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INSTITUTE OF FOOD SCIENCE AND BIOTECHNOLOGY
ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

DEVELOPMENT OF METHODS FOR GENETIC ANALYSIS OF PLANT FOODS
VÝVOJ METOD PRO GENETICKOU ANALÝZU POTRAVIN ROSTLINNÉHO PŮVODU

DOCTORAL THESIS
DIZERTAČNÍ PRÁCE

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AIMS OF THIS WORK

• Literary review: Methods for isolation and analysis of DNA from processed plant−based foods

• Optimization of DNA isolation methods from various types of plant−based foods, determination of concentration and purity

• Use of various methods of molecular biology for analysis of DNA isolates from plant−based foods and authenticity analysis

• Discussion of results

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ABSTRACT

Multiplex real-time PCR-HRM is an approach which has gained some attention in recent years. It has already found applications in clinical diagnostics and food authenticity and safety control. Compared to its corresponding singleplex PCR assays, an optimized multiplex PCR assay provides the same information in a fraction of time.

First part of this work dealt with isolation of DNA from both fresh fruits and processed commercial products. Six different DNA isolation protocols were tested with fresh fruits – three silica column-based kits, two magnetic carrier-based kits and one conventional protocol. One method was chosen as the most suitable and was applied to DNA isolation from commercial products. These experiments also involved optimisation of the chosen method.

The second part of this work was focused on the development of a triplex real-time PCR assay for simultaneous detection of blueberry, strawberry and raspberry, and its application on DNA isolated from commercial products. During DNA isolation, calcium chloride was shown to be a promising agent for removal of pectin from samples. In several samples, presence of raspberry DNA was confirmed by singleplex PCR and in several others, raspberry and blueberry DNA was tentatively identified. We found out that for accurate results of food analysis by this assay, further optimization of the PCR profile would be needed.

KEYWORDS

Real-time PCR, multiplex PCR, real-time PCR-HRM, food analysis, fruit, puree, tea

ABSTRAKT

Multiplex real-time PCR-HRM je analytická metoda, která v posledních letech získává pozornost. Našla si využití např. v diagnostických aplikacích, při kontrole bezpečnosti potravin, i při ověřování jejich autenticity. Optimalizovaná multiplex PCR reakce dokáže poskytnout stejné informace jako odpovídající singleplex PCR reakce za výrazně kratší dobu.

První část této práce byla zaměřena na izolaci DNA jak z čerstvého ovoce, tak i z vybraných průmyslově zpracovaných potravin. Na čerstvém ovoci bylo testováno šest různých metod pro izolaci DNA – tři kolonkové kity, dva kity s magnetickými nosiči a jedna konvenční metoda. Z těchto metod byla vybrána jedna, která byla využita pro izolace DNA z různých typů potravin rostlinného původu. Posledně zmíněné experimenty zahrnovaly i optimalizaci zvolené metody.

Druhá část této práce byla zaměřena na vývoj triplex PCR metody pro simultánní detekci borůvek, malin a jahod ve vybraných potravinách. Během izolace DNA se chlorid vápenatý ukázal jako vhodný prostředek k odstranění pektinu ze vzorků, a v několika komerčních výrobcích byla prokázána přítomnost malin pomocí sigleplex PCR. V několika dalších výrobcích byla zjištěna možná přítomnost DNA malin a borůvek. Z naměřených dat byl vyvozen závěr, že pro získání spolehlivějších výsledků jsou potřebné další optimalizace teplotního profilu triplex PCR.

KLÍČOVÁ SLOVA

Real-time PCR, multiplex PCR, real-time PCR-HRM, analýza potravin, ovoce, pyré, čaj

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CONTENTS

1   INTRODUCTION

Food fraud has long been a problem in food industry. Addition of undeclared dyes or flavouring agents, substitution of an expensive component for a cheaper one, or presence of an undeclared component are some of known types of food fraud. Foods which are subject to adulteration include plant-based foods, and plant species which may be subject to substitution by a cheaper variant include berries, such as blueberries, strawberries, and raspberries. Additionally, a product may contain a quantity of these species which is lower than declared.

The last two problems mentioned in previous paragraph are detectable, among other ways, by DNA-based analytical methods. These methods have several advantages over e. g. chromatographic or immunochemical methods. Firstly, DNA is more resistant to pH extremes, heat treatment and mechanical treatment than proteins. Secondly, a sequence of certain species' DNA does not change with climatic conditions during growth, or maturity stage of the species, unlike the content of certain metabolites.

On the other hand, isolation of sufficient quantity of DNA with sufficient purity out of plants and plant-based foods is difficult, and often unsuccessful. Enzymatic methods of DNA analysis, including PCR, are susceptible to contaminants from both sample matrix and agents used in DNA isolation protocols. However, the advantages mentioned in previous paragraph should not be forgotten.

One DNA-based approach which has gained attention in recent years is multiplex real-time PCR-HRM. This approach combines multiplex PCR, which may be used for simultaneous detection of several targets, thus saving time, and high-resolution melting analysis (HRM), which is able to detect even small differences in amplicon sequences. Additionally, HRM is a closed-tube method, and as such lessens the risk of sample contamination and consequent erroneous results.

In this work we attempted to develop a multiplex real-time PCR-HRM assay for simultaneous detection of blueberries, raspberries, and strawberries in various types of foods. In addition to the development of this assay, a DNA isolation protocol was also optimized for use with products such as purees, teas, or fruit bars.

2 THEORETICAL PART

2.1 DNA isolation from plant material

The isolation of DNA with sufficient concentration and purity is a starting point for every DNA-based analytical method. To obtain such DNA, a protocol suitable for the given matrix should be used [1]. DNA isolation protocols usually consist of several phases: Firstly, tissue disintegration and cell lysis. After these two phases a crude lysate is obtained. The lysate contains DNA, which is now released into the solution, but also other substances which were contained in various parts of the cells and must be removed. For this reason, the cell lysis is followed by DNA purification. Afterwards, the DNA is either captured on a solid carrier, or precipitated, cleaned of any residual impurities, and dissolved or eluted into water or a suitable (usually alkaline) buffer [2]. Plants and plant-based foods are complex matrices, which had even been labelled as recalcitrant samples, because isolating DNA from them is more difficult than from e. g. bacterial or animal cells [3, 4]. The following chapters focus on main phases of plant DNA isolation protocols and removal of contaminants contained in plants and plant-based food.

2.1.1 Tissue homogenization and cell lysis

DNA generally does not occur as a free molecule, but as a part of a cell, which means that a DNA isolation protocol should begin with a cell lysis step [5]. Because both plant tissues and cell walls are rigid [4], tissue homogenization and cell lysis in plant DNA isolation protocols are often more forceful than in isolation protocols for bacterial or animal DNA. The homogenization step often involves mechanical disruption of tissues, either by bead mills, or by mortar and pestle following the freezing of the tissue in liquid nitrogen [4, 6].

Plant cell lysis is achieved partly mechanically during tissue homogenization and partly by detergents or enzymes. A commonly used detergent is cetyl-trimethylammonium bromide (CTAB) [5, 6], but protocols using sodium lauryl sulfate (SDS) had also been reported [7, 8]. In case of enzymatic cell lysis, hydrolysing enzymes are often used. The choice of a particular enzyme depends on the composition of the cell wall – the use of cellulases, pectinases, cell wall macerases and even mixtures of several polysaccharide digesting enzymes had been reported [4, 9].

2.1.2 Removal of potentially inhibitory contaminants

While during isolation of DNA from bacterial or animal cells proteins are the main concern, in case of plant matrices, phenolic compounds and especially polysaccharides are the most problematic. These substances can either irreversibly bind to DNA, or coprecipitate with it, and cause problems when the contaminated DNA isolate is analysed – this is the case especially with enzymatic analytical methods [3, 4].

2.1.2.1 Polysaccharides

Polysaccharides had been described as "prime interferers" in DNA isolation by Varma et al. [4]. Kasem et al. claim that polysaccharides cause majority of problems related to the purity of isolated plant DNA [10]. Because of their chemical properties, polysaccharides can co-precipitate with DNA. A DNA isolate contaminated with polysaccharides can be viscous, with glue-like appearance [3]. The contaminating polysaccharides can interfere with electrophoresis, causing the DNA to remain in the well instead of migrating through the agarose gel, and they also inhibit activity of several enzymes, such as restriction endonucleases, ligases and DNA-polymerases, rendering the DNA unusable in enzymatic molecular methods [3, 4, 10].

A frequently used way of removing polysaccharides from plant cell lysates is their precipitation by CTAB [11].

At low ionic strength CTAB precipitates nucleic acids and acidic polysaccharides, such as pectin and xylan, and at high ionic strengths CTAB precipitates neutral polysaccharides, e. g. starch or inulin [6], although Varma et al. [4] mention that CTAB at high ionic strengths also precipitates *most* acidic polysaccharides. A sufficient ionic strength of the CTAB buffer is achieved by the addition of sodium chloride (NaCl). For efficient removal of polysaccharides, the concentration of NaCl in the CTAB buffer should be 0.5 M or higher, depending on the matrix [4, 10].

While the CTAB precipitation of polysaccharides during plant DNA isolation is a common way of removing them [11], other approaches had also been described, such as the use of ion exchangers and chromatography [10].

2.1.2.2 Phenolic compounds
Phenolic compounds pose a problem during plant DNA isolation because they can irreversibly bind to DNA, making them impossible to remove. During cell lysis vacuoles are disrupted and phenolic compounds are released, which makes them accessible to oxidases. The oxidised forms of phenolic compounds interact with DNA, and as mentioned above, these interactions are irreversible [4, 10]. Just as with polysaccharides, the presence of phenolic compounds in a DNA isolate makes it unusable for downstream applications, because phenolic compounds inhibit enzymes such as DNA-polymerases and endonucleases [3, 4, 10].

To prevent the oxidation of phenolic compounds during DNA isolation, the sample is kept at freezing temperatures prior to and during homogenization, and antioxidants and/or adsorbents are added to lysis buffers [10]. Polyvinylpyrrolidone (PVP) and polyvinylpolypyrrolidone (PVPP) are commonly used to adsorb polyphenols [4]. Such substances bind phenolic compounds by hydrogen bonds, forming complexes which precipitate at the interphase when the sample is extracted with chloroform [12].

β-mercaptoethanol is an antioxidant which is often used during plant DNA isolation [4, 10], although the use of others, such as diethyldithiocarbamic acid or bovine serum albumin, had been reported [10]. β-mercaptoethanol functions in two ways: Firstly, it prevents the polymerization of tannins. Secondly, it inhibits activity of polyphenol oxidases by reducing their intramolecular disulfide bonds, which results in the denaturation of these enzymes [4].

2.1.2.3 Proteins
While the "main interferers" in DNA isolation from plant matrices are polysaccharides [4], proteins can also cause problems. Protein contamination, more precisely enzyme contamination, can originate from sample matrix, or it can be introduced during DNA isolation [13]. Enzyme contamination is problematic, because it can interfere with downstream applications by digesting components used in these applications (e. g. a contaminating proteinase destroying DNA polymerase, thus causing inhibition of PCR, while a nuclease can digest template DNA or RNA) [14, 15].

Removal of proteins from the sample during DNA isolation can be achieved by two main ways. Firstly, proteins can be digested enzymatically. Proteinase K is an enzyme commonly used for this purpose [4, 10]. Secondly, proteins can be denatured non-enzymatically. Protein denaturing agents used in DNA isolation protocols are e. g. β-mercaptoethanol, phenol, or sodium dodecyl sulphate (SDS) [4, 10], although the use of phenol is not recommended when the sample is rich in phenolic compounds [4]. High concentration of salts such as NaCl may also be used to denature proteins [2].

2.1.3 DNA capture

In the final steps of an isolation protocol, DNA can be captured and purified from any residual contamination in several ways, such as alcohol precipitation or binding of DNA on a solid carrier, such as magnetic particles, resins, or glass fibres [1].

Ethanol and isopropanol, together with a salt (often sodium or ammonium acetate) are used for alcoholic precipitation of DNA. When the salt is added to an aqueous solution of DNA, it dissociates into a cation and an anion (in case of sodium acetate to Na^+ and $CH3COO^-$ ions). The positively charged ions are attracted to the negatively charged phosphate groups of DNA's sugar-phosphate backbone by electrostatic forces. As a result, the negative charge of the sugar-phosphate backbone is partially shielded and the molecules can aggregate. However, the dielectric constant of an aqueous solution is too high to allow precipitation of DNA and must be lowered. The lowering of the dielectric constant is achieved by addition of ethanol or isopropanol [16, 17]. While alcohol precipitation of DNA is a widely used method [16], it can be problematic when concentration of DNA in the solution is low, because the precipitated DNA does not form a clearly visible pellet after centrifugation [18]. To assist with precipitation of DNA from solutions with low concentration, co-precipitants such as linear polyacrylamide, glycogen, polyethylene glycol or starch nanoparticles may be used [16, 18].

For DNA capture on solid phase (or solid phase extraction, SPE) the commonly used solid support is silica [19, 20], which is used either in the form of a filter membrane, or as silica-coated magnetic particles [19]. One of proposed mechanisms for the binding of DNA on silica surfaces is a combination of electrostatic, or electrostatic and hydrogen bonding interaction [21]. In solutions with high ionic strength and low pH a chaotropic salt (e. g. guanidium thiocyanate) decreases solvation of DNA, which decreases the repulsive forces between its strands. At the same time the chaotropic salt produces silanol groups on the silica surface, which allows the DNA to bind to it through hydrogen bonds or salt bridges (Figure 1) [19, 22]. After the impurities are washed from the sample, DNA is eluted from the silica surface using a buffer with lower ionic strength and high (alkaline) pH [19, 20]. Capture of DNA on silica surface had been described as simple as fast [20]. On the other hand, a disadvantage of this approach is the inhibitory effect of chaotropic salts on PCR, if these salts are carried over to the eluted DNA [19, 21].

There are alternatives to the binding of DNA on silica surfaces, which do not require the use of chaotropic salts. One of them is anion-exchange technology, in which DNA binds to a positively charged surface through electrostatic interaction at low ionic strength and pH 6-9 [20]. Another alternative is a solid carrier coated with DNA-binding antibodies, or other substances which interact specifically and reversibly with DNA, such as aminosilane or polyethylenimine [20, 24]. Last, but not the least, alternative to silica surfaces, which is mentioned in this chapter, are charge-reversible particles. These particles are composed of a core coated with ligands containing pH-sensitive functional groups [25]. An example of this type of carriers are gold nanoparticles coated with mercaptoundecylamine and mercaptoundecanoic acid synthesized by Wang et al. [26].
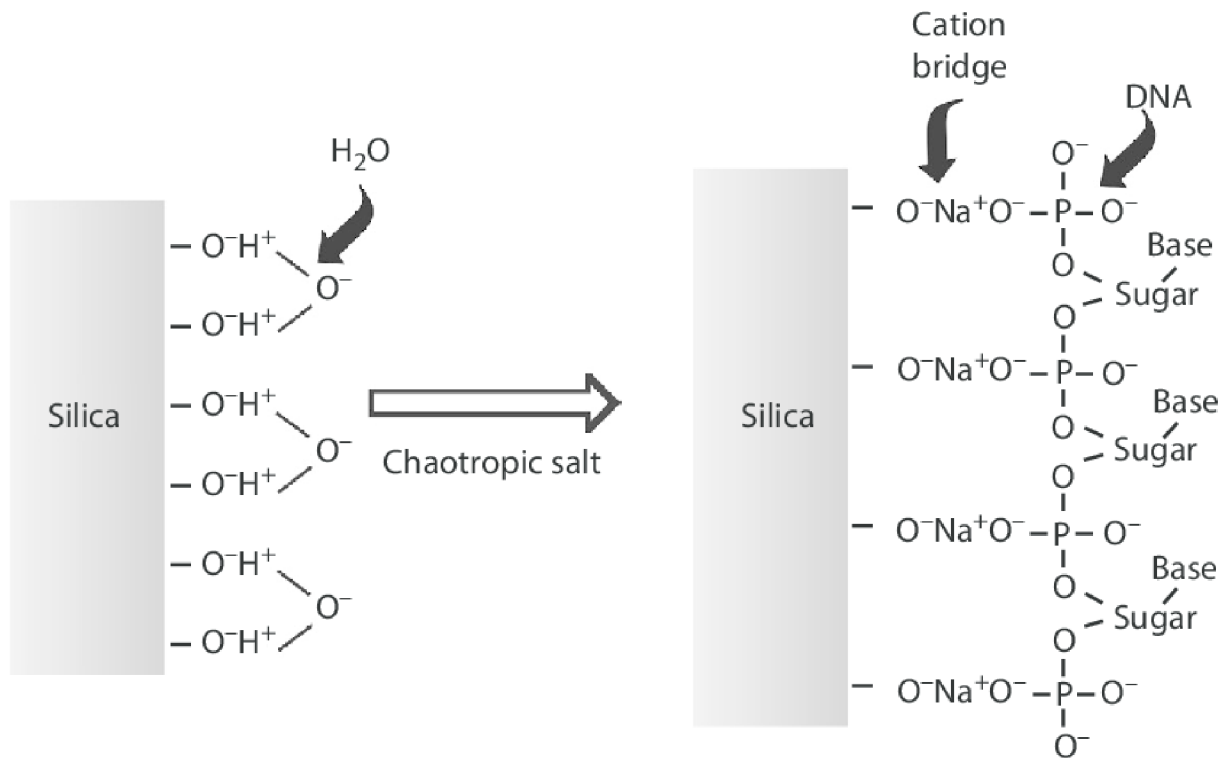
Figure 1: Binding of DNA to silica surface in the presence of a chaotropic salt [23]

The surface of these carriers contains amine and carboxylic groups and has a certain isoelectric point. At pH lower than its isoelectric point the surface is positively charged and DNA can be adsorbed on it through electrostatic interaction. At pH higher than its isoelectric point the surface becomes negatively charged and DNA is released into the solution by electrostatic repulsion [26].

2.2 Influence of food processing on DNA

Although DNA is more resistant to damage by food processing, such as cooking or sterilization, than other compounds, it still undergoes degradation [27]. Production of any plant-based processed food usually involves several steps which cause degradation of DNA [1]. For example, juices are extracted from grated or ground fruits by presses and clarified enzymatically, with the enzymatic clarification taking place at low pH (e. g. 2.5 − 6.0 when pectinase is used, with temperature 30 − 60 °C). These steps may be followed by pasteurization and concentration by evaporation [28, 29]. In case of purees and bars fruit disintegration and heat treatment also take place. Additives such as sucrose, pectin, acids or flavours might be added to both purees and bars, and the latter are also dried after blending of all required ingredients [30, 31], and during preparation of fruit teas the plant material also undergoes grinding and drying [32].

Out of the technological operation mentioned in previous paragraph, grating and grinding expose DNA to shear forces, which may fragment it. Furthermore, mechanical treatments disrupt cell walls and membranes, releasing both DNA and various enzymes. These enzymes may include e. g. nucleases, which degrade DNA [1].

Low pH, which is characteristic for fruit and vegetables [1], and which is also needed for processes such as enzymatic clarification [28], is known to cause depurination and subsequent cleavage of DNA. Furthermore, when operations such as grinding or boiling are performed at

acidic pH, they are more destructive to DNA than when performed in alkalic conditions [1, 33, 34].

Processes performed at heightened temperatures, such as drying and pasteurization, also have a detrimental effect on DNA. When exposed to high temperatures, DNA strands are destroyed through depurination. Although the temperatures used during drying or pasteurization are not sufficient for complete destruction of all DNA in the dried or pasteurized product, long strands of DNA are sheared into shorter fragments, which may reduce the sensitivity of PCR [1, 34].

2.3 DNA-based analytical methods in food authentication

DNA-based techniques for species identification had been introduced in the 1980s and since then, various approaches for DNA-based authentication of food had been developed [35]. Although DNA-based analytical methods have their weaknesses, such as lowered efficiency or even failure when the analysed DNA is degraded or contaminated [35, 36], they also have advantages over methods such as HPLC (influence of e. g. growing conditions or manufacturing process on the food's chemical profile, or complex chemical profile) [35], or ELISA (proteins less resistant to high temperatures or pH extremes during processing than DNA) [27, 37]. Lo and Shaw in their 2018 article divide DNA-based methods for food authentication into three main categories [35]:

- PCR-based techniques
- Hybridization based techniques
- Sequencing based techniques

DNA-based analytical techniques utilize polymorphisms in DNA between species. The PCR-based techniques either generate a specific fingerprint composed of several fragments, or produce a single specific fragment, which may be further analysed, e. g. by high resolution melting analysis (HRM) [35, 38].

Among PCR-based DNA fingerprinting techniques belong e. g. restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA (PCR-RAPD), amplified fragment length polymorphism (PCR-AFLP), single-stranded conformational polymorphism (PCR-SSCP), or inter-simple sequence repeat (PCR-ISSR). These techniques utilize either restriction enzymes in a certain phase of the protocol (restriction digestion of PCR products in PCR-RFLP, restriction digestion of template DNA before PCR in PCR-AFLP), or primers/primer sets which generate a characteristic fingerprint (PCR-RAPD, PCR-ISSR) [35]. In case of PCR-SSCP a DNA fingerprint is generated by combination of double-stranded PCR product denaturation and subsequent electrophoresis in non-denaturing polyacrylamide gel, during which single-stranded DNA fragments fold into different shapes with differing electrophoretic mobilities based on the fragment's sequence [39].

Regarding PCR-based techniques which utilize specific primers, this group of methods involves the use of sequence-characterized amplified regions (SCAR markers), multiplex PCR [35], real-time PCR [40] and HRM analysis [38].

The techniques mentioned above had been used for authentication of various plant-based products, such as pomegranate-containing drinks and jam (SCAR markers) [41], olive oils (PCR-AFLP, real-time PCR-HRM) [42, 43], wine must (PCR-ISSR) [44] and many others.

2.4 Real-time PCR

The amplification in real-time PCR is based on the same principles as in conventional PCR. The difference is in the methods used for the detection of amplicons. In conventional PCR the

amplicons are detected after all of the reaction's cycles are complete, while in real-time PCR the amplicons are detected in each cycle of the reaction [5, 45]. This chapter summarizes reaction components and principles of amplification, product detection and product quantification in real-time PCR.

2.4.1 Components

Reaction mixture for PCR consists of several components, in particular template DNA, a pair of primers, thermostable DNA polymerase, deoxyribonucleotide triphosphates (dNTPs) and a buffer which creates suitable conditions for DNA polymerase and usually also contains magnesium (Mg^{2+}) ions [5, 45, 46].

Template DNA (or in some cases RNA) contains the sequence which is to be amplified [5]. The template can be either double-stranded or single-stranded, and genomic DNA (gDNA), plasmid DNA and complementary DNA (cDNA) can all serve as a template. However, different starting concentration are recommended for different types of DNA. For genomic DNA, 5-50 ng are recommended, while for plasmid DNA 0.1-1 ng are recommended as a starting amount [45, 47].

Another component of PCR reaction mixture is thermostable DNA polymerase. The first thermostable polymerase used for PCR was the *Taq* polymerase [48]. First isolated in 1980 by Kaledin et al. [49], it is still widely used, although other thermostable DNA polymerases had been isolated in the 1990s and 2000s [48]. The DNA polymerase synthesises new DNA strands by linking nucleotides (deoxyribonucleotide triphosphates, usually in equimolar amounts in the reaction mixture) to the 3'-end of each primer, complementarily to the template DNA. However, to function properly, the DNA polymerase requires optimized concentration of Mg^{2+} ions [45].

Magnesium ions, which are usually a part of a buffer [5], have several functions. They are cofactors for DNA polymerase and they ease the formation of complexes between template DNA and primers. If the concentration of magnesium ions is too low, the activity of DNA polymerase will be lowered also, resulting in little or no product, while too high concentration of magnesium ions can cause synthesis of non-specific products [45].

Other components which determine the specificity and accuracy of a PCR reaction are primers [5]. These are oligonucleotides approximately 20 bp long (according to Carter et al. the ideal size range is 20-30 bp [5], Gökmen-Polar mentions size range 15-30 bp [45] and Bustin et al. [50] recommend primer size 18-24 bp). There are also several recommended parameters for primers, which include content of guanine and cytosine (around 50 %), melting temperatures (55-70 °C, with difference no higher than 5 °C between the primers) and sequence – the primers should not form secondary structures, and they should not be complementary to each other, especially at their 3'-end [45, 50].

2.4.2 Amplification steps

A basic PCR protocol consists of several steps (Figure 2), each of which is performed at a different temperature. These steps comprise a cycle, which is repeated 30 to 40 times [45]. The first step of a PCR cycle is denaturation of DNA, or in other words dissociation of double stranded template DNA into single strands. This is achieved by heating of the reaction mixture, usually to approximately 94-95 °C for 15-30 seconds [45], with the exact temperature and time depending on the length of the template and its content of guanine-cytosine (GC) and adenine-thymine (AT) pairs (the temperature is higher for longer templates and higher GC content). To ensure complete separation of template strands at the beginning of the reaction, and in some

14

cases to activate the DNA polymerase, a longer (several minutes) denaturation step is performed before the first PCR cycle [46, 47].
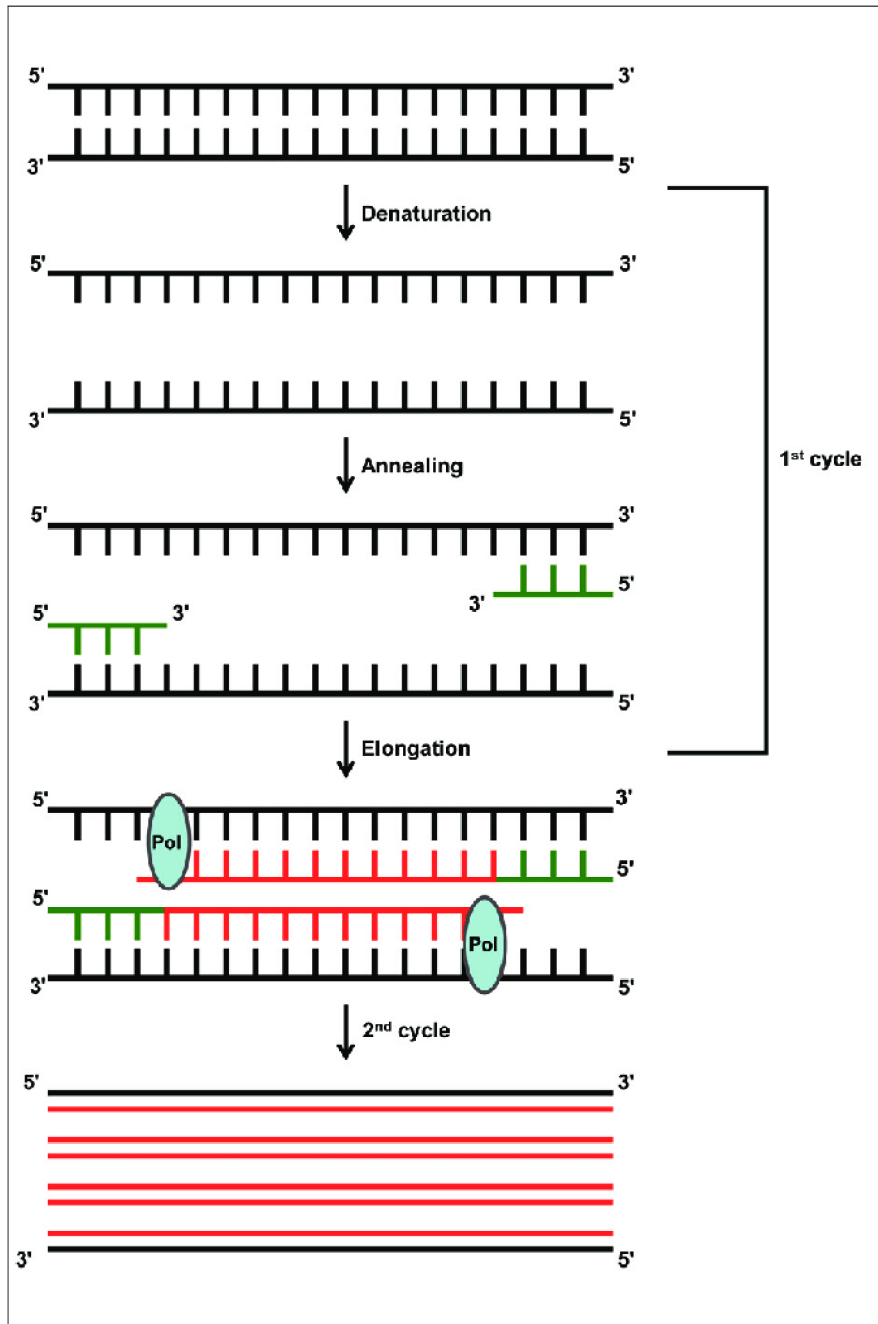
Figure 2: Schematic diagram of PCR steps [51]

Denaturation of DNA is followed by annealing of primers, which usually lasts 20-40 seconds [45]. The exact temperature of this step depends on the length and sequence of the primers [5, 46, 47], and it is usually several degrees below the melting temperature of the primers [46, 47]. The temperature at which annealing of primers takes place should allow them to "form stable complexes" with their target sequence, while preventing their binding to sequences which are not fully complementary to the primers [46, 47].

The last step of a PCR cycle is elongation, during which a polymerase synthesises new DNA strands complementary to the template, beginning at the 3´-end of an annealed primer [45]. This step often takes place at 72 °C (optimum temperature of DNA polymerase) [47], while its

duration depends on the length of the amplicon. Rahman et al. in their 2013 article [46] suggest 1 minute per 1 000 bp as a "rule of thumb".

2.4.3 Amplicon detection and quantitation

Real-time PCR has several advantages over conventional PCR, all of which are connected to the way amplicons are detected in real-time PCR. These advantages include precise quantification of the starting amount of template DNA, and lower risk of contamination due to real-time PCR being a closed-tube method [52]. The amplicon detection methods can be divided into two main groups according to the type of the "fluorescent agent": Non-specific methods, which use intercalating dyes, such as SYBR Green I, and specific methods, which use fluorophores attached to oligonucleotides [52, 53]. The principles of these methods are described in chapters 2.4.2.1 and 2.4.2.2.

2.4.3.1 Non-specific amplicon detection

Non-specific detection of amplicons is carried out by intercalating dyes, which bind to the minor groove of a double-stranded DNA [52]. When an intercalating dye is free in a solution, the intensity of its fluorescence is low. When the dye binds to a double-stranded DNA, the fluorescent signal increases significantly [5, 52]. The first intercalating dye used for the detection of amplicons in real-time PCR was ethidiumbromide [54], which was later replaced by other more sensitive intercalating dyes, most notably by SYBR Green I [53]. However, while SYBR Green I is still commonly used [55], it has some drawbacks which have been reported as early as the 2000s [56]. These drawbacks include inhibition of PCR at higher concentration of the dye [57] or limitations in the dye's usability in melting curve analysis [58].

Because of the above-mentioned limitations, other dyes belonging to newer generations of intercalating dyes, e. g. EvaGreen or SYTO9, have been proposed as alternatives to SYBR Green I due to their lower inhibitory effect on PCR, more consistent shapes of amplicon melting curves, lower affinity for single-stranded DNA and better suitability for melting curve and high resolution melting curve analysis [56, 59]. Recently, new dyes based on acridine orange had also been described [55].

2.4.3.2 Specific amplicon detection

Specific amplicon detection methods can be further divided into two main subgroups: Probe methods and primer-probe methods. Primer-probe methods include several types of fluorescent agents (hairpin primer-probes, cyclicons, Angler® technology) which employ different mechanisms of action [52].

In case of some types of hairpin primers, a fluorophore is attached to the 3'-end of the primer (e. g. LUX® technology), or to the 5'-end, with a quencher attached to the 3'-end of the hairpin (e. g. Amplifluor® technology). When the primer is not incorporated into an amplicon, the fluorophore is quenched by the hairpin structure of the primer. After the primer becomes a part of an amplicon, the hairpin structure is extended, the fluorophore is no longer quenched and the intensity of fluorescence increases. [52, 53]. Other types of hairpin primers function differently. For example, the Plexor® technology has isocytosine (a synthetic base) connected to a fluorophore at the 5'-end of the oligonucleotide. The reaction solution on the other hand contains isodeoxyguanosinetriphosphate (iso-dGTP) with a quencher linked to it. When the oligonucleotide labelled with isocytosine is incorporated into an amplicon, it pairs with iso-dGTP. When this happens, the fluorophore comes close to the quencher and the fluorescent signal decreases. The increase in concentration of the specific amplicon is then indicated by decrease in the intensity of fluorescence [53].

The use of another type of primer-probes, the cyclicons, was described by Kandimalla and Agrawal in 2000 [60]. Cyclicons consist of two oligonucleotides, one approximately 20 nucleotides long and complementary to the target sequence, and the other approximately 6-8 nucleotides long. The longer oligonucleotide is called the "probe or primer-probe", while the shorter oligonucleotide is called "modifier". The primer-probe and the modifier are linked at either their 5'-ends or their 3'-ends. The modifier is complementary to either the 3'-end or the 5'-end of the probe, forming a cyclic structure in the absence of target DNA. When a fluorophore is linked to the free end of the modifier and a quencher is linked to the free end of the primer-probe, in the absence of target DNA the fluorescence is quenched, because the cyclic structure of the cyclicon brings the fluorophore and the quencher close enough for Förster resonance energy transfer (FRET) quenching. When the primer-probe is hybridized to its target sequence, the cyclic structure is opened up and the FRET quenching is disrupted [52, 60].

The angler technology employs both labelled oligonucleotides and intercalating dye. An Angler primer-probe consists of a primer linked via hex-ethylene glycol (HEG) to a probe complementary to a sequence downstream of the primer. The probe has an acceptor fluorophore attached to its 3'-end. When the primer is incorporated into a newly synthesized DNA strand, and the amplicon is denatured, the probe hybridizes to a complementary sequence within this new DNA strand, creating a short double-stranded region. A fluorescent dye (SYBR Gold) intercalates into this region and emits fluorescence. Because the dye and the acceptor fluorophore of the probe are now close enough, a FRET transfer can take place, the intercalated dye serves as a donor and the acceptor fluorophore also emits fluorescence [52, 61].
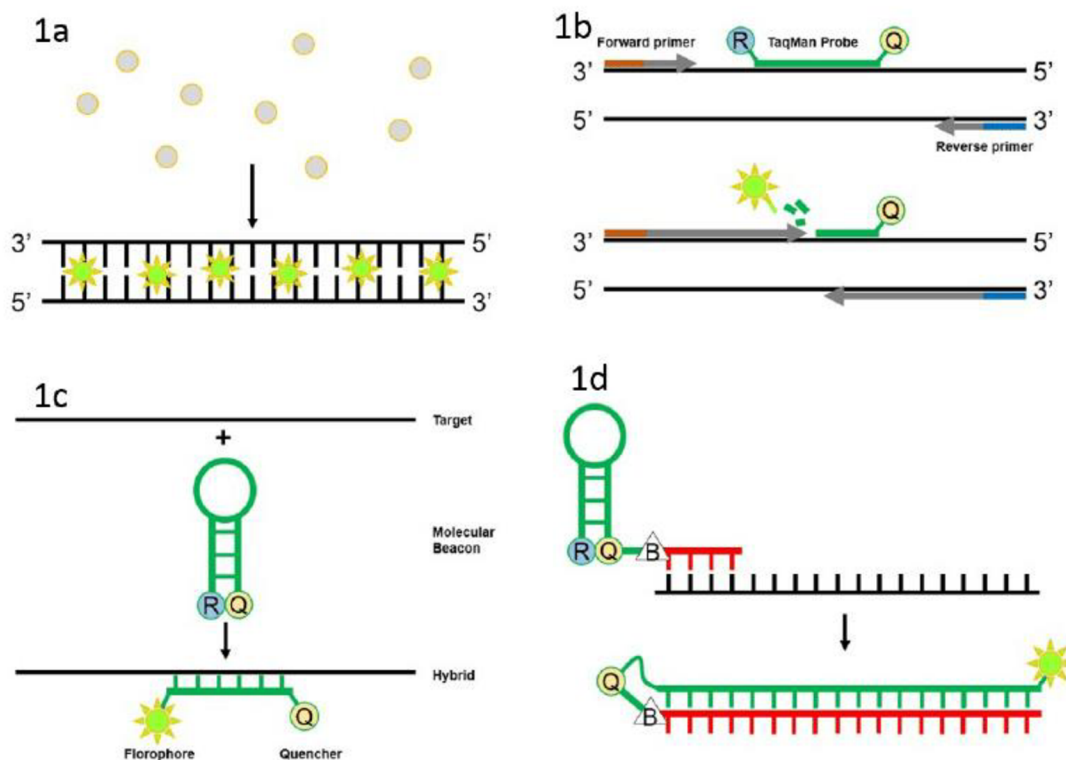

Figure 3: Mechanism of funtion for selected amplicon detection chemistries (1a = intercalating dye, 1b = TaqMan probe, 1c = molecular beacon, 1d = Scorpion technology). R = reporter, Q = quencher [62]

Probe methods can be divided into two groups according to the mechanism utilized: Hydrolysis probes and hybridization probes [5, 52]. Hydrolysis probes (i. e. 5'-nuclease probes) have a fluorophore linked to their 5'-end and a quencher linked to their 3'-end. An intact probe does not emit fluorescence, because the fluorophore and the quencher are in close proximity. A hydrolysis probe anneals to a complementary sequence within an amplicon. When primer extension takes place, the probe is hydrolysed through 5'-exonuclease activity of DNA polymerase (hence the term 5'-nuclease probes). After the hydrolysis of the probe the fluorophore and the quencher are separated, and fluorescent signal is released. The most common hydrolysis probes are the TaqMan® probes [5, 52].

Unlike hydrolysis probes, the hybridization probes stay intact throughout the whole PCR reaction. Some hybridization probes make use of a fluorophore and a quencher. These moieties can be linked to the same oligonucleotide. When that is the case, the unhybridized oligonucleotide forms a stem-loop structure, where the fluorophore and the quencher are close enough for FRET quenching. When the oligonucleotide hybridises to its target sequence, the stem-loop structure is extended/straightened, the fluorophore and the quencher are separated and fluorescent signal is emitted. The above-mentioned mechanism is employed by e. g. molecular beacons [5, 53].

Hybridization probes can also consist of two oligonucleotides, where one is linked to a fluorescent dye at the 3'-end (the donor probe) and the other is linked to a reporter dye at the 5'-end (the acceptor probe). When the donor and acceptor probes anneal to sequences which are in close proximity, the reporter dye of the acceptor probe absorbs energy from the donor probe and fluorescent signal is emitted. This mechanism is utilized by HybProbes [5, 52, 61].

The last type of hybridization probes described in this chapter are HyBeacons™. These probes consist of a single-stranded oligonucleotide, whose internal nucleotides are linked to fluorophores, and whose 3'-end is blocked either by a phosphate group or octanediol to prevent their extension during PCR. When the probe is hybridized to its target sequence, the fluorescent moieties bound to the probe's oligonucleotides emit stronger signal than when the probe is unbound in the solution [52, 63].

