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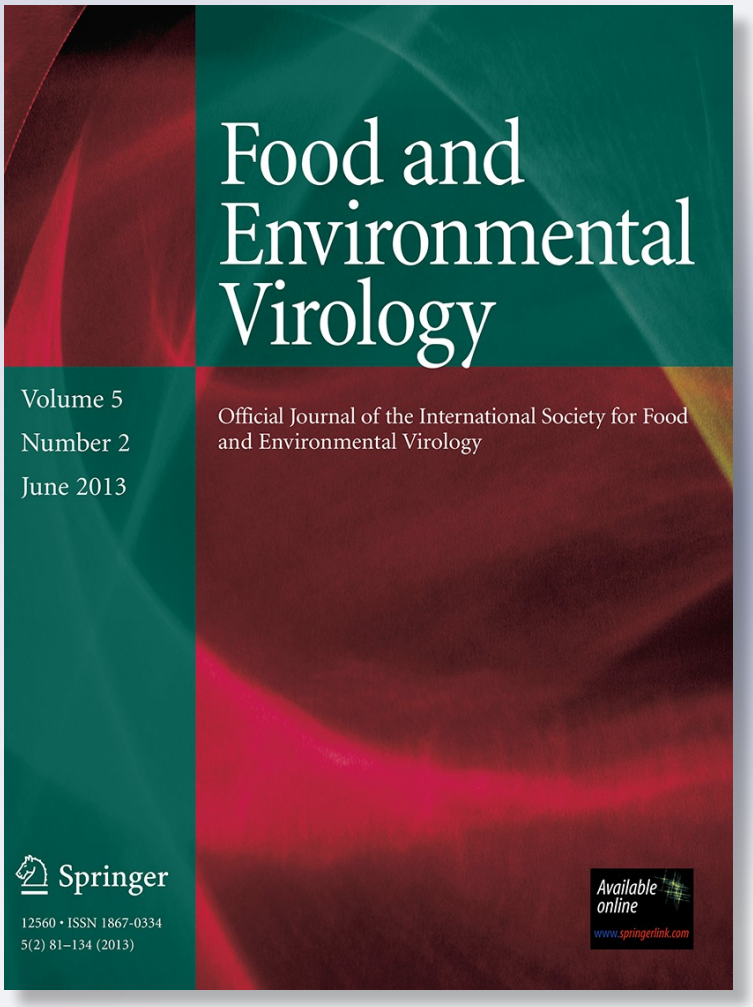
Food and Environmental Virology
The Official Journal of the International Society for Food and Environmental Virology

ISSN 1867-0334
Volume 5
Number 2

Food Environ Virol (2013) 5:103-109
DOI 10.1007/s12560-013-9107-3



Springer

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ORIGINAL PAPER

Impact of Milk Components on Recovery of Viral RNA from MS2 Bacteriophage

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Received: 9 August 2012 / Accepted: 12 February 2013 / Published online: 22 February 2013

Abstract Noroviruses are responsible for approximately 44 % of outbreaks involving dairy products for which causative agents are reported. Recovery of viruses from milk and dairy products is a difficult task. The role of different components of milk in the recovery of viral RNA was evaluated in this study. Four model milk formulations (A–D) were prepared by mixing different combinations of lactose, whey protein, casein, and fat in water. Each model formulation was spiked with five concentrations of bacteriophage MS2. The phenol-guanidine thiocyanate-chloroform protocol was used for extracting viral RNA from the model milk formulations and then extracted RNA was measured by a nanodrop spectrophotometer in ng/μl. The results showed that casein and whey protein had the highest negative impact on RNA yield, especially when the number of MS2 was less than 1.3 pfu/ml. The highest RNA recovery was obtained from the model milk formulation containing all four components; lactose, whey protein, casein, and fat. The amount of extracted RNA was closely correlated with the dry matter content of each formulation and the spiked concentration of coliphage using response surface modeling (R^2:0.93). It was determined that milk fat is the most effective component in facilitating RNA extraction and the highest RNA yield can be achieved via elimination of whey protein and casein from milk by centrifugation at $40{,}000 \times g$ for 60 min. To achieve the highest viral RNA recovery efficiency by the proposed method, milk fat must be recombined with the supernatant of the centrifuged sample and then homogenized before performing the extraction protocol.

