Evaluation of different methods for DNA extraction from milk

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Summary

The aim of this study was to compare six commercial, three non-commercial, and two combined methods for isolation of DNA from milk, on the basis of estimated quantity and quality, using spectrophotometric measurements and cow-specific real-time polymerase chain reaction (PCR). An evaluation of the associated time, cost and labour of each method was carried out as an additional assessment. The highest concentration of DNA was obtained by a phenol-chloroform protocol combined with DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), but the spectrophotometric measurements and real-time PCR parameters showed that the DNA quality was not good. According to the real-time PCR efficiency (E) and correlation coefficient (R^2), only three commercial kits, namely, QIAprep Spin Miniprep (Qiagen), DNeasy Blood and Tissue kit, (Qiagen) and SmartHelix First DNAid (ExVivon, Ljubljana, Slovenia), as well as one combined method (phenol-chloroform protocol and Nucleospin Food kit (Macherey-Nagel, Düren, Germany) produced DNA of good quality for real-time PCR. Given the estimated time required for DNA extraction, and the cost and labour requirements, the three commercial kits were faster, cheaper and simpler than the other methods, and can be recommended as applicable methods for the extraction of DNA from milk.

Keywords:

DNA extraction; real-time polymerase chain reaction; spectrophotometric measurement; milk; food authenticity

Quality and safety are the basic requirements that must be met for all sorts of foods. Substitution of ewes' and goats' milk by cows' milk is frequently reported, as the production of cows' milk is prevalent in many countries and there, cows' milk is cheaper than other types of milk [1–4]. The monitoring and control of milk and dairy product constituents requires availability of appropriate and adequate analytical methods.

A variety of methods have been applied to the identification of adulteration of milk and dairy products. The protein or fatty-acid composition can be analysed (e.g. using electrophoresis, isoelectric focusing, capillary electrophoresis, high-performance liquid chromatography, immunochemical methods (ELISA), mass spectrometry) and, on the basis of different protein or fatty acid profiles, various types of milk can be defined [1]. Another large group of methods is based on DNA analysis (e.g. using restriction fragment length polymorphism, randomly amplified polymorphic DNA, real-time polymerase chain reaction (PCR) [1, 5]. DNA molecules are thermally more stable than proteins, and this is one of the reasons why DNA-based methods have become of great interest in recent years [3, 6–8]. Another reason for the increasing number of studies on DNA-based analysis of milk and dairy products is the very good specificity and very low limits of detection that can be obtained by various PCR-based techniques [2, 5, 9–11]. DNA for these methods is usually extracted from somatic cells that are present in the milk in large but greatly variable numbers, such as $< 10^5$ cells per millilitre for a healthy animal, and potentially $> 10^6$ cells per millilitre during bacterial infection [12].

Numerous DNA extraction methods have been used for the preparation of DNA from milk, which generally provide more or less sufficient quantities of the isolated DNA, along with a reduction in potential inhibitors. However, most of these methods are time-consuming and technically demand-

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ing. The published methods for the extraction of DNA from milk include separation and purification steps using phenol-chloroform extraction and ethanol or isopropanol precipitation, or both [5, 13, 14]. Components of extraction mixtures that are known as PCR inhibitors include: chelators, such as ethylenediaminetetraacetic acid (EDTA), which can complex the Mg^{2+} ions that are necessary for polymerase activity; NaOH, which causes degradation of DNA and denaturation of polymerases; phenol, which also causes denaturation of the polymerase, as it binds to the enzyme molecule by hydrogen bonds; and ethanol and isopropanol, which cause precipitation of DNA [15, 16]. As indicated above, the calcium ions in milk have also been identified as a source of PCR inhibition [17]. The requirements that need to be fulfilled for a successful downstream PCR include the extraction of as much as possible pure DNA from milk, and the optimization of the reaction conditions.

As there are several methods for DNA extraction from milk and dairy products that include research protocols and commercial kits, the aim of our study was to evaluate different methods for the extraction of DNA from cows' milk. Another aim was to isolate DNA in a quality facilitating its direct use in real-time PCR without dilution. The DNA solutions obtained were compared on the basis spectrophotometrically determined concentration and purity, and on the basis of real-time PCR parameters, namely, amplification efficiency (E) and correlation coefficient (R^2). An evaluation of the costs of the extraction methods was also carried out, as comparisons of the time required for DNA extraction, the costs of the materials needed, and the labour required.