Several of the probes mentioned in this chapter also have modified versions. These modified probes have a minor groove binding (MGB) ligands (small molecule tripeptides) attached to their 3'-ends or 5'-ends. These ligands bind to AT-rich sequences through van der Waals interaction and stabilize the DNA structure [52, 53]. Apart from MGB ligands, nucleic acid analogues have also been used for probe synthesis. The mechanisms of action of these probes are the same as when conventional nucleic acids are used, but the analogues offer several advantages, such as higher stability and greater affinity for their target sequences [52].

2.4.3.3 Product quantification

Product quantification in real-time PCR utilizes threshold cycle (C_t) values detected during exponential phase of amplification [64]. C_t value marks the number of a cycle during which the concentration of a target amplicon gets high enough for its fluorescent signal to exceed the background noise. There are two main strategies for real-time PCR product quantification: Absolute quantification, which requires a calibration (standard) curve, and relative quantification, during which a comparison is made between the expression of a target gene and a reference gene [64, 65].

To construct a calibration curve for absolute quantification of a PCR product, a standard sample is needed. There may be several types of s standard, e. g. genomic DNA, recombinant plasmid DNA, or synthetic single-stranded oligonucleotides [66, 67]. Often, a series of

standards is prepared by 10-fold serial dilution, and the constructed calibration curve spans several (up to nine) orders of magnitude [65, 67]. To form a standard curve, the Ct values of standard samples are plotted against logarithms of their concentrations, and linear regression is performed [65]. For a reliable standard curve, a PCR efficiency of 80 – 115 % is needed and should be the same for both the unknown and standard samples. A quick way to check PCR efficiency was suggested by Zhang and Fang: A standard curve should have a slope of −3.0 - −3.9 [68].

Relative quantification is used when a change of expression of a target gene in relation to a reference gene is studied. The difference in expression between the target and reference gene may be calculated by several mathematical models [67]. Both relative and absolute quantification can be affected by various factors, such as sample and primer and probe storage, nucleic acid quality, or primer design [68, 69]. For easier replication of experiments and for easier design of reliable assays Bustin et al. designed a set of guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments, MIQE), which should be kept in mind if accurate data are to be produced [69].

2.5   Multiplex PCR

Multiplex polymerase chain reaction is a modification of PCR in which two or more targets are amplified simultaneously using two or more primer pairs [70]. Because multiplex PCR requires more complex reaction mixture than singleplex PCR, various problems may be encountered during a multiplex PCR assay development. Wei et al. even describe this process as "tedious and time consuming" [71].

Among the parameters which should be paid attention to are e. g. concentrations of PCR components such as DNA polymerase, dNTPs or magnesium ions, concentration of template DNA, and primer annealing temperature [70]. However, the "most crucial and critical step" during the development of a multiplex PCR assay is primer design [72], because primer sequences are directly linked to various problems which may be encountered during multiplex PCR assay design and use, such as formation of primer dimers or secondary structures [72], poor assay sensitivity or amplification of spurious products [73].

2.5.1   Primer selection

Primers for multiplex PCR should satisfy similar criteria as primers for singleplex PCR [73], such as their length, which should be 18–24 bp or longer, their GC content (30–60 %) and melting temperatures (55–58 °C), and if an end-point multiplex PCR assay is the method of choice, separability of the primers' amplicons by agarose gel electrophoresis should be possible [74].

In addition to the criteria mentioned above, attention should also be paid to PCR efficiencies, which should be similar for each primer pair [73], and also to the primers' complementarity to certain sequences: There should be no mismatches between the primers and their target sequences [72], while at the same time there should be no complementary sequences both within one oligonucleotide (risk of hairpin formation) and between two different oligonucleotides (possibility of primer dimer formation) [73]. Last, but not the least criterion is sufficient primer specificity, which should be verified using both in silico and in vitro approaches [72].

2.5.2   Multiplex PCR variations

Multiplex PCR assays can be divided into two categories according to the method used for amplicon detection: Multiplex end-point PCR and multiplex real-time PCR. The end-point variation of multiplex PCR makes use of amplicon detection by agarose gel electrophoresis. Even though it does not offer quantitative information (unlike the real-time variation) [72], it

may still be used in food safety and authenticity control, as evidenced by several recent publications (detection of *Vibrio vulnificus* in seafood, Roig et al. [75]; detection of adulteration in octopus products, Lee et al. [76]; simultaneous detection of three contaminating mold species in food, Rahman et al. [77] and others).

When compared to multiplex end-point PCR, the real-time multiplex PCR assays offer the same advantages as a real-time singleplex PCR assay offers over its conventional counterpart, such as quantitative information or lower risk of contamination (real-time PCR assays are single-tube methods) [52]. Multiplex PCR assays using both intercalating dyes (e. g. Zhong et al. [78], Sakalar and Abasiyanik [79], Gangwar et al. [80] and others) and specific probes (e. g. Dolch and Brügemann [81], Nakano [82], Kowada et al. [83] and others) for amplicon detection had been developed. Additionally, a multiplex PCR assay may be coupled to high resolution melting analysis (HRM), providing even more information about the amplified products (Rozej-Bielicka et al. [84], Mader et al. [85] and others).

2.6   High resolution melting analysis

High resolution melting analysis is an easy to use, low-cost and non-destructive analytical method. It is based on the same principles as the classical melting curve analysis used after real-time PCR, but it requires saturation dyes and instrumentation capable of more precise temperature measurements than in case of classical melting curve analysis [86]. This chapter focuses on the principles of HRM and on interpretation of its results.

2.6.1   Principle

High resolution melting analysis utilizes the capability of double-stranded DNA to dissociate with increase of temperature [87]. The melting behaviour of a double-stranded DNA fragment (amplicon) depends on two factors – the length of the amplicon and its content of guanine-cytosine (GC) pairs [38, 88]. Farrar and Wittwer [89] also mention the influence of the amplicon sequence on its melting behaviour. To perform a HRM analysis, a PCR amplification of the desired target in the presence of a saturating dye must first take place [88].

The outputs of HRM analysis are melting curves, which describe melting behaviour of the analysed amplicons. Melting curves are obtained when fluorescence intensity of the sample is plotted against temperature [38, 88]. They have pre-melt, melt and post-melt regions [38]. In the pre-melt and post-melt regions the concentration of double-stranded DNA is "effectively constant" and only small changes in the intensity of fluorescent signal take place. However, in the melt phase a sharp decrease (i. e. transition) in the intensity of fluorescent signal can be observed (Figure 4).

The location and slope of the fluorescence transition is a characteristic feature of a melting curve [90]. If a negative derivative of fluorescence intensity over temperature is plotted against temperature, the fluorescence transition in the melt region is represented by a peak. The temperature at which the negative derivative of fluorescence over temperature reaches a maximum (the top of the peak) describes the amplicon's melting point [91]. When a sample of double stranded DNA is at its melting point, half of the double strands of the sample are denatured [38]. While it is often possible to differentiate between samples simply based on their amplicon melting temperatures, this is not the case all the time [92].

20
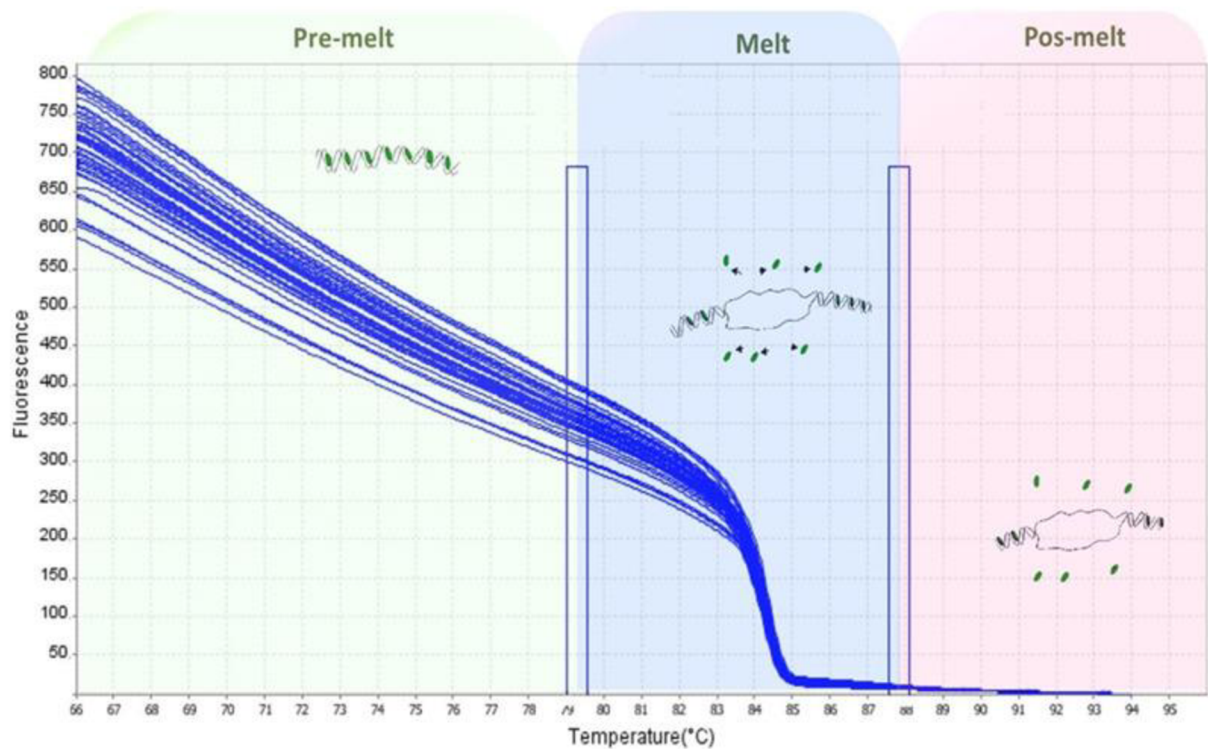
Figure 4: Raw melting curves with pre-melt, melt and post-melt regions highlighted [38]

Amplicons with a high content of GC pairs have higher melting temperatures than amplicons with low content of GC pairs. The reason for this is the way base pairs in double stranded DNA are linked together. GC pairs are bound by three hydrogen bonds, which makes them more stable than adenine-thymine (AT) pairs [38, 88]. Short amplicons melt with one fluorescence transition, while longer amplicons may melt in several stages, especially if they contain both regions rich in GC pairs and regions rich in AT pairs. In such cases a single T_m value for the amplicon cannot be defined [92].

2.6.2   Melting curve analysis

Because the absolute intensity of fluorescence may vary between samples, or even between replicates of one sample, a correction of raw melting curves is needed to ensure better data readability. This correction is achieved through normalization of the raw curves [38, 89]. A short part of the pre-melt region is used for the definition of 100% level of fluorescence for each sample. For 0% level of fluorescence of each sample, a short section of the post-melt region is selected [38]. The normalization of melting curves removes the differences in fluorescence intensity, making the differences in melting curve shapes easier to distinguish (Figure 5) [89].

If melting curves which represent amplicons with different sequences cannot be separated even after melting curve normalization, further data correction may be performed. One possible way of doing this is melting curve overlay [89] (i. e. temperature normalization [38] or temperature shift [88]), during which normalized melting curves are superimposed over each other in a high temperature region. While temperature shifting highlights some difference in melting curve shapes, other information may be lost [38, 89].
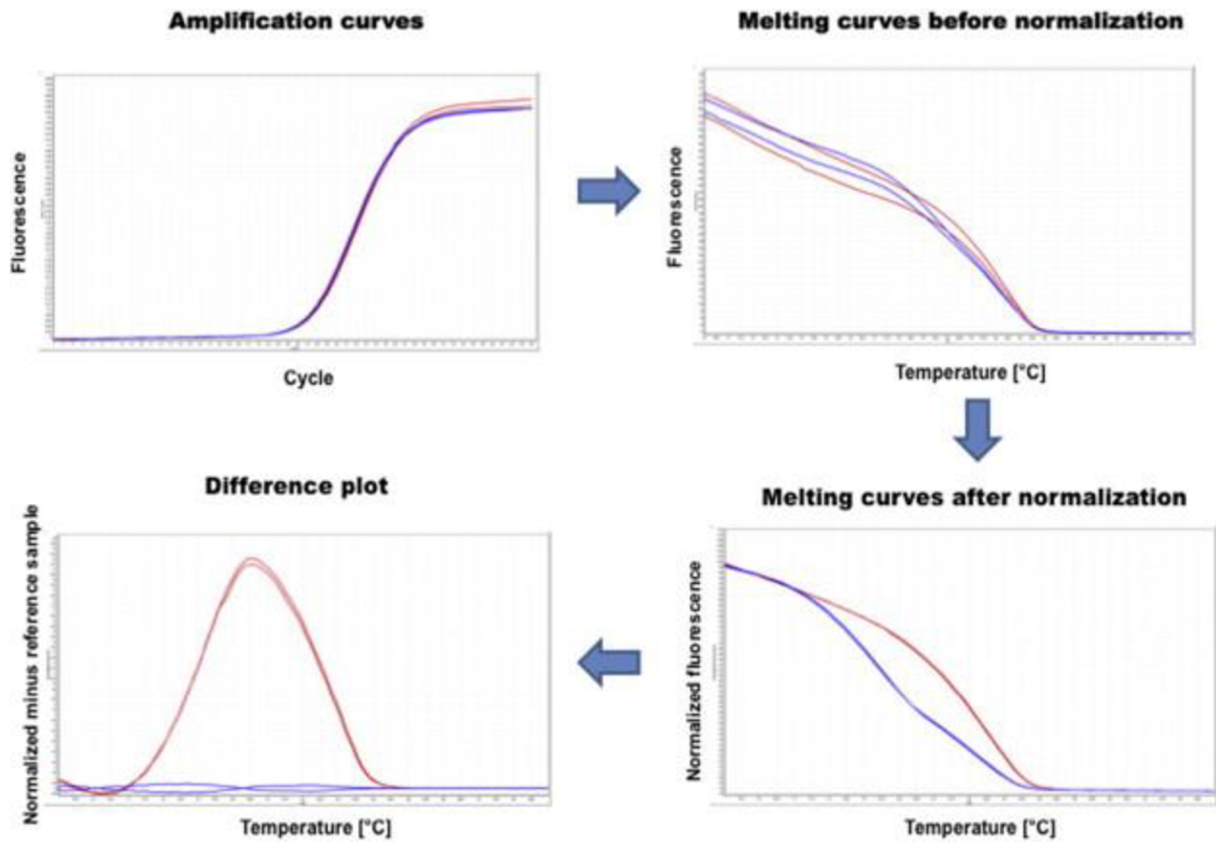
Figure 5: Amplification curves, raw and normalized melting curves, difference plots [88]

Other way of highlighting even small variations in shapes of melting curves are difference plots. These are obtained when the fluorescence data of a reference sample are subtracted from an unknown sample [38, 88]. The reference sample is then considered a baseline, and the melting curves which represent this sample are clustered around the horizontal axis [38, 89].

3   AIMS OF THIS WORK

- Literary review: Methods for isolation and analysis of DNA from processed plant-based foods
- Optimization of DNA isolation methods from various types of plant-based foods, determination of concentration and purity
- Use of various methods of molecular biology for analysis of DNA isolates from plant-based foods
- Discussion of results

4 EXPERIMENTAL PART

4.1 Fruit species

- Red raspberry (*Rubus idaeus*)
- Blueberry (*Vaccinium corymbosum*)
- Strawberry (*Fragaria ananassa*)
- Mango (*Mangifera indica*)
- Banana (*Musa acuminata*)
- Apricot (*Prunus armeniaca*)
- Peach (*Prunus persica*)
- Plum (*Prunus domestica*)

4.2 Commercial samples

All commercial samples were obtained from local distributors. In total, eleven products were analysed in this work. Five of them were teas with various compositions, two of which were intended for children. The remaining six samples consisted of one plant-based protein smoothie, three fruit purees sold as infant food, and two fruit bars, also sold as infant food. The teas and fruit bars were stored at room temperature until analysis, while the purees and smoothie were lyophilized and stored at −20 °C until analysis.

Table 1: Commercial products analysed in this work – Part 1

Sample	Composition as declared by manufacturer
dmBio fruit tea black currant and blueberries	Rosehip peels 32 %, hibiscus blossom 26 %, apples, orange peel, black currant 7 %, blueberries 6 %, elder 3 %, raspberries 2 %
Apotheke Bio tea, Forest blend with raspberry	Rosehips, hibiscus blossoms, seaberry fruit, blackberry leaves 10 %, natural aroma, strawberry leaves 5 %, mint leaves, liquorice root, raspberry fruit 2 %, blueberry fruit 1 %
Apotheke Bio children's herbal tea for imunity, with strawberry	Rosehip 25 %, seaberry fruit 10 %, hibiscus blossom, chamomile blossom, blackberry leaves, strawberry leaves, mint leaves, black currant fruit, liquorice root, marigold blossom
Leros herbal tea "Strong eyes" with blueberry and marigold	Blueberry fruit 20 %, chokeberry fruit 20 %, coriander fruit 20 %, carrot root 20 %, mallow blossom 10 %, marigold blossom 5 %, elder blossom 5 %
Majestic Tea herbal tea raspberry and camu camu	Hibiscus blossom, apple fruit, blackberry leaves, chokeberry fruit, aroma, cinnamon bark, raspberry fruit 2 %, camu camu extract 1 %, elderberries, orange pericarp

Table 2: Commercial products analysed in this work – Part 2

Sample	Composition as declared by manufacturer
Babylove bio baby food, strawberry and blueberry	Apple puree 76 %, strawberry puree 19 %, blueberry puree 5 %, antioxidant: ascorbic acid
Babylove bio baby food apple, strawberries and raspberries	Apples 83 %, strawberries 10 %, raspberries 5 %, blueberries 2 %
Relax 100% puree, raspberry	Apple puree 37 %, carrot puree 32 %, reconstituted apple juice 14 %, banana puree 10 %, raspberries 4 %, strawberries 2 %, reconstituted blueberry juice 1 %, aromas
dmBio Smoothie with protein, bananas, grapes and blueberries	Banana puree 27.5 %, white grape juice 22.75 %, blueberry juice 21.08 %, apple juice 17.25 %, raspberry juice 6 %, lemon juice 3 %, almond protein flour 2.4 %, dried vanilla 0.02 %
Hipp bio fruit bar apple banana and raspberries	Banana flakes 29 %, low acidity concentrated apple juice 29 %, wholegrain oat flour, ground wheat flakes, sunflower oil, wafers (wheat flour, starch, concentrated carrot juice), concentrated raspberry juice 3 %, concentrated chokeberry juice 2 %
dmBio fruit bar banana and blueberry	Banana flakes 33 %, wholegrain oat flour 26 %, concentrated white grape juice, concentrated pear juice, palm fat, concentrated blueberry juice 4 %, wafers (wheat flour, potato starch)

4.3   DNA isolation kits

- EliGene® Plant DNA Isolation Kit (Elisabeth Pharmacon, Czech Republic)
- DNeasy PowerPlant Pro Kit (Qiagen, Germany)
- ChargeSwitch™ gDNA Plant Kit (Invitrogen, USA)
- Chemagic DNA Plant kit (Perkin-Elmer, USA)
- EasyPrep Polyphenol Plant DNA Extraction Kit (Biotools Co., Taiwan)

4.4   Other chemicals

- Cetyl-trimethylammonium bromide (CTAB) (Sigma-Aldrich, Germany, molecular biology grade)
- Polyvinylpyrrolidone (PVP) (Sigma-Aldrich, Germany, molecular biology grade)
- Mercaptoethanol (Sigma-Aldrich, Germany, molecular biology grade)
- EDTA (Sigma-Aldrich, Germany, analytical grade)
- Proteinase K (Sigma-Aldrich, Germany, molecular biology grade)
- RNAse A (Serva, Germany, molecular biology grade)
- Tris-base (Serva, Germany, analytical grade)
- Pectinase (Sigma-Aldrich, Germany, suitable for plant cell cultures)
- Glycogen (Thermo Scientific, USA, molecular biology grade)
- PCR water (TopBio, Czech Republic)
- SYTO9 MasterMix (TopBio, Czech Republic)
- Oligonucleotide primers (Generi Biotech, Czech Republic)

- Agarose (Sigma-Aldrich, Germany, molecular biology grade)
- Midori Green gel dye (Nippon Genetics, Japan)
- Red Load loading dye (Top Bio, Czech Republic)
- 100 bp DNA ladder H3 RTU (Nippon Genetics, Japan)
- FastGene 50 bp DNA ladder (Nippon Genetics, Japan)

Other chemicals were of p. a. purity and were purchased from local distributors.

4.5 Laboratory equipment
- Freeze dryer FreeZone Triad (Labconco, USA)
- Incubator (Labnet, USA)
- Centrifuge Hermle Z216MK (Hermle Labortechnik GmbH, Germany)
- Magnetic rack (Thermo Fisher Scientific, USA)
- Spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific, USA)
- PCR box (Biosan, Latvia)
- Real-time PCR cycler RotorGene 6000 (Qiagen, Germany)
- Real-time PCR cycler LightCycler Nano (Roche Holding AG, Switzerland)
- Electrophoretic apparatus (Thermo Fisher Scientific, USA)
- Azure c200 gel imaging system (Azure Biosystems, Inc., USA)

4.6 Sequences of primers used in this work
All primer sequences used in this work were retrieved from literature. In case of species-specific primers, their specifity was verified before any experiments by the Primer-BLAST software (NCBI). The names, sequences, specifity and literary sources of all primers used in this work are summarised in Table 3 and Table 4

Table 3: Names and sequences of primers used in this work. AL = amplicon length, bp = base pairs

Name	Sequence	Specifity	AL (bp)	Ref.
S2F	ATGCGATACTTGGTGTGAAT	Plant ITS2 region	App. 500	93
S3R	GACGCTTCTCCAGACTACAAT			
VcBHLH003-F	AAATGGATTTGCTGTTATGGGTG	Blueberry	226	94
VcBHLH003-R	GGAATCATTAGGGAAACTGGGTA			
RiACO1-F	AATTGTTTGGAGCAGAGATTCAAGG	Raspberry	177	95
RiACO1-R	AAACTCCTTCATCACCTTCCTGTAG			
GAST1-F	GACAACATGCTTCACTTCTTGC	Strawberry	212	96
GAST1-R	TTAAGGACACTTGCGTTTGC			
APX1B-F	CTGGAGTTGTTGCTGTTGAGG		680	
APX1B-R	CTTTCCAGCATCAGGAAGACG			
APX1D-F	GAACGGTCTGGGTTTGAAGG		139	
APX1D-R	AGACAGGGTCTGACAGAAGAGC			
DFR1A-F	GGCTCGTCATGAGACTCCTC		100	
DFR1A-R	TGTGGTAACTCCAGCAGATGTC			

Table 4: Names and sequences of primers used in this work - continued. AL = amplicon length, bp = base pairs

Name	Sequence	Specifity	AL (bp)	Ref.
Prupe4053-F	ACCCACAACAAGAACAACAGTCC	Peach	171	97
Prupe4053-R	CCCTCGTGTAAAAATTCATCCAC			
AGS6-F	GAGTGGCCGATACCTGTTCT	Apricot	222	96
AGS6-R	AATGATGGGTTTTGGGTGTG			
Mito-Foc-S-001-F	CTCGCCGACACCTTACTTGAT	Banana	277	98
Mito-Foc-S-001-R	GGGGTCTCGTTGCTTGTCTC			
FT MDP-F	GCCAGCGAGGTTTCAACTTCTT	Apple	128	99
FT MDP-R	TGCCGCAGTAGTTGCTGGAATA			
PAL-F	TGGATTCAAGGGTGCTGAAATCGC	Mango	113	100
PAL-R	TCACATCTTGGTTGTGTTGCTCGG			
PdCass-F	CCGTGAGGGCAGAGAGGGGG	Plum	236	101
PdCass-R	GCTCCCAAAAGGCCTCGTGCT			
Pear-F	GACCTGCCAATGTTAATGC	Pear	115	102
Pear-R	CAGCAGTACTTCGAATCAC			

4.7 DNA isolation

4.7.1 EliGene® Plant DNA Isolation Kit

4.7.1.1 Unmodified manufacturer's protocol

Firstly, 0.2 g of homogenization sand and 0.05 g of plant material was added to a 1.5 ml tube. Then, 450 µl of homogenization buffer P1 was added to the tube and the plant material was homogenized using a homogenization pestle. After homogenization 50 µl of lysis buffer P2 was added. The sample was briefly vortexed and incubated at 70 °C for 10 minutes with occasional mixing. Following the incubation, the sample was centrifuged at 10 000×g for 3 minutes. The supernatant was transferred to a clean 1.5 ml tube and 175 ml of inhibitor removal buffer P3 was added. The sample was briefly vortexed and incubated on ice for 3 minutes. This step was followed by centrifugation at 10 000×g for 3 minutes. The supernatant (approximately 500 µl) was transferred to a clean 2 ml tube. 500 µl of binding buffer P4 was added, the sample was vortexed and shortly spinned. Then, 500 µl of binding buffer P5 was added and the sample was vortexed and shortly spinned again. Following the addition of binding buffers 750 µl of the sample was transferred onto a spin filter and centrifuged at 10 000×g for 1 minute. The supernatant was discarded and the rest of the sample was added to the same spin filter. The spin filter was centrifuged as in previous step and the supernatant was once again discarded. 500 µl of wash buffer P6 was added to the spin filter. The filter was centrifuged at 10 000×g for 1 minute. The flowthrough was discarded and 500 µl of wash buffer P7 was added. The filter was again centrifuged at 10 000×g for 1 minute and the flowthrough was discarded. The spin filter was then dried by centrifugation at 12 000×g for 2 minutes. After the drying step 100 µl of elution buffer P8. The spin filter was centrifuged at 10 000×g for 1 minute and the eluate containing the isolated DNA was transferred to a 1.5 ml tube and stored at 5 °C until further use.

4.7.1.2 Protocol with pectinase digestion of polysaccharides

Sample homogenization and cell lysis steps were performed the same way as in chapter 13. After the centrifugation of the sample after cell lysis the supernatant was transferred to a clean 1.5 ml tube and 12.8 µl of pectinase was added. The sample was briefly vortexed and incubated for 2 hours at 35 °C These conditions were selected based on the findings of Krall

and McFeeters [103]. After the incubation, 175 µl of inhibitor removal buffer P3 was added and the kit manufacturer's protocol was followed.

4.7.1.3   Protocol with calcium chloride precipitation of polysaccharides

Sample homogenization and cell lysis steps were performed as described in chapter 13. After the centrifugation of the sample after incubation at 70 °C the supernatant was transferred to a clean 1.5 ml tube and 100 µl of 300 mM calcium chloride ($CaCl_2$) was added. The sample was briefly vortexed and incubated for 15 minutes at room temperature. Afterwards, the sample was centrifuged at 10 000×g for 3 minutes. The supernatant was transferred to a clean 1.5 ml tube, 175 µl of inhibitor removal buffer P3 was added and the DNA isolation protocol was continued as described in chapter 13.

4.7.1.4   Protocol with calcium chloride precipitation of polysaccharides and additional purification of DNA isolates by a clean-up kit

All steps from sample homogenization to the elution of DNA from spin columns were performed as described in chapter 4.7.1.3. The eluted DNA was then purified by High Pure PCR Product Purification Kit (Roche) according to manufacturer's instructions: 500 µl of Binding Buffer was added to the isolated DNA and the sample was thoroughly mixed by inversion. The sample was transferred to the upper reservoir of a High Pure Filter Tube, which contained a spin column. This step was followed by centrifugation at 15 000×g for 60 seconds at room temperature. The supernatant was discarded and 500 µl of Wash Buffer was added onto the spin column. The sample was once again centrifuged at 15 000×g for 60 second at room temperature. The supernatant was discarded and the washing steps were repeated with 200 µl of wash buffer. The supernatant was discarded and the tube with spin column was placed into a clean 1.5 ml microcentrifuge tube. 100 µl of Elution Buffer was added onto the spin column and the sample was centrifuged at 15 000×g for 60 seconds at room temperature. The spin column was removed and the supernatant containing eluted DNA was centrifuged at 15 000×g for 70 seconds to remove any residual spin column glass fibres from the eluate. 10 µl of 3M sodium acetate, 5 µl of glycogen solution (20 µl·µl⁻¹) and 250 µl of ethanol was added to the eluted DNA. The sample was mixed and incubated at −20 °C for 15 minutes. The incubation was followed by centrifugation at 10 000×g for 15 minutes at room temperature. The supernatant was discarded and the pellet was air-dried at room temperature. After drying, the pellet was dissolved in 50 µl of buffer P8 from the EliGene Plant DNA Isolation Kit.

4.7.2   DNeasy PowerPlant Pro Kit

The DNA isolation was performed according to the manufacturer's protocol. 50 mg of plant material and 410 µl of Bead Solution and 40 µl of Phenolic Separation Solution was added to a 2 ml tube containing metal beads. The sample was then homogenized on a flat bed vortex at maximum speed for 10 minutes. Following the homogenization, the tube containing the sample was centrifuged at 13 000×g for 2 minutes. The supernatant was transferred into a clean 2 ml tube. 250 µl of IR solution was added to the supernatant. The sample was vortexed for 5 seconds and incubated at 5 °C for 5 minutes. The incubation was followed by centrifugation at 13 000×g for 2 minutes. The supernatant was transferred to a clean 2 ml tube and 600 µl of PB solution was added to it. The sample was vortexed for 5 seconds. 600 µl of the sample was then loaded onto a MB Spin Column. The column was centrifuged at 10 000×g for 30 seconds. The flowthrough was discarded, and the rest of the sample was loaded onto the same column. The column was again centrifuged at 10 000×g for 30 seconds. The flowthrough was again discarded. After all the sample was passed through the column, 500 µl of CB solution was loaded onto the spin column. The column was centrifuged at 10 000×g for 30 seconds and the flowthrough was discarded. 500 µl of ethanol was added to the spin column and the column was again centrifuged at 10 000×g for 30 seconds. The flowthrough

was again discarded. The empty spin column was centrifuged at 15 000×g for 2 minutes. After this centrifugation, the column was placed into a clean 2 ml collection tube, 100 µl of EB solution was loaded on it and the column was incubated for 2 minutes at room temperature. This step was followed by centrifugation at 10 000×g for 30 seconds. The column was discarded and the flowthrough containing elution buffer was stored at 5 °C until analysis.

4.7.3   ChargeSwitch™ gDNA Plant Kit

The DNA isolation was performed according to the manufacturer's protocol. Because the samples used were various fruits which are rich in polysaccharides and phenolic compounds [104], the manufacturer's recommendation of the use of Reagent A (chapter 15) was followed.

4.7.3.1   Preparation of reagent A

To prepare reagent A, 0.441 g of calcium chloride and 1.5 g of polyvinylpyrrolidone was added to 10 ml of ChargeSwitch® Lysis Buffer L18. The reagent was then thoroughly mixed by vortexing. The final concentration of calcium chloride in reagent A was 300 mM, and the final concentration of polyvinylpyrrolidone in reagent A was 15 %.

4.7.3.2   DNA isolation with ChargeSwitch^{TM} gDNA Plant Kit

The fruit used for DNA isolation was cut into small pieces. 900 µl of ChargeSwitch® Lysis Buffer L18 and 100 µl of Reagent A was added to the sample. RNAse A (2 µl) was also added. The sample was then homogenized by vortexing until it was completely resuspended. These steps produced a lysate. To this lysate, 100 µl of 10% SDS was added. The lysate was incubated at room temperature for 5 minutes. After the incubation 400 µl of ChargeSwitch® Precipitation Buffer N5 (previously chilled on ice) was added. The sample was vortexed 10 seconds until precipitate formed. This step was followed by centrifugation at 15 000×g for 5 minutes. The supernatant was transferred to a clean 1.5 ml tube and 100 µl of ChargeSwitch® Detergent D1 was added to it. 40 µl of resuspended ChargeSwitch® Magnetic Beads were also added at this stage. The sample was mixed by pipetting and incubated at room temperature for 1 minute. After the incubation the sample was placed into a magnetic rack until the magnetic beads formed a tight pellet. The supernatant was aspirated and the 1.5 ml tube was removed from the magnetic rack. 1 ml of ChargeSwitch® Wash Buffer (W12) was added and the magnetic beads with captured DNA were resuspended by pipetting. Afterwards, the tubes were again placed into a magnetic rack until the magnetic beads formed a tight pellet. The supernatant was aspirated, and the washing step was repeated one more time. After the second washing step the tube was placed into a magnetic rack until the magnetic beads formed a tight pellet. All the supernatant (washing buffer) was aspirated and 100 µl of ChargeSwitch® Elution Buffer E6 was added. The magnetic beads were resuspended by pipetting and the sample was incubated for 1 minute at room temperature. The tube was then placed into a magnetic rack until the magnetic beads formed a tight pellet. The supernatant, which now contained eluted DNA, was transferred to a clean 1.5 ml tube with a pipette, and stored at 5 °C until analysis.

4.7.4   Chemagic DNA Plant kit

The DNA isolation was performed according to the manufacturer's protocol. The fruits used for DNA isolation were ground with liquid nitrogen using mortar and pestle. 50 mg of the ground sample was added to a clean 2 ml tube. 400 µl of Lysis Buffer 1 and 2 µl of RNAse A (100 mg·ml^{-1}) were also added. The sample was thoroughly mixed by vortexing. This step was followed by centrifugation at 12 000×g for 5 minutes. The supernatant was transferred into a clean 1.5 ml tube and 30 µl and 320 µl of Binding Buffer 2 were added to it. The sample was mixed and incubated at room temperature for 5 minutes. After the incubation the tube containing the sample was placed into a magnetic rack for 2 minutes until the magnetic beads

formed a pellet. The supernatant was then aspirated and the tube was removed from the rack. 900 µl of Wash Buffer 3 was added to the sample and the magnetic beads were resuspended by pipetting. The tube was again placed into a magnetic rack for 2 minutes. The supernatant was aspirated and the washing step was first repeated with 900 µl of Wash buffer 4, and second time with 900 µl of 70% ethanol. After the separation of the magnetic beads from 70% ethanol and the aspiration of the ethanol, 1 ml of Wash Buffer 5 was added while the tube was kept in a magnetic rack. The sample was incubated in the magnetic rack for 1 minute. After the incubation the Wash Buffer 5 was pipetted off. The tube was removed from the magnetic rack and the magnetic beads were resuspended in 100 µl of Elution Buffer 6. The suspension was incubated for 10 minutes at 55 °C. The tube was placed into a magnetic rack for 1 minute until the magnetic beads were separated. The eluate, which now contained the isolated DNA, was then pipetted off into a clean 1.5 ml tube and stored at 5 °C until analysis.

4.7.5   EasyPrep Polyphenol Plant DNA Extraction Kit
The DNA isolation was performed according to the manufacturer's protocol. The fruits used for DNA isolation were ground in liquid nitrogen using mortar and pestle. 50 mg of the ground sample was used for DNA isolation. 700 µl of DGP1 (preheated to 65 °C and with β-mercaptoethanol added before use) was added to the ground sample. The sample was mixed by vortexing until all clumps were dispersed. This step was followed by incubation at 65 °C for 20 minutes with occasional mixing by inversion. After the incubation 700 µl of chloroform was added, the sample was mixed by inverting the tube and centrifuged for 5 minutes at 13 500×g. The supernatant was transferred into a clean 2 ml tube and 700 µl of buffer DGP2 was added. The sample was again mixed by inverting the tube several times. 700 µl of the sample from previous step was loaded onto a Spin Column CB3 and centrifuged at 13 500×g. The flowthrough was discarded and the previous two steps were repeated until all of the sample was passed through the spin column. 500 µl of Buffer DGD (with 96% ethanol added before use) was loaded onto the Spin Column CB3. The column was centrifuged at 13 500×g for 30 seconds and the flowthrough was discarded. 700 µl of Buffer DPW (with 96% ethanol added before use) was loaded onto the spin column. The column was centrifuged at 13 500×g for 30 seconds. The flowthrough was discarded and 500 µl of Buffer DPW was loaded onto the column. The column was again centrifuged at 13 500×g for 30 seconds and the flowthrough was discarded. The spin column was then centrifuged at 13 500×g for 2 minutes to remove any residual Buffer DPW. After the centrifugation the spin column was incubated at room temperature for 5 minutes with open lid (to completely dry the column). 100 µl of DTE buffer was added to the dry spin column. This step was followed by incubation at room temperature for 5 minutes and centrifugation at 13 500×g. The flowthrough, which now contained eluted DNA was stored at 5 °C until analysis.

4.7.6   In-house CTAB protocol
The CTAB protocol used in this work is a modified version of protocol used by Trojánek et al. in their 2018 article [105].

4.7.6.1   Preparation of CTAB buffer
To prepare the CTAB buffer, 30 ml of 10% CTAB was added to 28 ml of 5M NaCl, 4 ml of 0.5M EDTA and 10 ml of 1M Tris-HCl. 3 g of polyvinylpyrrolidone was added and dissolved. The total volume of the buffer was filled up with distilled water to 100 ml [106].

4.7.6.2   DNA isolation by in-house CTAB protocol
The fruits used for DNA isolation by this method were homogenized in liquid nitrogen using mortar and pestle. 50 mg of homogenized tissue were added to a clean 2 ml tube. 1 ml of CTAB buffer and 2 µl of β-mercaptoethanol were added. The sample was briefly mixed by

vortexing and incubated at 65 °C for 30 minutes with occasional mixing by inversion. After the incubation 800 μl of the mixture chloroform:octanol (24:1) was added. The sample was mixed by inverting the tube several times. The mixed sample was then centrifuged at 10 000×g for 10 minutes. After the centrifugation the sample separated into two phases. The upper (aqueous) phase was transferred to a clean 2 ml tube and isopropanol was added (0.6 of the volume of the aqueous phase). The sample was mixed by inverting the tube several times and centrifuged at 10 000×g for 5 minutes. The supernatant was poured off. 500 μl of 10 mM ammonium acetate in 70% ethanol was added to the sediment and the sample was incubated at room temperature for 30 minutes. After the incubation the supernatant was poured off, the sediment was dried at 37 °C and resuspended in 250 μl of TE buffer (1 ml of 1M Tris-HCl and 0.2 ml of 0.5M EDTA, with distilled water added to a total volume of 100 ml).