Keywords Viruses · Raw milk · Milk components · RNA extraction

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Introduction

Foodstuffs such as vegetables, fruits, shellfish, and milk products are commonly implicated in outbreaks of viral gastroenteritis (Dubois et al. 2002; Leggitt and Jaykus 2000). Between 1993 and 2006, a total of 48 disease outbreaks involving pasteurized dairy products were reported in the US. However, the causative agent was identified in only 30 of those outbreaks and 44 % (13/30) were caused by norovirus (Langer et al. 2012). Traditionally, food borne viruses are detected using cultural methods. However, application of molecular techniques facilitates rapid and sensitive viral detection and characterization. Despite many advances in molecular techniques the extraction of viral nucleic acids from food remains a difficult task (Richards 2001). In addition, viruses can be in low concentration in food samples and components of food matrices can interfere with amplification processes (Shieh et al. 1999). Typically, the extraction techniques are selected depending

on the food complexity and can be as simple as boiling to lyse the cells, or using detergents and proteinase K treatment, or organic extraction and ethanol precipitation (Atmar et al. 1993; Leggitt and Jaykus 2000; Mullendore et al. 2001). Efforts to recover microbial/viral genome from clinical specimens (Gouvea et al. 1990; Wilde et al. 1990), contaminated foods (Lees et al. 1994; Jiang et al. 1992) and environmental samples (Shieh et al. 1995; Rock et al. 2010) have always faced the challenges of inhibitory substances such as heavy metals, humic materials, and heparin. Removal of inhibitors is a key consideration for choosing a proper extraction method (Beutler et al. 1990; Wilson 1997; Rock et al. 2010).

In our previous work, it was shown that the milk components can interfere with MS2 coliphage recovery and enumeration (Yavarmanesh et al. 2010). Milk components such as casein, whey proteins, lactose, and fat belong to very different class of organic chemical structures and activities; therefore, they show different adsorption, sedimentation, and partitioning properties. Their physical–chemical properties even differ from their counter parts from other sources. Because of such differences, these molecules may have variable impacts on the recovery of microorganisms and their genomic materials. There still remains a need to investigate how these components interfere with microbial detection efforts using molecular techniques.

The objective of this study was to characterize the role of milk components in the recovery of viral RNA from milk and use this information to develop a suitable sample processing protocol for viral genome extraction from cow's milk.

Materials and Methods

Preparation of Model Formulations of Milk and Purified Milk for RNA Extraction

To identify the role of milk components on recovery of viral RNA, four model milk formulations: A (lactose), B (lactose + whey), C (lactose + whey + casein), D (lactose + whey + casein + fat), and purified milk (purified in that it was centrifuged to remove bacteria) were prepared as previously described (Yavarmanesh et al. 2010). For simplicity these formulations are referred as: model A (L), model B (L + W), model C (L + W + C), and model D (L + W + C + F); where L, W, C, and F, stands for lactose, whey, casein, and fat, respectively. Purified milk was included as reference matrix in this study. Samples of model formulations and purified milk were spiked with different concentration of MS2 and stored overnight at 4 °C. The following day, twelve aliquots from each formulation were collected and viral RNA was extracted from each of the aliquots.

Preparation of MS2 Stock for Spiking Model Formulations and Milk Samples

Male-specific bacteriophage MS2 (ATCC#15597-B1) and its host bacterium *E. coli* F_{amp} (ATCC#700891), were propagated as described in USEPA Method 1601 (EPA 2001). MS2 stocks were tittered using the double agar layer (DAL) method (EPA 2001). Viral stock was diluted in buffered phosphate solution (pH 7.2) to achieve the following concentrations: 1.3×10^4, 1.3×10^2, 1.3, 1.3×10^{-2}, and 1.3×10^{-4} pfu/ml.

