i>
Strip tubes and caps 0.1 mL	<i>Qiagen</i>

7.3.3 Equipment

Analytical balance	TE2144S	<i>Sartorius</i>
Ball mill	MM 2000	<i>Retsch</i>
Centrifuge	Centrifuge 5424	<i>Eppendorf</i>
Electrophoresis cell	<i>Mini Sub CellnGT</i>	<i>BioRad</i>
Mortar		
Nanodrop	2000c	<i>ThermoFisher Scientific</i>
PCR working station		<i>VWR Peqlab</i>
Power supply	Power Pac HV	<i>BioRad</i>
Qubit	2.0 Fluorometer	<i>ThermoFisher Scientific</i>

Thermomixer comfort		<i>ThermoFisher Scientific</i>
Thermo cycler	Rotor-Gene Q	<i>Qiagen</i>
Transilluminator		<i>Herolab GmbH</i>
Vortex mixer		<i>Janke & Kunkel, Velp Scientifica</i>
Pipettes		<i>Eppendorf</i>

7.3.4 Software

Mega 7
Nanodrop 2000c
PyroMark Assay Design 2.0
Rotor-Gene Q Series Software 2.1.0

7.3.5 Webservers

<i>Oligo Calc</i>	http://biotools.nubic.northwestern.edu/OligoCalc.html
<i>Oligo Analyzer 3.1</i>	https://eu.idtdna.com/calc/analyzer
<i>RNA fold</i>	http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi

7.4 List of abbreviations

%	Percent
°C	Degrees Celsius
μL	Microliter
μM	Micromolar
ANSES	French Agency for Food, Environment and Occupational Health and Safety
bp	Base pair
c	Concentration
COI	Cytochrome c oxidase 1
C _t	Cycle threshold
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dsDNA	Double stranded DNA
dTTP	Deoxythymidine triphosphate
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
et al.	Et alia/alii/aliae (English: and others)
EtBr	Ethidium bromide
GC	Gas chromatography
HPLC	High performance liquid chromatography
HRM	High resolution melting analysis

IR	Infrared spectroscopy
IRMS	Isotope ratio mass spectrometry
<i>ITS</i>	<i>Internal transcribed spacer</i>
kb	Kilobases
<i>matK</i>	<i>Maturase K</i>
mg	Milligram
Mg ²⁺	Magnesium
MgCl ₂	Magnesium chloride
mL	Milliliter
mm	Millimeter
mM	Millimolar
NaCl	Sodium chloride
nm	Nanometer
NTC	No template control
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Second
SSR	Simple Sequence Repeats
TAE	Tris-acetate-EDTA
Taq	<i>Thermus aquaticus</i>
T _m	Melting temperature
USA	United states of America
UTI	Urinary tract infections
UV	Ultra violet
V	Volt
v/v	Volume/volume

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References

1. Downey G. Advances in food authenticity testing. *Woodhead Publishing Ltd.* **2016**. 1-3.
2. Lees M. Food authenticity and traceability in Downey G. *Advances in food authenticity testing*. Woodhead Publishing Ltd. **2005**; 107-108.
3. Moore JC, Spink J, Lipp M. Development and application of a database of food ingredient fraud and economically motivated adulteration from 1980 to 2010. *Journal of Food Science*. **2012**; 77:R118-R126.
4. Twohig M, Krueger DA, Gledhill A, Yang J, Burgess JA. Super fruit juice authenticity using multivariate data analysis, high resolution chromatography, UV and Time of Flight MS detection. *Focus on Food Analysis*. **2011**; 22(5):23-26.
5. European Commission. Monthly summary of articles on food fraud and adulteration. JRC Food Fraud Monthly Report. **2017**; 1-4.
6. Codex Alimentarius commission. Codex general standard for fruit juices and nectars (Codex Stan 247-2005). **2005**; 1-19.
7. Codex Alimentarius commission. Codex standard for jams, jellies and marmalades (Codex Stan 295-2009). **2009**; 1-10.
8. Rinke P. Tradition meets high tech for authenticity testing of fruit juices in Downey G. *Advances in food authenticity testing*. Woodhead Publishing Ltd. **2016**; 626-638.
9. FÜgel R, Carle R, Schieber A. Quality and authenticity control of fruit purées, fruit preparations and jams—a review. *Trends in Food Science & Technology*. **2005**; 16(10):433-441.
10. Ericales. Available from: <https://www.britannica.com/plant/Ericales#toc191380main>. 11th October 2017.
11. Vander Kloet SP. The taxonomy of the highbush blueberry, *Vaccinium corymbosum*. *Canadian Journal of Botany*. **1980**; 58:1187-1201.
12. Müller D, Schantz M, Richling E. High performance liquid chromatography analysis of anthocyanins in bilberries (*Vaccinium myrtillus* L.), blueberries (*Vaccinium corymbosum* L.), and corresponding juices. *Journal of Food Science*. **2012**; 77(4):C340-C345.