MATERIALS AND METHODS

Milk samples

Samples of fresh whole milk from cows (*Bos taurus*) were obtained from a local Slovenian producer, and were transferred to the laboratory in a cool box (1–4 °C). The milk samples were divided into 1 ml, 2 ml, 10 ml and 50 ml aliquots under aseptic conditions and used for DNA extraction or frozen at –80 °C.

DNA extraction methods

Eleven different DNA extraction methods were used to extract total DNA from the milk samples, with at least three replicates performed for each milk sample. Six commercially available kits were included in the evaluation: NucleoSpin Food kit (Macherey-Nagel, Düren, Germany); QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany); DNeasy Blood and Tissue kit (Qiagen); DNeasy *mericon* Food kits (Qiagen); Smarthelix First DNAid kits (ExVivon, Ljubljana, Slovenia); and SmartHelix Complex Samples kits (ExVivon). Three published non-commercial methods were also evaluated: phenol-chloroform extraction [5]; extraction following the protocol described by Bonaïti et al. [13], which is here designated as guanidine thiocyanate (GITC) extraction; and extraction following the protocol described by Murphy et al. [14], which is here designated as a Tween-based extraction. Furthermore, two combined methods were included in the study: phenol-chloroform extraction combined with NucleoSpin Food kit, and phenol-chloroform extraction combined with DNeasy Blood and Tissue kit.

For DNA extraction with NucleoSpin Food kit, QIAprep Spin Miniprep kit and DNeasy Blood and Tissue kit, the milk samples (1 ml) were centrifuged for 10 min at 10 000 ×g, for 3 min at 11 337 ×g, and for 5 min at 4 294 ×g, respectively. The upper fatty layer was then removed using a sterile inoculation loop, and the supernatant was discarded. The pellet that remained was used as the starting sample material and DNA extraction with the relevant kits was carried out as described in the manufacturer's instructions. In order to achieve higher purity of the extracted DNA using the NucleoSpin Food kit, a washing step with NucleoSpin Food kit buffer was performed twice. After pelleting the somatic cells in case of DNeasy Blood and Tissue kit, the Protocol for purification of total DNA from animal blood or cells was performed [18].

The extraction of DNA using DNeasy *mericon* Food kit was performed with milk samples of 2 ml, and followed the standard manufacturer's protocol for large-scale samples. After the first centrifugation step (2 500 ×g, 5 min), the fatty layer was removed using a sterile inoculation loop, and then the manufacturer's protocol was followed. Samples of 2 ml milk were also used to extract total DNA with SmartHelix First DNAid kit (ExVivon), following the protocol for liquid samples. With SmartHelix Complex Samples kit (ExVivon), DNA was extracted from milk samples of 500 μl. The manufacturer's protocol was followed, using a bead beater cell disruptor (Bullet blender; Next Advance, Averill Park, New York, USA), at speed 8.

The protocol for classical phenol-chloroform extraction was based on that described by De et al. [5]. Briefly, 1 ml milk sample was centrifuged for 2 min at 6 708 ×g, the upper fatty layer was re-

moved with a sterile disposable inoculation loop, and the supernatant was pipetted off and discarded. Five-hundred microliters of 0.9% NaCl (Merck, Darmstadt, Germany) solution was added, with the samples mixed for 30 s, and centrifuged for 2 min at 6708 ×*g*. Then, 500 μl TENS buffer made up of 10 mmol·l^{-1} Tris-HCl pH 8.0 (Promega, Madison, Wisconsin, USA), 1 mmol·l^{-1} EDTA (Sigma, St. Louis, Missouri, USA), 100 nmol·l^{-1} NaCl, 0.5% SDS (Promega) and 25 mg·ml^{-1} proteinase K (Roche Diagnostics, Mannheim, Germany) was added to the pellet, the samples were again mixed for 30 s, and incubated in a water bath for 3 h at 50 °C. After digestion of proteins, 500 μl of a mixture of phenol (Invitrogen, Carlsbad, California, USA) : chloroform (Merck) 1 : 1 was added, and the samples were mixed for 30 s and then centrifuged for 2 min at 12000 ×*g*. The clear upper water phase was transferred to a new microtube and 500 μl chloroform was added. The samples were mixed, centrifuged for 2 min at 12000 ×*g*, and the upper water phase was again transferred to a new microtube. Then, 50 μl 3 mol·l^{-1} Na-acetate (Sigma) and 400 μl isopropanol (Kemika, Zagreb, Croatia) were added, the samples were mixed by turning, and then incubated for a minimum of 30 min at –20 °C. Following a 10-min centrifugation at 13000 ×*g*, the supernatant was carefully pipetted off and discarded. After the remaining pellet was dried, it was resuspended in 25 μl sterile double-distilled water (dd H_2O).