250 μl of 5M sodium chloride, 25 μl of 3M sodium acetate and 1.25 ml of 96% ethanol was added. The sample was mixed by inverting the tube several times and incubated at −20 °C for 15 minutes. The sample was then centrifuged at 10 000×g for 15 minutes. The supernatant was poured off and the sediment was dried at 37 °C. The dried sediment was resuspended in 500 μl of TE buffer and 4 μl of RNAse A (10 mg·ml⁻¹) was added. The sample was incubated at 37 °C for 15 minutes. 4 μl of proteinase K (20 mg·ml⁻¹) was added and the sample was incubated at 37 °C for 1 hour. After the incubation, 200 μl of phenol and 200 μl of mixture chloroform:isoamylalcohol (24:1) was added. The sample was mixed by inverting the tube several times and centrifuged at 10 000×g for 2 minutes. The upper (aqueous) phase was transferred into a clean 2 ml tube and 700 μl of the mixture chloroform:isoamylalcohol (24:1) was added. The sample was mixed by inverting the tube several times and centrifuged at 10 000×g for 2 minutes. The upper (aqueous) phase was transferred into a clean 2 ml tube and the alcohol precipitation described at the beginning of this paragraph was repeated. The sediment was dried at 37 °C and resuspended in 100 μl of TE buffer. The isolated DNA was stored at 5 °C until analysis.

4.8   Concentration and purity measurements of DNA isolates

The concentration and purity of all DNA isolates was evaluated by UV-VIS spectrophotometry using the NanoDrop 2000. The volume of sample used was 2 μl. For DNA isolates obtained by isolation kits, elution buffers from the kits were used as blank samples. For the CTAB protocol, TE buffer was used as blank. The concentration of DNA isolates was determined based on their absorbance at 260 nm and the purity of DNA isolates was assessed based on the absorbance ratios of 260 nm/230 nm and 260 nm/280 nm.

4.9   Amplification of DNA isolates from fruits by real-time PCR

Real-time PCR mixtures were prepared the same way for every primer pair. The mixture composition for one sample is summarized in Table 5. The PCR profile used for the reaction can be found in

Table *6*. The concentration of primer stock solutions was 10 pmol·ml⁻¹ and the total volume of each PCR mixture was 25 μl.

Table 5: Composition of PCR mixture for primers S2F and S3R and all species specific primers used for amplification of DNA isolated from fruits

Component	Volume (μl)
PCR water	9.5
SYTO9 MasterMix	12.5
S2F primer	1
S3R primer	1
Template DNA	1

Table 6: PCR profiles for plant-specific PCR assay, and for species-specific assays

Primers	Temperature (°C)	Time		Ref.
S2F + S3F	95	5 minutes		93
	95	30 seconds	35x repeated	
	52	30 seconds		
	72	45 seconds		
	72	10 minutes		
VcBHLH003	95	5 minutes		94
	95	30 seconds	30x repeated	
	55	30 seconds		
	72	30 seconds		
	72	5 minutes		
RiACO1	95	10 minutes		107
	95	2 seconds	35x repeated	
	60	15 seconds		
	72	25 seconds		
	72	5 minutes		
GAST1, APX1B, APX1D, DFR1A, AGS6	94	2 minutes		96
	94	30 seconds	40x repeated	
	60 to 55 in 10 cycles with 0.5 °C step	45 seconds		
	72	60 seconds		
	72	5 minutes		
Prupe4053	95	30 seconds		97
	95	5 seconds	40x repeated	
	55	30 seconds		
	72	30 seconds		
	72	5 minutes		
Mito-Foc-S-001	94	5 minutes		98
	94	30 seconds	35x repeated	
	58	30 seconds		
	72	30 seconds		
	72	7 minutes		
FT MDP	94	2 minutes		99
	94	30 seconds	40x repeated	
	55	30 seconds		
	72	30 seconds		
	72	2 minutes		
PAL	95	5 minutes		100
	95	10 seconds	40x repeated	
	60	10 seconds		
	72	30 seconds		
	72	5 minutes		

Table 7: PCR profiles for assays specific for plum and pear

Primers	Temperature (°C)	Time		Ref.
PdCass	95	5 minutes		101
	95	10 seconds	35x repeated	
	58	10 seconds		
	72	30 seconds		
	72	5 minutes		
Pear	95	5 minutes		102
	95	15 seconds	40x repeated	
	55	30 seconds		
	72	30 seconds		
	72	5 minutes		

4.10 PCR assays for analysis of commercial foods

4.10.1 Singleplex PCR assays

The composition of PCR mixtures differed for each species-specific primer pair. The PCR mixtures were prepared according to *Table 8*. Total volume of the PCR mixtures was 25 µl. The concentration of primer stock solutions was 10 pmol·ml⁻¹. The reaction profile was the same for all three species-specific assays and the temperatures and durations of its steps can be found in *Table 9*.

Table 8: Composition of PCR mixtures for species-specific PCR assays

Primer pair	VcBHLH3	RiACO1	GAST1
Component	**Volume (µl)**	**Volume (µl)**	**Volume (µl)**
PCR water	9	9.75	7.5
SYTO9 MasterMix	12.5	12.5	12.5
Forward primer	0.75	0.375	1.5
Reverse primer	0.75	0.375	1.5
Template DNA	2	2	2

Table 9: PCR profile for assays with species-specific primers

Temperature (°C)	Time		Ref.
95	5 minutes		107
95	2 seconds	35x repeated	
53	15 seconds		
72	25 seconds		
72	5 minutes		

4.10.2 Triplex real-time PCR with species specific primers

The compositions of triplex PCR mixtures can be found in *Table 10*, Table 11 and *Table 12*. The composition in *Table 10* was used during the selection of a primer set which would be

appropriate for analysis of commercial plant-based products. The compositions in Table 11 were used during optimizations of primer concentration of the selected primer set.

Table 10: Composition of triplex PCR mixtures for selection of the most appropriate primer set

Component	Volume (µl)
PCR water	3.5
SYTO9 MasterMix	12.5
Forward primer – pair 1	1
Reverse primer – pair 1	1
Forward primer – pair 2	1
Reverse primer – pair 2	1
Forward primer – pair 3	1
Reverse primer – pair 3	1
Template DNA	3×1 µl

Table 11: Composition of triplex PCR mixtures for primer concentration optimization and method sensitivity determination. Concentration of primers in PCR reaction mixtures is also shown.

	Mixture 1	Mixture 2	Mixture 3	Mixture 4	Mixture 5
Component	**Volume (µl)**				
PCR water	5.0	4.5	4.0	3.75	4.25
SYTO9 MasterMix	12.5	12.5	12.5	12.5	12.5
Primer VcBHLH003-F	0.75 (300 nM)	0.75 (300 nM)	0.75 (300 nM)	0.875 (350 nM)	0.75 (300 nM)
Primer VcBHLH003-R	0.75 (300 nM)	0.75 (300 nM)	0.75 (300 nM)	0.875 (350 nM)	0.75 (300 nM)
Primer RiACO1-F	0.5 (200 nM)	0.5 (200 nM)	0.5 (200 nM)	0.5 (200 nM)	0.375 (150 nM)
Primer RiACO1-R	0.5 (200 nM)	0.5 (200 nM)	0.5 (200 nM)	0.5 (200 nM)	0.375 (150 nM)
Primer GAST1-F	1.0 (400 nM)	1.25 (500 nM)	1.5 (600 nM)	1.5 (600 nM)	1.5 (600 nM)
Primer GAST1 -R	1.0 (400 nM)	1.25 (500 nM)	1.5 (600 nM)	1.5 (600 nM)	1.5 (600 nM)
Template DNA	3.0	3.0	3.0	3.0	3.0
Total volume (µl)	25	25	25	25	25

Table 12: Composition of triplex PCR mixtures used for analysis of commercial foodstuffs

Component	Volume (µl)
PCR water	5.25
SYTO9 MasterMix	12.5
Primer Vc-BHLH003-F	0.75
Primer VcBHLH003-R	0.75
Primer RiACO1-F	0.375
Primer RiACO1-R	0.375
Primer GAST1-F	1.5
Primer GAST1-R	1.5
Template DNA	2

Table 12 shows composition of PCR mixtures used for triplex PCR assays with DNAs from commercial products. The composition in this table was chosen after optimization of concentration of all three primer pairs.

4.11 High resolution melting analysis

High resolution melting analysis (HRM) was performed in LightCycler Nano (Roche). The temperature ranged from 60 °C to 99 °C, with the temperature increasing by 0.01 °C increments. The raw data were analysed using the software LightCycler Nano SW 1.1.

4.12 Agarose gel electrophoresis

All gels in this work were prepared using 0.5x TBE buffer, which was prepared by tenfold dilution of 5x TBE stock solution (54 g of tris-base, 27.5 g of boric acid and 20 ml of 0.5M EDTA dissolved in distilled water, with total volume adjusted to 1 dm^3). Gels with two different agarose concentrations were used in this work. For electrophoresis of amplicons obtained in singleplex PCR assays 1.2% gel was used (1.2 g of agarose dissolved in 100 ml of 0.5x TBE buffer). To assess amplicon size a 100 bp ladder was used (Figure 6).
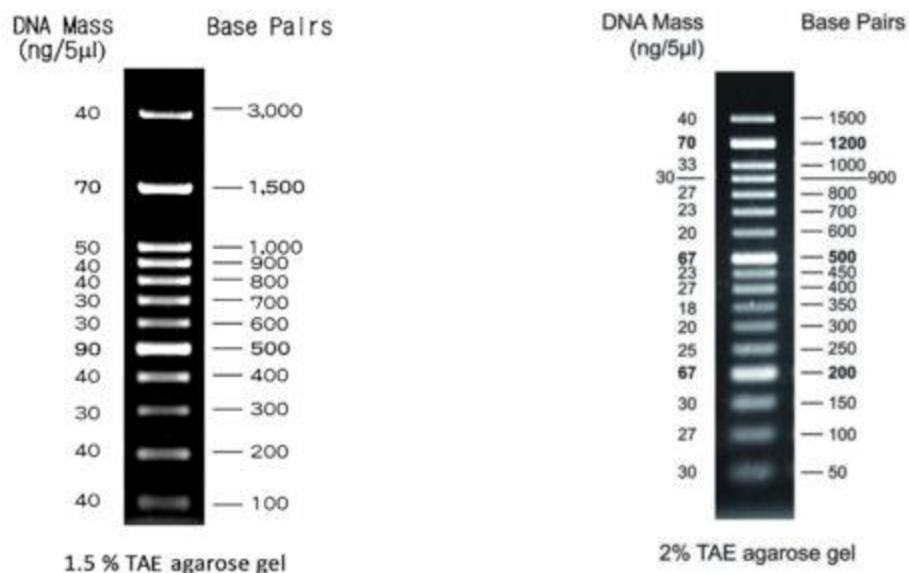

Figure 6: DNA markers used in this work – 50 bp ladder on the right [108], 100 bp ladder on the left [109]

For amplicons obtained in species-specific singleplex PCR assays and triplex PCR assays 1.5% gel was used (1.5 g of agarose dissolved in 100 ml of 0.5x TBE buffer) and for assessment of amplicon lengths a 50 bp ladder was used (Figure 6). Each sample was mixed with Red Load loading dye in a ratio dye:sample 1:5. 5 μl of samples mixed with loading dye were used for each run. Electrophoresis with 1.2% gel was run at 80 V for 1 hour 45 minutes, and electrophoresis with 1.5% gel was run at 60 V for 3 hours. Amplicons and ladders were visualised using the Midori Green intercalating dye according to the manufacturer's instructions (5 μl of dye per 100 ml of agarose). The amplicons were visualised using UV light with wavelength of 302 nm. For both visualisation and photographing of the gels the Azure c200 documenting system was used.

5   RESULTS AND DISCUSSION

5.1   Selection of DNA isolation method

In this section of experiments six DNA isolation methods were tested. Five were commercial kits. Out of these, three used silica columns (DNeasy PowerPlant Pro Kit, EliGene Plant DNA Isolation Kit, EasyPrep Polyphenol Plant DNA Extraction Kit) and two used magnetic carriers (Chemagic DNA Plant Kit and ChargeSwitch gDNA Plant Kit). The sixth DNA isolation method was an in-house CTAB protocol which used alcohol precipitation for DNA capture and purification.

All six DNA isolation methods were tested on ten fruit species commonly occurring in fruit purees and smoothies: Apple, pear, apricot, peach, plum, mango, banana, blueberry, raspberry and strawberry. Out of the six protocols mentioned above, one was chosen as the most suitable for DNA isolation from fruit-based foods based on the purity of DNA isolates and their behaviour in plant-specific and species-specific PCR assays. The results which are discussed in this part of the work were also published as an article [110].

5.1.1   DNA concentration and purity measurements

Concentration and purity of all DNA isolates was assessed by UV spectrophotometry using the NanoDrop 2000 spectrophotometer. Concentrations of DNA obtained by all isolation protocols are summarized in Figure 7, Figure 8 and Table 13. For better readability of both figures, the concentrations of DNA isolates obtained by CTAB protocol are divided by 10.

The isolates with highest concentration of DNA were obtained by the CTAB protocol. The concentration of these isolates ranged from 53.8 ng·µl^{-1} (DNA from pear) to 681.1 ng·µl^{-1} (DNA from raspberry). The concentrations of DNA isolates obtained by commercial kits were generally not higher than 20 ng·µl^{-1}, however, there were exceptions to this. In case of the Invitrogen kit the concentrations of apricot, blueberry and strawberry DNA were higher than 20 ng·µl^{-1}, and in case of the Qiagen kit concentrations of apricot, blueberry, pear, plum and raspberry DNA were higher than 20 ng·µl^{-1} (Table 13).
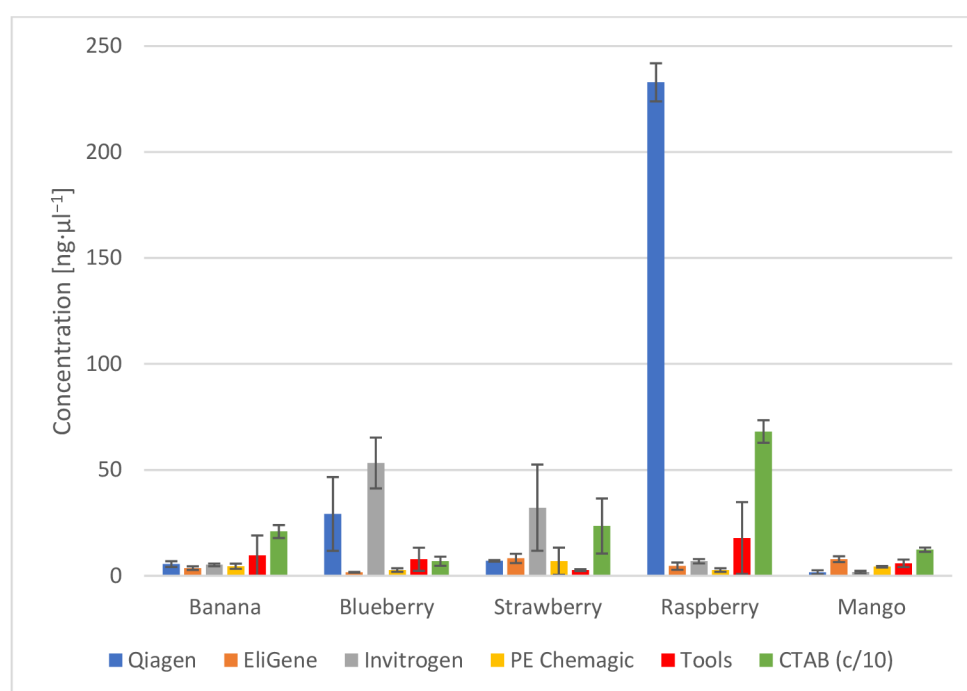

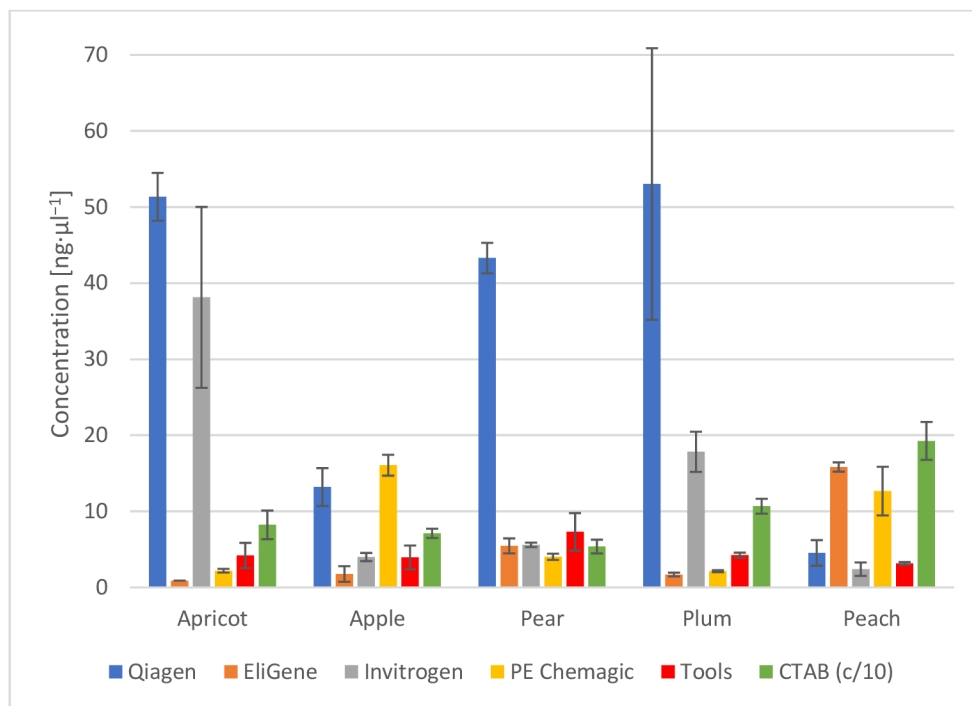*Figure 7: Concentrations of DNA from tropical fruits and berries*

Figure 8: Concentrations of DNA from pome fruits (apple and pear) and stone fruits (peach, apricot and plum)

If concentration of DNA was the only parameter considered, the CTAB protocol would seem the most appropriate method for DNA isolation from ten fruit species used, the Qiagen kit would be appropriate for DNA isolation from apricot, blueberry, pear, plum and raspberry, and the Invitrogen kit would be suitable to isolate DNA from apricot, blueberry and strawberry.

Table 13: Concentrations of DNA isolates from fruits

	Qiagen	EliGene	Invitrogen	PE Chemagic	Tools	CTAB
Banana	5.5 ± 1.4	3.6 ± 0.8	5.2 ± 0.6	4.5 ± 1.2	9.5 ± 9.5	208.8 ± 30.6
Apricot	51.4 ± 3.2	0.9 ± 0.0	38.1 ± 11.9	2.2 ± 0.2	4.2 ± 1.7	82.3 ± 18.8
Apple	13.2 ± 2.5	1.8 ± 1.0	4.0 ± 0.5	16.1 ± 1.4	3.9 ± 1.6	71.1 ± 6.1
Blueberry	29.2 ± 17.4	1.6 ± 0.2	53.2 ± 12.0	2.7 ± 0.8	7.8 ± 5.5	68.9 ± 21.6
Pear	43.3 ± 2.0	5.5 ±. 1.0	5.6 ± 0.3	4.0 ± 0.4	7.3 ± 2.5	53.8 ± 9.0
Plum	53.0 ± 17.9	1.7 ± 0.2	17.8 ± 2.6	2.1 ± 0.1	4.2 ± 0.3	106.8 ± 9.8
Strawberry	7.1 ± 0.4	8.2 ± 2.2	32.2 ± 20.3	6.9 ± 6.4	2.7 ± 0.5	234.9 ± 129.9
Raspberry	232.9 ± 9.0	4.6 ± 1.7	6.9 ± 1.0	2.7 ± 0.8	17.8 ± 17.0	681.1 ± 53.3
Peach	4.5 ± 1.7	15.8 ± 0.6	2.4 ± 0.9	12.7 ± 3.2	3.2 ± 0.2	192.6 ± 24.9
Mango	1.7 ± 1.0	7.8 ± 1.4	1.8 ± 0.5	4.3 ± 0.4	5.9 ± 1.8	123.2 ± 10.2

However, plant matrices contain substances (e. g. polysaccharides and phenolic compounds) which may be inhibitory to DNA based analytical methods [3]. This is a reason why DNA purity should also be considered, with some authors even considering sufficient DNA purity more important than high DNA concentration [111].

When purity of DNA was taken into consideration (assessed in this part of the thesis by absorbance ratios A260/A230 – indicator of phenolic and polysaccharide contamination and A260/A280 – indicator of protein or RNA contamination [112]), the conclusion reached was different than when DNA concentration was the only consideration. In case of the Invitrogen kit it is apparent that while DNA isolates from apricot, blueberry and strawberry had the highest concentrations of all isolates obtained by this kit (Figure 8 and Table 13), they also had the lowest purity out of all DNA isolates obtained by the Invitrogen kit. The absorbance ratios A260/A230 and A260/A280 were even the lowest measured for all apricot, blueberry and strawberry DNA isolates (Figures 9-12, Table 14 and Table 15).
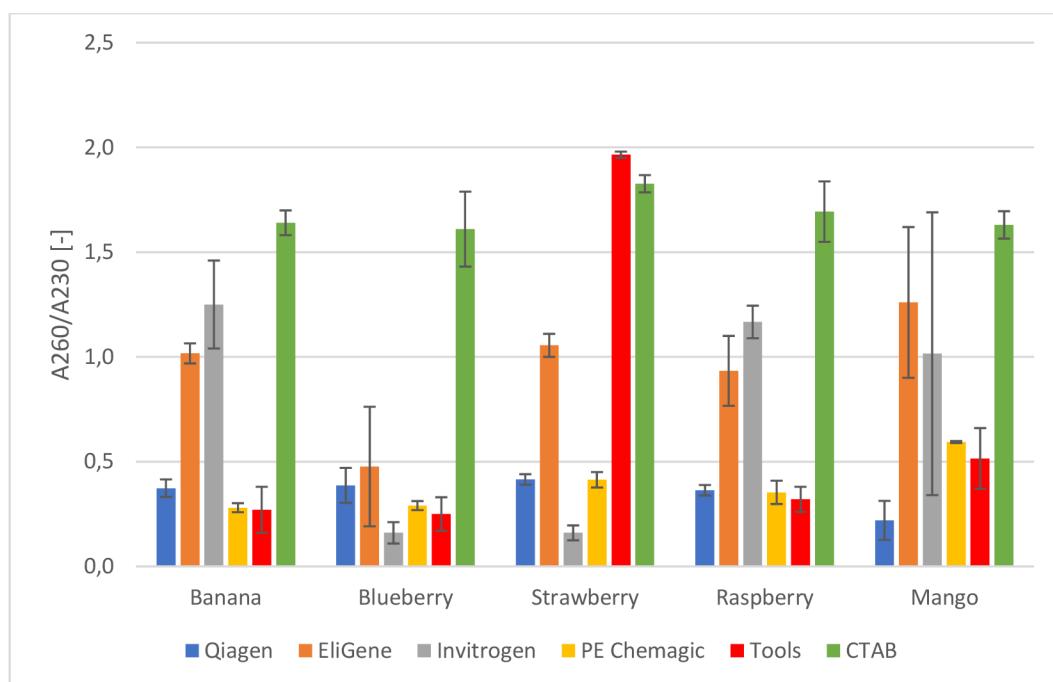

Figure 9: A260/A230 absorbance ratios of DNAs from tropical fruits and berries

In case of the Qiagen kit, the absorbance ratio A260/A230 was 0.5 or lower (1.7 – 2.0 is the range for DNA sufficiently pure for downstream applications [113]) for all DNA isolates, including apricot, blueberry, pear, plum and raspberry DNA, whose concentration was higher than the concentrations of DNA obtained by the remaining methods, except the CTAB protocol (Table 14). The absorbance ratio A260/A280 was generally between 1.1 and 1.5, except for mango DNA, where this ratio was 1.75 (Table 15). These values were also out of range (1.8 to 2.0, [113]) for "pure" DNA.

Regarding the CTAB protocol, the absorbance ratio A260/A230 of DNA isolates obtained by this method came close to the recommended range of 1.7 – 2.0, except for plum DNA (Figure 9 and Table 14). This did not happen with any of the remaining five DNA isolation methods. In case of the Qiagen, Invitrogen and Perkin-Elmer kits the recommended range for the A260/A230 ratio was never reached (although for banana, raspberry and mango DNA isolated by the Invitrogen kit this ratio was higher than, or close to 1.0 – better result than the Qiagen and Perkin-Elmer kits), or it was reached only with DNA from one fruit species out of ten (strawberry DNA isolated by the Tools kit, peach DNA isolated by the EliGene kit). Similarly to

the Invitrogen kit, the A260/A230 absorbance ratios of several isolates obtained by the EliGene kit (banana, strawberry, raspberry and mango DNA) at least came close, or were higher than 1.0 (Figure 10 and Table 14).
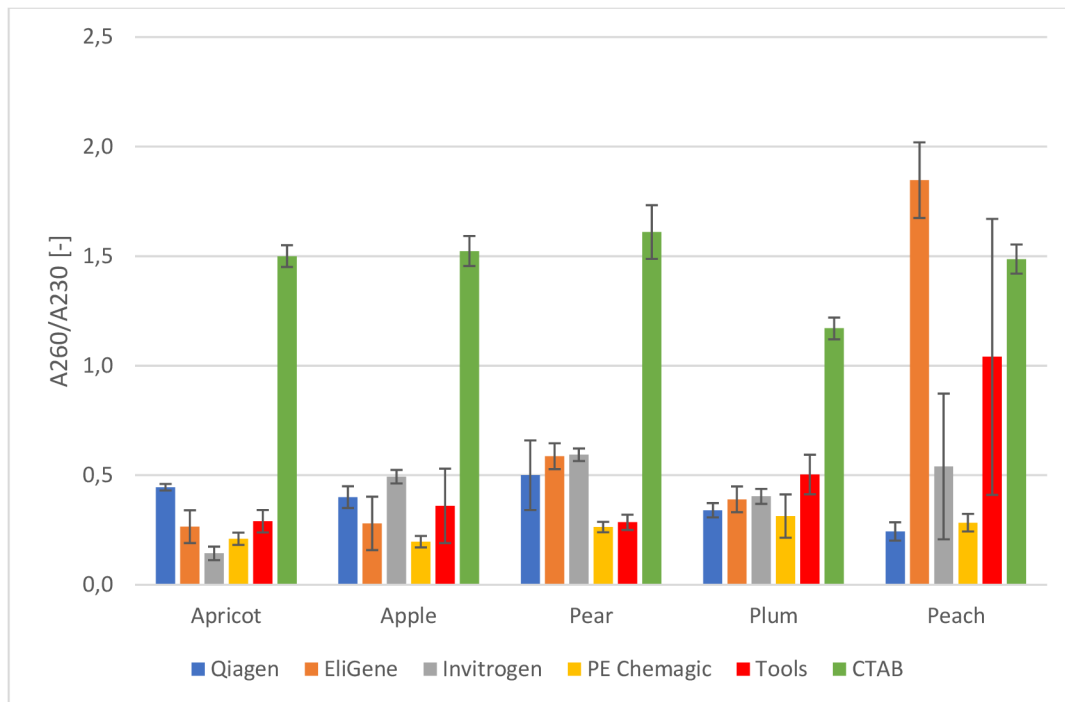

Figure 10: A260/A230 absorbance ratios of DNAs from pome fruit (apple and pear) and stone fruit (apricot, peach and plum)

Table 14: Absorbance ratios A260/A230 of DNA isolates from fruits

	Qiagen	EliGene	Invitrogen	PE Chemagic	Tools	CTAB
Banana	0.37 ± 0.04	1.02 ± 0.05	1.25 ± 0.21	0.28 ± 0.02	0.27 ± 0.11	1.64 ± 0.06
Apricot	0.45 ± 0.02	0.27 ± 0.08	0.14 ± 0.03	0.21 ± 0.03	0.29 ± 0.05	1.50 ± 0.05
Apple	0.40 ± 0.05	0.28 ± 0.12	0.49 ± 0.03	0.20 ± 0.03	0.36 ± 0.17	1.52 ± 0.07
Blueberry	0.39 ± 0.08	0.48 ± 0.29	0.16 ± 0.05	0.29 ± 0.02	0.25 ± 0.08	1.61 ± 0.18
Pear	0.50 ± 0.16	0.59 ± 0.06	0.59 ± 0.03	0.26 ± 0.02	0.29 ± 0.03	1.61 ± 0.12
Plum	0.34 ± 0.16	0.39 ± 0.06	0.40 ± 0.03	0.31 ± 0.02	0.50 ± 0.09	1.17 ± 0.05
Strawberry	0.42 ± 0.16	1.06 ± 0.06	0.16 ± 0.04	0.41 ± 0.04	1.97 ± 0.02	1.83 ± 0.04
Raspberry	0.36 ± 0.02	0.93 ± 0.17	1.17 ± 0.08	0.35 ± 0.06	0.32 ± 0.06	1.69 ± 0.14
Peach	0.24 ± 0.04	1.85 ± 0.17	0.54 ± 0.33	0.28 ± 0.04	1.04 ± 0.63	1.49 ± 0.07
Mango	0.22 ± 0.09	1.26 ± 0.36	1.02 ± 0.68	0.59 ± 0.00	0.52 ± 0.15	1.63 ± 0.07

Regarding the A260/A280 absorbance ratio, the values measured showed some differences among the various DNA isolation protocols (Figure 11 and Table 15). For the Qiagen kit the

A260/A280 value for mango DNA was close to the range indicating pure DNA (A260/A280 = 1.75 ± 0.41), while DNA isolates from the remaining nine fruit species showed protein contamination, with their A260/A280 ratios being out of range for pure DNA. The results were similar for both Perkin-Elmer Chemagic kit and the CTAB protocol. The A260/A280 values ranged from 0.99 ± 0.05 to 1.20 ± 0.22 for the Perkin-Elmer kit and from 1.36 ± 0.01 to 1.57 ± 0.03 for the CTAB protocol, which means that all DNA isolates obtained by both the Perkin-Elmer kit and the CTAB protocol showed protein contamination.
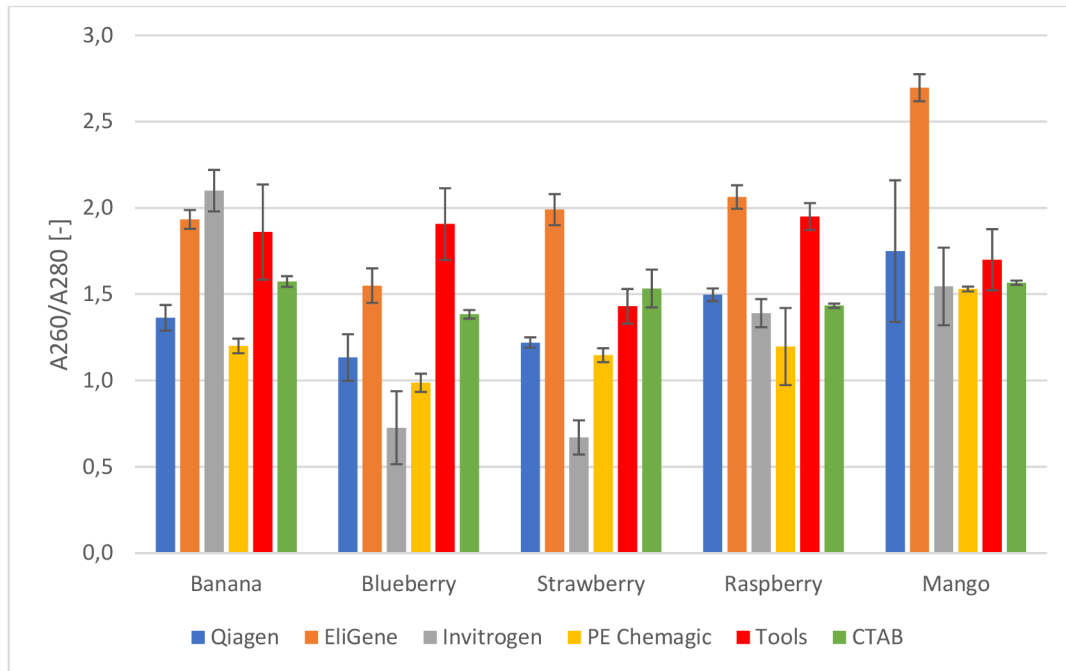

Figure 11: Absorbance ratios A260/A280 of DNAs isolated from tropical fruits and berries
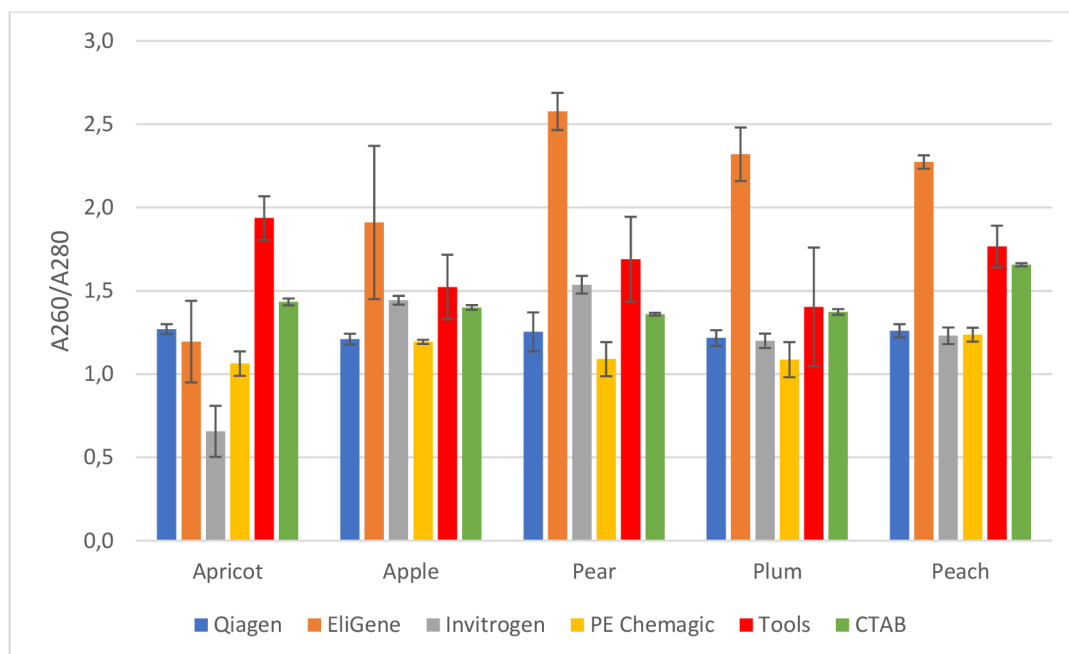

Figure 12: Absorbance ratios A260/A280 of DNAs isolated from pome fruit (apple and pear) and stone fruit (apricot, peach and plum)

Table 15: Absorbance ratios A260/A280 of DNA isolates from fruits

	Qiagen	EliGene	Invitrogen	PE Chemagic	Tools	CTAB
Banana	1.36 ± 0.07	1.93 ± 0.05	2.10 ± 0.12	1.20 ± 0.04	1.86 ± 0.28	1.57 ± 0.03
Apricot	1.27 ± 0.03	1.20 ± 0.25	0.66 ± 0.15	1.06 ± 0.07	1.94 ± 0.13	1.43 ± 0.02
Apple	1.21 ± 0.03	1.91 ± 0.46	1.44 ± 0.03	1.19 ± 0.01	1.52 ± 0.19	1.40 ± 0.01
Blueberry	1.13 ± 0.13	1.55 ± 0.10	0.73 ± 0.21	0.99 ± 0.05	1.91 ± 0.21	1.38 ± 0.02
Pear	1.25 ± 0.12	2.58 ± 0.11	1.54 ± 0.05	1.09 ± 0.10	1.69 ± 0.25	1.36 ± 0.01
Plum	1.22 ± 0.05	2.32 ± 0.16	1.20 ± 0.04	1.09 ± 0.11	1.40 ± 0.36	1.37 ± 0.02
Strawberry	1.22 ± 0.03	1.99 ± 0.09	0.67 ± 0.10	1.15 ± 0.04	1.43 ± 0.10	1.53 ± 0.11
Raspberry	1.50 ± 0.04	2.06 ± 0.07	1.39 ± 0.08	1.20 ± 0.22	1.95 ± 0.08	1.43 ± 0.01
Peach	1.26 ± 0.04	2.27 ± 0.04	1.23 ± 0.05	1.24 ± 0.04	1.77 ± 0.12	1.66 ± 0.01
Mango	1.75 ± 0.41	2.70 ± 0.08	1.55 ± 0.23	1.53 ± 0.01	1.70 ± 0.18	1.57 ± 0.01

In case of EliGene, Invitrogen and Tools kits, the A260/A280 values varied between fruit species (Table 15). From the samples obtained by the EliGene kit, DNAs from banana, apple and strawberry were free from protein contamination (if the A620/A280 absorbance ratio is taken as an indicator), DNAs from apricot and blueberry showed protein contamination and DNAs from pear, plum, raspberry, peach and mango showed RNA contamination. Out of the samples obtained by the Invitrogen kit, apricot, apple, blueberry, pear, plum, strawberry, raspberry, peach, and mango DNAs showed varying levels of protein contamination, while DNA from banana showed slight RNA contamination. In case of samples obtained by the Tools kit, the A260/A280 values of banana, apricot, blueberry, and raspberry DNAs were in the 1.8 to 2.0 range, and therefore free of protein contamination. Apple, pear, plum, strawberry, peach and mango DNAs showed protein contamination comparable to the DNAs isolated by the CTAB protocol.

To sum up, if the purity of DNA isolates assessed by the A260/A230 and A260/A280 absorbance ratios is considered in addition to the concentration of the isolates, the Qiagen kit and the Perkin-Elmer kit seem to be less appropriate for DNA isolation from various fruits than the remaining four methods, especially due to the consistently low A260/A230 values, which indicate contamination by phenolic compounds and/or polysaccharides, both of which are known PCR inhibitors [13]. Regarding the remaining four methods, the samples obtained by the CTAB protocol showed higher A260/A230 values (and therefore lower levels of phenolic and/or polysaccharide contamination) than the samples obtained by the EliGene, Invitrogen and Tools kits. The exceptions were strawberry DNA obtained by the Tools kit and peach DNA obtained by the EliGene kit. Taken together with the A260/A280 absorbance ratios, the CTAB protocol seems to be the most appropriate method for DNA isolation from various fruit species.

5.1.2   Amplifiability of DNA isolates – primers specific for plant ITS2 region
To further assess the quality of DNA isolated by the six tested protocols, all isolates were amplified in a PCR assay specific for plant ITS2 region and a PCR assay specific for the given fruit species. Because of our intention to analyse fruit-based infant foods in later phases of this

work, special attention was paid to the behaviour of DNA isolates from apple, which is a widely used basic material for production of foods intended for children and infants [114]. This chapter summarizes the results of the assay specific for plant ITS2 region.

The isolated DNAs were analysed in a real-time PCR assay specific for plant ITS2 region according to the protocol described in chapter 4.9. If a specific product was detected for two or all three replicate samples, the DNA was considered amplifiable. If a specific product was not detected or detected only in one replicate, DNA was considered not amplifiable. The results are summarized in Table 16.