13. Upton R. Bilberry Fruit: *Vaccinium myrtillus* L.: Standards of analysis, quality control, and therapeutics. *American Herbal Pharmacopoeia and Therapeutic Compendium*. **2001**.
14. Neto CC, Vinson JA. Cranberry in Benzie IFF, Wachtel-Galor S. Herbal medicine: biomolecular and clinical aspects. 2nd edition. *Boca Raton (FL): CRC Press/Taylor & Francis*. **2011**; 26(3).
15. European Food Safety Authority (EFSA). Scientific Opinion on the substantiation of health claims related to proanthocyanidins from cranberry (*Vaccinium macrocarpon* Aiton) fruit and defence against bacterial pathogens in the lower urinary tract (ID 1841, 2153, 2770, 3328), “powerful protectors of our gums” (ID 1365), and “heart health” (ID 2499) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal*. **2011**; 9(6):2215.
16. French Agency for Food, Environment and Occupational Health and Safety (ANSES). Opinion of the French agency for Food, Environmental and Occupational Health and Safety No. 2010-SA-0214. **2004**; 1-17.
17. Janick J. The origins of fruits, fruit growing, and fruit breeding. *Plant Breeding Reviews*. **2005**; (25):255-321.
18. Vyas P, Curran NH, Igamberdiev AU, Debnath SC. Antioxidant properties of lingonberry (*Vaccinium vitis-idaea* L.) leaves within a set of wild clones and cultivars. *Canadian Journal of Plant Science*. **2015**; 95:663-669.
19. Brown PN, Turi CE, Shipley PR, Murch SJ. Comparisons of large (*Vaccinium macrocarpon* Ait.) and small (*Vaccinium oxycoccos* L., *Vaccinium vitis-idaea* L.) cranberry in British Columbia by phytochemical determination, antioxidant potential, and metabolomic profiling with chemometric analysis. *Planta Medica*. **2012**; 78:630-640.
20. Eid HM, Ouchfoun M, Brault A, Vallerand D, Musallam L, Arnason JT, Haddad PS. Lingonberry (*Vaccinium vitis-idaea* L.) exhibits antidiabetic activities in a mouse model of diet-induced obesity. *Evidence-Based Complementary and Alternative Medicine*. **2014**; 2014:1-10.
21. Lee J. Anthocyanin analyses of *Vaccinium* fruit dietary supplements. *Food Science and Nutrition*. **2016**; 4(5):742-752.