MS2 Coliphage RNA Extraction

The viral RNA was extracted using a commercially available kit (Tripure, Roch applied science, Indianapolis, USA) by following the manufacturer's instruction. In brief, each sample (1 ml) was mixed with 1 ml Tripure reagent and extracted with 200 µl of chloroform and the sample was then centrifuged at 40,000×*g* for 1 h at 4 °C to separate RNA into the aqueous phase (Yavarmanesh et al. 2010). Then, the aqueous phase containing viral RNA was precipitated with 500 µl of isopropanol. The RNA pellet was then washed with 1 ml of 75 % ethanol and resuspended in 50 µl of sterile DEPC water and stored in a liquid nitrogen tank until use.

Quantification of MS2 Coliphage Total RNA

Total RNA in each sample was measured in ng/µl using a nanodrop spectrophotometer (ND1000, Thermoscientific, Delaware, USA). For control samples, total background RNA concentration was measured before spiking MS2 in the samples of model milk formulations. MS2 RNA concentration in each extracted sample was determined after adjusting for background concentration. The purity of the measured RNA was determined by absorbance ratio of 260/280 nm wave length.

The molecular weight of the MS2 genome was calculated to be 3,569 bp (accession number V00642) with 52.1 % GC content. Based on the sequence, each MS2 particle contains approximately 1.9 ag of RNA resulting in 0.019 pg of RNA for 10^4 MS2 particles http://www.changbioscience.com/genetics/mw.html (accessed 14/01/2011).

Confirmation of Extraction of MS2 Bacteriophage RNA

Two-step RT-PCR protocol was used in this study. In the first step, RT was carried out in a volume of 11 µl that included 5 µl RNA template (previously denatured for

5 min at 65 °C), 1 µl antisense primer at final concentration of 25 mM and 5 µl RT diluents buffer (Gen pack RT core, Isogen, Russia). The temperature profile for RT was 42 °C for 60 min and 80 °C for 10 min (enzymatic inactivation). In the second step, the PCR was performed in a 20 µl reaction that were 2 µl RT reaction, 2 µl of sense (2,717, CTGGGCAATAGTCAAA) and antisense (3,031, CGTGGATCTGACATAC) primer (25 mM), 10 µl PCR diluents buffer, and 6 µl sterile deionized water (Gen pack PCR core, Isogen, Russia). The PCR conditions included 95 °C for 10 min followed by 40 cycles of 1 min at 95 °C, 1 min at 55 °C, and 45 s at 72 °C. A final extension was performed for 7 min at 72 °C. The PCR reaction resulted in the amplification of a 314-bp product.

Statistical Analyses

Data were analyzed using the generalized additive model (Sigma Stat Software (version 2.0, Jandel Corporation, SanRafael, CA, USA) for approaching the best functions, and Slide Write software (plus 2.0, Landbouw University, Wageningen, the Netherlands) for exhibiting the functions. In addition, the Slide Write software was used for predicting the RNA yield using a counter isoline technique.

Results

Viral Total RNA Yield in Each Model Formulation

The amounts of total RNA recovered in each model milk formulation (spiked and non-spiked) are shown in Table 1. Model D (L + W + C + F) had the highest total RNA recovery and model C (L + W + C) had the lowest total RNA recovery.

Viral Total RNA Yield in Purified Milk

Purified milk was used as a reference sample for the recovery of viral RNA. The total amount of RNA recovered from the non-spiked and spiked samples of purified milk is presented in Table 2. The total RNA recovered from spiked purified milk and the supernatant of spiked purified milk was more than model formulations A, B, and C but less than model D (Tables 1, 2).

Purity of Total RNA Recovered from Spiked Samples of Model Formulations and Purified Milk

Before RT-PCR amplification, the purity of RNA extracted from various milk samples was determined by the