Table 16: Amplifiability of DNA isolated from fruits - PCR assay specific for plant ITS2 region. Amplifiable DNA = "+", not amplifiable DNA = "-"

	Qiagen	EliGene	Invitrogen	PE Chemagic	Tools	CTAB
Banana	+	+	+	+	+	+
Apricot	-	+	+	+	+	+
Apple	+	+	+	+	+	+
Blueberry	+	+	+	+	+	-
Pear	+	+	+	+	+	+
Plum	-	+	-	+	+	+
Strawberry	+	+	+	+	+	-
Raspberry	-	+	+	+	+	+
Peach	+	+	+	+	+	+
Mango	+	+	+	+	+	+

In case of the Qiagen kit the results of the plant-specific PCR assay confirm the conclusion drawn in chapter 5.1.1, that out of the five kits tested, the Qiagen kit is the least suitable for DNA isolation from various fruit species. Out of the DNAs isolated by this kit, those from apricot, plum and raspberry were not amplifiable.

In case of the Perkin-Elmer kit and the CTAB protocol, the conclusions made in chapter 5.1.1 were not confirmed. All DNA isolates obtained by the Perkin-Elmer kit were amplifiable in the plant-specific PCR assay. This also happened with the DNA isolates obtained by kits EliGene and Tools, while out of DNA isolates obtained by the Invitrogen kit only those from plum were not amplifiable. This means that the performance of the Perkin-Elmer kit was better than that of the Qiagen kit and comparable to kits EliGene, Invitrogen and Tools. Out of DNAs isolated by the CTAB protocol, blueberry and strawberry DNA were not amplifiable. This result makes the CTAB protocol more suitable for DNA isolation from fruits than the Qiagen kit, but less than kits EliGene, Invitrogen, Perkin-Elmer and Tools. Following real-time PCR, electrophoresis of the amplicons was also performed. The results can be found in supplementary material (Chapter 8, Figures 41-46)

5.1.3 Amplifiability of DNA isolates – species specific primers

Apart from plant-specific real-time PCR assay, the isolated DNAs were also amplified in species specific assays according to protocols described in chapter 4.9. Electrophoresis of the amplicons was also performed, the results of which can be found in supplementary material (Chapter 8, Figures 47-51). The results of species-specific assays confirm the conclusion about the Qiagen kit reached in chapter 5.1.2. Out of DNAs isolated by this kit, only half were amplifiable in species-specific PCR assays (banana, blueberry, pear, strawberry and peach DNA). The DNAs which were not amplifiable included apricot, plum and raspberry DNAs, which were also not amplifiable in the plant-specific PCR assays. In addition, mango and apple DNA were not amplifiable in their respective species-specific assays (Table 17). These results made the Qiagen kit unsuitable for our intended purpose of analysing plant-based foods, including fruit-based ones.

Table 17: Amplifiability of DNA isolated from fruits - species-specific PCR assays. Amplifiable DNA = "+", not amplifiable DNA = "-"

	Qiagen	EliGene	Invitrogen	PE Chemagic	Tools	CTAB
Banana	+	+	+	+	+	+
Apricot	-	+	+	+	+	-
Apple	-	+	+	-	+	-
Blueberry	+	+	+	+	+	-
Pear	+	+	+	-	+	-
Plum	-	+	-	+	+	-
Strawberry	+	+	+	+	+	+
Raspberry	-	+	+	+	+	+
Peach	+	+	+	+	+	+
Mango	-	+	+	+	+	+

In case of CTAB protocol, the results of species-specific PCR assays were similar to those of the Qiagen kit in the sense that DNA isolates from only five fruit species (banana, strawberry, raspberry, peach and mango) out of ten were amplifiable. It is possible that in case of the CTAB protocol the amplifiability of DNA isolates was influenced by polysaccharide contamination, or by residues of chemicals used during DNA isolation, e. g. phenol or ethanol. To sum up, the results of PCR assays with DNAs isolated by the CTAB protocol make this particular protocol unsuitable for DNA isolation from fruit-based foods.

Out of the remaining DNA isolation methods, the Perkin-Elmer kit was found to be unsuitable for DNA isolation from apple and pear (Perkin-Elmer kit), because apple and pear DNAs isolated by this kit were not amplifiable in their respective species-specific assays. However, while this kit did not provide apple and pear DNA of sufficient quality, it did provide amplifiable DNA from other fruit species.

We would also like to point out the result of strawberry DNA isolation by the Tools kit. It is especially interesting both because of the amplifiability of the DNA isolates in both plant-specific and species-specific PCR assays and because their A260/A230 absorbance ratios were in range for pure DNA, which was a rare result in this work (Table 15). This results also makes the Tools kit a possible choice for DNA isolation in works which focus on strawberry fruits or products made mainly out of strawberry fruits.

The selection of DNA isolation method was narrowed down to the EliGene, Tools and the Invitrogen kit. All these kits provided apple DNAs which were amplifiable in both plant-specific and species-specific PCR assays – this did not happen with the remaining three DNA isolation methods. The final selection of DNA isolation method for our further work was based mainly on the results of PCR assays, especially those with apple DNA isolates. These results indicated that the EliGene kit, the Tools kit and the Invitrogen kit were the most suitable for our further work. Other factors considered while making the choice among these three kits were the ease of use and price per sample. Because the EliGene kit was easier to use than the Invitrogen kit, and the price per sample was lower than both the Tools and Invitrogen kit, we chose the EliGene kit for DNA isolations in later parts of this work.

5.1.4 Partial summary of results

The aim of the first part of this work was to select a DNA isolation method suitable for DNA isolation from various fruit-based foods. Five commercial kits and one conventional (CTAB-based) method were tested. DNA was isolated from ten fruit species commonly occurring in fruit-based baby food, and special attention was paid to the behaviour of apple DNA isolates in both plant-specific and species-specific PCR assays.

Out of the six DNA isolation methods tested, three were considered suitable for analysis of foods which contain apple as a major component. These protocols were the EliGene kit, the Tools kit and the Invitrogen kit. While the principles of DNA capture were different for each kit, there were also some similarities between the EliGene and the Invitrogen kit. Both utilize SDS at some point in the protocol. In case of the EliGene kit, SDS is a component of the lysis buffer (chapter 4.7.1.1), while in case of the Invitrogen kit SDS is added to the cell lysate after it is treated with RNase A (chapter 4.7.3.2). Protocols for both kits also include a step designed to remove polysaccharides – in the EliGene kit it is an inhibitor removal buffer P3 (composition not stated by the manufacturer), while in the Invitrogen kit it is possibly the addition of calcium chloride, as calcium ions are known to precipitate pectins [115]. Pectins are components of cell walls in higher plants, including fruits [116, 117], and they also belong among acidic polysaccharides, which are known inhibitors of enzymatic analytical methods [118]. Additionally, protocols for both the EliGene and Invitrogen kit involve more washing steps than protocols for the Qiagen, Perkin-Elmer and Tools kits (chapter 4.7).

The results achieved with the CTAB protocol correspond to the work of Turci et al [119]. In their article focusing on comparison of several DNA isolation kits and conventional protocols Turci et al. reported that a CTAB based protocol yielded highest concentrations of DNA compared to two other conventional protocols and three kits when applied to tomatoes and processed tomato products, but the lowest proportion (12 %) of successful PCR reactions was achieved with these DNAs. They also reported better results with an SDS-based protocol (75 % of PCR reactions successful) and one column-based kit (100 % of PCR reactions successful) [119].

On the other hand, while Sovová et al. in their 2018 work [120] reported that a commercial kit was more suitable for DNA isolation from jams than a CTAB-based protocol, they encountered

problems with spectrophotometric measurements of DNA concentrations rather than with DNA amplifiability.

5.2 Multiplex PCR – method optimisation

During multiplex PCR optimization two main tasks were performed. First of these tasks was the selection of an appropriate set of three primer pairs, which was followed by the adjustment of the primer pair's concentrations. As a final step, method sensitivity was determined.

5.2.1 Selection of appropriate primer set

Four different primer sets (triplexes) were tested. In these triplexes, the primers specific for raspberry (RiACO1, Table 3) and blueberry (VcBHLH003, Table 3) were always the same, as was their concentration (200 nM of each primer in reaction mixture for both primer pairs). The triplexes differed in the primers specific for strawberry (APX1B, DFR1A, APX1D and GAST1). The concentration of strawberry-specific primer pairs in the reaction mixtures was 200 nM of each primer. The desired outcome was to find a triplex which would provide three amplicons distinguishable by high resolution melting analysis. In other words, each of the three amplicons should be represented by a clearly distinguishable peak on a differential melting curve, or by a clearly distinguishable pre-melt and post-melt regions in the raw melting curve.
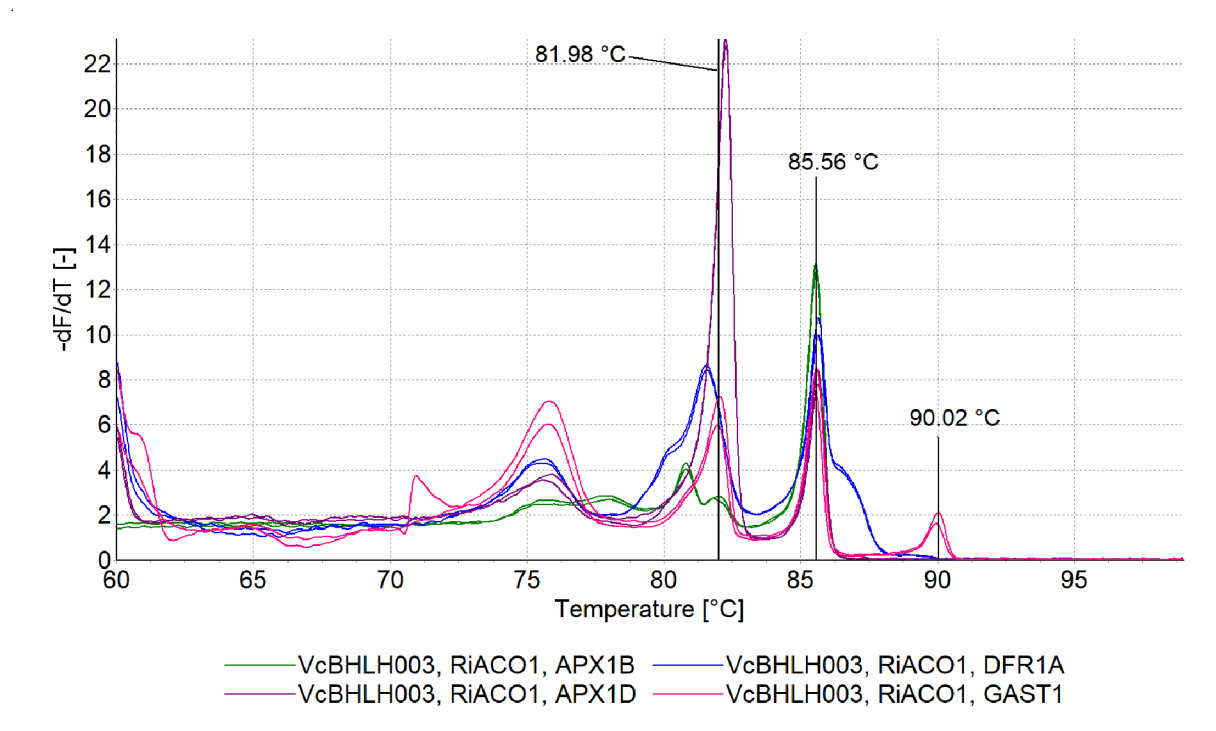
Figure 13: Comparison of four primer sets for triplex PCR, differential amplicon melting curves. Melting temperatures of the VcBHLH003 amplicon (81.98 °C, blueberry-specific), RiACO1 amplicon (85.56 °C, raspberry specific) and GAST1 amplicon (90.02 °C, strawberry-specific) are marked in the figure.

Above mentioned goal was achieved with the primer set containing GAST1 primers (Figure 13). In this primer set, the VcBHLH003 (blueberry-specific) amplicon is represented by a peak at approximately 81.7 °C, the RiACO1 (raspberry-specific) amplicon is represented by a peak at approximately 85.5 °C, while the GAST1 (strawberry-specific) peak can be seen at approximately 89.8 °C.

Regarding the remaining three primer sets, the RiACO1 amplicon is distinguishable every time (Figure 13). However, in case of the primer set containing the APX1D primers the melting

peaks of VcBHLH003 and APX1D amplicons are merged into one, which makes them indistinguishable from each other. It is also possible that preferential amplification of the APX1D amplicon over the VcBHLH003 amplicon took place, as the leftmost melting peak in the corresponding melting curve has a higher melting temperature than 81.98 °C, which was the measured melting point of the VcBHLH003 amplicon (Figure 13).With the triplex containing the DFR1A primers, it may be deduced from the shape of corresponding melting curves, that the melting peak of the DFR1A amplicon was merged with either the VcBHLH003 or RiACO1 peak, and that a non-specific amplification possibly took place. The melting curves which represent this triplex show a leftmost peak, whose melting temperature differs from that of the VcBHLH003 amplicon, while there is also a possible unresolved melting peak next to the melting peak of the RiACO1 amplicon. Finally, while the triplex containing the APX1B primers did provide amplicons represented by three distinguishable peaks (Figure 13, green melting curves), with the APX1B peak visible to the left of VcBHLH003 peak, the shape of the melting curves shows preferential amplification of the RiACO1 amplicons (RiACO1 peak higher than VcBHLH003 and APX1B peaks). Additionally, the melting peaks of the triplex containing the GAST1 primers are more clearly separated than melting peaks of the triplex with APX1B primers.

5.2.2 Primer concentration optimization

Because the differential amplicon melting curves of our chosen triplex showed a preferential amplification of the VcBHLH003 amplicon over the GAST1 amplicon and preferential amplification of RiACO1 amplicons over both VcBHLH003 and GAST1 amplicons, optimization of primer concentrations took place, with five different combinations of primer concentrations tested (Figure 14).
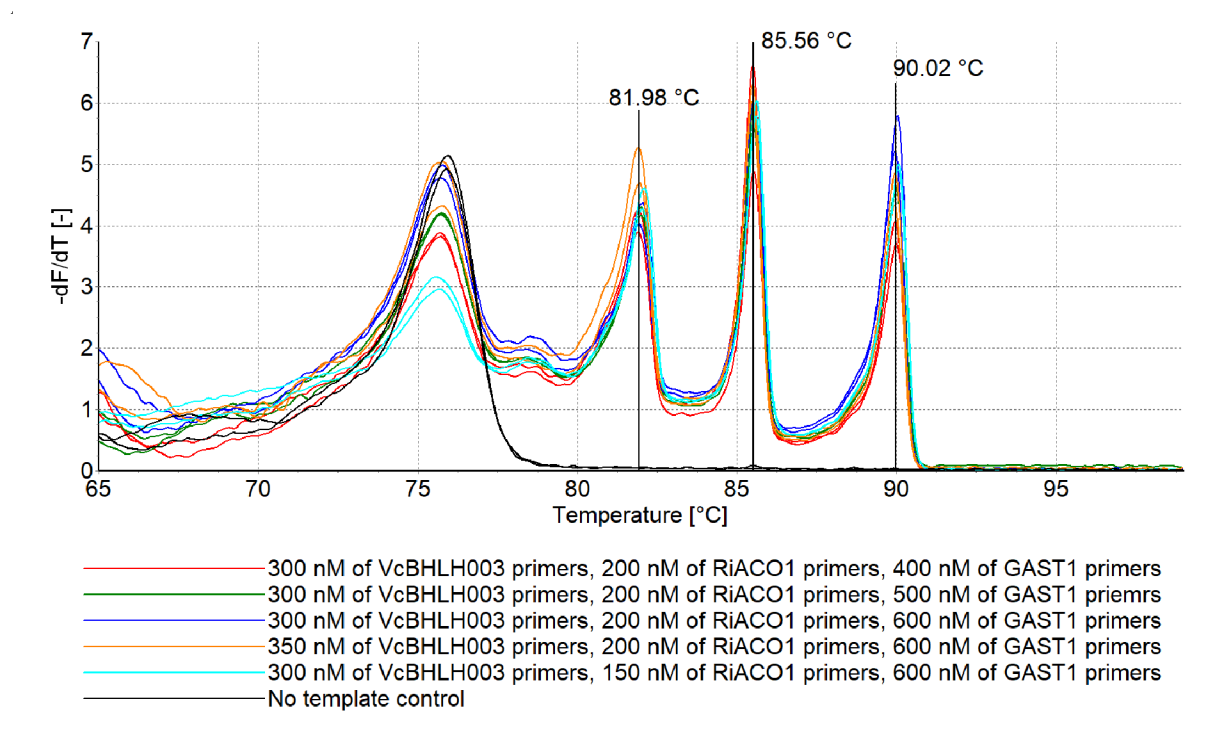

Figure 14: Differential melting curves of triplex PCR mixtures, varying concentrations of primers. Melting temperatures of all amplicons are marked in the figure (81.98 °C for VcBHLH003 amplicon, 85.56 °C for RiACO1 amplicon and 90.02 °C for GAST1 amplicon)

Because preferential amplification of the RiACO1 amplicons over both VcBHLH003 and GAST1 amplicons was observed, the concentration of primers VcBHLH003 was raised to

300 nM of each primer in reaction mixture, and the concentration of GAST1 primers was raised to 400 nM in reaction mixture. With this reaction mixture composition, the heights of the three amplicon melting peaks were more even than when the concentration of all primer pairs were equal (Figures Figure 13 and Figure 14). Following this experiment, two other concentrations of GAST1 primers were tested, namely 500 nM and 600 nM in reaction mixture.

Due to the preferential amplification observed during the primer set selection, the primer concentrations were adjusted in favour of the GAST1 primer pair and 600 nM in reaction mixture was the concentration selected for this primer pair. To further favour the GAST1 primers, 300 nM of VcBHLH003 primers in reaction mixture were used instead of 350 nM, and in case of the RiACO1 primers, their concentration in reaction mixture was decreased to 150 nM in reaction mixture, with no noticeable influence on the shape of the melting curves.

5.2.3 Method sensitivity

To determine the method sensitivity, triplex PCR mixtures were prepared with primer concentrations selected during the method optimisation (300 nM of VcBHLH003 primers, 150 nM of RiACO1 primers and 600 nM of GAST1 primers in reaction mixture). Blueberry, strawberry and raspberry DNAs were serially diluted to concentrations of 10 ng·µl^{-1}, 1 ng·µl^{-1}, 100 pg·µl^{-1} and 10 pg·µl^{-1} and the method sensitivity was tested in this concentration range.
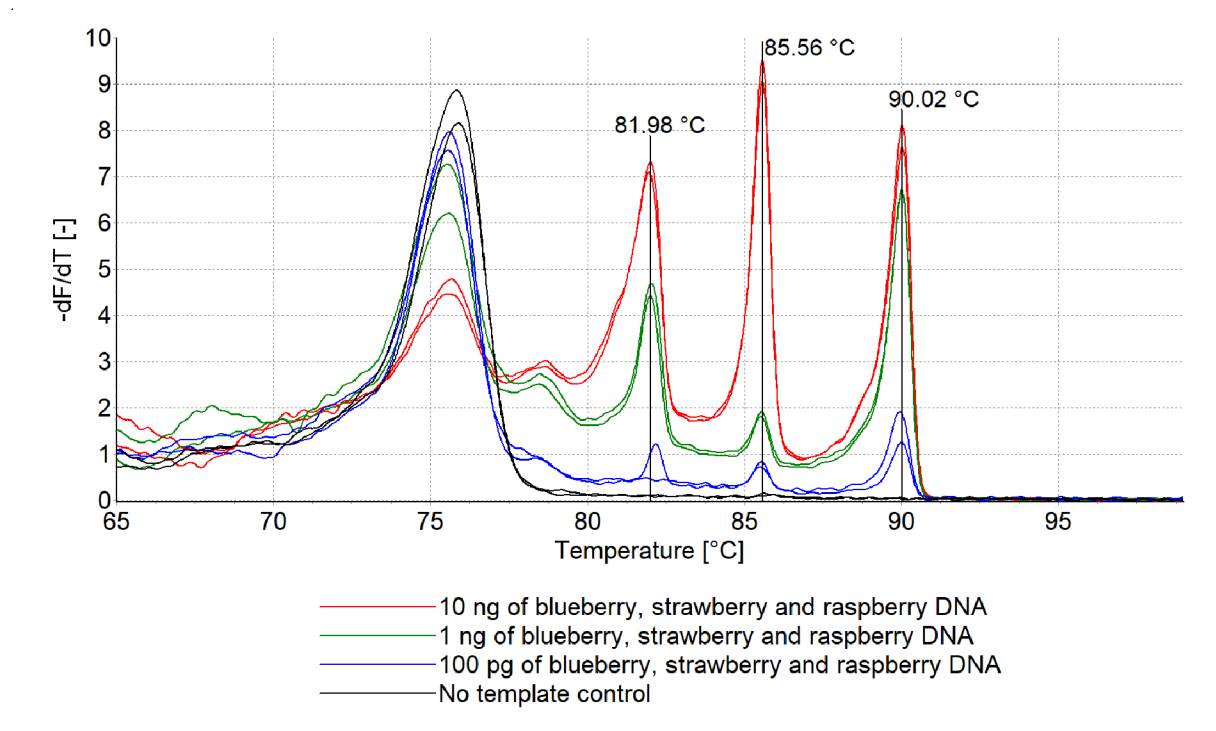

Figure 15: Differential melting curves of triplex PCR mixtures with 10 ng, 1 ng and 100 pg of blueberry, strawberry and raspberry DNA. Melting peaks of VcBHLH003 amplicons (81.98 °C), RiACO1 amplicons (85.56 °C) and GAST1 amplicons (90.02 °C) may be seen in all melting curves.

Specific amplicons were detected only on reaction mixtures containing 10 ng, 1 ng and 100 pg of DNA (Figure 15), while no specific products were detected in reaction mixtures containing 10 pg of DNA. The sensitivity of the method was therefore determined to be 100 pg for each primer pair. The sensitivity of this assay is comparable to several multiplex PCR assays used to detect plant DNA. Suh et el. [121] reported 80 pg sensitivity for an assay detecting allergen coding genes of five fruit species, while Hubalkova and Rencova [122] reported detection limit

of 100 pg for an assay simultaneously detecting pecan and Brazil nut, and Zhang et al. [123] reported sensitivities from 10 to 100 pg for triplex real-time PCR assay detecting peanut, soybean and sesame.

5.3 Optimization of DNA isolation protocol for commercial foods

In this chapter, the results of DNA isolation from five teas, three purees, one smoothie and two fruit bars are discussed. The chosen isolation protocol was the EliGene kit. Four sets of DNA isolates were obtained: The first with the unmodified kit, the second with pectinase digestion of polysaccharides introduced into the DNA isolation protocol, the third with polysaccharide precipitation by calcium chloride, and the last set of samples was obtained by the EliGene kit with polysaccharide precipitation by calcium chloride, purified by a clean-up kit and concentrated by alcohol precipitation with co-precipitant (chapter 4.7.1). Detailed composition of all commercial samples is shown in Table 1.

Table 18: Commercial samples used in this work and the codes used to label them

Product	Code
dmBio fruit tea black currant and blueberries	T1
Apotheke Bio tea, Forest blend with raspberry	T2
Apotheke Bio children's herbal tea for imunity, with strawberry	T3
Leros herbal tea "Strong eyes" with blueberry and marigold	T4
Majestic Tea herbal tea raspberry and camu camu	T5
Babylove bio baby food, strawberry and blueberry	P1
Babylove bio baby food apple, strawberries and raspberries	P2
Relax 100% puree, raspberry	P3
dmBio Smoothie with protein, bananas, grapes and blueberries	S
Hipp bio fruit bar apple banana and raspberries	B1
dmBio fruit bar banana and blueberry	B2

The short codes used to label the samples during experiments are summarized in Table 18. The teas were poured from their bags and used for DNA isolation directly, as they were already dry and ground to small pieces during the manufacturing process. The fruit bars were also use directly, while purees and smoothie were lyophilized before DNA isolation.

5.3.1 DNA concentration and purity

The concentrations of all DNA isolates from commercial products are summarized in Table 19. The DNA concentrations varied among different sample types and among the four DNA isolation protocols. In case of the unmodified kit the DNA concentrations ranged from units of nanograms to tens of nanograms. The lowest amounts of DNA were obtained from purees P1

and P2 (2.2 ng·μl^{-1} and 3.1 ng·μl^{-1}, respectively), both of which contained apple puree as the majority component (76 % and 83 %, respectively). The DNA obtained from puree P3, where apple puree content was lower (37 %) had several times higher concentration (13.2 ng·μl^{-1}). The DNA isolated from smoothie (S, with banana puree, white grape and blueberry juice making up approximately 70 % of the product) had also higher concentration than DNAs isolated from purees P1 and P2.

Regarding the DNA isolated from teas (samples T1 – T5), the isolates from teas T1, T2 and T5 had similar concentrations (8.0 ng·μl^{-1}, 10.0 ng·μl^{-1} and 8.72 ng·μl^{-1}, respectively). The concentration of DNA isolated from teas T3 and T4 was 22.4 ng·μl^{-1} and 44.9 ng·μl^{-1}, respectively. The presence or absence of hibiscus blossom in the teas might have had an influence on the DNA yield, because in teas T1, T2 and T5, where the DNA yields were lower, hibiscus blossom is either in the first (T1, T2), or second place (T5) in the composition. In tea T3 the hibiscus blossoms are in the third place in the composition, while tea T4, where the DNA yield was highest, did not contain any hibiscus blossom.

In case of fruit bars (samples B1 and B2) a difference in DNA concentrations was also observed. The concentration of DNA isolated from bar B2 was approximately seven times higher than concentration of DNA isolated from bar B1 (Table 19). A possible reason for this result might again be a different composition of these bars. Wile both bars contained similar amounts of banana flakes (29 % in bar B1 and 33 % in bar B2), the B1 bar contained also 29 % of apple juice, while the B2 bar contained 26 % of wholegrain oat flour.

Table 19: Concentration of DNA isolated from commercial foods by unmodifed and modified EliGene kit

Product	Unmodified kit	Pectinase	Calcium chloride	Calcium chloride, clean-up kit, co-precipitant
T1	8.0 ± 0.2	3.0 ± 0.2	5.9 ± 0.2	9.5 ± 0.24
T2	10.0 ± 0.1	2.7 ± 0.2	8.3 ± 0.1	19.6 ± 0.24
T3	22.4 ± 0.1	3.7 ± 0.3	14.3 ± 0.1	9.8 ± 0.38
T4	42.9 ± 0.4	6.1 ± 0.0	3.1 ± 0.1	11.9 ± 0.33
T5	8.72 ± 0.3	3.5 ± 0.2	5.4 ± 0.3	7.5 ± 0.00
P1	2.2 ± 0.2	2.0 ± 0.1	1.7 ± 0.1	10.0 ± 0.14
P2	3.1 ± 0.1	5.5 ± 0.2	1.5 ± 0.1	11.7 ± 0.25
P3	13.2 ± 0.7	2.7 ± 0.1	3.5 ± 0.2	13.1 ± 0.21
S	18.1 ± 0.1	0.9 ± 0.0	4.5 ± 0.1	14.4 ± 0.08
B1	11.0 ± 0.2	1.9 ± 0.1	4.5 ± 0.3	8.7 ± 0.08
B2	77.5 ± 1.0	4.8 ± 0.1	10.6 ± 0.4	11.4 ± 0.29

In case of the three modified DNA isolation protocols the differences in DNA yields were smaller than in case of the unmodified kit (Table 19), i. e. the influence of the sample matrix was less pronounced. The protocol with pectinase and the protocol with calcium chloride also yielded DNA isolates whose concentrations were generally lower than the concentrations of DNA isolates obtained by the unmodified kit. However, the DNAs isolated by the protocol with calcium chloride, clean-up kit and alcohol precipitation with co-precipitant had in some cases higher concentrations than those obtained by the unmodified kit. This was the case with tea T2, and purees P1 and P2. For teas T1 and T5, puree P3, smoothie (S) and bar B1 the concentrations of isolated DNAs were similar to those obtained by the unmodified kit and for tea T4 and bar B2 the DNA concentrations were lower than those obtained by the unmodified kit.

Regarding the purity of DNA isolates obtained by the four tested protocols, a certain influence of sample matrix can be seen with the unmodified kit. The A260/A280 absorbance ratios of DNAs isolated from teas T1, T2 and T3 have similar values (0.82 to 1.07, Table 20) and are out of the 1.8 − 2.0 range which indicates no protein or RNA contamination [113]. The A260/A230 absorbance ratios have also similar values (0.16 to 0.23, Table 21) and are also out of range for (1.7 − 2.0) for "pure" DNA [113]. All three of these teas contain rosehips at the first place in composition. In case of tea T5, which contains hibiscus at the first place in the list of components (Table 1) the A260/A280 absorbance ratio is slightly higher (1.28, Table 20), while the value of its A260/A230 ratio is similar to those of teas T1, T2 and T3.

Table 20: A260/A280 absorbance ratios of DNAs isolated from commercial foods

Product	Unmodified kit	Pectinase	Calcium chloride	Calcium chloride, clean-up kit, co-precipitant
T1	1.01 ± 0.03	1.07 ± 0.04	0.88 ± 0.03	1.51 ± 0.02
T2	0.82 ± 0.00	0.91 ± 0.06	0.92 ± 0.05	1.47 ± 0.00
T3	1.05 ± 0.01	1.02 ± 0.08	1.04 ± 0.02	1.62 ± 0.06
T4	1.93 ± 0.03	1.21 ± 0.04	1.45 ± 0.10	1.57 ± 0.03
T5	1.28 ± 0.04	1.08 ± 0.08	1.21 ± 0.03	1.64 ± 0.01
P1	0.80 ± 0.11	1.15 ± 0.05	1.05 ± 0.24	1.56 ± 0.04
P2	0.99 ± 0.05	0.63 ± 0.00	1.40 ± 0.29	1.60 ± 0.02
P3	1.79 ± 0.02	1.05 ± 0.08	2.65 ± 0.02	1.54 ± 0.02
S	2.08 ± 0.04	0.80 ± 0.07	1.83 ± 0.19	1.54 ± 0.01
B1	2.04 ± 0.02	0.85 ± 0.03	2.14 ± 0.20	1.58 ± 0.03
B2	2.04 ± 0.01	1.01 ± 0.03	1.84 ± 0.06	1.61 ± 0.07

In case of T5, which does not contain either rosehips or hibiscus (Table 1), the A260/A280 ratio is in range for pure DNA (Table 20), and while this sample's A260/A230 ratio is out of range for pure DNA, it is approximately six times higher than the A260/A230 ratios of teas T1,

T2, T3 and T5 (Table 21). In case of DNAs from purees, those isolated from purees P1 and P2 (samples with high content of apples, Table 2) have lower A260/A280 and A260/A230 ratios than DNA isolated from puree P3 (sample with lower content of apples than purees P1 and P2, Table 1). However, while the A260/A280 ratio of puree P3 borders the range of values for pure DNA, unlike the the A260/280 ratios of purees P1 and P2, the A260/A230 ratio of puree P3 is solidly out of range of values for pure DNA and only slightly higher than A260/A230 ratios of DNAs isolated from purees P1 and P2 (Table 21). In case of the three samples with similar contents of bananas (smoothie and both fruit bars) the A260/A280 have identical (bars B1 and B2) or nearly identical (smoothie) values, indicative of slight RNA contamination. The A260/A230 ratios of DNAs from these samples are out of range for pure DNA, although for the DNAs isolated from the fruit bars the values of this absorbance ratios are higher than for the DNA isolated from smoothie.

Certain sample matrix influence on DNA purity was observed also in DNA isolates obtained by the protocol modified with calcium chloride (Table 20 and Table 21). The A260/A280 ratio of tea T4 is higher than A260/A280 ratios of the other teas, although this time it is also out of range of values for pure DNA. The A260/A230 absorbance ratio of tea T4 is similar to A260/A230 absorbance ratios of the other teas, a result different from the one obtained with the unmodified kit (Table 21). In case of purees, the A260/A280 ratios of samples P1 and P2 are out of range of values for pure DNA and indicate protein contamination, while for puree P3 the A260/A280 indicates RNA contamination (Table 20). The A260/A230 values of DNA isolates from all three purees are solidly out of the range of values for pure DNA, similarly to the DNA isolates obtained from the purees by the unmodified kit (Table 21).

Table 21: A260/A230 absorbance ratios of DNAs isolated from commercial foods

Product	Unmodified kit	Pectinase	Calcium chloride	Calcium chloride, clean-up kit, co-precipitant
T1	0.19 ± 0.00	0.22 ± 0.01	0.16 ± 0.00	0.44 ± 0.01
T2	0.16 ± 0.00	0.27 ± 0.01	0.18 ± 0.00	0.50 ± 0.00
T3	0.23 ± 0.00	0.26 ± 0.01	0.21 ± 0.00	0.46 ± 0.00
T4	1.16 ± 0.00	0.35 ± 0.00	0.14 ± 0.00	0.49 ± 0.00
T5	0.23 ± 0.00	0.31 ± 0.02	0.17 ± 0.01	0.46 ± 0.00
P1	0.13 ± 0.01	0.08 ± 0.00	0.10 ± 0.00	0.48 ± 0.01
P2	0.15 ± 0.00	0.12 ± 0.00	0.15 ± 0.01	0.45 ± 0.00
P3	0.27 ± 0.00	0.13 ± 0.00	0.19 ± 0.01	0.44 ± 0.00
S	0.54 ± 0.00	0.19 ± 0.01	0.22 ± 0.00	0.52 ± 0.01
B1	1.41 ± 0.09	0.22 ± 0.01	0.26 ± 0.01	0.48 ± 0.02
B2	1.23 ± 0.01	0.21 ± 0.00	1.84 ± 0.06	0.51 ± 0.00

In case of smoothie and both fruit bars, which contained similar amounts of bananas, the A260/A280 ratios indicate pure DNA in isolates from smoothie and from bar B2. For DNA from bar B1 the A260/A280 ratio indicates RNA contamination. The value of A260/A230 ratio of DNA obtained from bar B2 indicates pure DNA, while the values of this ratio for DNAs from smoothie and bar B1 are similar to values determined for DNAs from teas and purees and indicate phenolic and/or polysaccharide contamination (Table 21).

In case of DNA isolates obtained by the protocol modified with pectinase and the protocol modified with calcium chloride, clean-up procedure and alcohol precipitation with co-precipitant, no significant sample matrix influence was observed. The values of A260/A280 ratios of DNA isolates obtained by the protocol modified with pectinase were similar for all samples (0.80 – 1.15), except puree P2, where the value of A260/A280 ratio was 0.63. The A260/A280 values for all samples indicate protein contamination (Table 20). The A260/A230 absorbance ratios of DNA isolates obtained by the protocol with pectinase ranged from 0.08 to 0.35, out of range of values for pure DNA and indicative of phenolic or polysaccharide contamination (Table 21).

The A260/A280 values of DNA isolates obtained by the protocol followed by a clean-up procedure ranged from 1.47 to 1.64. These values are higher than those measured for DNA isolates obtained by the protocol with pectinase, but still indicative of protein contamination in all DNA isolates. The A260/A230 ratios of DNAs isolated by the protocol with clean-up procedure ranged from 0.44 to 0.52. These values were again higher than those measured in DNAs isolated by the protocol with pectinase, although they also indicate phenolic or polysaccharide contamination, and they could also have been influenced by the presence of glycogen, which was used as a co-precipitant.

5.3.2 Amplifiability of DNA isolates – primers specific for plant ITS2 region

5.3.2.1 Unmodified kit

Real-time PCR assay with primers specific for plant ITS2 region was performed as an additional way of assessing the quality of DNAs isolated from commercial samples by both the unmodified and modified protocols. Figure 16 shows differential meting curves of ITS2 amplicons in PCR mixtures which contained DNA from teas isolated by the unmodified protocol. Figure 17 shows electrophoresis of the amplicons represented by the melting curves in Figure 16. The positive control is represented by a melting peak at approximately 90 °C. This peak is represented by a sharp band of approximately 500 bp on the gel.