DNA extraction with guanidine thiocyanate was performed following the protocol of Bonaïti et al. [13]. Briefly, each 2 ml milk sample was mixed with 4 ml of 4 mol·l^{-1} guanidine thiocyanate (Promega), 0.1 mol·l^{-1} Tris-HCl pH 7.5 (Promega) and 250 μl 10% N-lauroylsarcosine (Sigma). Then, 200 mg glass beads (diameter 0.5 mm) and alkaline extraction solution were added to 350 μl of prepared sample. After disruption of the cells by prolonged mixing of the samples on a vortex, the sample was centrifuged at 20800 ×*g* for 45 min, and the upper water phase was transferred to a new microtube. A two-step washing process with phenol : chloroform : isoamyl alcohol (Kemika) (25 : 24 : 1, pH 8.0) and chloroform : isoamyl alcohol (24 : 1, pH 8.0) was carried out. DNA was precipitated using ethanol precipitation with an incubation at –20 °C for a minimum of 2 h, and then it was resuspended in 100 μl Tris-EDTA (5 mmol·l^{-1} Tris, 2 mmol·l^{-1} EDTA).

The Tween-based DNA extraction protocol was according to Murphy et al. [14]. Milk samples (50 ml) were centrifuged for 5 min at 2200 ×*g*, the supernatant was removed while leaving 1 ml of liquid that contained somatic cells and casein at the bottom of the tubes, and this was transferred to a new microtube. Then, 300 μl casein-dissolving mixture was added (0.5 mol·l^{-1} EDTA, pH 8.0), followed by 200 μl of TE buffer (10 mmol·l^{-1} Tris-HCl, 1 mmol·l^{-1} EDTA, pH 7.6), with vortexing. After a few minutes, the samples became clear as the casein micelles dissolved. Following further centrifugation at 14000 ×*g* for 1 min, the supernatant was discarded, and the pellet was washed twice with 1.5 ml TE buffer. Digestion of somatic cells was performed in 100 μl digestion mix made up of 40 mmol·l^{-1} Tris-HCl pH 8.3, 50 mmol·l^{-1} KCl (Sigma), 3 mmol·l^{-1} $MgCl_2$ (Sigma), 1 μl Tween 20 (Merck), and 20 μl of 20 mg·ml^{-1} proteinase K (Roche Diagnostics), which was incubated for 3 h at 55 °C. The extraction of DNA was continued using the classical phenol-chloroform extraction protocol, starting from the addition of phenol : chloroform (1 : 1).

The last two DNA extraction methods were the combination of phenol-chloroform extraction with NucleoSpin Food kit or DNeasy Blood and Tissue kit. After the isolation of DNA using the phenol-chloroform extraction, 25 μl DNA solution was diluted in dd H_2O and purified following the NucleoSpin Food kit protocol. The second combination was phenol-chloroform extraction and DNeasy Blood and Tissue kit. Here, 25 μl DNA diluted in dd H_2O that had been extracted with the classical phenol-chloroform isolation was used as the material for purification with DNeasy Blood and Tissue kit. The protocol was carried out as described by the manufacturer, without digestion of proteins with proteinase K.

Spectrophotometric measurement

DNA yield and purity was determined using a Lambda Bio spectrophotometer (Perkin Elmer, Waltham, Massachusetts, USA) with a Hellma TrayCell measuring cell (Hellma Analytics, Müllheim, Germany). Elution buffers from commercial kits or sterile dd H_2O were used as the control. Absorbance (*A*) at 230 nm, 260 nm and 280 nm was measured and DNA concentration and purity were calculated [19, 20]. In order to compare DNA concentrations between various extraction methods, spectrophotometrically determined DNA concentrations were normalized by re-calculation according to a volume of milk sample of 1 ml and an elution volume of 100 μl.

Real-time PCR

Species-specific primers and probes described by López-Calleja et al. [9] were used for specific detection of cow (*B. taurus*) DNA, with a nu-

Tab. 1. Primers and probe specific for cow (*Bos taurus*) [9].