Table 1 Total RNA yields in different model milk formulation

RNA yield(pg/µl)								
	Total RNA detected in milk formulations				Viral RNA calculated in milk formulations (spiked–non-spiked)			
MS2 spike concentration	A (L)	B (L + W)	C (L + W + C)	D (L + W + C + F)	A (L)	B (L + W)	C (L + W + C)	D (L + W + C + F)
0	6.5 ± 0.6	19.5 ± 1.1	9.2 ± 0.3	17.4 ± 0.9	0	0	0	0
1.3×10^4 (ln:9.47)	23.45 ± 1.9	30.97 ± 1.3	13.45 ± 1.4	68.05 ± 2.4	16.95 ± 1.2	11.47 ± 0.2	4.25 ± 1.0	50.65 ± 1.5
1.3×10^2 (ln:4.86)	18.52 ± 1.4	25.42 ± 1.3	11 ± 1.2	55.16 ± 1.7	12.05 ± 0.8	5.92 ± 0.3	1.8 ± 0.8	37.76 ± 0.8
1.3 (ln:0.26)	7.4 ± 0.4	18.9 ± 1.3	10.55 ± 0.3	47.9 ± 1.3	0.9 ± 0.3	−0.6 ± 0.3	1.35 ± 0.05	30.5 ± 0.4
1.3×10^{-2} (ln:−4.34)	6.1 ± 0.3	17 ± 1	10.17 ± 0.15	43.4 ± 1.8	−0.4 ± 0.4	−2.5 ± 0.07	0.975 ± 0.2	26 ± 0.9
1.3×10^{-4} (ln:−8.94)	4.6 ± 0.15	8 ± 0.1	9.95 ± 0.2	34.25 ± 0.3	−1.9 ± 0.5	−11.5 ± 0.9	0.75 ± 0.07	16.85 ± 0.5

Table 2 Total RNA yields in purified natural milk

RNA yield in purified milk(pg/µl)				
	Total RNA detected in milk		Viral RNA calculated in milk sample (spiked–non-spiked)	
MS2 spike concentration	Purified milk	Supernatant of purified milk	Purified milk	Supernatant of purified milk
0	3.9 ± 0.27	4.2 ± 0.29	0	0
1.3×10^4 (ln:9.47)	18.95 ± 0.65	37.5 ± 1.2	15.05 ± 0.38	33.3 ± 0.91
1.3×10^2 (ln:4.86)	16.1 ± 0.33	35 ± 1	12.2 ± 0.06	30.8 ± 0.71
1.3 (ln:0.26)	12.35 ± 0.56	30.5 ± 0.86	8.45 ± 0.29	26.3 ± 0.57
1.3×10^{-2} (ln:−4.34)	9.3 ± 0.22	15 ± 0.96	5.4 ± 0.05	10.8 ± 0.67
1.3×10^{-4}(ln:−8.94)	5.5 ± 0.26	9.5 ± 0.52	1.6 ± 0.01	5.3 ± 0.23

Table 3 Quality of total RNA recovered from spiked model milk formulation and spiked purified milk

Purity of total RNA recovered from spiked model formulations and spiked purified milk (absorbance at 260/280 nm)						
Model milk formulation	A (L)	B (L + W)	C (L + W + C)	D (L + W + C + F)	Purified milk	Supernatant of purified milk
Spiked initial MS2						
1.3×10^{4} (ln:9.47)	1.89 ± 0.01	1.77 ± 0.03	1.61 ± 0.09	1.86 ± 0.04	1.7 ± 0.04	1.86 ± 0.02
1.3×10^{2} (ln:4.86)	1.7 ± 0.05	1.7 ± 0.05	1.44 ± 0.02	1.74 ± 0.03	1.68 ± 0.01	1.78 ± 0.00
1.3 (ln:0.26)	1.65 ± 0.08	1.64 ± 0.01	1.32 ± 0.04	1.68 ± 0.07	1.63 ± 0.06	1.77 ± 0.00
1.3×10^{-2} (ln:−4.34)	1.52 ± 0.05	1.59 ± 0.03	1.29 ± 0.06	1.64 ± 0.03	1.52 ± 0.02	1.71 ± 0.04
1.3×10^{-4} (ln:−8.94)	0.83 ± 0.07	1.4 ± 0.02	1.12 ± 0.03	1.65 ± 0.03	1.54 ± 0.06	1.69 ± 0.01

absorbance ratio (Table 3). The highest quality RNA was recovered from Model D (L + W + C + F); whereas, RNA of the least purity was recovered from Model C (L + W + C) (Table 3). In terms of milk fractions, the milk supernatant yielded RNA of greater purity than the whole purified milk.