22. Wang C, Yuegang Z. Ultrasound-assisted hydrolysis and gas chromatography-mass spectrometric determination of phenolic compounds in cranberry products. *Food Chemistry*. **2011**; 128(2):562-568.
23. Huopalahti R, Järvenpää EP, Katina K. A novel solid-phase extraction-HPLC method for the analysis of anthocyanin and organic acid composition of Finnish cranberry. *Journal of Liquid Chromatography & Related Technologies*. **2000**; 23(17):2695-2701.
24. Hong V, Wrolstad RE. Use of HPLC Separation/Photodiode array detection for characterization of anthocyanins. *Journal of Agricultural and Food Chemistry*. **1990**; 38(3):708-715.
25. Määttä-Riihinen KR, Kamal-Eldin A, Mattila PH, González-Paramás AM, Törrönen AR. Distribution and contents of phenolic compounds in eighteen Scandinavian berry species. *Journal of Agricultural and Food Chemistry*. **2004**; 52:4477-4486.
26. Paliyath G, Whiting MD, Stasiak MA, Murr DP, Clegg BS. Volatile production and fruit quality during development of superficial scald in red delicious apples. *Food Research International*. **1997**; 30(2):95-103.
27. Sývacý A, Sökmen M. Seasonal changes in antioxidant activity, total phenolic and anthocyanin constituent of the stems of two *Morus* species (*Morus alba* L. and *Morus nigra* L.). *Plant Growth Regulation*. **2004**; 44:251-254.
28. Drake SR, Eisle TA. Influence of harvest date and controlled atmosphere storage delay on the color and quality of 'Delicious' apples stored in a purge-type controlled-atmosphere environment. *Hort Technology*. **1994**; 260-263.
29. Holland D., Hatib K., Bar-Ya'akov I., Pomegranate: botany, horticulture, breeding. *Horticulture*. **2009**; 35:128–178.
30. Melgarejo P, Salazar DM, Artés F. Organic acids and sugars composition of harvested pomegranate fruits. *European Food Research and Technology*. **2000**; 211(3):185-190.
31. Koppel K, Chambers E. Development and application of a lexicon to describe the flavor of pomegranate juice. *Journal of Sensory Studies*. **2010**; 25(6):819-837.
32. Fischer UA, Carle R, Kammerer DR. Identification and quantification of phenolic compounds from pomegranate (*Punica granatum* L.) peel, mesocarp, aril and

- differently produced juices by HPLC-DAD-ESI/MSⁿ. *Food Chemistry*. **2011**; 127(2):807-821.
33. Akhtar S, Ismail T, Fraternale D, Sestili P. Pomegranate peel and peel extracts: Chemistry and food features. *Food Chemistry*. **2015**; 174:417-425.
 34. Al-Maiman SA, Ahmad D. Changes in physical and chemical properties during pomegranate (*Punica granatum L.*) fruit maturation. *Food Chemistry*. **2002**; 76(4):437-441.
 35. Fuhrman B, Volkova N, Aviram M. Pomegranate juice inhibits oxidized LDL uptake and cholesterol biosynthesis in macrophages. *The Journal of Nutritional Biochemistry*. **2005**; 16(9):570-576.
 36. Malik A, Afaq F, Sarfaraz S, Adhami VM, Syed DN, Mukhtar H. Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America*. **2005**; 102(41): 14813-14818.
 37. Lansky EP, Jiang WG, Mo HB, Bravo L, Froom P, Yu WP, Harris NM, Neeman I, Campbell MJ. Possible synergistic prostate cancer suppression by anatomically discrete pomegranate fractions. *Investigational New Drugs*. **2005**; 23(1):11-20.
 38. Adams LS, Seeram NP, Aggarwal BB, Takada Y, Sand D, Heber D. Pomegranate Juice, Total Pomegranate Ellagitannins, and Punicalagin Suppress Inflammatory Cell Signaling in Colon Cancer Cells. *Journal of Agricultural and Food Chemistry*. **2006**; 54(3):980-985.
 39. Bajec MR, Pickering GJ. Astringency: mechanisms and perception. *Critical Reviews in Food Science and Nutrition*. **2008**; 48(9):858-857.
 40. Turfan Ö, Türkyılmaz M, Yemiş O, Özkan M. Anthocyanin and colour changes during processing of pomegranate (*Punica granatum L.*, cv. Hicaznar) juice from sacs and whole fruit. *Food Chemistry*. **2011**; 129(4):1644-1651.
 41. Zhang Y, Krueger D, Durst R, Lee R, Wang D, Seeram N, Heber D. International multidimensional authenticity specification (IMAS) algorithm for detection of commercial pomegranate juice adulteration. *Journal of Agricultural and Food Chemistry*. **2009**; 57:2550-2557.