Designation	Sequence (5'–3')
BOS-F	AAA GGA CTT GGC GGT GCT T
BOS-R	TGG TTT CAT AAT AAC TTT CGT* GCT
BOS-P	FAM-TAG AGG AGC CTG TTC TAT AAT CGA TAA ACC CCG-TAMRA

F – forward primer; R – reverse primer; P – TaqMan probe; * – in our study the primer with T instead of C was used.

cleotide change on the reverse primer to achieve 100% homology (Tab. 1). Real-time PCR amplification was performed using ABI Prism 7500 (Life Technologies, Carlsbad, California, USA). The temperature programme was 50 °C, 2 min; 95 °C, 10 min; followed by 40 cycles of 95 °C, 15 s; and 60 °C, 1 min. The reaction volume was 25 μl, and the reaction mixture contained 2.5 μl extracted DNA, 12.5 μl 2× TaqMan Universal PCR Master Mix (Life Technologies), concentration of BOS-F primer (Life technologies) was 900 nmol·l^{-1}, concentration of BOS-R primer (Life technologies) was 600 nmol·l^{-1} and concentration of BOS-P probe (Life technologies) was 50 nmol·l^{-1}, and dd H_2O as needed. The sequences of primers and the probe are presented in Tab. 1. All reactions were run in duplicate and a no-template control was included. The fluorescence in the amplification reactions was analysed using the Sequence Detection Software, v1.4 (Life Technologies). The threshold line for the calculation of the threshold cycle number (*Ct*) was fixed at 0.02 for 6-carboxyfluorescein (FAM) dye. Samples with *Ct* values of less than 35 cycles were considered as positive. The linearity and the efficiency of the real-time PCR assays were determined for each extraction of DNA. Ten-fold serial dilutions (in dd H_2O) of different extractions of DNA were used to create the standard curves. After amplification, the data were plotted against the logarithm of the initial DNA concentration. The detection window (i.e., linear range of detection) was determined from the graphs. After this, linear regression was used to calculate the slope (*S*) of the standard curve. From the slope, the amplification efficiency (*E*) and the correlation coefficient (R^2) were calculated. The efficiency of amplification was calculated according to Eq. 1 [21, 22] and expressed in percent:

$$E = [(10^{-1/S}) - 1] \times 100 \quad (1)$$

Economical evaluation

Evaluation of each extraction method was also carried out according to the time required for each method, and the difficulty of its performance. The required time was calculated as the sum of the time needed for each of the steps in the protocol, including the centrifugations and incubations. These calculations were carried out on five samples extracted simultaneously. As part of the comparison, we also calculated the material costs of each method.

Statistical analysis

The experimental data for the different DNA extraction methods were evaluated statistically using the SAS/STAT programme (SAS Software; SAS Institute, Cary, North Carolina, USA). The differences according to the DNA extraction methods were analysed through a general linear model procedure and least squares mean tests (SAS/STAT), with a level of significance of 0.05.

RESULTS AND DISCUSSION

Yield and quality of DNA prepared with the different extraction methods

Milk sampling is an easy method of acquisition of samples, although the molecular biology analysis can be quite a challenge to obtain good samples of extracted nucleic acids. Several different methods for DNA extraction have been described [2, 5, 9–11, 13, 14], and also several commercial kits are available for this purpose. In the present study, we compared 11 different DNA extraction protocols, in terms of quantity, quality, purity and detection of the extracted DNA. The evaluation was based on spectrophotometric measurements and real-time PCR *Ct* values. The mean values of the absorbance ratios (A260/A280 and A260/A230), which indicate the purity and concentration of DNA, typical volume of the obtained DNA solutions and DNA concentrations in these solutions are shown along with the statistical comparisons in Tab. 2. In order to compare different DNA extraction methods, the measured concentrations of DNA were also re-calculated to the same starting volume of milk sample (1 ml) and the same final volume of DNA solution (100 μl). These data are shown in Tab. 2 as normalized

DNA concentrations (c_n). The results of real-time PCR are presented as correlation coefficient and efficiency for each DNA extraction protocol.