DNAs from teas T1, T2, T3 and T4 were amlifiable, with specific products detected in both replicate samples for each of these four teas. The amplicon melting curves of these samples contain peaks in the region between 80 °C and 95 °C, which are also represented by sharp bands of approximately 500 bp on the gel. Samples containing DNA from teas T3 and T4 only specific products were observed, while in samples containing DNA from teas T1 and T2 non-specific amplification was also observed. This is represented by short peaks at approximately 75 °C, which are not visible in the melting curve of positive control. The melting curves of samples containing DNA from tea T5 overlap the melting curve of no template control and show no melting peaks of specific products (Figure 16). No bands of specific products were detected by electrophoresis, too. Because the concentration and purity of DNA isolated from tea T5 by unmodified kit was similar to concentrations of DNA from teas T1 and T2, a similar result of PCR amplification was expected. However, the DNA from tea T5 was not amplifiable. A possible cause of this result might have been the combination of hibiscus blossom and apple fruit in this tea, as hibiscus blossoms are rich in polysaccharides and phenolic compounds

[124], with the same being true for apples [125]. These compounds might have remained in the DNA isolate and could have caused inhibition of PCR [13].
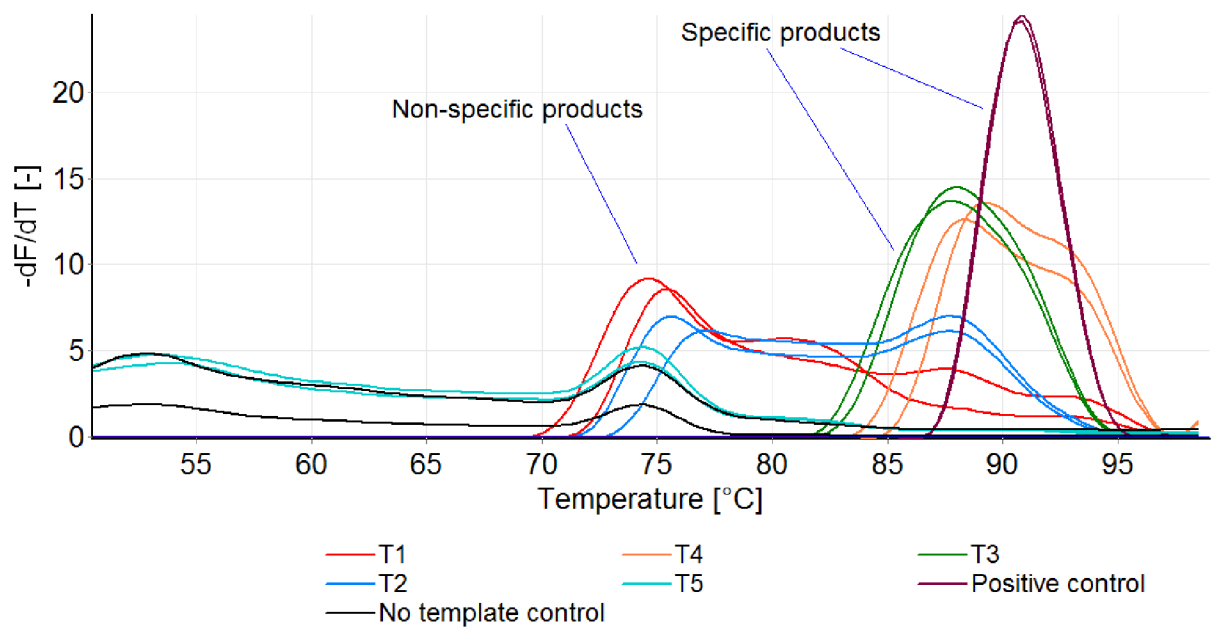

Figure 16: Differential melting curves of ITS2 amplicons, DNA from teas isolated by unmodified kit (T1 = dmBio fruit tea, T2 = Apotheke Bio Forest blend, T3 = Apotheke Bio herbal tea for imunity, T4 = Leros herbal tea "Strong eyes", T5 = Majestic Tea)
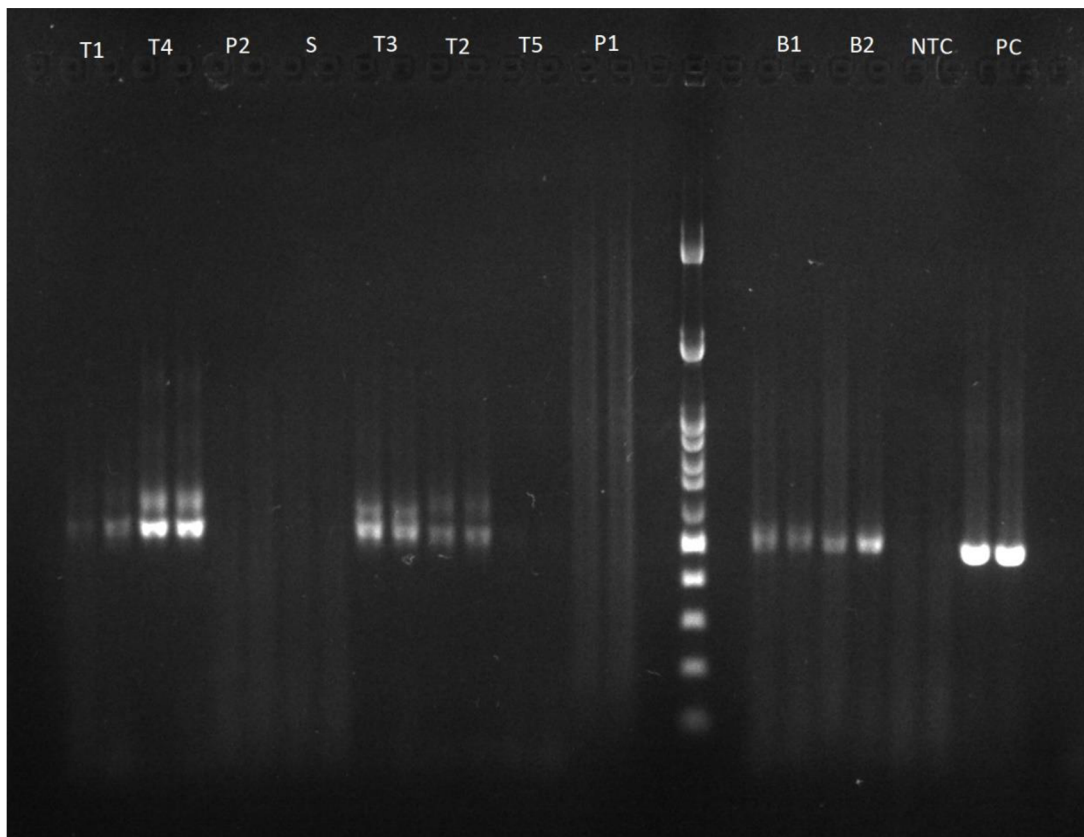

Figure 17: ITS2 amplicons of samples isolated by unmodified kit (sample codes are the same as in Figure 16 and Figure 18)

The results of plant-specific PCR assay with DNA isolated from purees, smoothie and fruit bars by the unmodified kit are shown in Figure 18. Specific amplicons were detected in samples containing DNAs from both fruit bars (melting peaks between 85 and 95 °C), while in samples containing DNAs from purees and smoothie only non-specific products were detected (melting peaks at approximately 75 °C). In case of puree P1, a melting peak was detected at approximately 85 °C. However, this peak represents non-specific products, because only a smear may be seen on the gel instead of a sharp 500 bp band (Figure 17). Similar smears may be seen also in the runs which represent purees P2 and P3, and smoothie. The approach used for analysis of results in this chapter is used also in following chapters which focus on real-time PCR with plant-specific primers.
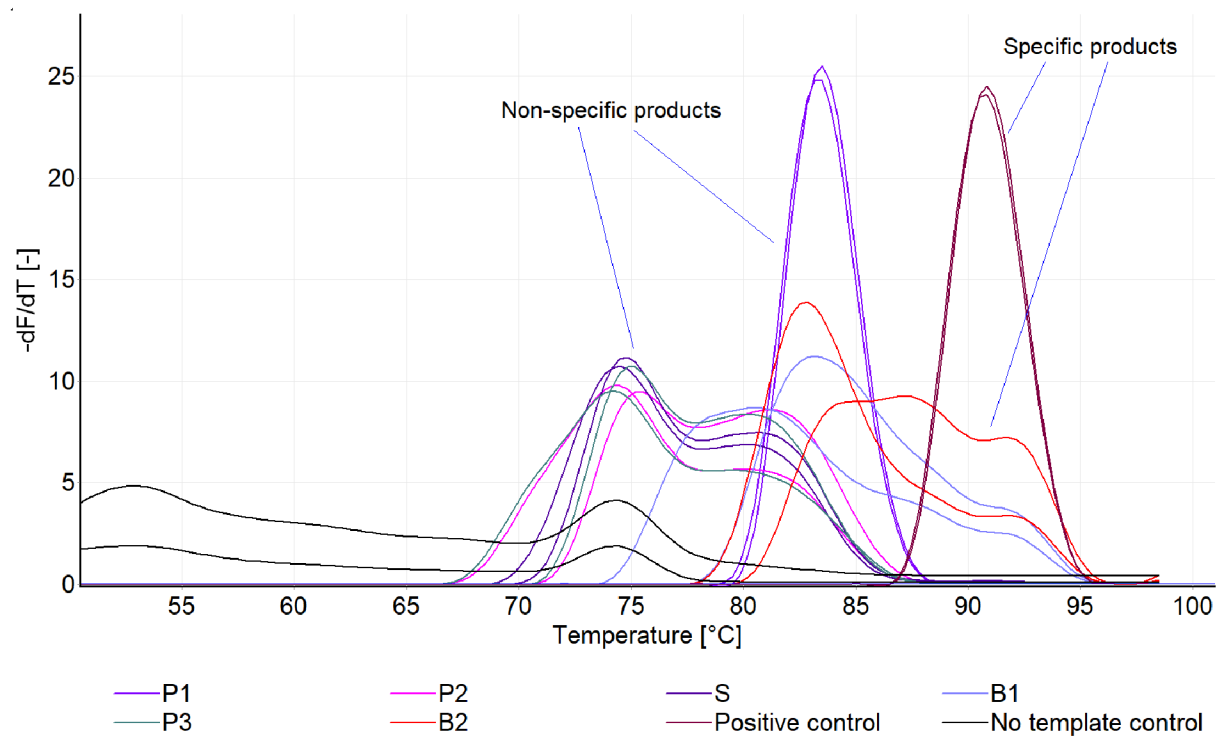
Figure 18: Differential melting curves of ITS2 amplicons, DNA from purees, smoothie and fruit bars isolated by unmodified protocol (P1 = Babylove baby food, strawberry and blueberry, P2 = Babylove baby food apple, strawberries and raspberries, P3 = Relax 100 % puree, raspberry, B1 = Hipp fruit bar, B2 = dmBio fruit bar)

There are several possible reasons for this result. Contamination with inhibitors of PCR is likely, as was indicated by the low A260/A230 ratios of DNA isolates from purees and smoothie (Table 21). Additionally, because the manufacturing process of purees and smoothies involves crushing of fruit [104], mechanical shearing of DNA might have occurred. Lastly, DNA is susceptible to acidic hydrolysis [126], which might have taken place before the purees and smoothie were purchased and lyophilised.

5.3.2.2 Kit modified by pectinase digestion of polysaccharides

The results of plant-specific PCR assay with DNAs isolated from teas by the protocol with pectinase digestion of polysaccharides are shown in Figure 19. Out of the five DNA isolates from teas, four (from teas T1 – T4) were amplifiable, with melting peaks in the region between 85 °C and 95 °C visible, while in the samples containing DNA from tea T5 only non-specific products were detected. These no-specific products are represented by melting peaks at temperatures lower than 85 °C. In case of samples containing DNA from tea T2 some non-specific products were detected, while in case of sample T1 only specific products were

detected. It may be concluded that in case of sample T1 the amplifiability of DNA was improved (no non-specific products detected, unlike the result with DNA obtained by unmodified kit), while in case of sample T5 it remained the same, and the pectinase treatment was not sufficient for removal of inhibitory compounds from the DNA isolated from this sample.
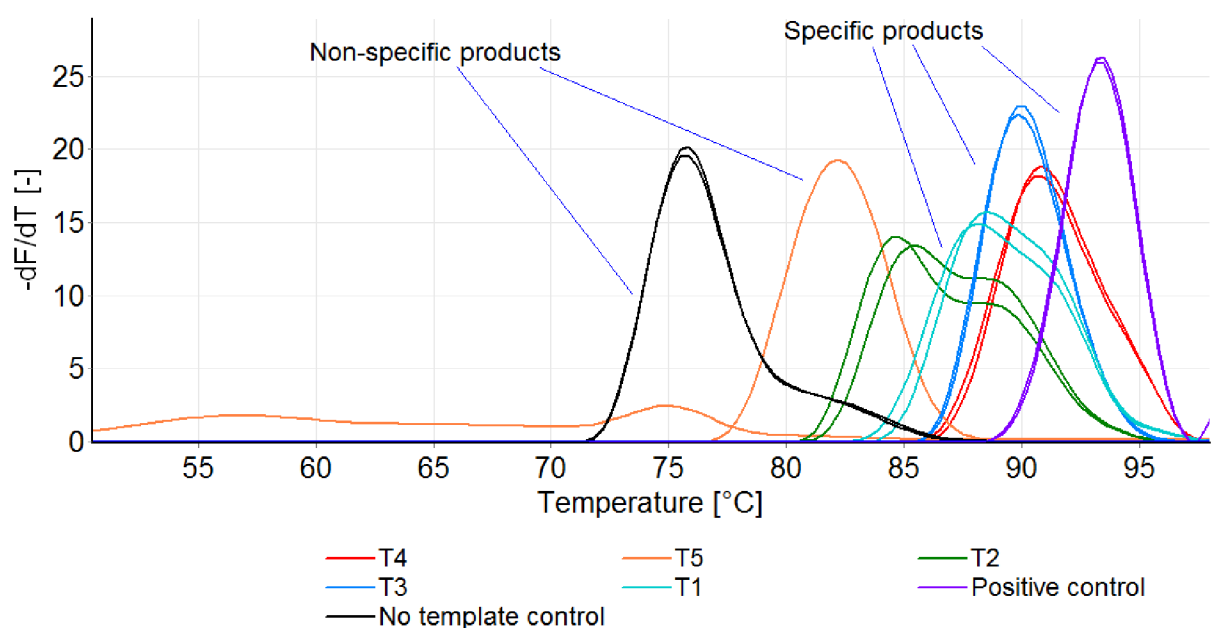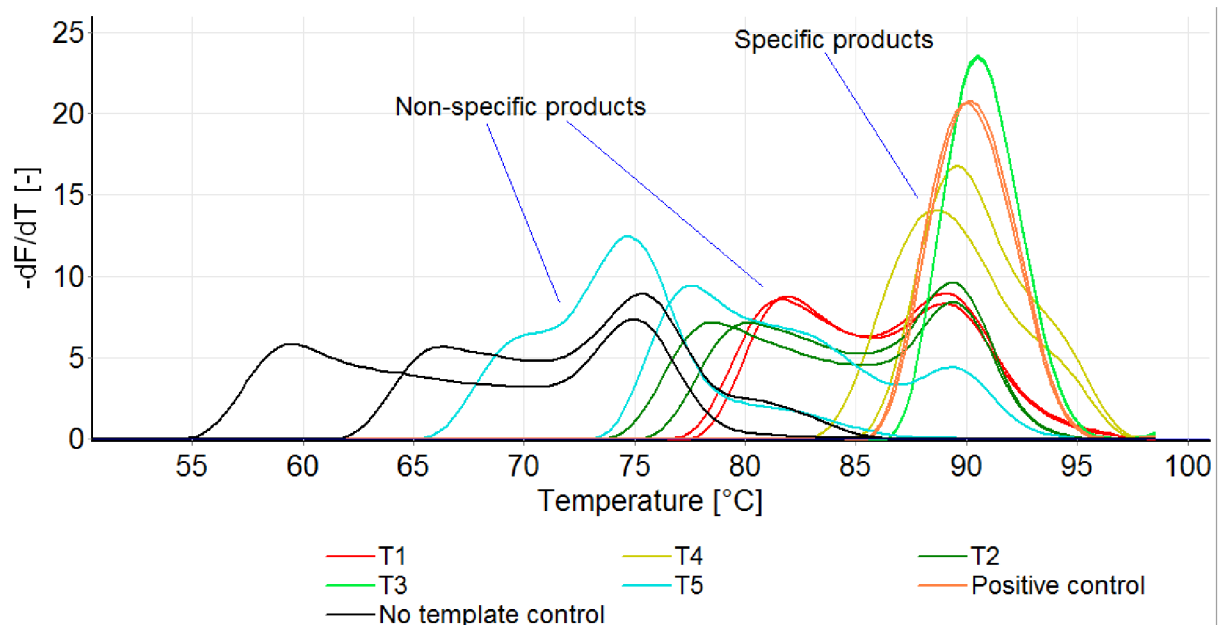

Figure 19: Differential melting curves of ITS2 amplicons, DNA from teas isolated by modified protocol with pectinase (T1 = dmBio fruit tea, T2 = Apotheke Bio Forest blend, T3 = Apotheke Bio herbal tea for imunity, T4 = Leros herbal tea "Strong eyes", T5 = Majestic Tea)
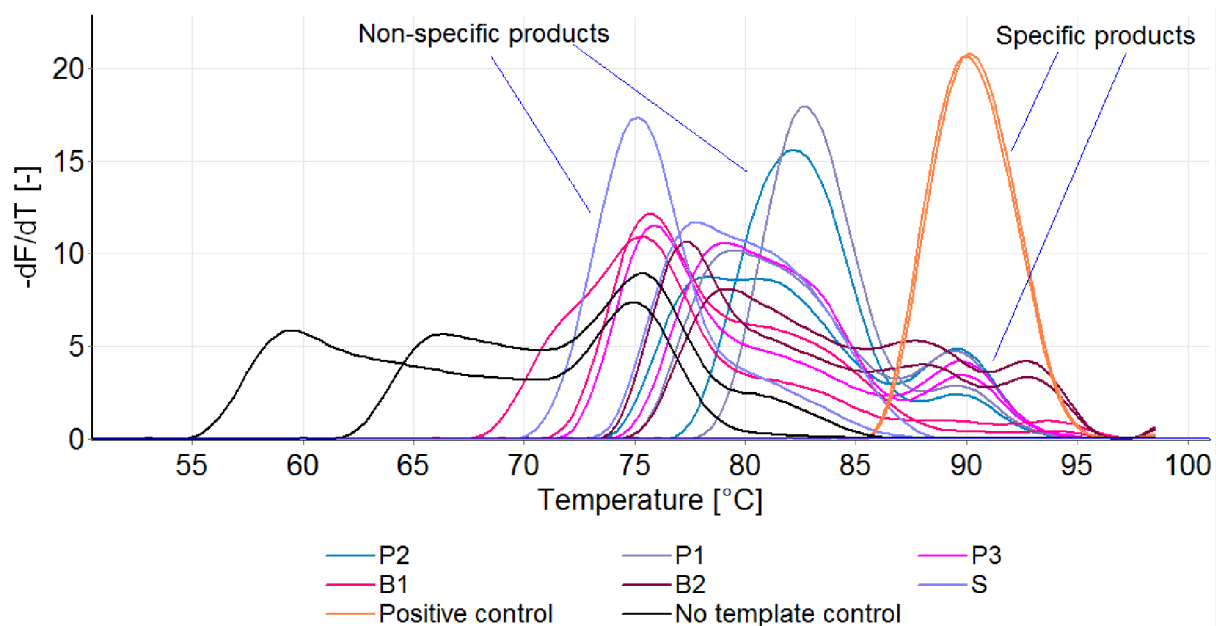
The results of plant-specific PCR assay with DNA isolated from purees, smoothie and fruit bars are shown in Figure 20. Out of six DNA isolates, only two (from both fruit bars) were amplifiable (melting peaks in the region between 85 °C and 95 °C, although in case of bar B1 the signal of this peak is only slightly higher than the background noise), as was the case with the isolates from purees, smoothie and fruit bars obtained by the unmodified kit (chapter 5.3.2.1). In melting curves representing all three purees and smoothie, only peaks with melting temperatures lower than 85 °C may be seen. As in previous chapter, these peaks were represented by smears on the electrophoretic gel (supplementary material – chapter 8, Figure 56). This result confirms that the pectinase digestion of polysaccharides is not appropriate or sufficient modification of the isolation protocol. The inclusion of pectinase digestion of polysaccharides did not solve the problem with amplifiability of DNAs from tea T5, neither of the three fruit purees, or smoothie.
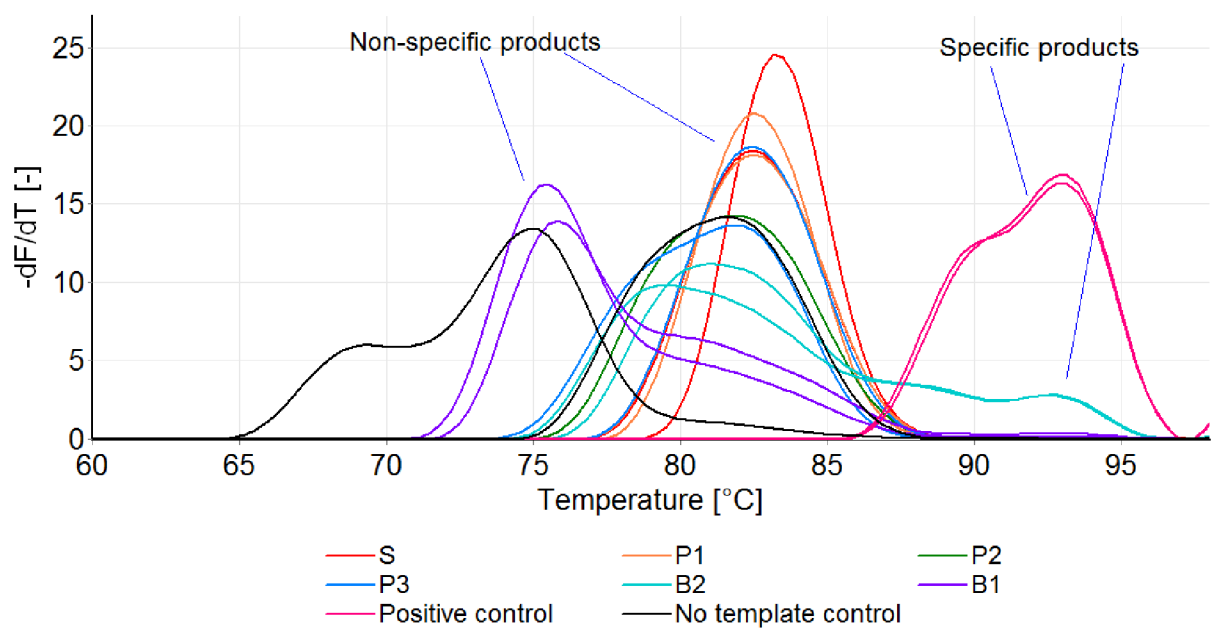
Figure 20: Differential melting curves of ITS2 amplicons, DNA isolated from purees, smoothie and fruit bars using modified protocol with pectinase (P1 = Babylove baby food, strawberry and blueberry, P2 = Babylove baby food apple, strawberries and raspberries, P3 = Relax 100 % puree, raspberry, B1 = Hipp fruit bar, B2 = dmBio fruit bar)

5.3.2.3 Kit modified by precipitation of polysaccharides by calcium chloride

The results of plant-specific PCR assay with DNAs isolated from teas are shown in Figure 21. As was the case with previous two protocols, DNAs from teas T1 – T4 were amplifiable, with amplicon melting peaks visible in the region between 85 °C and 95 °C. In case of tea T5 specific products were detected in one replicate sample.

Figure 21: Differential melting curves of ITS2 amplicons, DNA isolated from teas by modified protocol with calcium chloride (T1 = dmBio fruit tea, T2 = Apotheke Bio Forest blend, T3 = Apotheke Bio herbal tea for imunity, T4 = Leros herbal tea "Strong eyes", T5 = Majestic Tea)

Figure 22: Differential melting curves of ITS2 amplicons, DNA from purees, smoothie and fruit bars isolated by modified protocol with calcium chloride (P1 = Babylove baby food, strawberry and blueberry, P2 = Babylove baby food apple, strawberries and raspberries, P3 = Relax 100 % puree, raspberry, B1 = Hipp fruit bar, B2 = dmBio fruit bar)
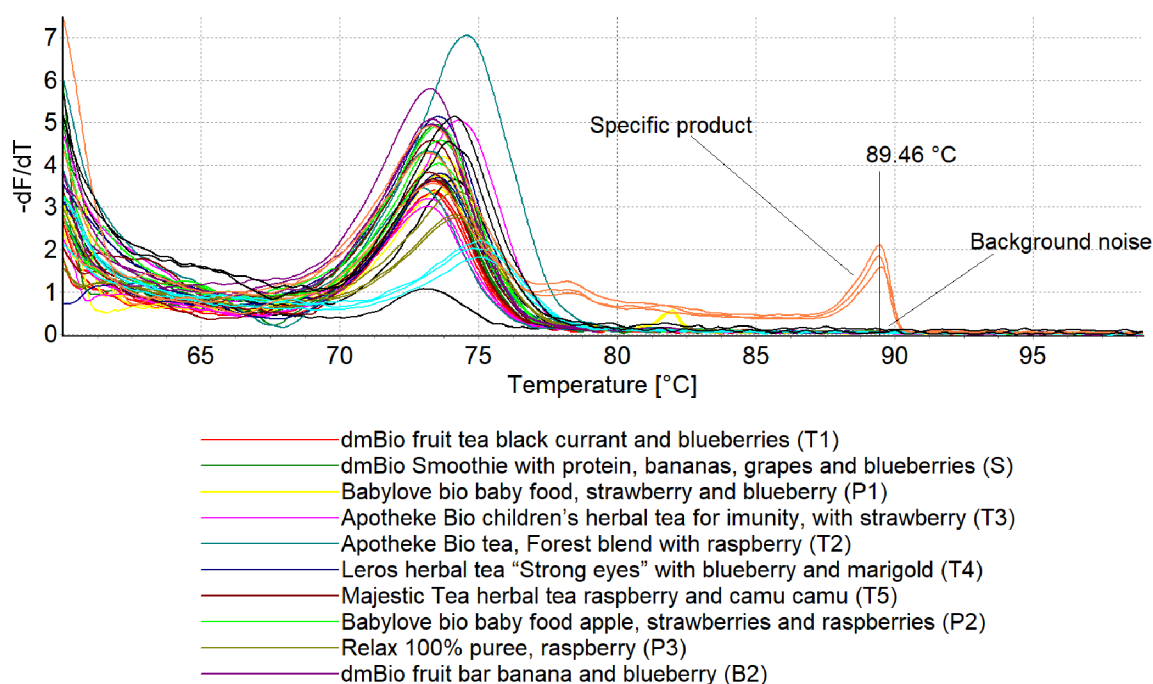
Figure 23: Differential melting curves of GAST1 amplicons, DNA from all commercial samples isolated by modified protocol with calcium chloride

The results of plant-specific PCR assay with DNAs isolated from purees, smoothie and fruit bars are shown in Figure 22. Specific products were detected in samples containing DNA from all three purees and both fruit bars. A single melting peak in the region between 85 °C and 95 °C may be seen in melting curves which represent replicate samples containing DNA from

all purees. Two melting peaks in the same region may be seen in melting curves which represent replicate samples containing DNA isolated from both fruit bars. In samples containing DNA from smoothie only non-specific products were detected (melting peak with melting temperature lower than 85 °C). These results show improved amplifiability of DNAs from some of the more problematic samples, particularly from purees. Electrophoresis of the amplicons was also performed, which confirmed the result of melting curve analysis (supplementary material, Figure 57)

The calcium chloride treatment was initially considered to be a modification whose inclusion into the DNA isolation protocol would improve the amplifiability of certain DNA isolates. However, when the DNAs isolated by the protocol with calcium chloride treatment were used in a strawberry-specific real-time PCR assay, only background noise was detected, regardless of the sample type and its amplifiability in the plant-specific PCR assay (Figure 23). Because of this result, a further modification of the DNA isolation protocol took place.

5.3.2.4 Kit modified by calcium precipitation of polysaccharides, clean-up procedure and alcoholic precipitation with co-precipitant

The results of plant-specific PCR assays with DNAs isolated from teas are shown in Figure 24. As was the case with previous protocols, DNAs from teas T1 – T4 were amplifiable (specific products visible in the region between 85 °C and 95 °C), while in samples containing DNA from tea T5 only non-specific products were detected (melting peaks with temperatures lower than 85 °C). This tea may be labelled a "recalcitrant sample", possibly due to the presence of the combination of hibiscus blossoms and apple fruit, and the contaminating substances (phenolic compounds and pectin) which they contain [124, 125].
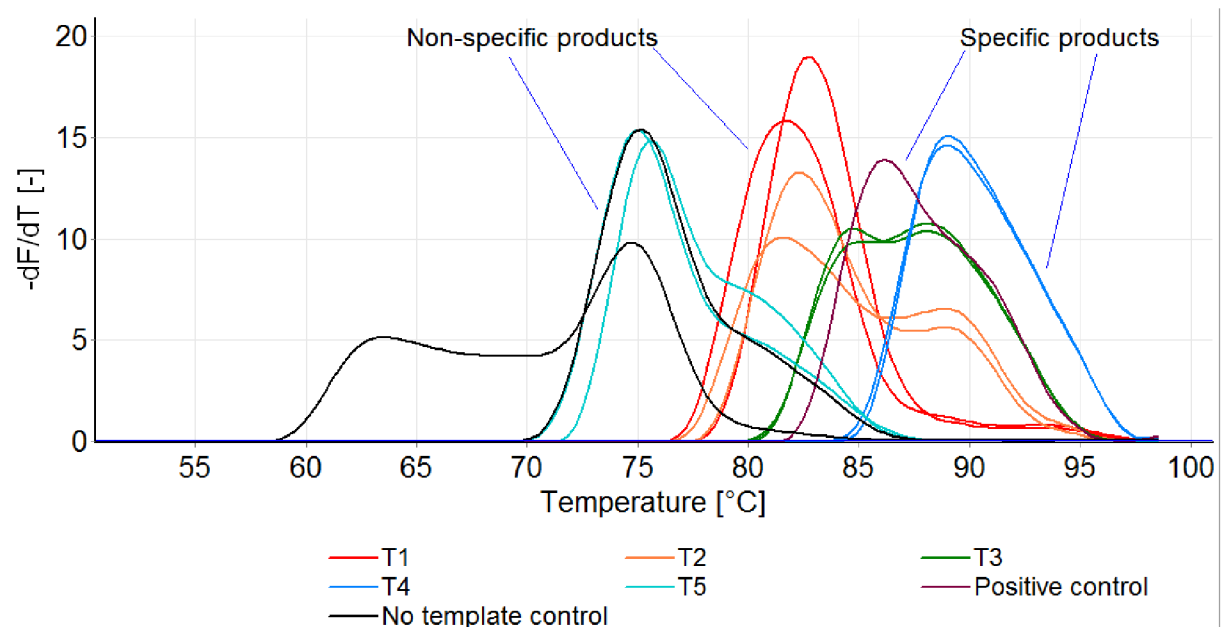

Figure 24: Differential melting curves of ITS2 amplicons, DNA from teas isolated by modified protocol with calcium chloride, clean-up kit and co-precipitant - glycogen (T1 = dmBio fruit tea, T2 = Apotheke Bio Forest blend, T3 = Apotheke Bio herbal tea for imunity, T4 = Leros herbal tea "Strong eyes", T5 = Majestic Tea)

The results of plant-specific PCR assays with DNAs isolated from purees, smoothie and fruit bars are shown in Figure 25. The result of electrophoresis of the amplicons may be seen in supplementary material (Figure 58). At first glance, specific products seem to be present only in one replicate sample containing DNA from fruit bar B2 (a melting peak in the region between

85 °C and 95 °C), with only non-specific products detected in samples containing DNA from purees, smoothie and fruit bar B1 (melting peaks with temperatures lower than 85 °C). However, specific products were detected in both replicate samples containing DNA from bars B1 and B2, puree P2 and one replicate sample containing DNA from smoothie, as is shown in the detail of the melting curves which represent these samples (Figure 26).
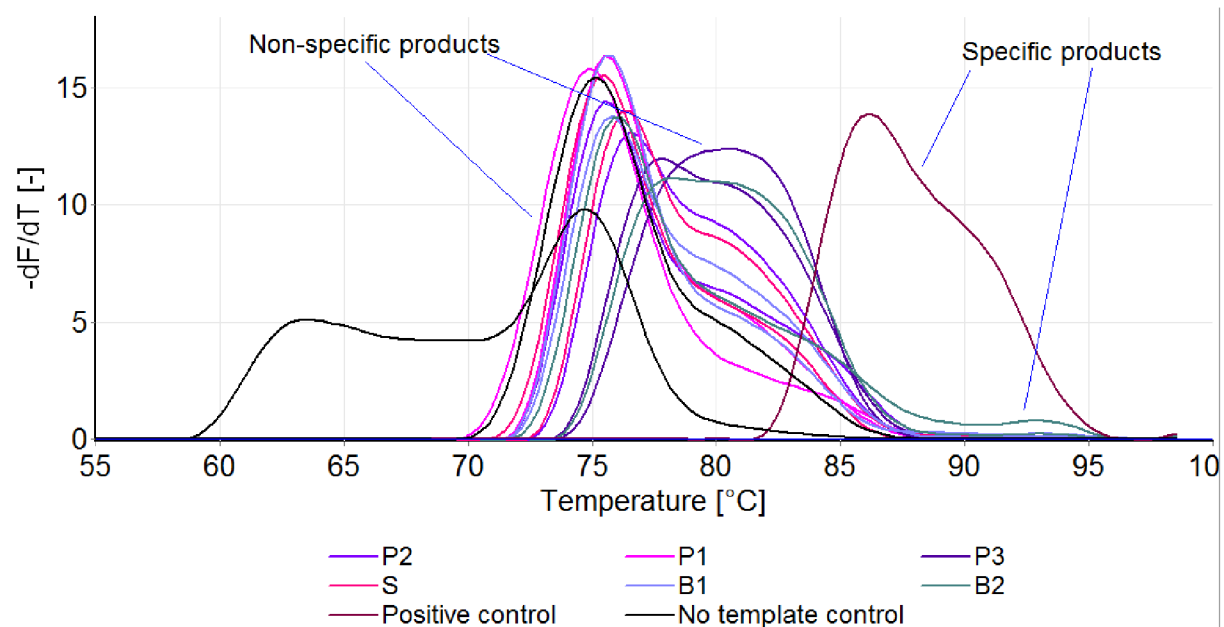

Figure 25: Differential melting curves of ITS2 amplicons, DNA from purees, smoothie and fruit bars isolated by modified protocol with calcium chloride, clean-up kit and co-precipitant (P1 = Babylove baby food, strawberry and blueberry, P2 = Babylove baby food apple, strawberries and raspberries, P3 = Relax 100 % puree, raspberry, B1 = Hipp fruit bar, B2 = dmBio fruit bar)

Although the results of plant-specific PCR assays with DNAs obtained by the protocol with calcium chloride treatment, clean-up procedure and alcoholic precipitation with co-precipitant (glycogen) were seemingly not as good as the results of PCR assays with DNAs obtained by the protocol with calcium chloride treatment only (lower signal from melting peaks of specific products in samples containing DNA isolated by the protocol with clean-up procedure and co-precipitant), the former DNAs were also tested in species-specific real-time PCR assays.

It has been shown that DNAs isolated by protocol with calcium chloride treatment only could not be amplified in the strawberry-specific PCR assay. A possible cause of this result might have been residual calcium ions present in the DNA isolates. Opel et al. in their 2010 article [127] showed that calcium ions inhibit Taq polymerase.
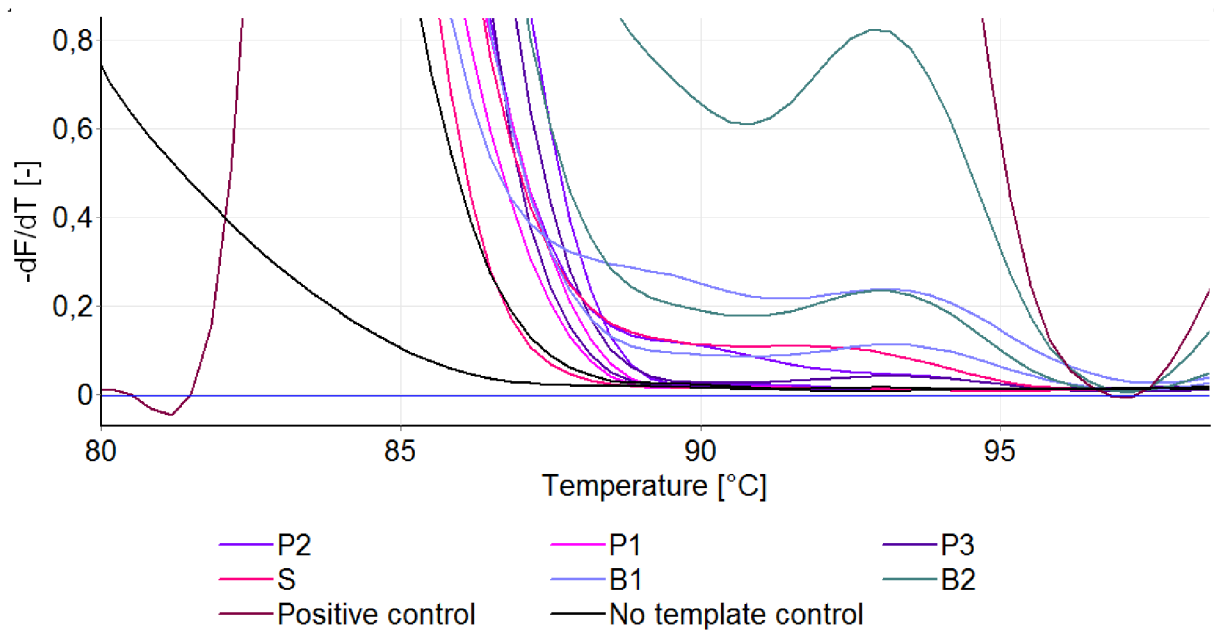
Figure 26: Detail of Figure 25 (P1 = Babylove baby food, strawberry and blueberry, P2 = Babylove baby food apple, strawberries and raspberries, P3 = Relax 100 % puree, raspberry, B1 = Hipp fruit bar, B2 = dmBio fruit bar)

If residual calcium ions remained in the DNA isolates obtained by the protocol modified by calcium chloride precipitation of polysaccharides, further purification of the isolates would be required. The last modified protocol tested in this work contained such step. For this reason, the DNAs isolated by the last protocol with the most modifications were used for further analyses in species-specific singleplex and multiplex PCR assays.

5.4 Analysis of commercial products by species-specific PCR assays

The composition of reaction mixtures for this analysis is described in chapter 4.10.1. The amounts of DNA used ranged from 15.0 ng to 39.2 ng for teas and from 17.4 ng to 28.8 ng for purees, smoothie, and fruit bars. HRM analysis was performed as described in chapter 4.11. DNAs isolated from blueberry (*Vaccinium corymbosum*), red raspberry (*Rubus idaeus*) and strawberry (*Fragaria ananassa*) were used as reference samples. As a verification of the assays' specificity DNAs from all commercial samples were used in all species specific PCR assays, even if the presence of the species was not declared in a product.

5.4.1 Detection of blueberry DNA in commercial foodstuffs – singleplex real-time PCR-HRM

The results obtained with DNAs isolated from teas are shown in Figure 27 (differential melting curves). The specific product of primers VcBHLH003 (specific for blueberry) is represented by a peak with melting temperature $T_m = 81.70$ °C (green curves in Figure 27). In differential melting curves representing DNAs from teas T3 and T5, where blueberry was not declared, no melting peaks of specific products are visible. In case of teas T1, T2 and T4 the result was the same.
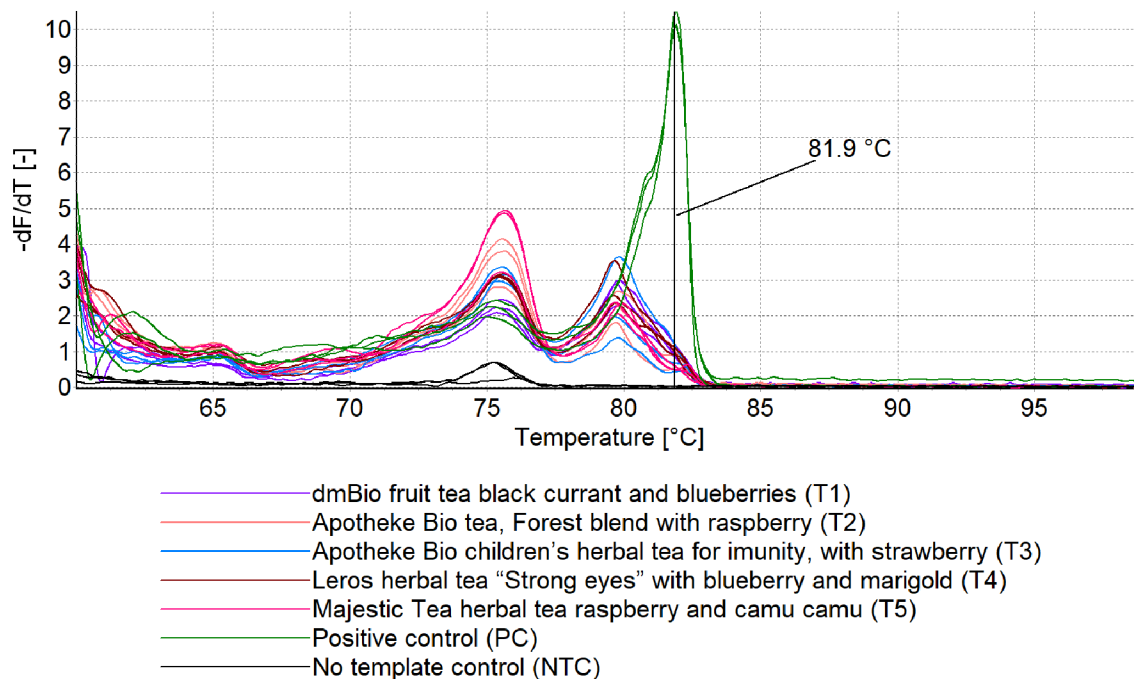
Figure 27: Differential melting curves of VcBHLH003 amplicons, DNA from teas

Apart from inhibition of PCR by contaminants, there are several possible reasons for this outcome. Firstly, bilberry (also known as European blueberry, *Vaccinium myrtillus*), might have been added to the teas instead of "Canadian" blueberry (*Vaccinium corymbosum*). Secondly, the concentration of template sequences for primers VcBHLH003 might have been under the limit of detection of these primers – this might be the case especially for teas T1 and T2, where the content of blueberry was declared to be 6 % and 1 %, respectively. With tea T4, the previous reason for no amplification of specific product is more likely, as the content of blueberries in this tea was declared to be 20 % (Table *1*). Because no melting peaks of specific products were observed in the samples containing DNAs isolated from teas, HRM analysis was not performed.