Effect of Dry Matter on the Yield and Purity of RNA Extracted from Milk

Based on the response surface curve in Fig. 1a, there was a close mathematical correlation among total RNA yield, initial bacteriophage concentration and dry matter contents of model milk formulation (polynomial function). Also there was a similar mathematical correlation between total RNA purity and dry matter content (Fig. 2a). Total RNA yield and purity can be predicated by the dry matter content of milk using a counter isoline technique (Figs. 1b, 2b).

Evaluation of the Limit of Detection Using the Newly Optimized RNA-Extraction Method

The optimal RNA-extraction method identified in this study was used to recover target RNA from the samples spiked with a gradient of MS2 concentrations and the RT-PCR results are presented in Fig. 3. The RNA-extraction procedure followed by the RT-PCR resulted in the detection of target amplicons of 314-bp from a milk sample spiked at 1.3×10^{-2} pfu/ml.

Discussion

For this study, MS2 was chosen as a surrogate for human enteric viruses and it is widely used as viral surrogate in studies focusing on food (Alum et al. 2011), water (Abbaszadegan et al. 2008), and biosolids (Rock et al. 2010). Among all spiked model milk formulations, the highest yield of total RNA was obtained in model D (L + W + C + F) which contains fat, followed by model A (L), model B (L + W), and model C (L + W + C). Fat milk is mainly polar phospholipid, which orient into micelle forms and may play a role in capturing RNA in a solution. The captured RNA on charged surfaces of fat milk globules are further purified by liquid–liquid extraction before used as template in RT-PCR. Therefore, better recovery of RNA from model D

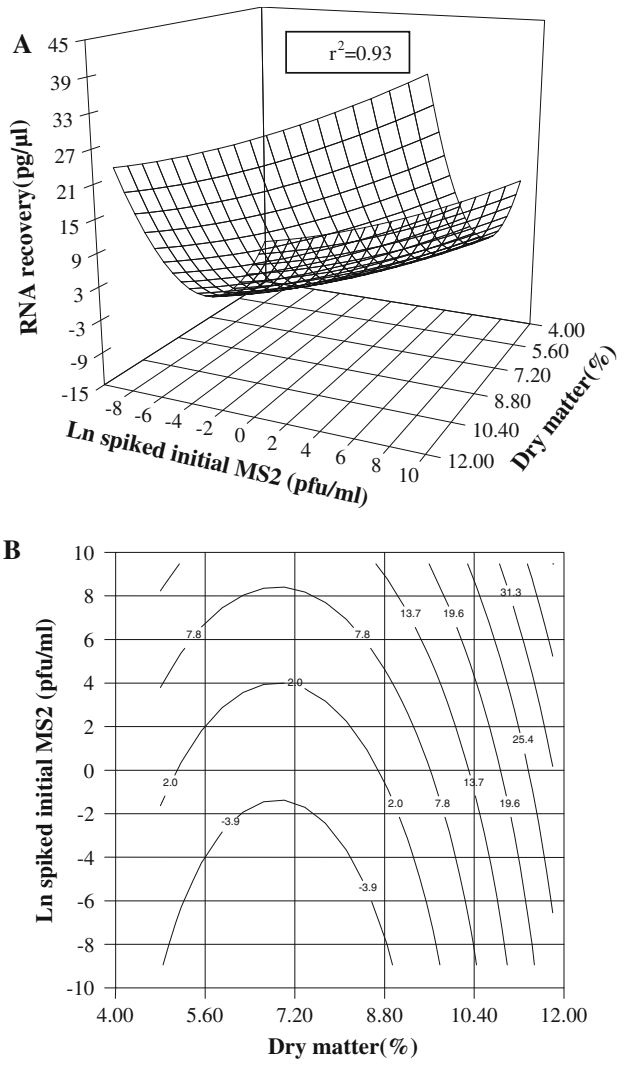

Fig. 1 Relationship between the total RNA yield, coliphage concentration, and dry matter contents; response surface (**a**) and counter isoline (**b**) curve related to polynomial equation