The highest amount of DNA from 1 ml milk was isolated with the phenol-chloroform protocol combined with DNeasy Blood and Tissue kit. As expected, high amount of DNA was also isolated with the classical phenol-chloroform protocol [5]. Surprisingly, two spectrophotometric measurements (A260/A280 and A260/A230 ratios) showed better quality of the isolated DNA when only phenol-chloroform extraction was used. Pure DNA solutions have an A260/A280 ratio of ≥ 1.8 [19, 20]. Lower A260/A280 ratios can indicate contamination by proteins. However, repeatability of these DNA extractions was not good. For the phenol-chloroform extraction combined with NucleoSpin Food kit as an additional step for DNA cleaning, the repeatability was better, but concentrations of the obtained DNA were low. When the phenol-chloroform DNA extractions were included, the laboratory work was much more labour-demanding and the error was increased. Indeed, spectrophotometric measurements showed poor quality of the extracted DNA in all cases. High amount of DNA was also isolated with Tween-based method [14]. Addition of the casein-dissolving mixture was performed to improve the quality of isolated DNA. However, A260/A280 and A260/A230 ratios showed poor quality of DNA isolated by this protocol. The repeatability of the DNA extractions with the Tween-based method was very good, although the DNA concentrations were lower than those obtained by methods that included phenol-chloroform. DNA could not be used for direct application to real-time PCR amplification and required dilution. For comparison, we also included DNA extraction with guanidine thiocyanate, where DNA was isolated by a combined chemical and mechanical procedure (30 s mixing cells in SDS extraction solution with glass beads, with vortexing) [13]. The results showed low quantity and quality of the isolated DNA in comparison with the other tested methods.

According to A260/A280 ratio and the repeatability of the extractions, two commercial methods, namely. NucleoSpin Food kit and SmartHelix Complex Samples kit, yielded relatively pure DNA in the present study. The high purity of DNA isolated with SmartHelix Complex Samples kit was surprising. As this kit involves phenol extraction, we expected to achieve results similar to those obtained by the classical extraction methods. Unfortunately, chemical reasons of the good performance of this kit cannot be evaluated since the other components of the kit were not revealed by the producer. However, better extraction of DNA could also be related to the breakage of the cells with the bead beater, which was used only with the SmartHelix Complex Samples kit. The highest A260/A230 ratio was obtained for DNA extracted with SmartHelix Complex Samples kit, while all other tested extraction methods showed lower A260/A230 ratios than the desired level. It is known that samples that have an A260/A230 ratio below 1.5–1.8 have a significant presence of contaminants, such as urea, phenol and other aromatic compounds [22]. All DNA solutions obtained with commercial kits had DNA concentrations in the same range except of those obtained with SmartHelix Complex Samples kit and SmartHelix First DNAid kit. In recent years, real-time PCR has become a reliable tool for assessing DNA quantity and quality for downstream applications. The values of *Ct*, *E* and R^2 directly reflect the utility of the extracted sample for molecular analysis [21, 22]. As shown in Tab. 2, DNA extracted from the milk could be amplified with real-time PCR [9] with the following DNA extraction methods: NucleoSpin Food kit; QIAprep Spin Miniprep kit; DNeasy Blood and Tissue kit; SmartHelix First DNAid kit; SmartHelix Complex Samples; the phenol-chloroform protocol; and the phenol-chloroform protocol with NucleoSpin Food kit. No amplification was achieved with DNA isolated by the other methods: DNeasy *mericon* Food kit; GITC extraction; Tween-based extraction; and phenol-chloroform protocol with DNeasy Blood and Tissue kit. The highest concentrations of isolated DNA did not mean successful real-time PCR, as the amplification also depends on the co-extracted compounds [5, 11, 16].

According to the real-time PCR measurements and calculations of the correlation coefficients and reaction efficiencies, the three commercial kits, namely, QIAprep Spin Miniprep kit, DNeasy Blood and Tissue kit, and SmartHelix First DNAid kit, as well as the combined method with phenol-chloroform and NucleoSpin Food kit, were the most successful DNA extraction methods for raw cows' milk.

The samples extracted by the non-commercial methods (Tween-based extraction, phenol-chloroform protocol combined with DNeasy Blood and Tissue kit) indicated high DNA quantities but, based on the poor real-time PCR performance, these extracted DNA samples apparently contained inhibitors. It is known that foods are complex samples that contain many inhibitors such as the calcium ions in milk [16, 17]. As we used the same food matrix, row cows' milk, as a starting material for all DNA extraction methods, the

Tab. 2. Purity, concentration and real-time PCR parameters of the DNA obtained from milk with the different extraction methods.