42. Dalmia A, Perkins GL. Rapid screening of adulteration in pomegranate juice with grape juice using DSA/TOF with no sample preparation. *Perkin Elmer*. **2013**; 1-3.
43. Hmid I, Elothmani D, Hanine H, Oukabli A, Mehinagic E. Comparative study of phenolic compounds and their antioxidant attributes of eighteen pomegranate (*Punica granatum L.*) cultivars grown in Morocco. *Arabian Journal of Chemistry*. **2013**; 1878-5352.
44. Basharat Y, Khalid G, Ali Abas W, Preeti S. Health benefits of anthocyanins and their encapsulation for potential use in food systems: A review. *Critical Reviews in Food Science and Nutrition*. **2016**; 56(13):2223-2230.
45. Jaakola L, Suokas M, Häggman H. Novel approaches based on DNA barcoding and high-resolution melting of amplicons for authenticity analyses of berry species. *Food Chemistry*. **2010**; 123(2):494-500.
46. Marmioli N, Peano C, Maestri E. Advanced PCR techniques in identifying food components in Lees M. Food authenticity and traceability. *Woodhead Publishing Ltd*. 2003;3-9.
47. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. **1988**; 239(4839):448-87.
48. Cichna-Markl M, Druml B. High resolution melting (HRM) analysis of DNA - its role and potential in food analysis. *Food Chemistry*. **2014**; 158:245-154.
49. Applied Biosystems. A guide to high resolution melting (HRM) analysis. **2009**; 1-20.
50. Ganopoulos I, Argiriou A, Tsaftaris A. Microsatellite high resolution melting (SSR-HRM) analysis for authenticity testing of protected designation of origin (PDO) sweet cherry products. *Food Control*. **2011**; 22(3):532-541.
51. Mader E, Ruzicka J, Schmiderer C, Novak J. Quantitative high-resolution melting analysis for detecting adulterations. *Analytical Biochemistry*. **2011**; 409(1):153-155.
52. Savolainen V, Cowan RS, Vogler AP, Roderick GK, Lane R. Towards writing the encyclopedia of life: an introduction to DNA barcoding. *Philosophical Transactions of the Royal Society B*. **2005**; 360:1805-1811.

53. Hebert PDN, Ratnasingham S, de Waard JR. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society London B*. **2003**; 270:96-99.
54. Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN. DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B*. **2005**; 360:1847-1857.
55. Lambert DM, Baker A, Huynen L, Haddrath O, Hebert PDN, Millar CD. Is it a large-scale DNA-based inventory of ancient life possible? *Journal of Heredity*. **2005**; 96(3):279-284.
56. CBOL plant working group. A DNA barcode for land plants. *Proceedings of the National Academy of Sciences*. **2009**; 106(31):12794-12797.
57. Staats M, Arulandhu AJ, Gravendeel B, Holst-Jensen A, Scholtens I, Peelen T, Prins TW, Kok E. Advances in DNA metabarcoding for food and wildlife forensic species identification. *Analytical and Bioanalytical Chemistry*. **2016**; 408:4615-4630.
58. Fazekas AJ, Kuzmina ML, Newmaster SG, Hollingsworth PM. DNA barcoding methods for land plants. *Methods of Molecular Biology*. **2012**; 858:223-252.
59. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH. Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences*. **2005**; 102(23):8369-8374.
60. Madesis P, Ganopoulos I, Bosmali I, Tsaftaris A. Barcode high resolution melting analysis for forensic uses in nuts: a case study on allergenic nuts (*Corylus avellana*). *Food Research International*. **2013**; 50:351-360.
61. Osathanunkul M, Madesis P, Ounja S, Pumiputavon K, Soomboonchai R, Lithanatudom P, Chaowasku T, Wipasa J, Suwannapoom C. Identification of *Uvaria sp* by barcoding coupled with high-resolution melting analysis (Bar-HRM). *Genetic and Molecular Research*. **2016**; 15(1):1-12.
62. Osathanunkul M, Suwannapoom C, Osathanunkul K, Madesis P, de Boer H. Evaluation of DNA barcoding coupled high resolution melting for discrimination of closely related species in phytopharmaceuticals. *Phytomedicine*. **2016**; 23(2):156-165.