The results obtained with DNAs isolated from purees, smoothie and fruit bars are shown in Figure 28. The melting peak of the specific product of primers VcBHLH003 is represented by the red curves. Melting peaks with slightly different melting temperatures are visible in curves representing bar B1, puree P3 one replicate sample containing DNA from puree P2, and one replicate sample containing DNA isolated from smoothie. In the curves representing puree P1, bar B2, one replicate sample containing DNA from puree P2 and one replicate sample containing DNA from smoothie only non-specific products were observed.

Because specific amplicons of primers VcBHLH003 were detected in some samples, HRM analysis was performed. The data were normalized in the region between 77.3 and 85.0 °C. Bilinear normalization without temperature shifting was performed. The result is represented in the form of difference plots of normalized melting curves (Figure 29). The curves representing positive control were used as a reference, and in Figure 29 they can be seen clustered around the baseline.
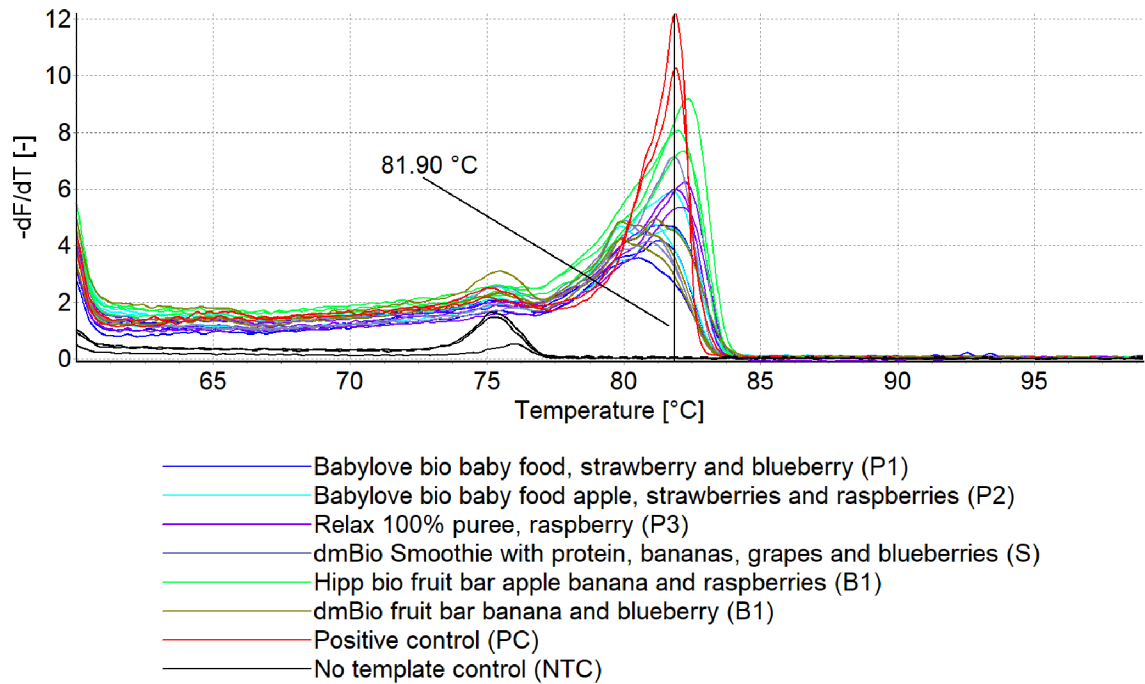
Figure 28: Differential melting curves of VcBHLH003 amplicons, DNA from purees, smoothie and fruit bars

Ideally, if two amplicons have the same sequence, the curves which represent them should completely overlap [88]. In practice, melting curves of replicate samples may not overlay completely due to small differences in shapes caused by e. g. temperature variability among individual wells of an instrument [128].
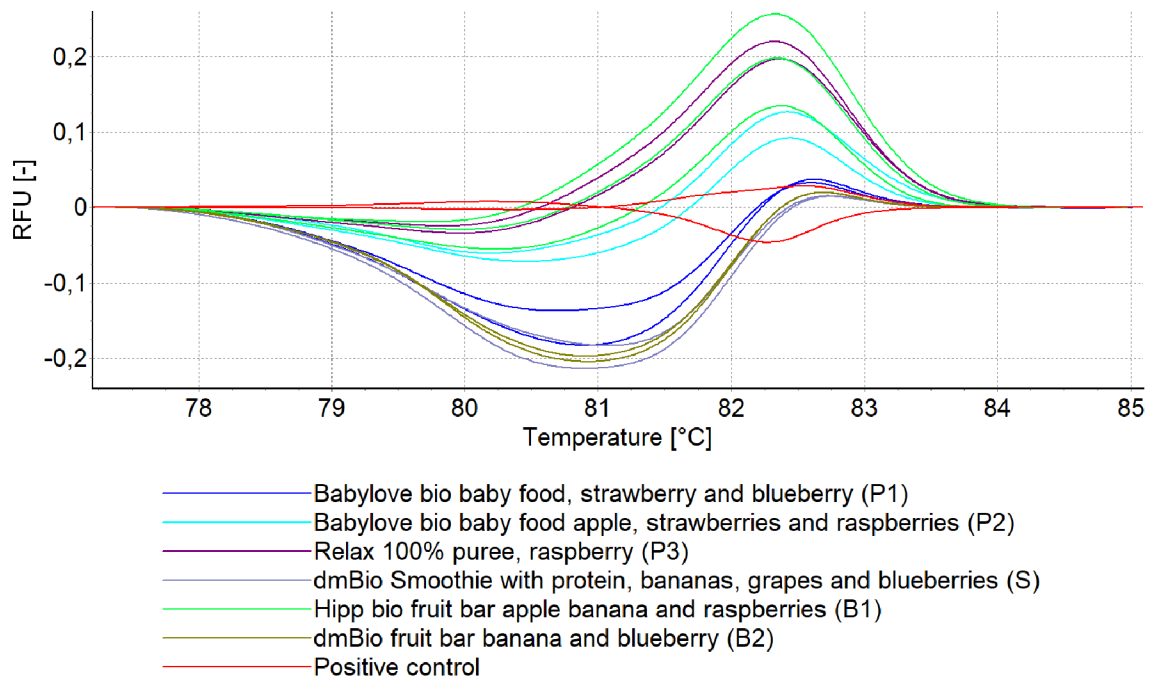

Figure 29: Difference plots of normalized melting curves, VcBHLH003 amplicons, DNA from purees, smoothie and fruit bars

Figure 29 shows the curves which represent puree P1, fruit bar B2 and smoothie clustered together. Because no melting peaks of specific products were observed in the differential melting curves of these samples (Figure 28), it can be concluded that in these samples only non-specific products with identical or similar sequences were amplified. Another group of samples visible in Figure 29 consists of curves which represent fruit bar B1, puree P3 and puree P2. In these samples melting peaks of specific products were observed (Figure 28). If no melting peak was observed in sample B1, in which blueberries were not declared by the manufacturer (Table 1), we would be able to say that puree P2 and puree P3 contain blueberries, but different variety than the one used as reference sample. However, because melting peaks of specific amplicons were observed in a sample where they should not be, and because these amplicons showed similar melting behaviour as amplicons detected in purees P2 and P3, we were not able to confirm if these purees contained blueberries or not.

5.4.2   Detection of raspberry DNA in commercial foodstuffs – singleplex PCR

The results obtained with DNAs isolated from teas are shown in Figure 30 and Figure 31. The specific product of positive control had melting temperature $T_m = 85.52$ °C. In the differential melting curves of samples containing DNA from teas similar peaks were observed, even in teas T3 and T4, which did not have raspberries declared in their composition (Table 1). The raw melting curves were normalized in region between 83.5 and 87.7 °C and underwent HRM analysis.
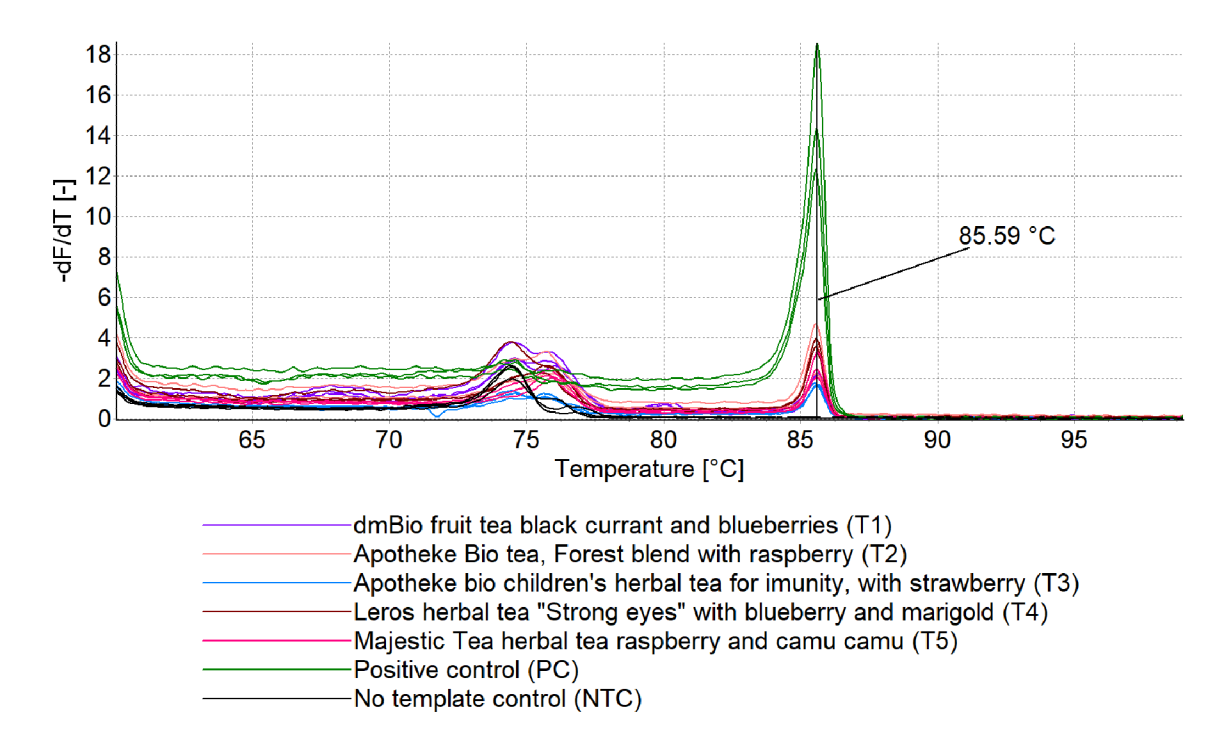

Figure 30: Differential melting curves of RiACO1 amplicons, DNA from teas

Figure 31 shows difference plots of the amplicon's melting curves. The curves which represent teas T2, T3, T4 and T5 are grouped together and have a shape different from the positive control. The exception are curves which represent tea T1. Each of the three replicate samples has a different shape, two are different from each other and the positive control and one is grouped together with curves of samples T2 – T5. In case of tea T1 the presence of raspberries was not confirmed.
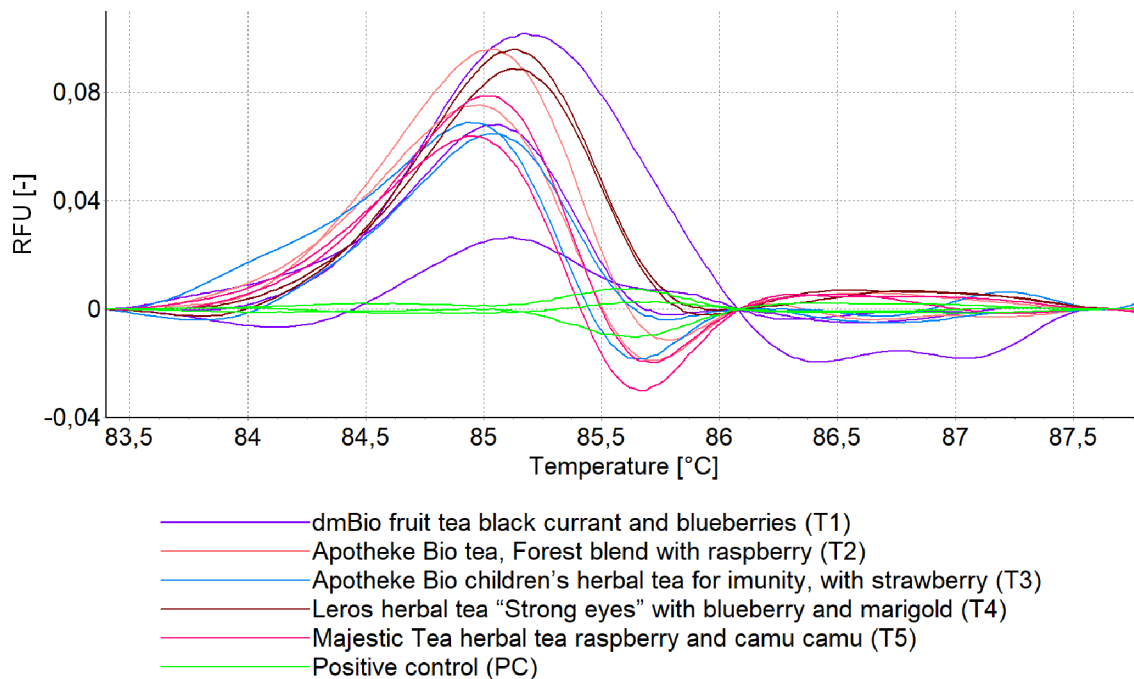
Figure 31: Difference plots of normalized and shifted melting curves, RiACO1 amplicons, DNA from teas

In case of teas T2 and T5, where raspberry was declared by the manufacturer, we were also not able to confirm whether these teas indeed contained raspberry, as the difference plots of the curves which represent these samples have shapes similar to the difference plots of curves which represent teas T3 and T4, where raspberries were not declared.

The results obtained with DNAs from purees, smoothie and fruit bars are shown in Figure 32 and Figure 33. The positive control is represented by red curves and the melting temperature of its amplicon was once again $T_m = 85.52$ °C. Melting peaks of specific products can be observed in all samples containing DNA from commercial samples, but especially in melting curves which represent puree P3 and bar B1, both of which had raspberries declared in their composition.

The HRM analysis confirmed the presence of raspberries in puree P3 (purple curves) and in bar B1 (green curves), as the curves which represent these samples are grouped together with the positive control, whose curves (red) are clustered around the baseline. In puree P3, cyanidine-3-o-rutinoside, which occurs in fruits of *Rubus* genus [129], was also tentatively identified by HPLC with PDA detector (Figure 59 in supplementary material). In case of puree P1 spurious amplification products were detected, as this sample did not have raspberries declared in its composition (Table 1) and the curves which represent this sample in Figure 33 are not aligned and each of the three replicate curves has a different shape from the other two. The situation is similar with bar B2, in which raspberries were also not declared. In case of puree P2, a raspberry variety different from the one used as positive control might have been present, and might also be true for smoothie, but further verification (such as PCR with more stringent conditions) would be advisable, as some of the melting curves representing these samples have shapes similar to curves representing puree P1, where spurious amplification was observed (Figure 33).
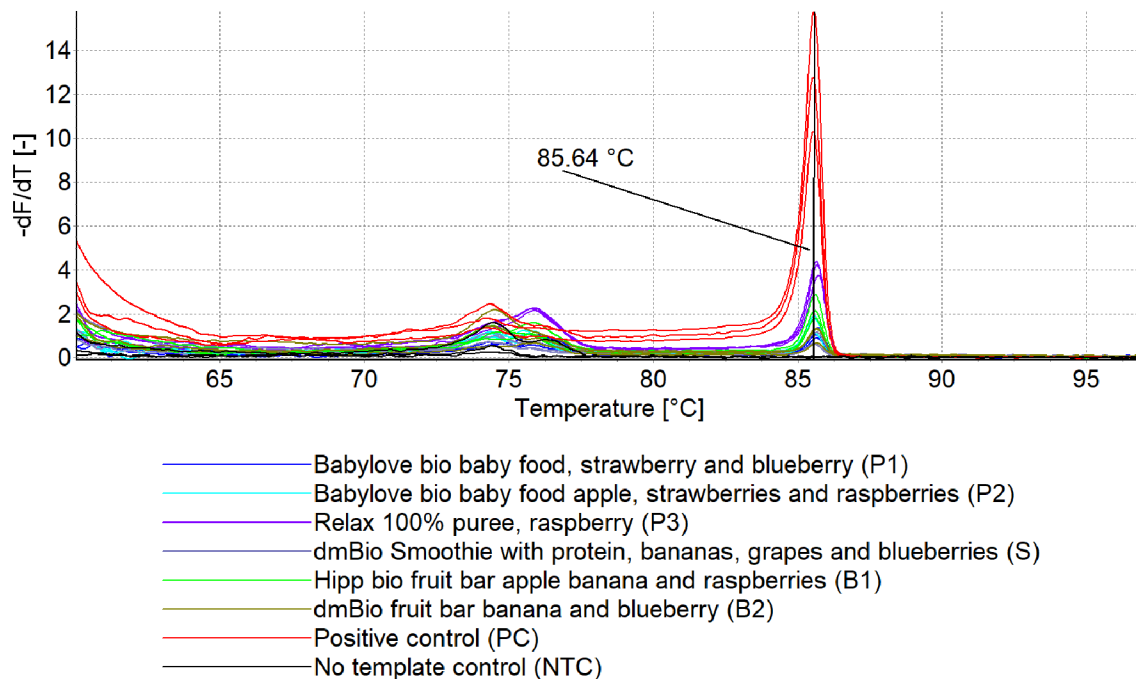
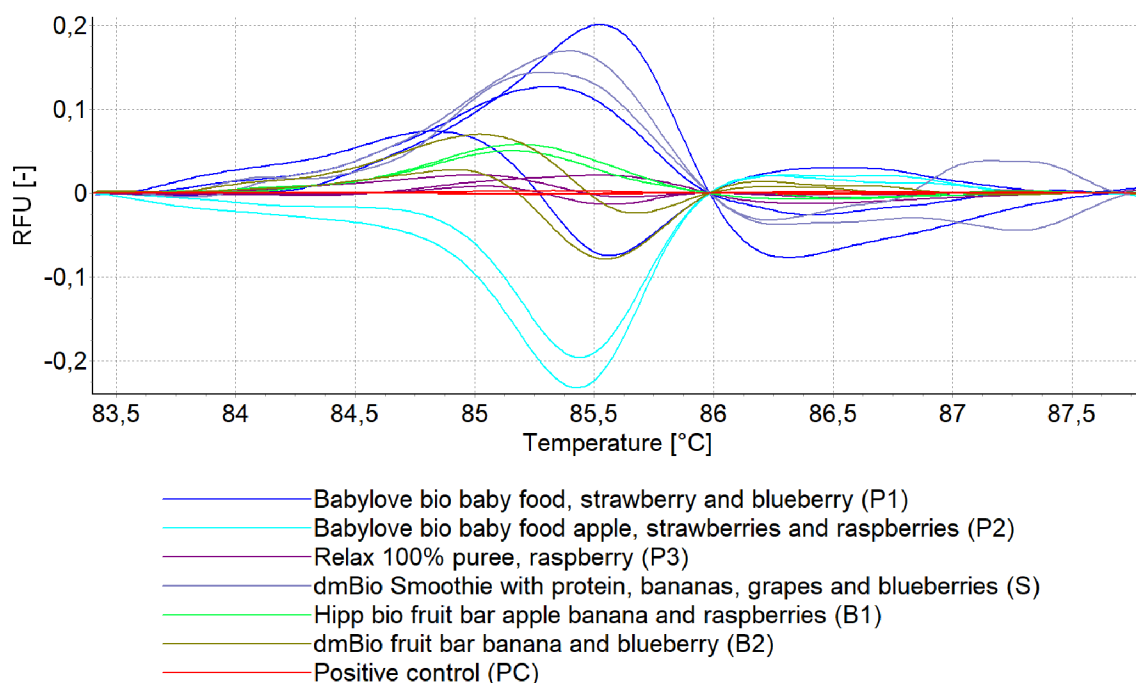*Figure 32: Differential melting curves of RiACO1 amplicons, DNA from purees, smoothie and fruit bars*

Figure 33: Differential plot of normalized and shifted melting curves, RiACO1 amplicons, DNA from purees, smoothie and fruit bars

5.4.3 Detection of strawberry DNA in commercial foodstuffs – singleplex PCR

The results obtained with DNAs isolated from teas are shown in Figure 34 and Figure 35. The melting peak of positive control (green curves) had melting temperature T_m = 89.38 °C. Similar peaks were observed in the samples containing DNA from teas, however, the signal of these peaks was barely higher than the background noise (Figure 34).
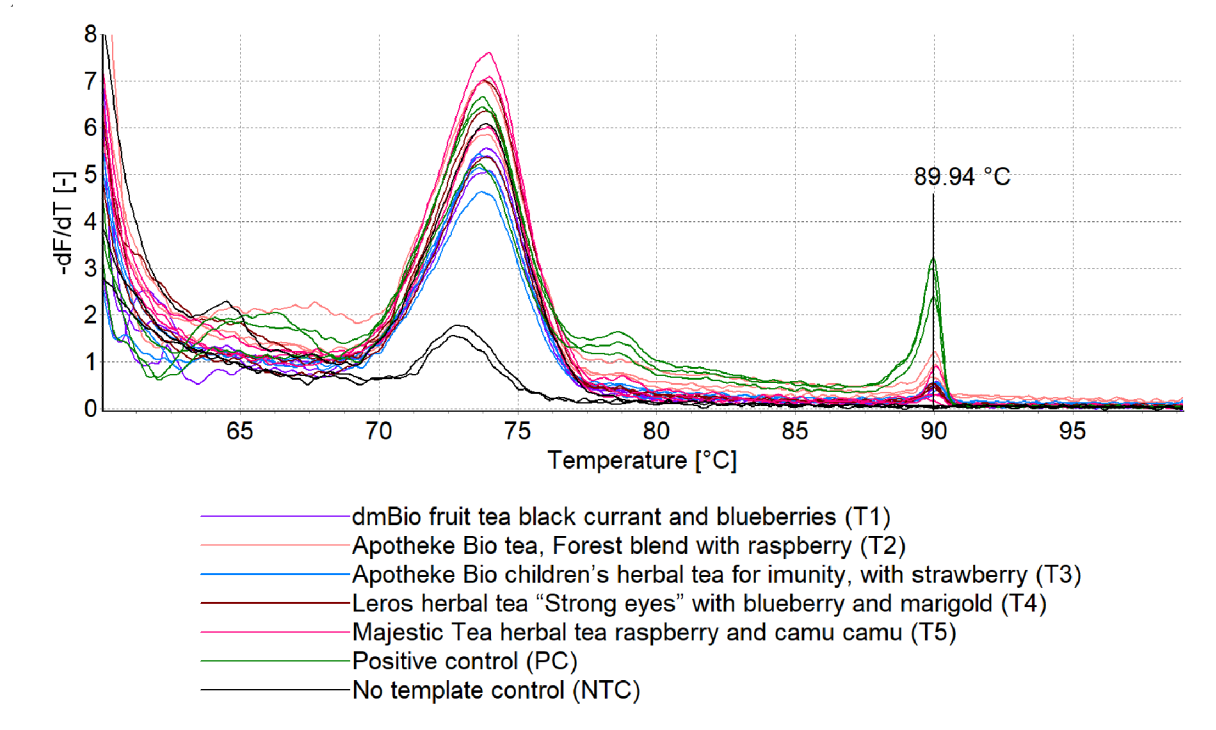

Figure 34: Differential melting curves of GAST1 amplicons, DNA from teas

The raw melting curves of detected amplicons were normalized in the region of 87.5 – 92 °C. Bilinear normalization was used, with no temperature shifting. The only curve grouped together with the positive control is one replicate sample containing DNA from tea T5, which did not have strawberry declared in its composition (Table 1), which means that in this case spurious amplification occurred. The same conclusion might be made about the remaining samples. The signal from the products detected in these samples was poor (Figure 34) and the curves which represent samples where strawberry was declared have similar shapes to curves which represent samples where strawberry was not declared. This means that we were not able to confirm the presence of strawberry in any of the five analysed teas.
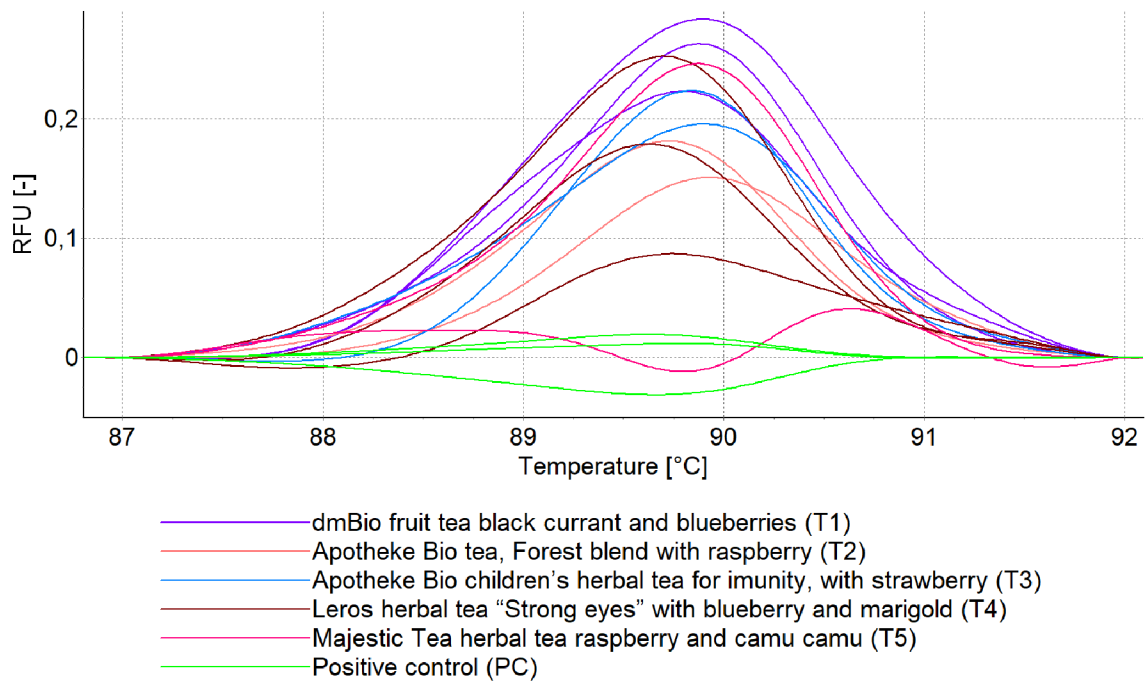
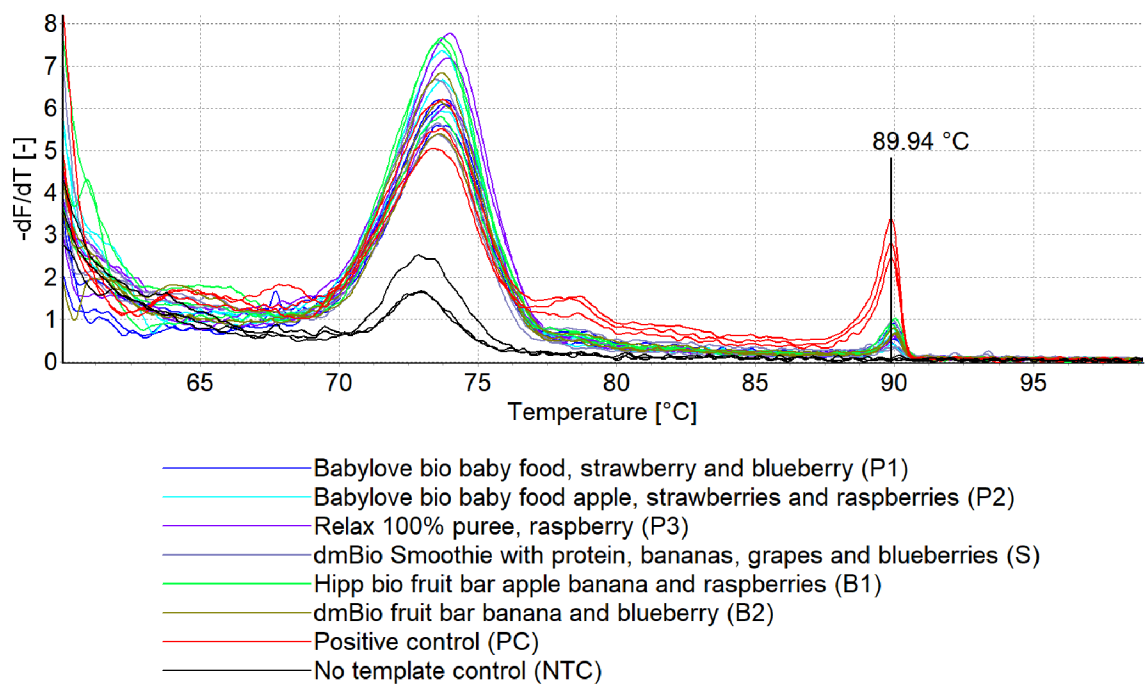*Figure 35: Difference plots of normalized melting curves, GAST1 amplicons, DNA from teas*

Figure 36: Differential melting curves of GAST1 amplicons, DNA from purees, smoothie and fruit bars

The results obtained with DNAs from purees are shown in Figure 36 and Figure 37.Similarly to the results observed in samples which contained DNAs from teas, amplicon melting peaks with temperatures similar to that of the positive control were observed in all samples containing DNA from purees, smoothie and fruit bars. The signal of these amplicons was once again poor (Figure 36). Furthermore, melting peaks of amplicons with melting temperatures similar to the

positive control were also observed in samples which did not have strawberries declared in their composition, in particular smoothie, fruit bar B1 and fruit bar B2.
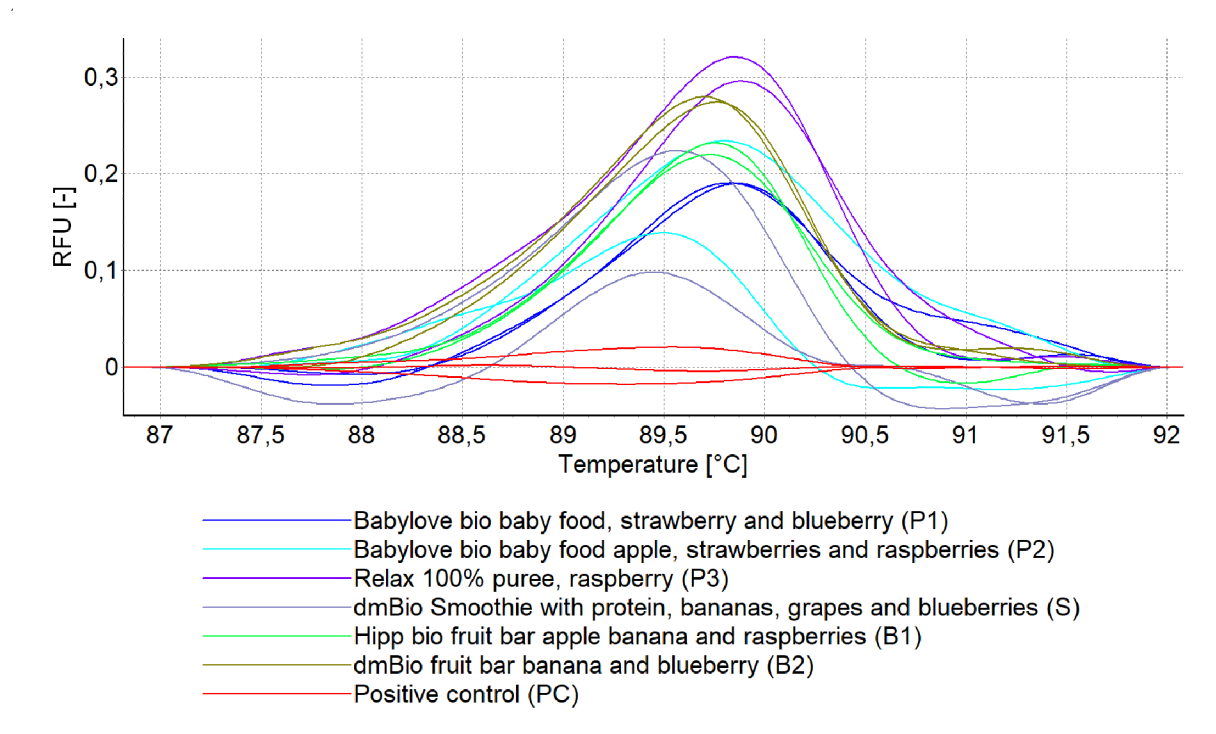

Figure 37: Difference plots of normalized melting curves, GAST1 amplicons, DNA from purees, smoothie and fruit bars

Although the signal of amplicons detected in samples containing DNAs from commercial products was poor, the raw melting curves were still normalized, again in region of 87.5 – 92 °C. The difference plots of the normalized melting curves (Figure 37) show that none of the curves which represent commercial samples are grouped together with the positive control. The curves of samples where strawberries were not declared in the composition also have the same or similar shape as curves of samples where strawberries were declared. This means that we were not able to confirm the presence of strawberries in purees P1, P2 and P3, even though strawberries were declared to be in these products and amplicon melting peaks were observed in samples which contained DNA from these three purees.

5.4.4 Simultaneous detection of blueberry, raspberry and strawberry DNA in commercial foodstuffs – triplex PCR

The differential melting curves of samples containing DNAs from teas are shown in Figure 38. The positive control is represented by green curves and melting peaks of specific amplicons can be seen at 81.98 °C (primers VcBHLH003, blueberry specific), 85.66 °C (primers RiACO1, raspberry specific) and 90.04 °C (primers GAST1, strawberry specific). In addition to positive control containing template DNAs for all three primer pairs of the triplex, another set of controls was prepared. These controls consisted of PCR reaction mixtures containing components for triplex PCR, but template for only one primer pair in the triplex. The controls containing template DNA for only one primer pair were used as baselines in HRM analyses of amplicons obtained in the triplex PCR assay. Three separate HRM analyses were performed, each for amplicons of different primer pair in the triplex.