Extraction method		A260/A280	A260/A230	*V* [µl]	*c* [ng·µl^{-1}]	c_n [ng·µl^{-1}]	Real-time PCR	
							R2	*E* [%]
Commercial kits	NucleoSpin Food kit	1.86 ± 0.53^{c}	0.64 ± 0.07^{bc}	100	7.3 ± 1.1	7.3 ± 1.1^{b}	0.836	135.2
	QIAprep Spin Miniprep kit	1.58 ± 0.12^{cde}	0.22 ± 0.15^{cd}	50	3.2 ± 0.7	6.4 ± 1.4^{b}	0.996	82.1
	DNeasy Blood and Tissue kit	1.57 ± 0.25^{cde}	0.75 ± 0.12^{b}	200	3.0 ± 1.0	1.5 ± 0.5^{b}	0.981	100.1
	DNeasy *mericon* Food kit	1.34 ± 0.32^{cde}	0.52 ± 0.08^{bcd}	150	3.9 ± 2.4	1.3 ± 0.8^{b}	nd	nd
	SmartHelix First DNAid kit	1.77 ± 0.03^{cd}	0.77 ± 0.15^{b}	100	11.8 ± 1.8	5.9 ± 0.4^{b}	0.995	117.6
	SmartHelix Complex Samples kit	1.87 ± 0.01^{c}	1.67 ± 0.15^{a}	50	60.0 ± 4.2	240.0 ± 17.0^{ab}	0.998	80.8
Non-commercial methods	Phenol-chloroform protocol	1.14 ± 0.11^{de}	0.40 ± 0.06^{bcd}	25	76.6 ± 53.4	306.6 ± 213.6^{ab}	0.971	134.6
	GITC extraction	0.48 ± 0.14^{f}	0.11 ± 0.15^{cd}	100	7.0 ± 0.4	3.5 ± 0.2^{b}	nd	nd
	Tween-based extraction	1.07 ± 0.00^{e}	0.27 ± 0.15^{cd}	25	192.0 ± 0.4	15.4 ± 0.1^{b}	nd	nd
Combined methods	Phenol-chloroform protocol plus NucleoSpin Food kit	2.5 ± 0.71^{b}	0.45 ± 0.15^{bcd}	100	1.8 ± 1.0	1.8 ± 1.0^{b}	0.982	90.5
	Phenol-chloroform protocol plus DNeasay Blood and Tissue kit	4.40 ± 0.08^{a}	0.63 ± 0.15^{bc}	100	651.3 ± 171.7	651.3 ± 171.7^{a}	nd	nd

Values are given as mean ± standard deviation. Values with the different superscript letter are significantly different ($p < 0.05$).
A260/A280, A260/A230 – ratio of absorbances measured at 260 nm and 280 nm, respectively at 260 nm and 230 nm, *V* – volume of DNA solution, *c* – concentration of DNA solution, c_n – normalised DNA concentration, i.e. recalculated DNA concentration to the same starting volume of milk sample (1 ml) and the same final volume of the DNA extracted (100 µl), R^2 – correlation coefficient, *E* – PCR efficiency, nd – not detected (no readable threshold cycle number).

reason for poor real-time PCR results obtained by GITC and Tween-based extraction methods could be the presence of inhibitors in extracted DNA that originated from the sample. The other reason could be also that some components (e.g. EDTA, isopropanol) used for extraction of DNA remained as traces in the extracted DNA [15, 16]. No improvement was achieved using the combinations of extraction methods. Among classical methods, phenol-chloroform protocol yielded DNA at high concentrations and also facilitated a good performance of real-time PCR [5].

Economical evaluation

The DNA extraction methods were also compared in terms of the labour requirements, throughput time, and material costs (Tab. 3). The required time for DNA extraction from milk samples with the different extraction methods varied from 1 h up to 7.5 h, with an average time of 2 h for the commercial kits and 5.6 h for the non-commercial extractions. Two of the commercial kits were the fastest, namely, QIAprep Spin Miniprep kit and DNeasy Blood and Tissue kit. Although these two commercial kits provided DNA in low concentrations and not ideal purity, their real-time PCR parameters were good (Tab. 2). Some researchers have already successfully applied these methods to isolate DNA from milk and cheese samples for real-time PCR analysis [7, 23, 24]. Similarly, Olexová et al. [25] tested different methods of DNA extraction from foods, looking for the maximum yield also for the fastest performance.

Another parameter to consider when choosing an extraction method is the difficulty of performing the methodology. In general, all commercial kits for DNA extraction were not labour-demanding, with the exception of the SmartHelix Complex Samples kit, which required somewhat more concentration and labour. There were no significant differences between the other five commercial kits. All commercial kits had simple and easy-to-follow protocols. On the other hand, all three published non-commercial methods demanded more skill and attention. The Tween-based extraction [14] was the most complex to perform, followed by the GITC extraction [13] and the phenol-chloroform extraction [5].