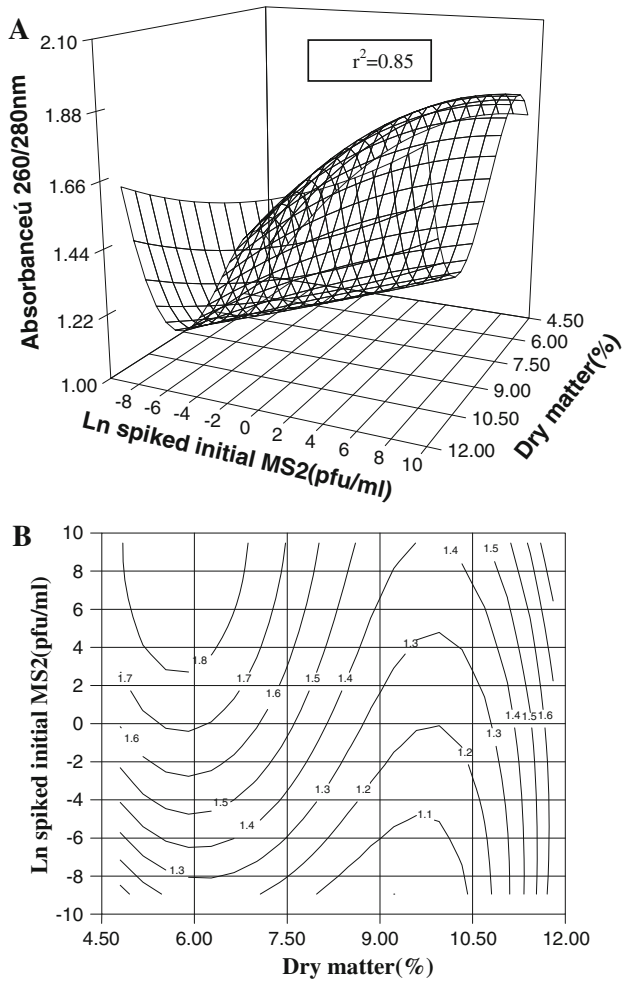

Fig. 2 Relationship between the quality of RNA extract, coliphage concentration, and dry matter contents; response surface (**a**) counter isoline (**b**) curve related to polynomial equation

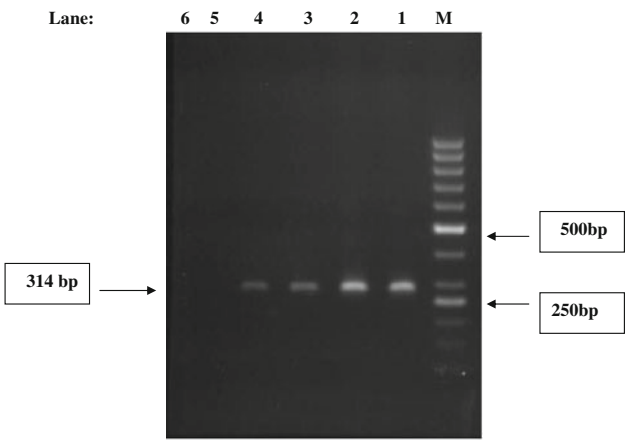

Fig. 3 RT-PCR detection limit for the optimized method for RNA extraction (*M*, markers; *Lane1*, MS2 1.3 × 10^4 pfu/ml; *Lane2*, MS2 1.3 × 10^2 pfu/ml; *Lane3*, MS2 1.3 pfu/ml; *Lane4*, MS2 1.3 × 10^{-2} pfu/ml; *Lane5*, MS2 1.3 × 10^{-4}; *Lane6*, negative control)

Table 4 Comparison between density of components of model milk formulations and organic solvents used for RNA extraction