63. Faria MA, Magalhaes A, Nunes ME, Oliveira MBPP. High resolution melting of *trnL* amplicons in fruit juices authentication. *Food Control*. **2013**; 33(1):136-141.
64. Osathanunkul M, Suwannapoom C, Ounjai S, Rora JA, Madesis P, de Boer H. Refining DNA barcoding coupled high resolution melting for discrimination of 12 closely related *Croton* species. *Plos One*. **2015**; 10(9):1-14.
65. Ganopoulos I, Aravanopoulos F, Madesis P, Pasentsis K, Bosmali I, Ouzounis C, Tsaftaris A. Taxonomic identification of Mediterranean pines and their hybrids based on the high resolution melting (HRM) and *trnL* approaches: From cytoplasmic inheritance to timber tracing. *Plos One*. **2013**; 8(4):1-12.
66. Thermo fisher. Technical bulletin NanoDrop 1000 & 8000.
67. Desjardins P, Conklin D. NanoDrop microvolume quantitation of nucleic acids. *Journal of Visualized Experiments*. **2010**; (45):1-4 e2565.
68. Invitrogen™. Comparison of fluorescence-based quantitation with UV absorbance measurements. 1-4.
69. Müller HJ, Prange DR. PCR - Polymerase-Kettenreaktion. 2nd edition. *Springer Spektrum*. **2016**; 2-7.
70. Polymerase chain reaction. Available from: https://www.abmgood.com/marketing/knowledge_base/polymerase_chain_reaction_introduction.php. 11th October 2017.
71. Kubista M, Andrade JS, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N. The real-time polymerase chain reaction. *Molecular Aspects of Medicine*. **2006**; 27(2-3):95-125.
72. Mao F, Leung WY, Xin X. Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications. *BMC Biotechnology*. **2007**; 7:76 1-16.
73. Garritano S, Gemignani F, Voegelé C, Nguyen-Dumont T, Le Calvez-Kelm F, De Silva D, Lesueur F, Landi S, Tavtigian SV. Determining the effectiveness of high resolution melting analysis for SNP genotyping and mutation scanning at the TP53 locus. *BMC Genetics*. **2009**; 10(5):1-12.
74. Reed GH, Kent JO, Wittwer CT. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics*. **2007**; 8(6):597–608.

75. Lee PY, Costumbrado J, Hsu C, Kim YH. Agarose gel electrophoresis for the separation of DNA fragments. *Journal of Visualized Experiments*. **2012**; 62: 1-6.
76. Smith SB, Aldridge PK, Callis JB. Observation of individual DNA molecules undergoing gel electrophoresis. *Science*. **1989**; 243:2-3-206.
77. Oligo Calc: Oligonucleotide Properties Calculator. Available from: <http://biotools.nubic.northwestern.edu/OligoCalc.html>. 11th October 2017.
78. RNAfold Webserver. Available from: <http://rna.tbi.univie.ac.at/cgi-bin/RNA-WebSuite/RNAfold.cgi>. 11th October 2017.
79. Oligo Analyzer 3.1. Available from: <https://eu.idtdna.com/calc/analyzer>.
80. Särkinen T, Staats M, Richardson JE, Cowan RS, Bakker FT. How to open the treasure chest? Optimising DNA extraction from herbarium specimens. *Plos One*. **2012**; 7(8): 1-9.