Tab. 3. Estimation of the labour intensity, time and material costs required for the DNA extraction with the different extraction methods.

Extraction method		Labour requirements	Time for 5 samples [h]	Cost per sample [€]
Commercial kits	NucleoSpin Food kit	+	1.8	3.32
	QIAprep Spin Miniprep kit	+	1	1.80
	DNeasy Blood and Tissue kit	+	1	1.44
	DNeasy mericon Food kit	+	1.7	3.47
	SmartHelix First DNAid kit	+	2	3.27
	SmartHelix Complex Samples kit	++	4.4	3.17
Non-commercial methods	Phenol-chloroform protocol	++	5.7	0.37
	GITC extraction	+++	6	2.14
	Tween-based extraction	++++	5.2	0.37
Combined methods	Phenol-chloroform protocol plus NucleoSpin Food kit	++	7.5	3.69
	Phenol-chloroform protocol plus DNeasy Blood and Tissue kit	++	6.5	1.81

(+) – very easy, (++) – easy, (+++) – difficult, (++++) – very difficult.

Both combined methods for DNA extraction used the phenol-chloroform protocol, and consequently these were the slowest of all tested methods.

The comparison of material costs showed large differences between the methods used in this study. The cheapest methods were, as expected, the phenol-chloroform and Tween-based extractions, followed by DNeasy Blood and Tissue kit, GITC extraction and QIAprep Spin Miniprep kit. The next group of methods included the much more expensive methods: SmartHelix Complex Samples kit, SmartHelix First DNAid kit, NucleoSpin Food kit and DNeasy *mericon* Food kit, which costed more than 3 € per sample.

Despite quite a wide range of the material costs of the commercial methods, the great advantage of using them is the easier and faster performance, compared to the non-commercial and combined protocols.

CONCLUSIONS

PCR-based methods have been applied over the last two decades in different food areas for the determination of different food contaminants and constituents, such as pathogens and toxin-producing organisms, food ingredients, allergens and adulteration. The successful extraction of DNA from food is one of the parameters that affect the successful implementation of these methods. In this study, selected methods for extracting DNA from milk were found to produce DNA of very different quantity and quality. According to the evaluated parameters (spectrophotometric measurements, real-time PCR parameters, estimated time, material costs, and labour requirements), three commercial kits , namely, QIAprep Spin Miniprep kit (Qiagen), DNeasy Blood and Tissue kit (Qiagen), and SmartHelix First DNAid kit (ExVivon), provided the best results.

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REFERENCES

1. Borková, M. – Snášelová, J.: Possibilities of different animal milk detection in milk and dairy products – a review. Czech Journal of Food Sciences, *23*, 2005, pp. 41–50.
2. Darwish, F. S. – Allam, H. A. – Amin A. S.: Evaluation of PCR assay for detection of cow's milk in water buffalo's milk, *4*, 2009, pp. 461–467.
3. Cottenet, G. – Blancpain, C. – Golay, P. A.: Simultaneous detection of cow and buffalo species in milk from China, India, and Pakistan using multiplex real-time PCR. Journal of Dairy Science, *94*, 2011, pp. 3787–3793.
4. Zachar, P. – Šoltés, M. – Kasarda, R. – Novotný, J. – Novikmecová, M. – Marcinčáková, D.: Analytical methods for the species identification of milk and milk products. Mljekarstvo, *61*, 2011, pp. 199–207.
5. De, S. – Brahma, B. – Polley, S. – Mukherjee, A. – Banerjee, D. – Gohaina, M. – Singh, K. P. – Singh, R. –