In the samples containing DNA from teas where blueberry was declared in the composition (T1, T2 and T4, red, pale pink and brown curves, respectively), only non-specific products were

detected, with melting temperatures close to non-specific products detected in teas in whose composition blueberry was not declared (T3, and T5). The reasons for this outcome might have been again presence of inhibitors in the template DNA, low quantities of blueberries in teas T1 and T2, and presence of bilberry in tea T4 instead of blueberry. Combination of two of these factors is likely, too.
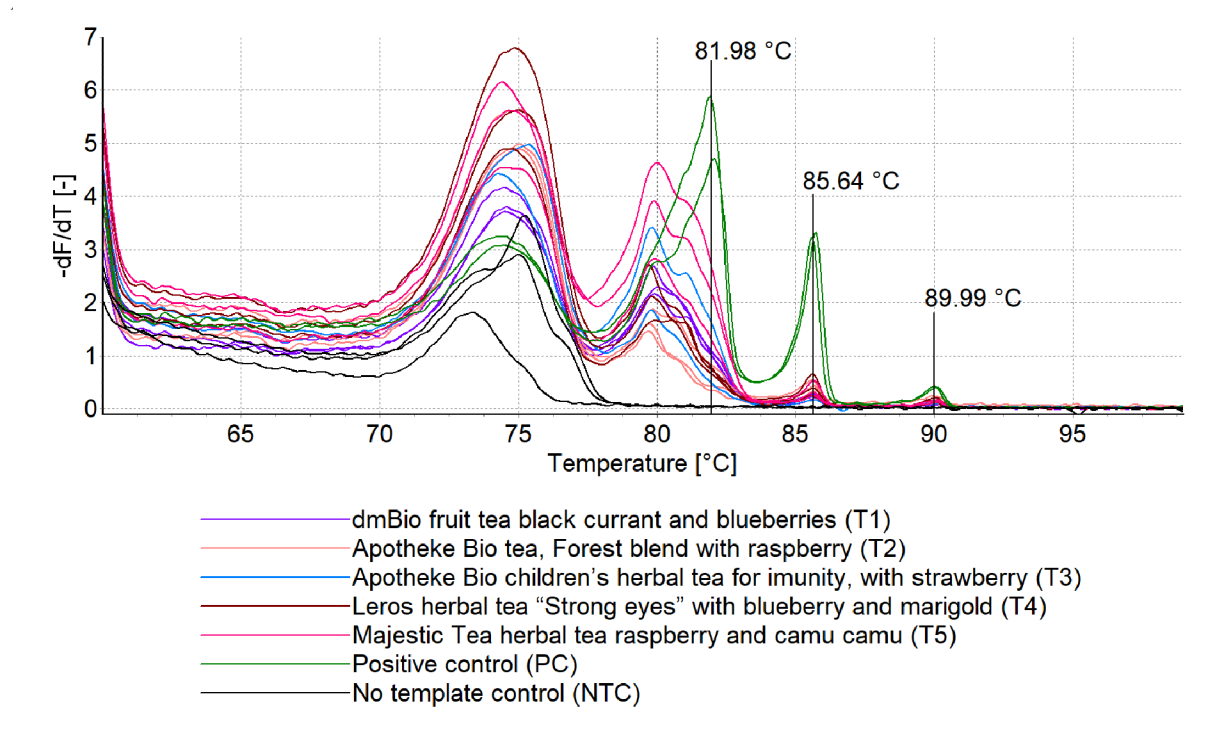

Figure 38: Differential melting curves of VcBHLH003, RiACO1 and GAST1 amplicons obtained in triplex PCR, DNA from teas

In case of primers RiACO1, amplicon melting peaks were observed in all samples containing DNA from teas (Figure 38). Curves representing two replicate samples of tea T2 (pale pink) and one replicate sample of tea T5 (fuchsia) are clustered together with the positive control (green) around the baseline (Figure 39). In these teas raspberries were declared (2 % in both T2 and T5). However, we were not able to confirm the presence of raspberries in tea T2, because apart from the curves mentioned above, those representing tea T4 (brown) are also clustered around the baseline with the positive control. Further analysis would be needed to confirm the result obtained with sample T2, such as PCR with a different primer pair specific for raspberry, or singleplex PCR with primers RiACO1 in more stringent conditions. Regarding tea T1, we were not able to confirm if the curves shown in Figure 39 represent a variety of raspberry different from that in the positive control, again because the curves representing tea T4 (brown) are clustered together with curves representing samples where raspberry was declared, instead of being clustered separately from these samples.
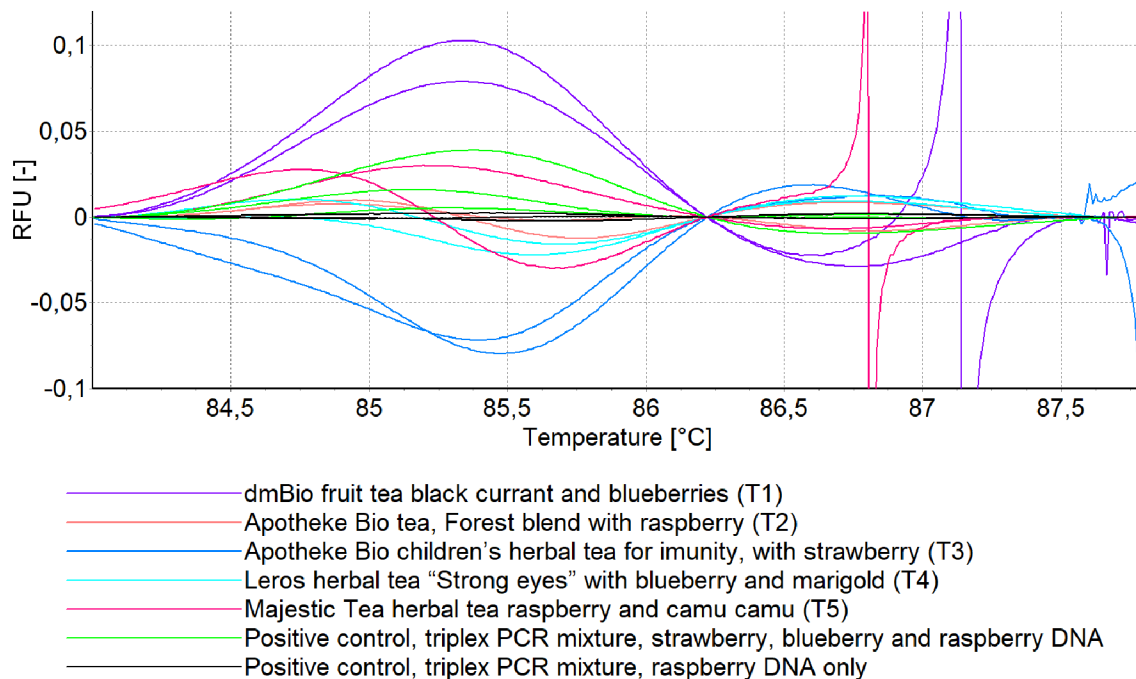
Figure 39: Difference plots of normalized melting curves, RiACO1 amplicons, triplex PCR, DNA from teas

In case of primers GAST1 we were not able to confirm presence of strawberries in any of the five analysed teas. Except for two replicate samples containing DNA from tea T2 (pale pink curves) and two replicate samples with DNA from tea T4 (light blue curves), all curves representing the commercial teas are clustered together with the positive control (green curves) (Figure 40). It may be concluded that only spurious amplicons were detected in the samples containing DNA from commercial teas, and more stringent PCR conditions and possibly a different strawberry specific primer pair is needed for better result. Adjustment of DNA isolation protocol may be needed, as inhibition of PCR may also have played a role in the results obtained with DNAs from teas T2 and T3, where strawberries were declared in the composition.

The results of triplex PCR with DNAs from purees, smoothie and fruit bars are shown in the form of differential melting curves in Figure 41. Positive control containing template DNA for all three primer pairs in the triplex is represented by red curves. Melting peaks of VcBHLH003 amplicons can be seen at 81.71 °C, peaks of RiACO1 amplicons are visible at 85.4 °C and peaks of GAST1 amplicon can be seen at 89.78 °C. Like with analysis of DNAs from teas, in this case a second set of controls was prepared. This set consisted of three triplex PCR mixtures prepared in triplicates. Each of these mixtures contained either blueberry, strawberry or raspberry DNA, and these mixtures were used as baseline in HRM analyses.
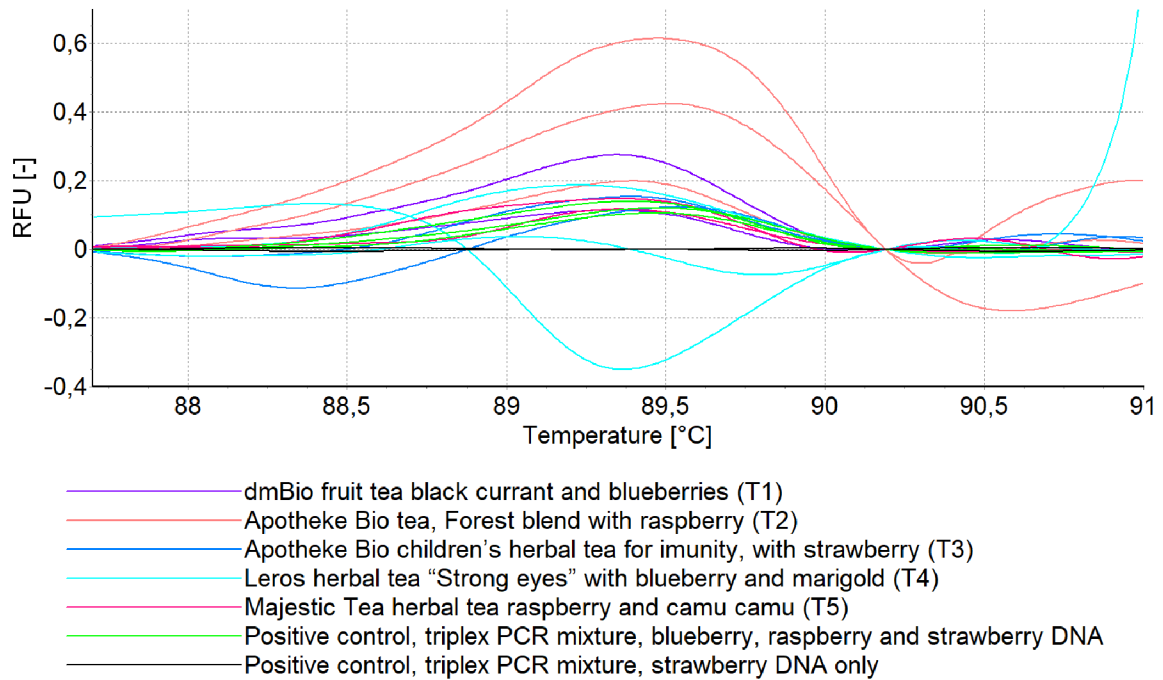
Figure 40: Difference plots of normalized melting curves, GAST1 amplicons, triplex PCR, DNA from teas

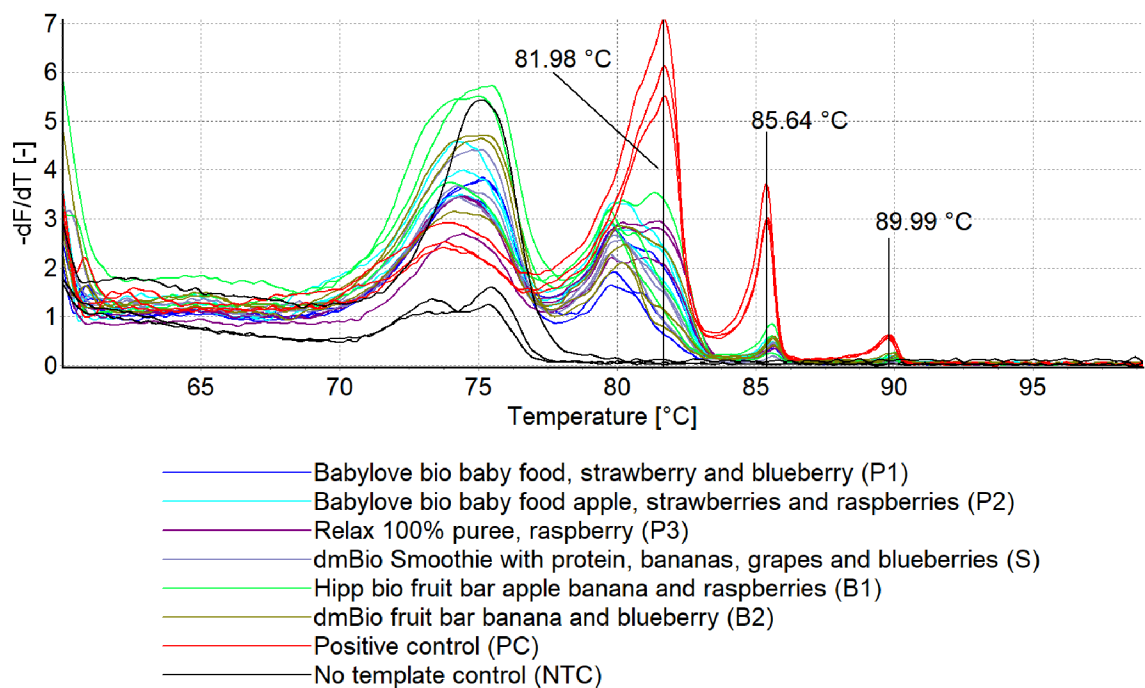*Figure 41: Differential melting curves of VcBHLH003, RiACO1 and GAST1 amplicons obtained in triplex PCR, DNA from purees, smoothie and fruit bars*

Regarding VcBHLH003 amplicons, in case of purees P1 and P2, smoothie, bar B2 and two replicate samples with DNA from bar B1 these amplicons were not observed (Figure 41). It is also not entirely clear whether the products detected in samples with DNA from puree P3 are

specific VcBHLH003 amplicons, or non-specific products whose melting temperature is close to that of the specific products.

From the difference plots of the normalized amplicon melting curves (Figure 42) it may be concluded that the products detected in samples with DNA from puree P3 are indeed non-specific. The curves representing these samples (purple) have similar shape to the curves representing samples where only non-specific products were detected, and they are also clustered together with these curves.

Possible reasons why blueberry was not detected in product where it was declared (purees P1, P2 and P3, smoothie and bar B2) involve low quantities of blueberry matrix in the purees (5 % in puree P1, 2 % in puree P2, 1% in puree P3, 4 % in bar B2), as well as the character of blueberry matrix in these samples, as in smoothie, puree P3 and bar B2 blueberry juice was declared, rather than blueberry fruits or puree. Other factor which may have played a role were contaminants present in the DNA isolates from commercial products, which might have partially inhibited DNA polymerase.
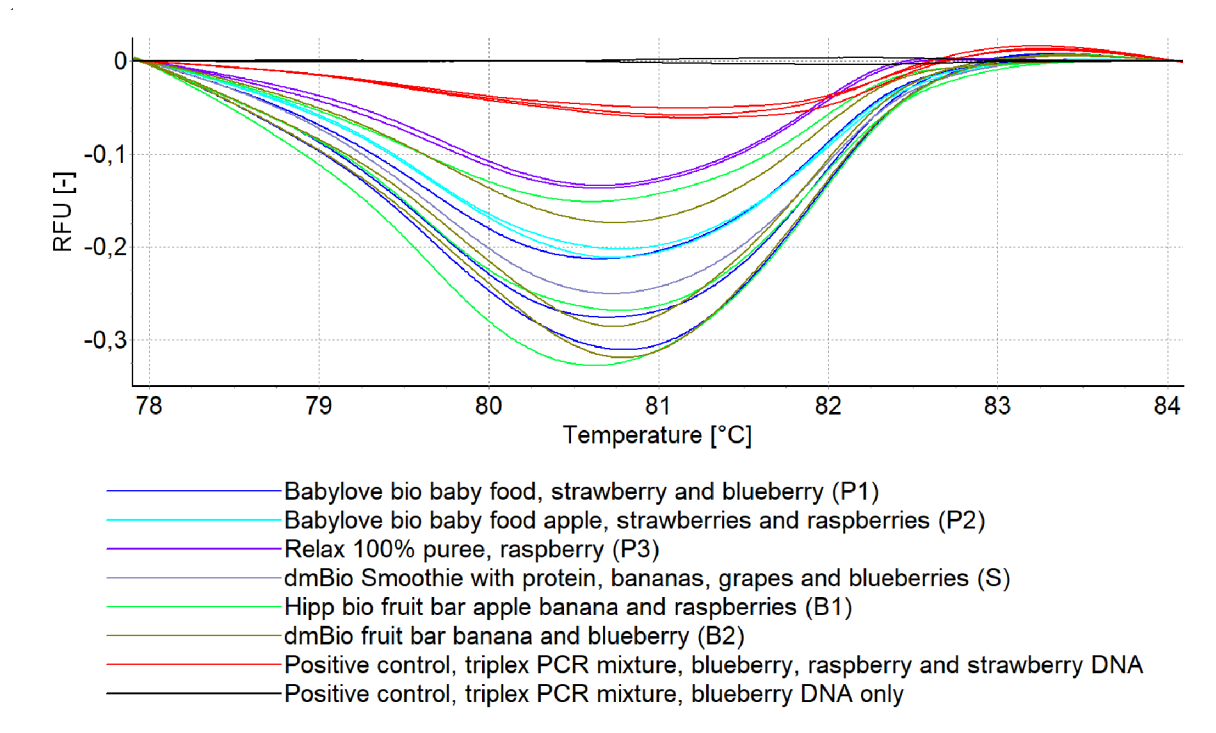

Babylove bio baby food, strawberry and blueberry (P1)
Babylove bio baby food apple, strawberries and raspberries (P2)
Relax 100% puree, raspberry (P3)
dmBio Smoothie with protein, bananas, grapes and blueberries (S)
Hipp bio fruit bar apple banana and raspberries (B1)
dmBio fruit bar banana and blueberry (B2)
Positive control, triplex PCR mixture, blueberry, raspberry and strawberry DNA
Positive control, triplex PCR mixture, blueberry DNA only

Figure 42: Difference plots of normalized melting curves, VcBHLH003 amplicons, DNA from teas

In case of RiACO1 amplicons, intended for detection of raspberry DNA, the results achieved in triplex PCR assay differed from that achieved in singleplex PCR assay. In the latter, we were able to confirm the presence of raspberry in puree P3 and bar B1, while in case of puree P2 and smoothie we concluded that raspberry-specific amplicons might have been detected, but further verification of the result would be needed (chapter 5.4.2).

In case of triplex PCR assay, the curves representing puree P3 differ from each other in shape and two of them have a shape similar to curves representing puree P1, where no raspberries were declared (Figure 43). With puree P2 the result was similar to the one achieved with puree P3. In case of smoothie, the curves representing replicate samples each have a different

shape. The curves representing bar B1 are clustered with the positive control around the baseline, but the same is true for curves representing bar B2, in whose composition no raspberries were declared. From these results it may be concluded that only spurious amplification took place in the samples containing DNA from commercial products. A likely reason why raspberries were not detected in puree P3 and bar B1, even though in singleplex PCR we were able to detect them in these samples, is primer sensitivity loss which may occur when a primer pair is used in a primer set for multiplex PCR. The sensitivity loss might be by one, or even two orders of magnitude for some primer pairs, as was shown by Suh et al. [121] and Lee et al. [130].
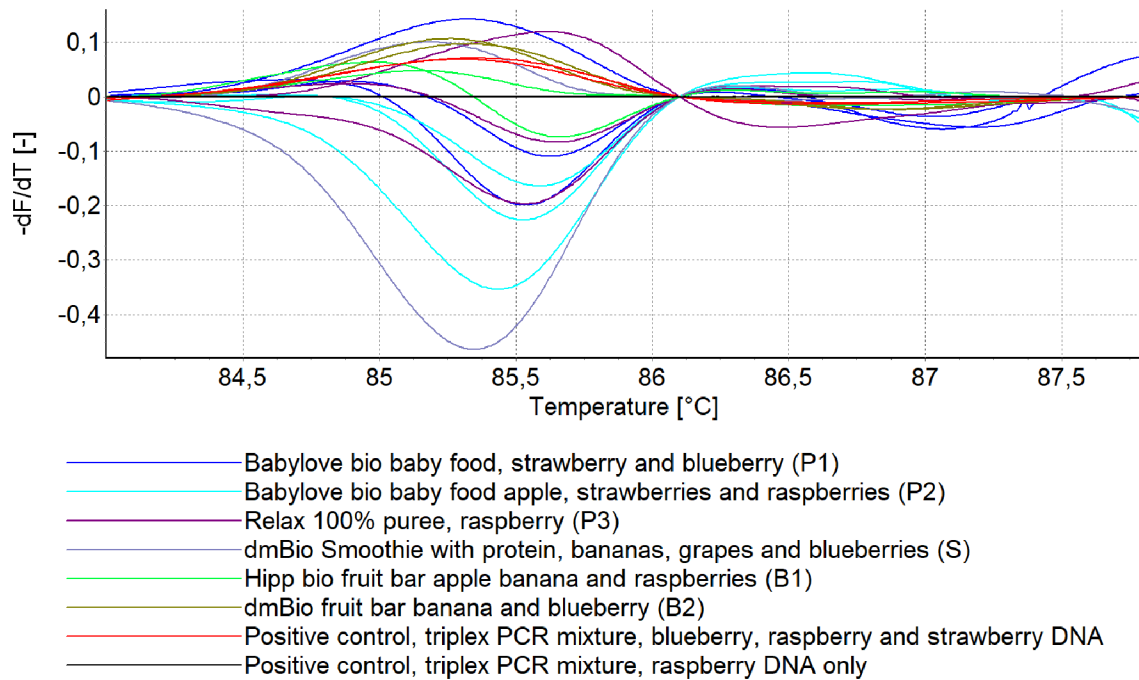

Babylove bio baby food, strawberry and blueberry (P1)
Babylove bio baby food apple, strawberries and raspberries (P2)
Relax 100% puree, raspberry (P3)
dmBio Smoothie with protein, bananas, grapes and blueberries (S)
Hipp bio fruit bar apple banana and raspberries (B1)
dmBio fruit bar banana and blueberry (B2)
Positive control, triplex PCR mixture, blueberry, raspberry and strawberry DNA
Positive control, triplex PCR mixture, raspberry DNA only

Figure 43: Difference plots of normalized melting curves, RiACO1 amplicons, triplex PCR, DNA from purees, smoothie and fruit bars

In case of GAST1 primers, the signal of products detected in samples containing DNA from purees, smoothie and fruit bars was poor (Figure 41). Based on the differential curves alone it would not be possible to conclude if specific or spurious products were detected. Based on the result of HRM analysis, presented here as difference plots of normalized amplicon melting curves, it may be concluded that the detected products were spurious. The curves which represent replicate samples differ in shapes, except for bar B2 and bar B1, and none of the curves representing commercial products are grouped with positive control around the baseline. We therefore conclude that we were not able to confirm the presence of strawberries in any of the purees, smoothie, or fruit bars.
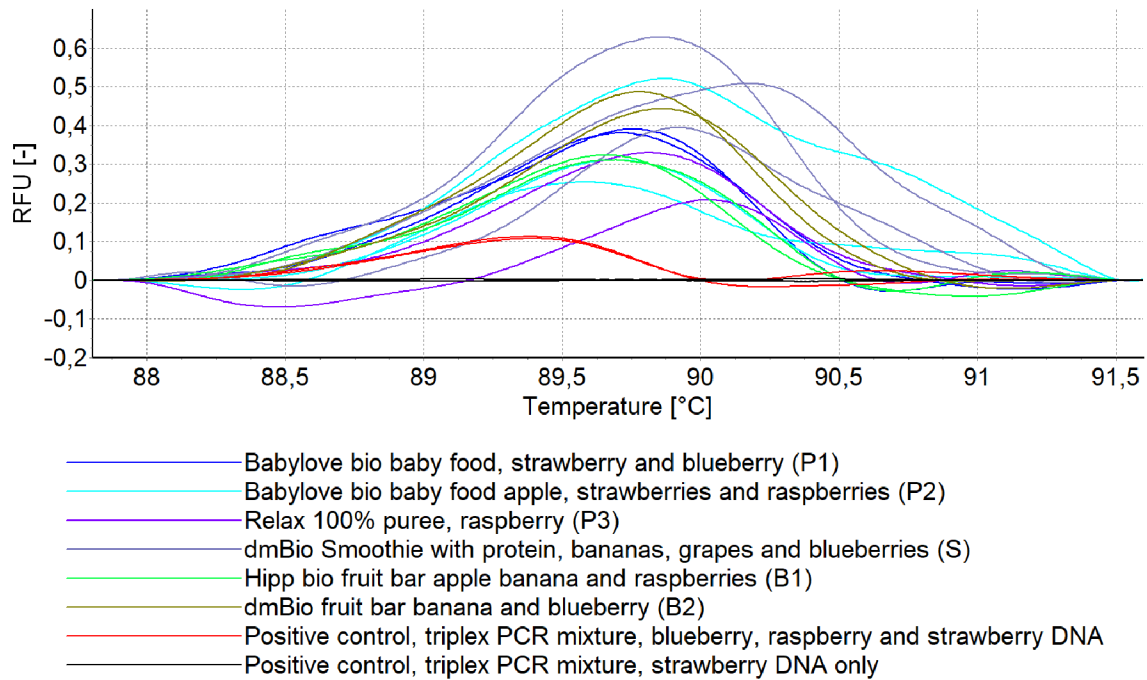
Figure 44: Difference plots of normalized melting curves, GAST1 amplicons, triplex PCR, DNA from purees, smoothie and fruit bars

To sum up, while in case of singleplex PCR we were able to confirm, or at leas tentatively identify raspberry or blueberry DNA in some of the analysed commercial products, in case of multiplex PCR the achievement of identical results was hampered by the combination of PCR inhibition and possible sensitivity loss of the primer pairs used for the multiplex PCR assay.

6 CONCLUSIONS

The main objective of this work was to develop a DNA-based method for analysis of various plant-based foods. Development of such method involved testing and selection of an appropriate DNA isolation protocol and optimisation of this protocol, selection of an appropriate primer set for triplex PCR specific for blueberry, raspberry, and strawberry, the optimisation primer concentration, and application of the method on commercial samples. These samples included liquid (wet) matrices, in particular fruit purees and a smoothie, and dry matrices which underwent varying technological procedures (teas and fruit bars).

Six methods for DNA isolation were tested on ten fruit species. Three methods, two of which (EliGene Plant DNA Isolation Kit and Invitrogen ChargeSwitch gDNA Plant Kit) utilized SDS at some point in the protocol, contained steps intended specifically for removal of polysaccharides, and had more extensive purification procedures than the other methods tested, were proven to be the most suitable for DNA isolation from various fruit species. The final choice of method was made from among the EliGene, Invitrogen and Tools kits based on the ease of use and price per sample.

When the chosen DNA isolation method was applied to commercial samples, DNA amplifiable in real-time PCR was isolated from four teas and both fruit bars, but not from one tea, all three purees and smoothie. An optimisation of the DNA isolation method was therefore needed. Based on the composition of the analysed samples, polysaccharides (especially pectin) were considered the most likely cause for the inhibition of PCR assays with DNAs isolated from the five problematic samples.

Two ways of pectin removal were tested: Enzymatic digestion of pectin with pectinase, and precipitation of pectin by calcium chloride. When calcium chloride was used, the DNA isolates showed higher purity and better amplifiability than when pectinase was used. However, calcium ions might have remained in the DNA isolates, and while the plant-specific PCR assay was not inhibited by them, the opposite was likely the case with the strawberry-specific PCR assay. Huggett et al. [131] also described such a phenomenon, although they found no definite relationship between composition of PCR mixtures for different assays and the assays' susceptibility to inhibition.

To remove any residual calcium ions from DNA isolates, we used a spin-column based clean-up procedure to remove any residual calcium ions from the DNA isolates, which improved their amplifiability in species-specific PCR assays. It may be concluded that while precipitation by calcium chloride is a possible way to remove pectin from the sample during DNA isolation, attention should be paid also to the removal of any residual calcium ions, as they may inhibit certain PCR assays [127].

Regarding results of species-specific PCR assays with DNAs isolated from commercial products, we were able to confirm presence of raspberry DNA by singleplex PCR in two samples – puree P3 and fruit bar B1. In case of other samples, non-specific and spurious products were detected both in singleplex and triplex PCR assays.

These results might have been caused by combination of several factors. First of these is the presence of contaminants with inhibitory effect on PCR in the DNA isolates, as the A260/A280 and A260/A230 absorbance ratios of the DNA isolates were generally out of range of values for pure DNA. This, together with low quantities of blueberry, strawberry and raspberry matrices could be the reason while only non-specific or spurious PCR were often detected.

There are several ways to improve the performance of the method developed in this work. To increase purity, during DNA isolation by the EliGene kit calcium chloride should be used for removal of polysaccharides from the samples. The DNA captured on spin column should then be washed more times than recommended by the kit manufacturer. This might remove any residual calcium chloride from the DNA isolates, and at the same time replace the column-based clean-up procedure, thus shortening the whole DNA isolation protocol. To improve yield of DNA, several lysates could be prepared from one sample, and DNA from these lysates could be captured on a single spin-column. The increased purity and concentration of isolated DNA would then also have a beneficial effect on the species-specific PCR assays.

Apart from using less contaminated and more concentrated DNA, the performance of species-specific PCR assays used in this work could be improved in other ways. To prevent the amplification of non-specific and spurious products, more stringent conditions (i. e. higher primer annealing temperature) could be used. The efficiency of the strawberry-specific PCR assay could be improved by prolonging the denaturation step in the PCR profile. The GAST1 (strawberry specific) amplicons have high melting temperature, which is indicative of high content of GC pairs, and 2 seconds might have been insufficient for complete denaturation of template DNA for primers GAST1.

In this work, calcium chloride precipitation of pectin in the samples was successfully used and was shown to be a promising way of removing pectin from the samples during isolation of DNA. Regarding the PCR analyses of commercial products, raspberry and blueberry were either confirmed or tentatively confirmed by several of the commercial samples in singleplex PCR. However, problems were encountered while trying to replicate the results in multiplex PCR.

To sum up, several modifications to the triplex PCR assay developed in this work should be implemented to improve its performance. The template DNA should be prepared as described above, and more stringent conditions, together with a longer denaturation step should be used.

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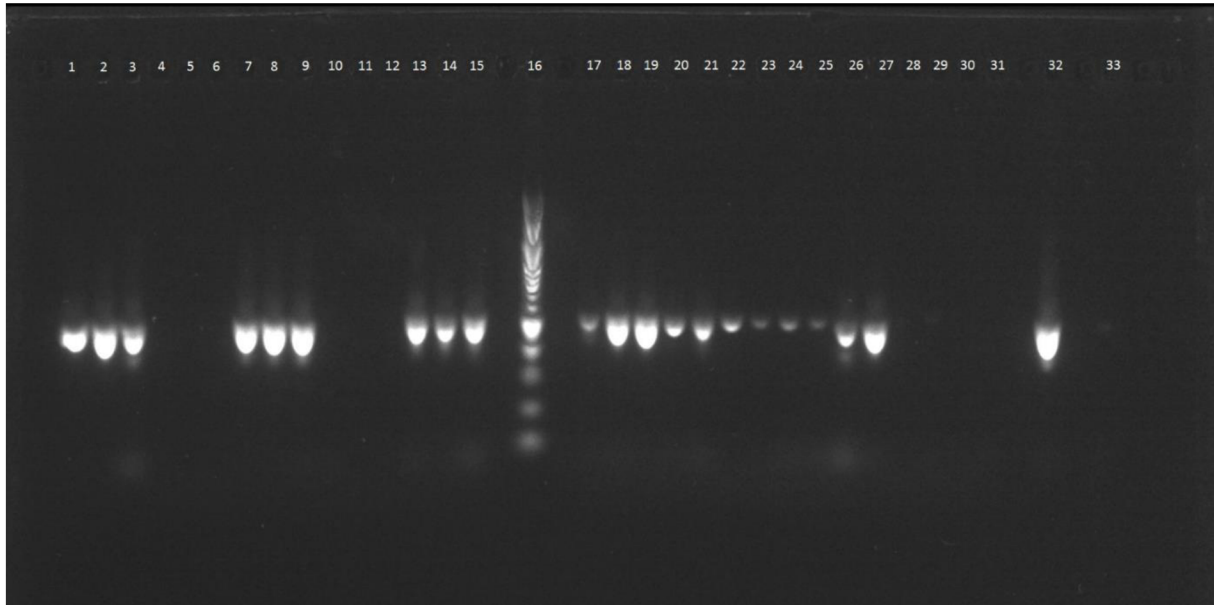
8 SUPPLEMENTARY MATERIALS

Figure 45: Result of plant-specific PCR assay with DNA isolates obtained by kit 1 (Qiagen). Samples: 1-3 banana, 4-6 raspberry, 7-9 peach, 10-12 apricot, 13-15 blueberry, 16-DNA ladder, 17-19 mango, 20-22 pear, 23-25 apple, 26-28 strawberry, 29-31 plum, 32-positive control, 33-no template control [110]
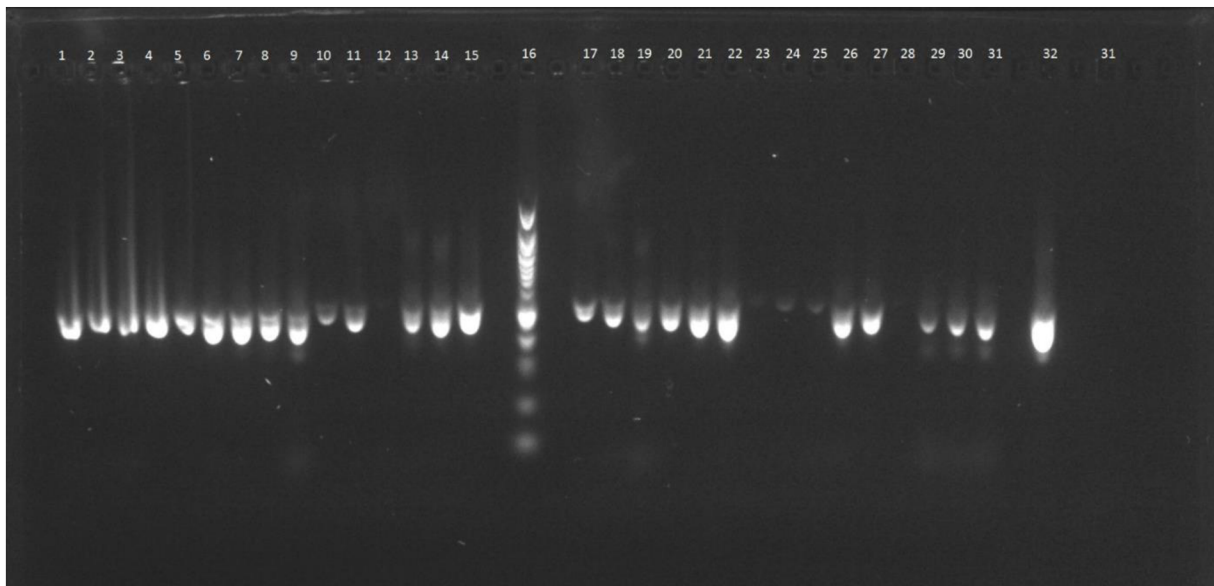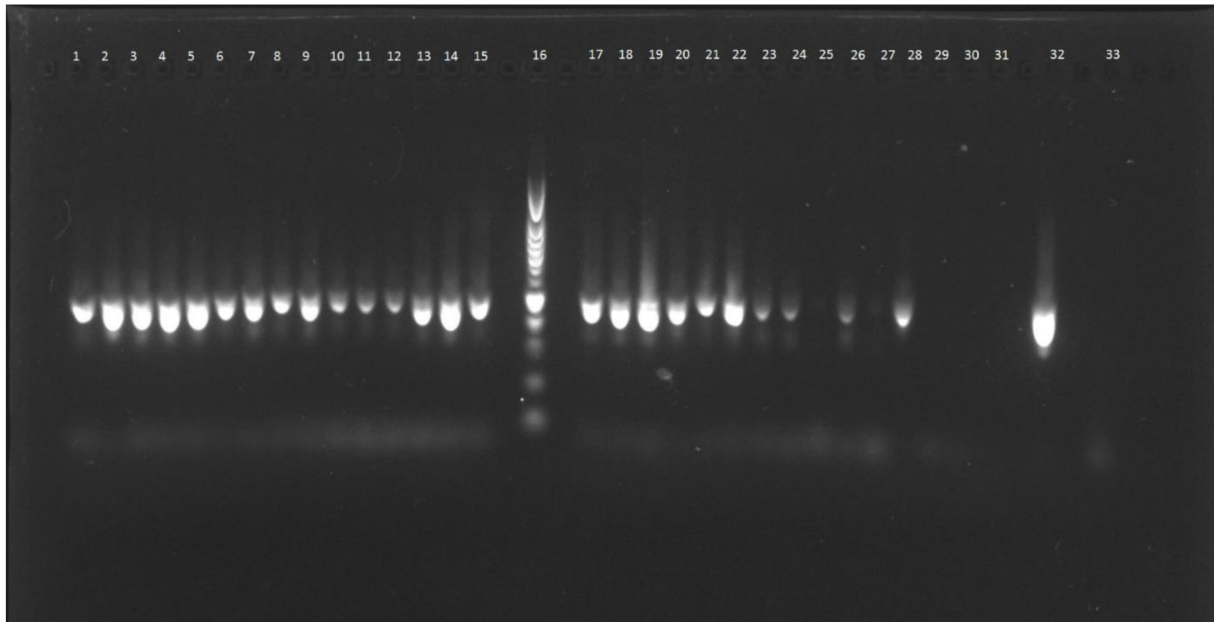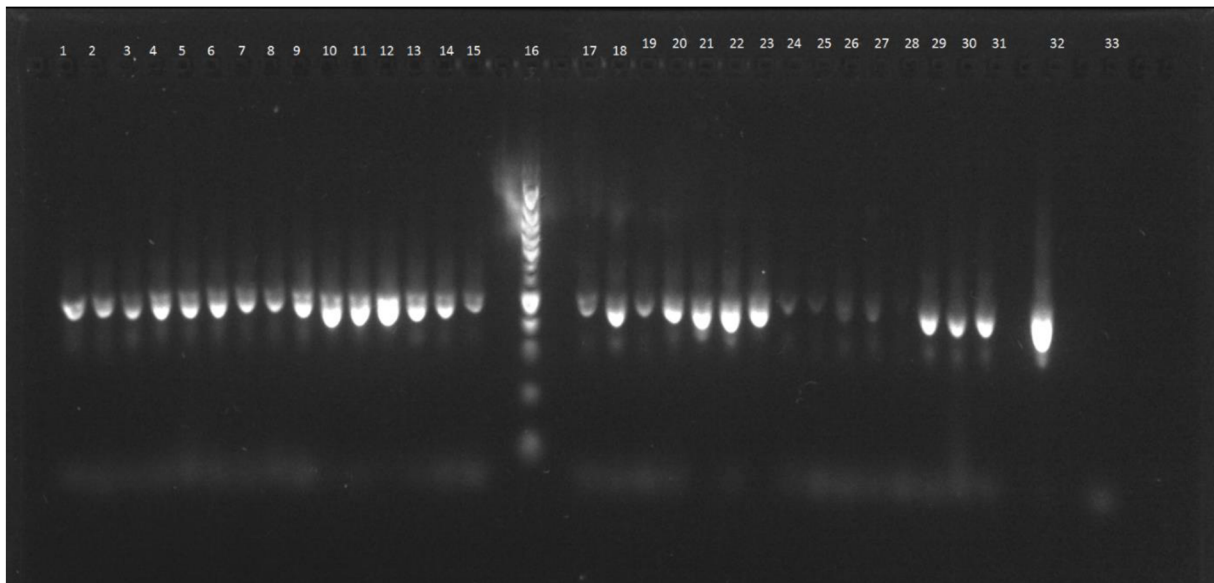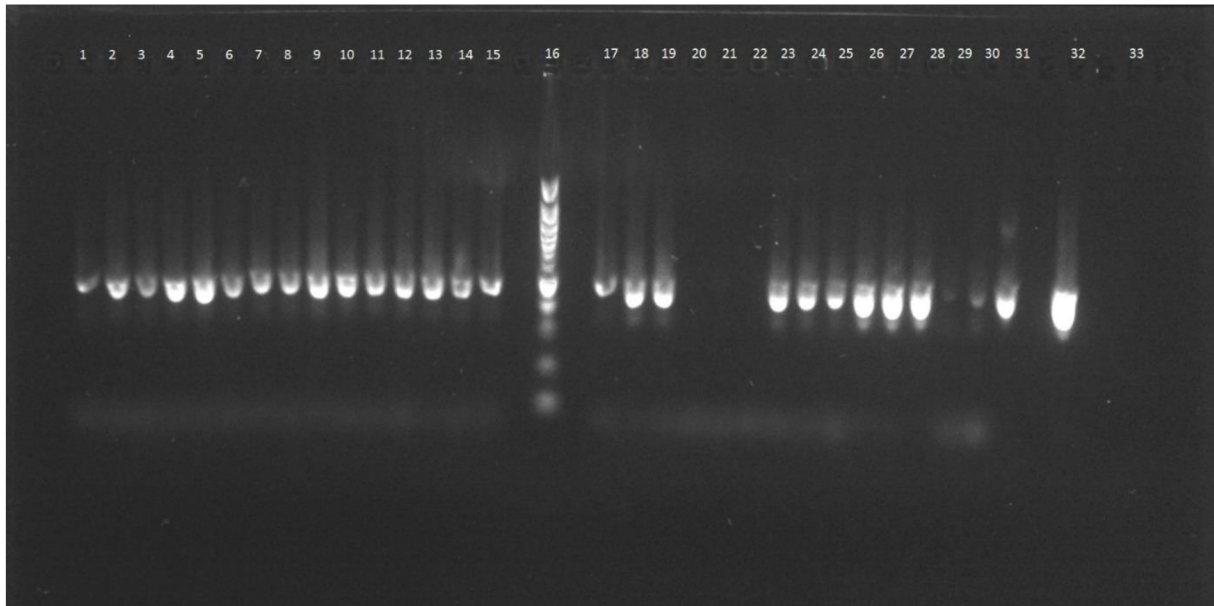

Figure 46: Result of plant-specific PCR assays with DNA isolates obtained by kit 2 (EliGene). Samples: 1-3 banana, 4-6 raspberry, 7-9 peach, 10-12 apricot, 13-15 blueberry, 16-DNA ladder, 17-19 mango, 20-22 pear, 23-25 apple, 26-28 strawberry, 29-31 plum, 32-positive control, 33-no template control [110].
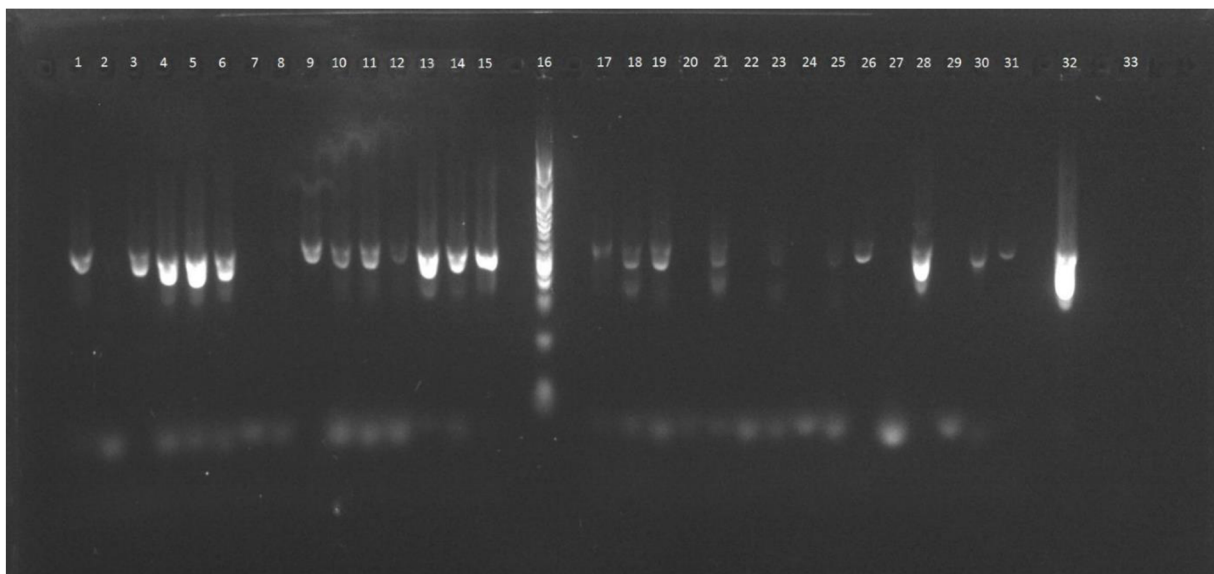
Figure 47: Results of plant-specific PCR assay with DNA isolates obtained by kit 3 (Invitrogen). Samples: 1-3 banana, 4-6 raspberry, 7-9 blueberry, 10-12 mango, 13-15 peach, 16-DNA ladder, 17-19 apricot, 20-22 strawberry, 23-25 pear, 26-28 apple, 29-31 plum, 32 positive control, 33 no template control [110].