Solvents and milk components	Density (g/ml) at 20 °C
Isopropanol	0.786
Fat globule	0.92
Phenol	1.07
Guanidine thiocyanate	1.103
Casein micelle	1.11
Chloroform	1.483

can be ascribed to the hydrophilic–hydrophobic interaction of a single strand of positive sense RNA with milk fat (Eichler 2006; Hadad 2002; VanVoorthuizen et al. 2001). The structure of the ssRNA molecule contains a base consisting of a phosphate group and ribose sugar, therefore, RNA possesses both hydrophobic and hydrophilic properties (Eichler 2006). Milk fat globules are aggregates of phospholipid micelles, which contain a negative (ζ) potential that can facilitate hydrophobic interaction with ssRNA (Corroding and Dalgleish 1997; Dewettinck et al. 2007; Kanno et al. 1991; Michalski et al. 2001). Homogenization of the milk fat globules produce submicron sized micelles with increased (negative) surface potential (ζ) and a decrease in pH (Miller et al. 1980). This characteristic of lipid micelles has been used to construct lipid–nucleic acid particles for gene delivery (Morille et al. 2008). Lipid–nucleic acid particles can be formed using organic solvents in processes such as phenol–chloroform extraction (Wheeler et al. 2004). The conventional nucleic acid extraction procedures rely on phenol and similar organic solvents to disrupt or denature microbial cell wall/membrane/capsid for releasing nucleic acids before liquid-phase separation in the isolation process. The negatively charged milk fat globules do not play a role in releasing nucleic acid from microbial structures; however, during the phase separation step they behave similarly to an organic solvent and it is attributed to their partitioning characteristics and specific densities (Table 4) (Smallwood 1996). This is a significant characteristic, which enables a positive interaction between these structures and ssRNA. Similarly, an interaction between ssRNA and polar lipids of whey protein can facilitate RNA extraction and recovery from milk (Rombaut and Dewettinck 2006). This explains the positive correlation between the milk fat and the RNA-extraction efficiency, which is demonstrated in purified milk (Table 2).

In model D (which contains butter fat) the high yield of total RNA can be related to high load of polar lipids in triglyceride butter fat. The polar lipids are assumed to facilitate total RNA extraction because of surfactant properties (Rombaut and Dewettinck 2006). In addition, the positive role of milk fat in increasing the quality of extracted RNA can be based on hydrophilic–hydrophobic interactions (Eichler 2006;

Hadad 2002; VanVoorthuizen et al. 2001). Unlike milk fat, the presence of milk proteins (whey and casein) negatively impact the total RNA yield, which can be due to the fact that at neutral pH, MS2 and RNA molecules are negatively charged thereby moving to hydrophobic phase in model D (L + W + C + F) (Swaisgood 1996). In addition, phenol and chloroform used for the RNA extraction are known to denature milk proteins, and such organic solvents are also used for the purification of fat from milk (Chomczynski and Sacchi 1987; Miller et al. 1980). Denaturation of milk protein and fat contents by organic solvent used at the early stages of RNA extraction may be the main cause in decreasing the yield and quality of RNA extracted from milk (Miller et al. 1980). On the other hand, the apparent increase in the yield of total RNA in non-spiked milk model formulations B and D can be related to the presence of some components in milk fat (carotenoids and trans-double bonds fatty acids) and whey proteins (soluble proteins). The presence of these components in a sample increases the absorbance at 260 nm, thereby causing a bias (false positive) in the RNA measurements (Swaisgood 1996). In this study, we also succeeded to find a direct correlation (R^2:0.93) between total RNA yield, initial concentration of coliphage, and the amount of model milk dry matter. Thus, the highest recovery of RNA was achieved by adding milk fat to the milk model formulation (Table 1; Fig. 1). The positive effect of fat on RNA recovery was confirmed in purified milk and the highest total RNA yield was achieved in the homogenized supernatant of milk which contains milk fat (Table 2). The procedure described in this study can be recommended as a method for optimal recovery of viral RNA in milk. The detection limit of PCR using the extraction method was 1.3×10^{-2} pfu/ml which is similar to other extraction methods adopted for different food and water samples (Abbaszadegan et al. 1993; Mullendore et al. 2001; Rombaut and Dewettinck 2006).

Conclusion

1. Among the milk components, fat was the most facilitating in the recovery of viral RNA from milk using the phenol-guanidine thiocyanate-chloroform method.
2. The Casein content of milk was the highest inhibitory factor in the recovery of viral RNA from milk using phenol–guanidine thiocyanate–chloroform.
3. There was a direct correlation between the recovery of viral RNA and the initial concentration of coliphage and the amount of dry matter in milk (R^2:0.93).
4. Homogenization of milk supernatant containing milk fat results in higher RNA yield.

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