Datta, T. K. – Goswami, S. L.: Simplex and duplex PCR assays for species specific identification of cattle and buffalo milk and cheese. Food Control, *22*, 2011, pp. 690–696.
6. Dabrowska, A. – Wałecka, E. – Bania, J. – Zelazko, M. – Szołtysik, M. – Chrzanowska, J.: Quality of UHT goat's milk in Poland evaluated by real-time PCR. Small Ruminant Research, *94*, 2010, pp. 32–37.
7. Dalmasso, A. – Civera, T. – La Neve, F. – Bottero, M. T.: Simultaneous detection of cow and buffalo milk in mozzarella cheese by real-time PCR assay. Food Chemistry, *124*, 2011, pp. 362–366.
8. Sakaridis, I. – Ganopoulos, I. – Anagnostis, A. – Athanasios, T.: High resolution melting analysis for quantitative detection of bovine milk in pure water buffalo mozzarella and other buffalo dairy products. International Dairy Journal, *28*, 2013, pp. 32–35.
9. López-Calleja, I. – González, I. – Fajardo, V. – Martín, I. – Hernández, P. E. – García, T. – Martín, R.: Real-time TaqMan PCR for quantitative detection of cows' milk in ewes' milk mixtures. International Dairy Journal, *17*, 2007, pp. 729–736.
10. Mafra, I. – Roxo, Á. – Ferreira, I. M. P. L. V. O. – Oliveira, M. B. P. P.: A duplex polymerase chain reaction for the quantitative detection of cows' milk in goats' milk cheese. International Dairy Journal, *17*, 2007, pp. 1132–1138.
11. Drummond, M. G. – Brasil, B. S. A. F. – Dalsecco, L. S. – Brasil, R. S. A. F. – Teixeira, L. V. – Oliveira, D. A. A.: A versatile real-time PCR method to quantify bovine contamination in buffalo products. Food Control, *29*, 2013, pp. 131–137.
12. Sharma, N. – Singh, N. K – Bhadwal, M. S.: Relationship of somatic cell count and mastitis: An overview. Asian – Australasian Journal of Animal Sciences, *24*, 2011, pp. 429–438.
13. Bonaïti, C. – Parayre, S. – Irlinger F.: Novel extraction strategy of ribosomal RNA and genomic DNA from cheese for PCR-based investigations. International Journal of Food Microbiology, *107*, 2006, pp. 171–179.
14. Murphy, M. A. – Shariflou, M. R. – Moran, C.: High quality genomic DNA extraction from large milk samples. Journal of Dairy Research, 69, 2002, pp. 645–649.
15. Bar, T. – Kubista, M. – Tichopa, A. Validation of kinetics similarity in qPCR. Nucleic Acids Research, *40*, 2012, pp. 1395–1406.
16. Hedman, J. – Rådström, P.: Overcoming inhibition in real-time diagnostic PCR. Methods in Molecular Biology, *943*, 2013, pp. 17–48.
17. Bickley, J. – Short, J. K. – McDowell, D. G. – Parkes, H. C.: Polymerase chain reaction (PCR) detection of *Listeria monocytogenes* in diluted milk and reversal of PCR inhibition caused by calcium ions. Letters in Applied Microbiology, *22*, 1996, pp. 153–158.
18. Protocol: Purification of total DNA from animal blood or cells (DNeasy 96 Protocol). In: DNeasy® Blood & Tissue Handbook. Sine loco : Qiagen, July 2006, pp. 31-35. Available also at <http://www.qiagen.com/resources/download.aspx?id=6b09dfb8-6319-464d-996c-79e8c7045a50&lang=en>
19. Luebbehusen, H.: The significance of the 260/230 ratio in determining nucleic acid purity. In: Genomic and RNA profiling core [online]. Houston : Baylor College of Medicine, 2006 [cit. 12 May 2013].<http://www.bcm.edu/mcfweb/?PMID=3100>
20. Nucleic acid purity assessment using a260/280 ratios. In: BioTek [online]. Vermont : BioTek Instruments, 2001 [cit. 12 May 2013]. <http://www.biotek.com/resources/articles/nucleic-acid-purity-a260a280.html>
21. Rutledge, R. G. – Cote, C.: Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Research, *31*, 2003, pp. 1–6.
22. Pfaffl, M. W.: Quantification strategies in real-time PCR. In: Bustin, S. A. (Ed.): A–Z of quantitative PCR. La Jolla : International University Line, 2004, pp. 87–112.
23. Santos Guerreiro, J. – Fernandes, P. – Bardsley, R. G.: Identification of the species of origin of milk in cheeses by multivariate statistical analysis of polymerase chain reaction electrophoretic patterns. International Dairy Journal, *25*, 2012, pp. 42–45.
24. Pinto, A. D. – Forte, V. T. – Guastadisegni, M. C. – Martino, C. – Schena, F. P. – Tantillo, G.: A comparison of DNA extraction methods for food analysis. Food Control, *18*, 2007, pp. 76–80.
25. Olexová, L. – Dovičovičová, L. – Kuchta, T.: Comparision of three types of methods for the isolation of DNA from flours, biscuits and instant paps. European Food Research and Technology, *218*, 2004, pp. 390–393.

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