Figure 48: Results of plant-specific PCR assay with DNA isolates obtained by kit 4 (Perkin-Elmer). Samples: 1-3 banana, 4-6 raspberry, 7-9 blueberry, 10-12 mango, 13-15 peach, 16-DNA ladder, 17-19 apricot, 20-22 strawberry, 23-25 pear, 26-28 apple, 29-31 plum, 32 positive control, 33 no template control [110].

Figure 49: Results of plant-specific PCR assay with DNA isolates obtained by kit 5 (Tools). Samples: 1-3 banana, 4-6 raspberry, 7-9 blueberry, 10-12 mango, 13-15 peach, 16-DNA ladder, 17-19 apricot, 20-22 apple, 23-25 plum, 26-28 strawberry, 29-31 pear, 32 positive control, 33 no template control [110].

Figure 50: Results of plant-specific PCR assay with DNA isolates obtained by the CTAB protocol. Samples: 1-3 banana, 4-6 raspberry, 7-9 blueberry, 10-12 mango, 13-15 peach, 16-DNA ladder, 17-19 apricot, 20-22 strawberry, 23-25 pear, 26-28 apple, 29-31 plum, 32 positive control, 33 no template control [110].
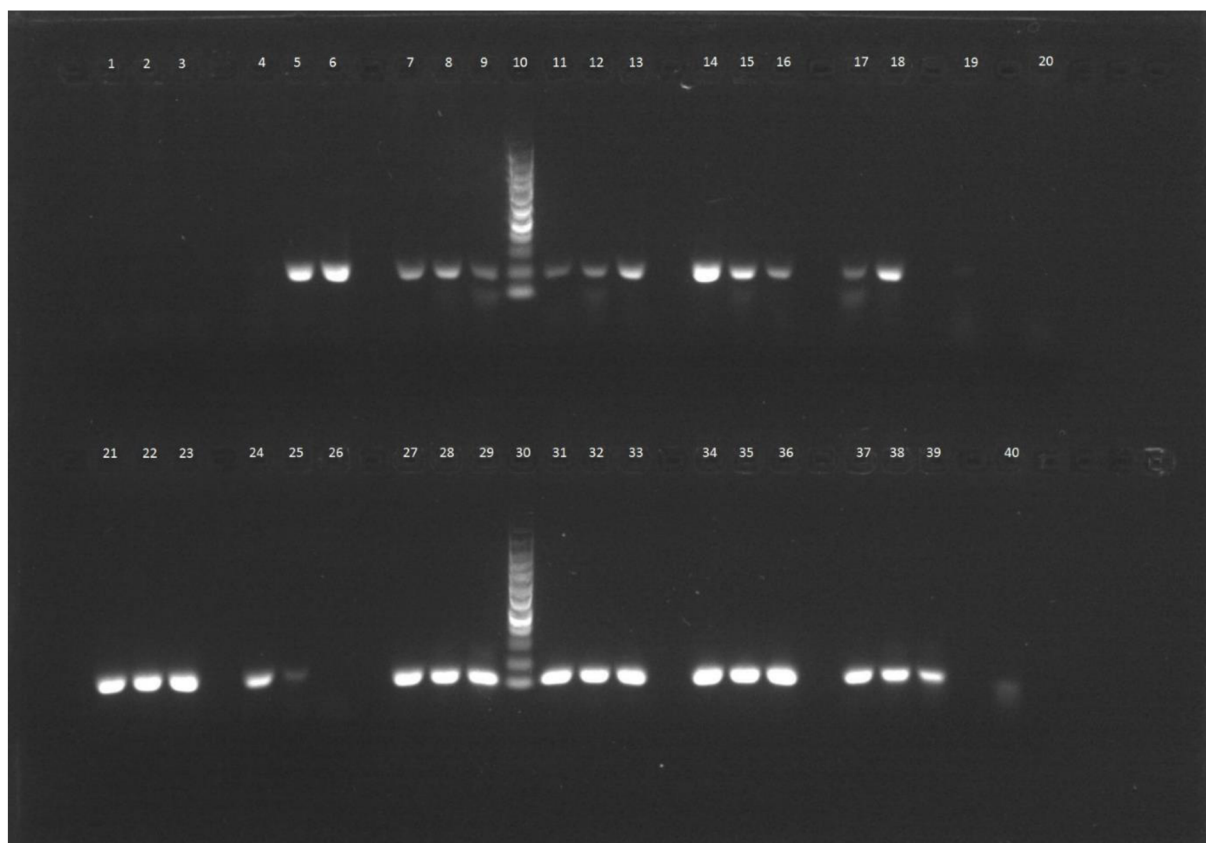
Figure 51: Results of raspberry-specific and mango-specific PCR assays with DNA isolates obtained by all six methods. Samples: Upper row: Raspberry, 1-3 kit 1, 4-6 kit 2, 7-9 kit 3, 10 DNA ladder, 11-13 kit 4, 14-16 kit 5, 17-19 CTAB protocol, 20 no template control. Lower row: Mango, 21-23 kit 1, 24-26 kit 2, 27-29 kit 3, 30 DNA ladder, 31-33 kit 4, 34-36 kit 5, 37-39 CTAB protocol, 40 no template control [110].
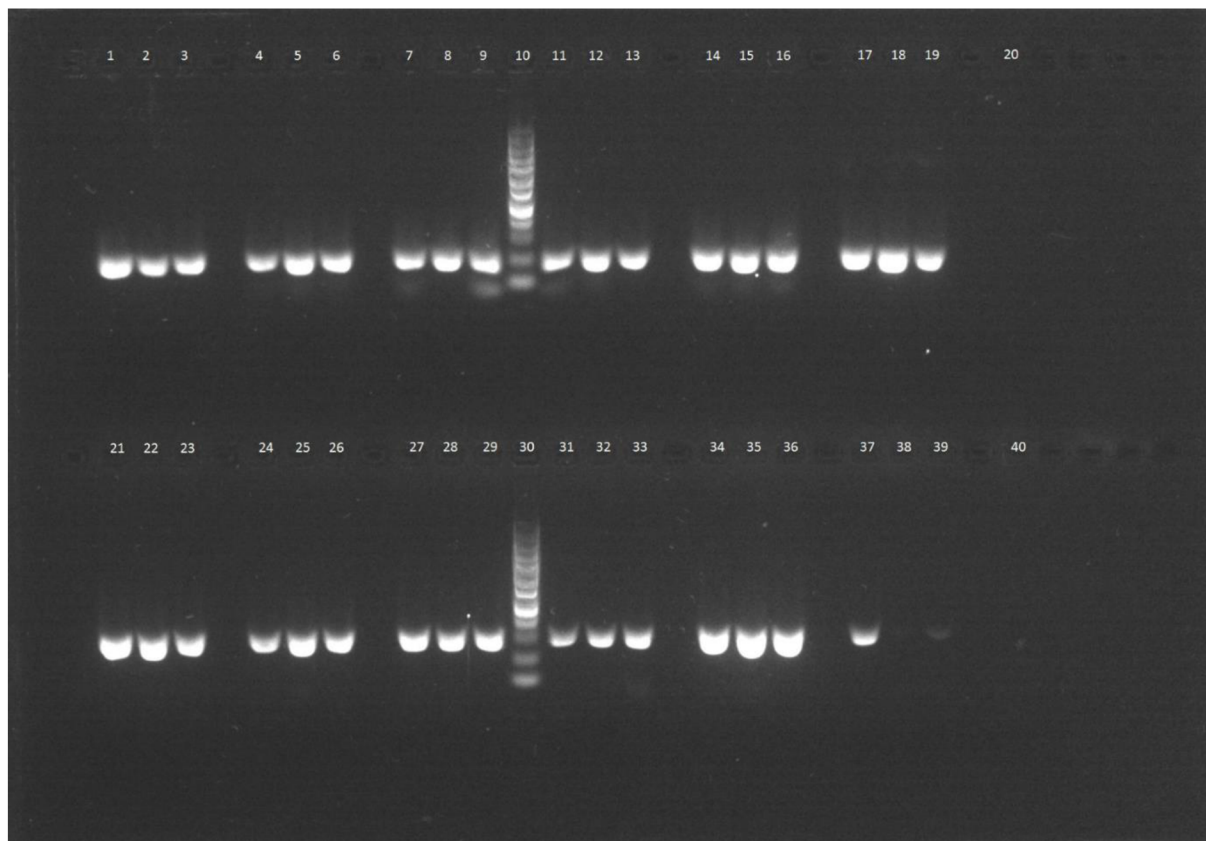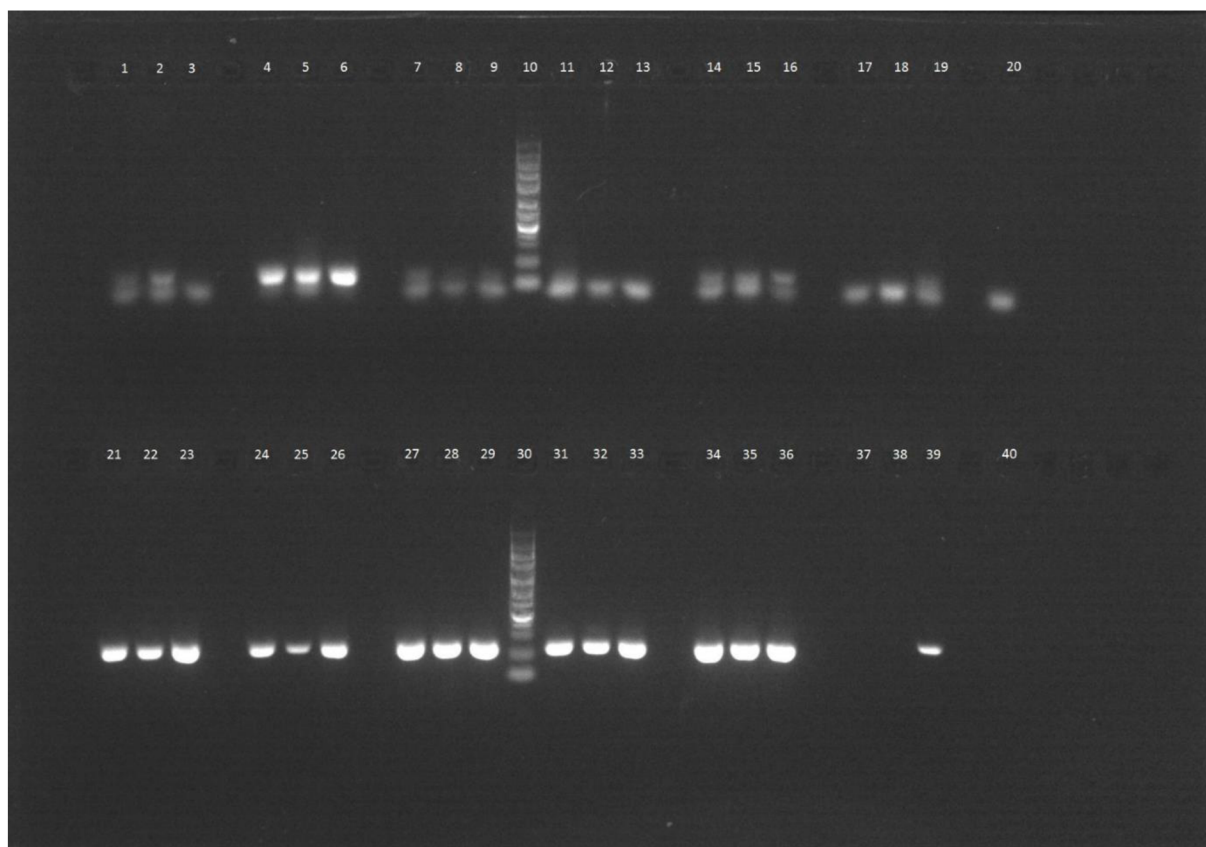
Figure 52: Results of peach-specific and banana-specific PCR assays with DNA isolates obtained by all six methods. Samples: Peach: 1-3 kit 1, 4-6 kit 2, 7-9 kit 3, 10 DNA ladder, 11-13 kit 4, 14-16 kit 5, 17-19 CTAB protocol, 20 no template control. Banana: 21-23 kit 1, 24-26 kit 2, 27-29 kit 3, 30 DNA ladder, 31-33 kit 4, 34-36 kit 5, 37-39 CTAB protocol, 40 no template control [110].

Figure 53: Results of pear-specific and blueberry specific PCR assays with DNA isolates obtained by all six methods. Samples: Pear: 1-3 kit 1, 4-6 kit 2, 7-9 kit 3, 10 DNA ladder, 11-13 kit 4, 14-16 kit 5, 17-19 CTAB protocol, 20 no template control. Blueberry: 21-23 kit 1, 24-26 kit 2, 27-29 kit 3, 30 DNA ladder, 31-33 kit 4, 34-36 kit 5, 37-39 CTAB protocol, 40 no template control [110].
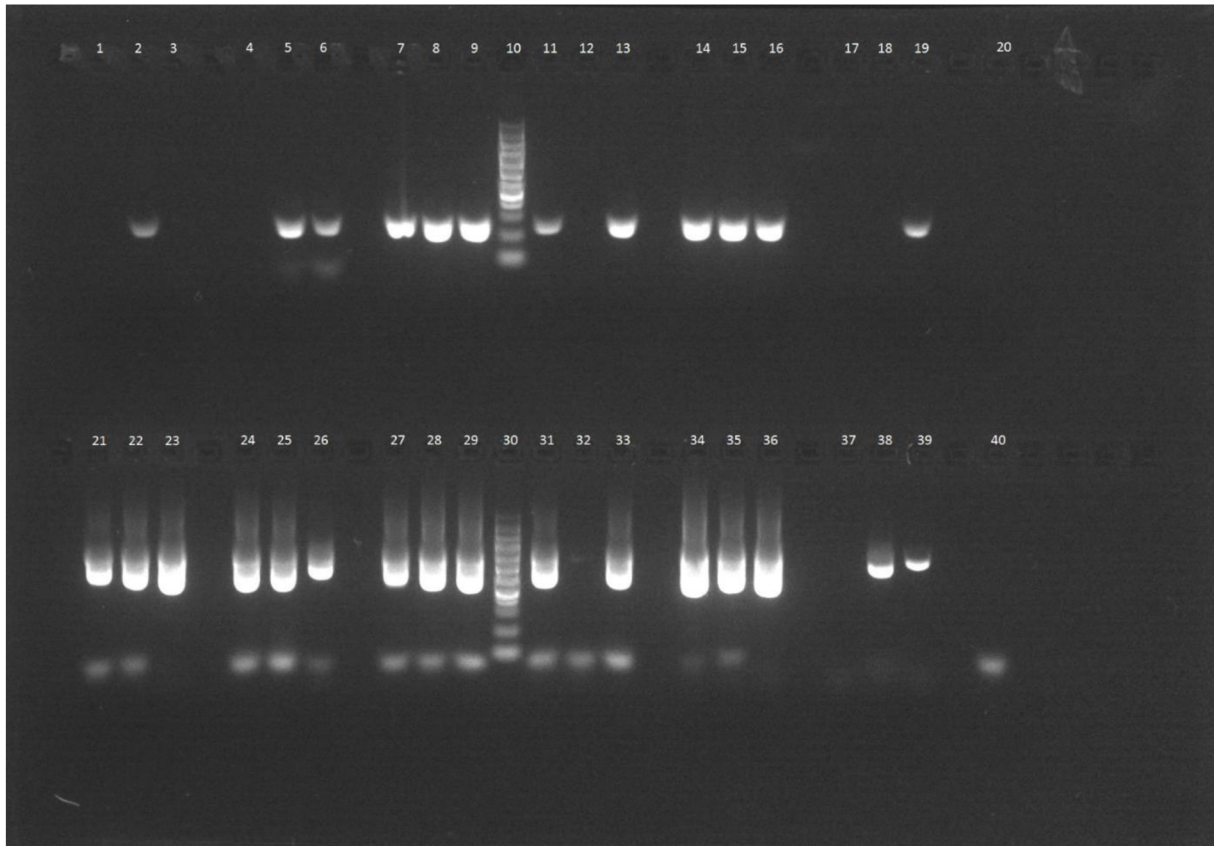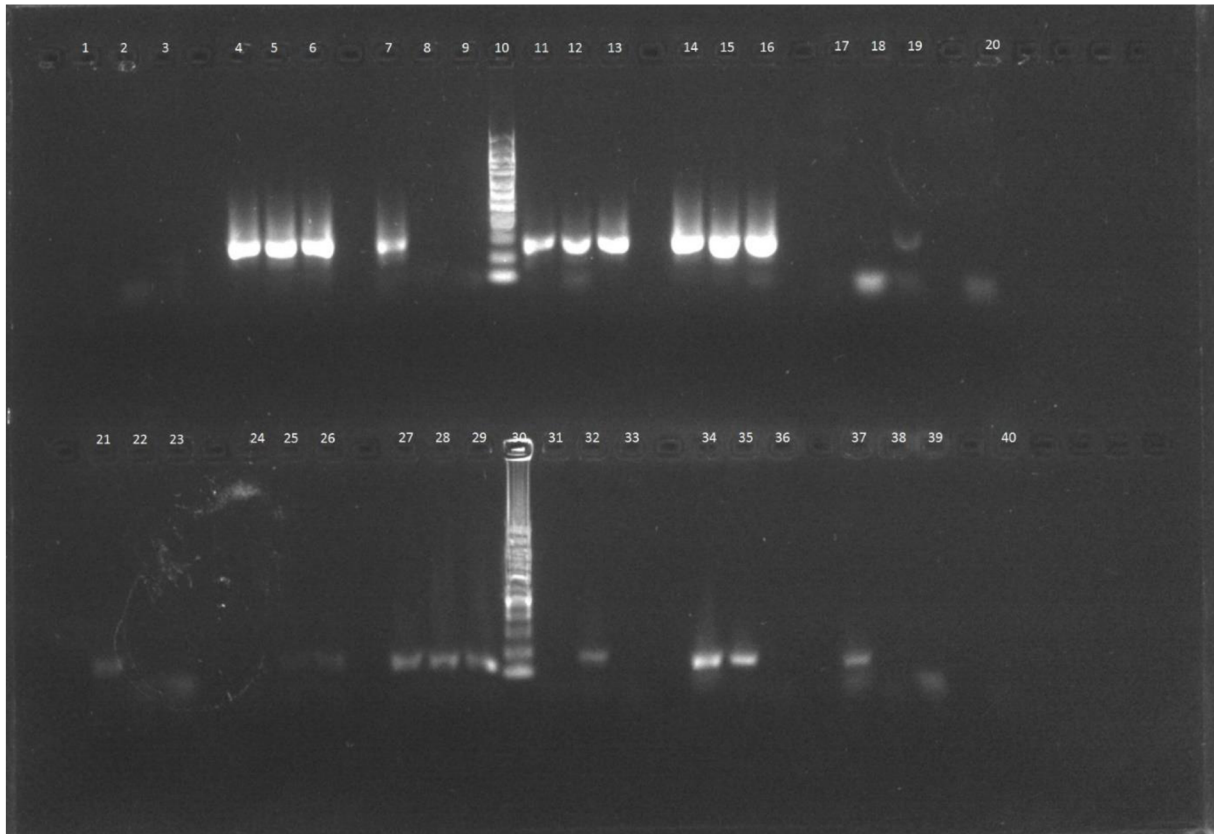
Figure 54: Results of apricot-specific and strawberry specific PCR assays with DNA isolates obtained by all six methods. Samples: Apricot: 1-3 kit 1, 4-6 kit 2, 7-9 kit 3, 10 DNA ladder, 11-13 kit 4, 14-16 kit 5, 17-19 CTAB protocol, 20 no template control. Strawberry: 21-23 kit 1, 24-26 kit 2, 27-29 kit 3, 30 DNA ladder, 31-33 kit 4, 34-36 kit 5, 37-39 CTAB protocol, 40 no template control [110].

Figure 55: Results of plum-specific and apple-specific PCR assays with DNA isolates obtained by all six methods. Samples: Plum: 1-3 kit 1, 4-6 kit 2, 7-9 kit 3, 10 DNA ladder, 11-13 kit 4, 14-16 kit 5, 17-19 CTAB protocol, 20 no template control. Apple: 21-23 kit 1, 24-26 kit 2, 27-29 kit 3, 30 DNA ladder, 31-33 kit 4, 34-36 kit 5, 37-39 CTAB protocol, 40 no template control [110].
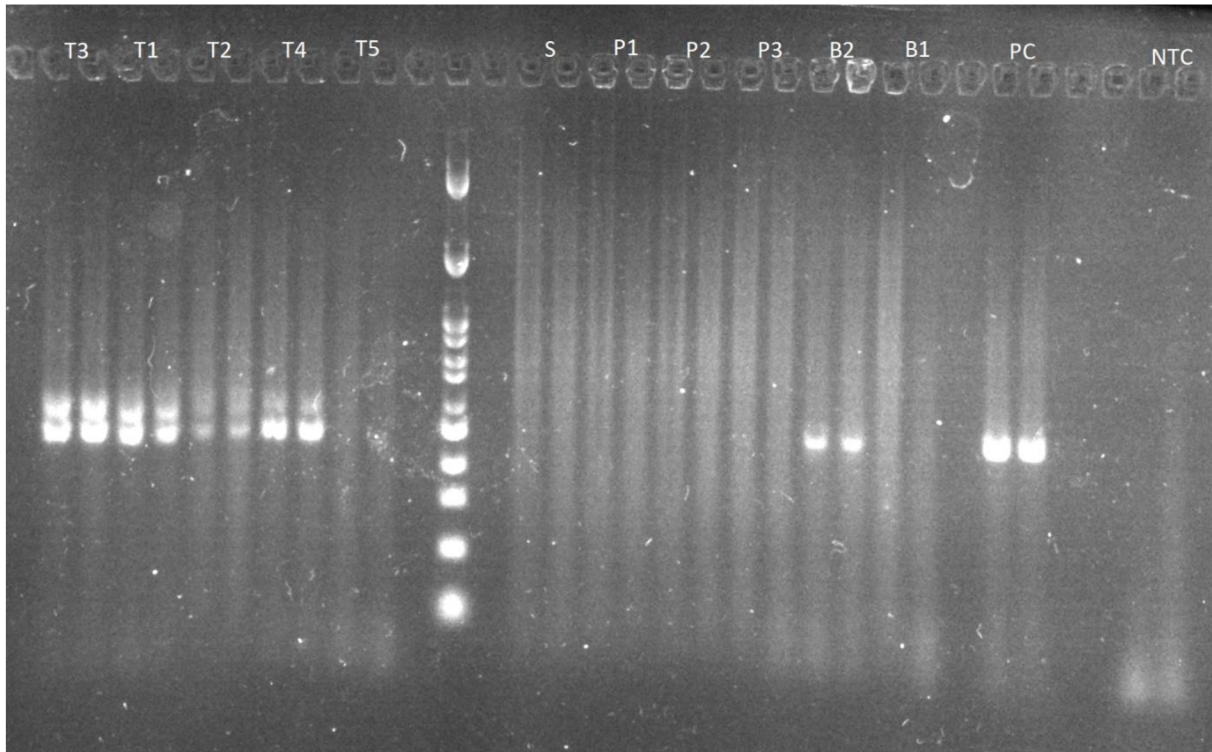
Figure 56: ITS2 amplicons of samples isolated by kit modified with pectinase (T1 = dmBio fruit tea, T2 = Apotheke Bio Forest blend, T3 = Apotheke Bio herbal tea for imunity, T4 = Leros herbal tea "Strong eyes", T5 = Majestic Tea, P1 = Babylove baby food, strawberry and blueberry, P2 = Babylove baby food apple, strawberries and raspberries, P3 = Relax 100 % puree, raspberry, B1 = Hipp fruit bar, B2 = dmBio fruit bar, PC = positive control, NTC = no template control)
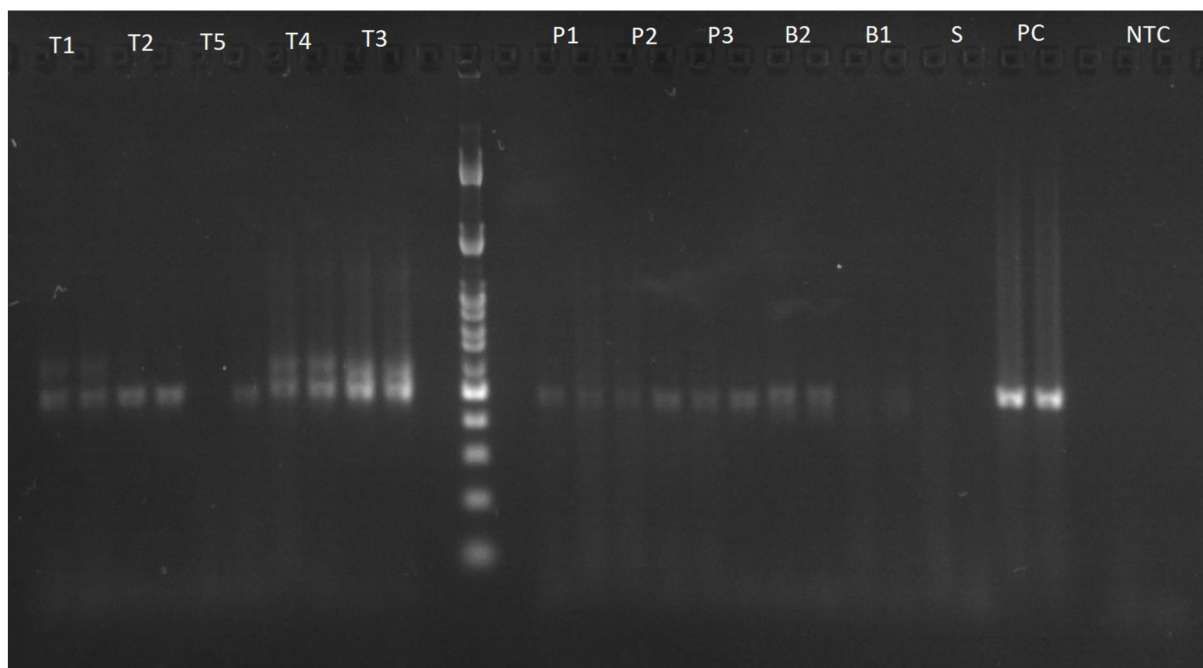
Figure 57: ITS2 amplicons of samples isolated by kit modified with calcium chloride (T1 = dmBio fruit tea, T2 = Apotheke Bio Forest blend, T3 = Apotheke Bio herbal tea for imunity, T4 = Leros herbal tea "Strong eyes", T5 = Majestic Tea, P1 = Babylove baby food, strawberry and blueberry, P2 = Babylove baby food apple, strawberries and raspberries, P3 = Relax 100 % puree, raspberry, B1 = Hipp fruit bar, B2 = dmBio fruit bar, PC = positive control, NTC = no template control)
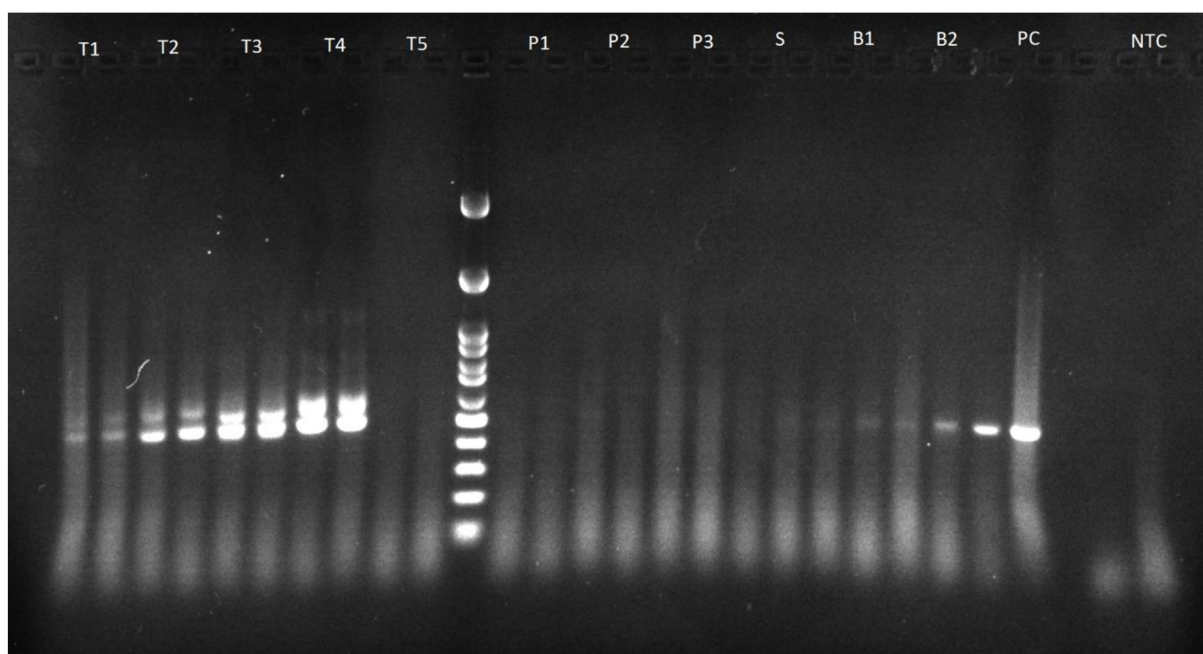

Figure 58: ITS2 amplicons of samples isolated by kit modified with calcium chloride and clean-up procedure (T1 = dmBio fruit tea, T2 = Apotheke Bio Forest blend, T3 = Apotheke Bio herbal tea for imunity, T4 = Leros herbal tea "Strong eyes", T5 = Majestic Tea, P1 = Babylove baby food, strawberry and blueberry, P2 = Babylove baby food apple, strawberries and raspberries, P3 = Relax 100 % puree, raspberry, B1 = Hipp fruit bar, B2 = dmBio fruit bar, PC = positive control, NTC = no template control)
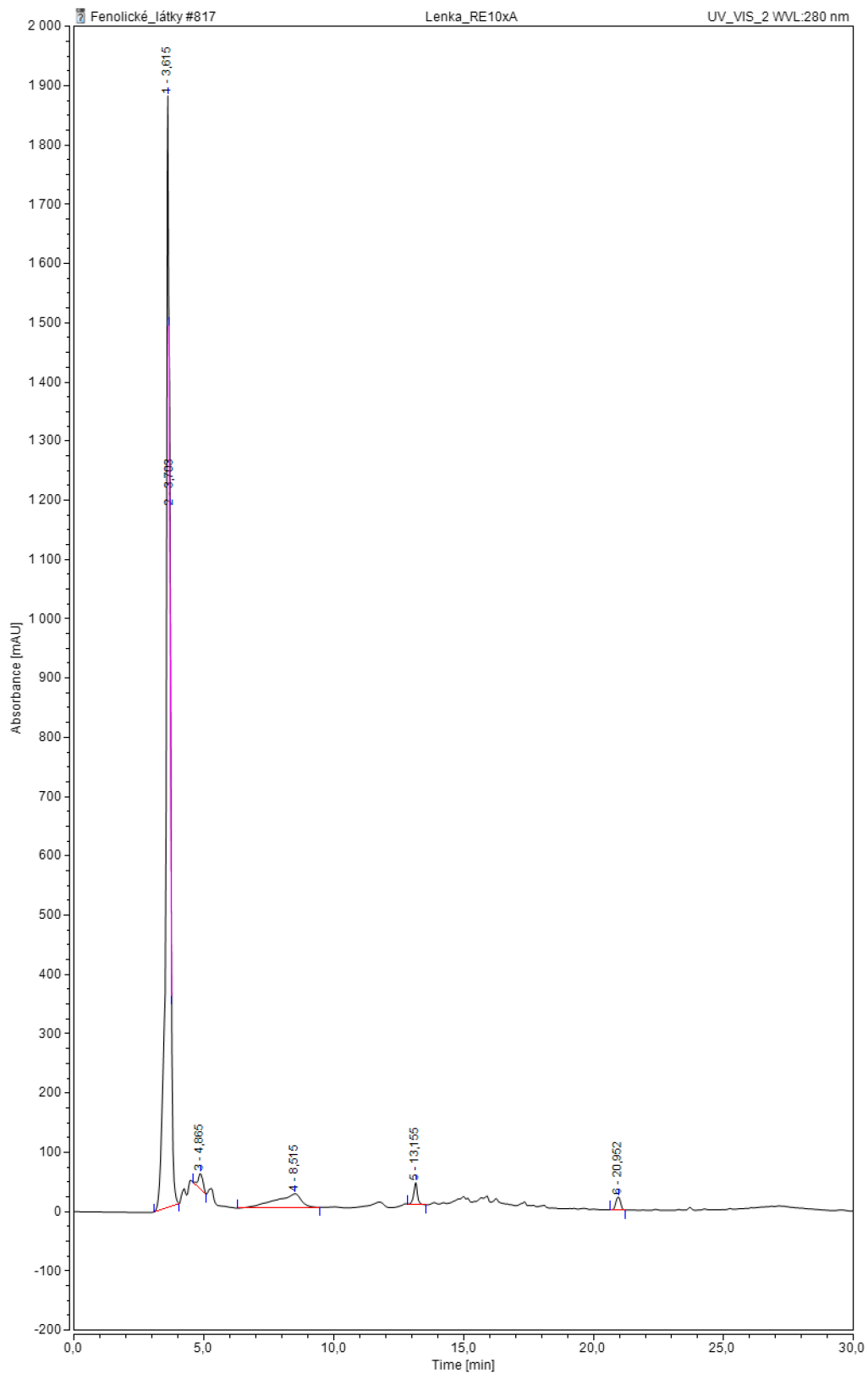
Figure 59: Chromatogram of phenolic compounds detected in puree P3 (Relax 100 % puree, raspberry)

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Článek v recenzním řízení:

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FIALOVÁ, L.; LANGOVÁ, D.; PRACHÁROVÁ, A.; MÁROVÁ, I. Development of a Multiplex PCR Assay for Simultaneous Detection of Closely Related Fruit Species in Food. Studentská odborná konference CHEMIE JE ŽIVOT 2021 Sborník abstraktů. 2021. s. 34-35. ISBN: ISBN 978-80-214-6002.

ROMANOVSKÁ, D.; FIALOVÁ, L.; MÁROVÁ, I. Newly designed primers targeting limonene synthase. Biotechnology & Biotechnological Equipment, Special Issue:European Biotechnology Congress 2020. Biotechnology and Biotechnological Equipment. 35. Prague: Taylor and Francis, 2021. s. 119-119. ISBN: 1310-2818. ISSN: 1314-3530.

FIALOVÁ, L.; ROMANOVSKÁ, D.; NĚMCOVÁ, A.; MÁROVÁ, I. Multiplex qPCR-HRM with intercalating dye: A possible way to verify red fruit presence in food. Biotechnology and Biotechnological Equipment. 2020. s. 400-400. ISSN: 1314-3530.

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FIALOVÁ, L.; ROMANOVSKÁ, D.; MÁROVÁ, I. SIMULTANEOUS DETECTION OF PEACH AND APRICOT DNA BY MULTIPLEX REAL-TIME PCR-HRM WITH INTERCALATING DYE. 2019. CECE 2019. 16th International Interdisciplinary Meeting on Bioanalysis. Gdańsk, 24-26 September 2019. Poster abstracts.

FIALOVÁ, L.; ŠPANOVÁ, A.; RITTICH, B. Polylysine Coated Magnetic Nanoparticles As A Tool For DNA Isolation From Real Samples. 7th Meeting on Chemistry and Life 2018. Book of abstracts. 1. Brno: Brno University of Technology, Faculty of Chemistry, Purkyňova 464/118 612 00 Brno, 2018. s. 134-134. ISBN: 978-80-214-5488-0.