Single step fractionation of raw milk with FraMiTrACR® prior to detection of the residual contaminants chlorate and perchlorate

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Abstract

Residues of chlorate and perchlorate in dairy products pose a challenge in the dairy industry. Both residues almost exclusively enter dairy food production as a disinfection or cleaning by-product. Since these substances can jeopardize food safety, we developed a cost-effective, passive and rapid workflow for raw milk sample preparation prior to determination of chlorate and perchlorate content. By means of centrifugal ultrafiltration, unprocessed raw milk was fractionated into its constituent phases (water and fat/protein), using a FraMiTrACR®°unit. When the water phase was analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS), we were able to demonstrate quantification and detection levels of 0.001 mg/kg and 0.0005 mg/kg, respectively, for perchlorate, and 0.01 mg/kg and 0.005 mg/kg, respectively, for chlorate.

Keywords: Milk, Raw Milk, Chlorate, Perchlorate, Sample Preparation, Fractionation, LC-MS/MS

Introduction

Chlorate and perchlorate are oxyanions of chlorine and are highly soluble in water. Both residues, when found in dairy products, almost exclusively originate from processing water treated with chlorine-containing disinfectants and chlorine-containing cleaning agents. The use of chlorine-free lye can also be an unexpected source of chlorate and perchlorate, since these substances can be carried over during lye manufacturing, leading to contamination in products which are labelled chlorine-free [1]. Animal feed and water can also be a natural, not insignificant source of perchlorate in milk. Public awareness of the risks these substances pose was raised when it was shown that chlorate and perchlorate can enter the thyroid gland and inhibit the synthesis of thyroid hormones [2, 3]. This is a particular concern for infants and young children because, unlike adults, their lower levels of stored thyroid hormones may fail to inhibit the uptake of chlorate and perchlorate into the thyroid [2, 3]. Therefore, new limits for food have been established with the help of the European regulations 2020/749 for chlorate and 2020/685 for perchlorate [4, 5]. In addition, the European Food Safety Authority (EFSA) has set a tolerable daily intake (TDI) of 0.003 mg/kg or 0.0003 mg/kg for chlorate or perchlorate, respectively [6]. Since infants typically consume higher volumes of milk, especially through milk-based formulae, the TDI is easily exceeded for this group [6,7]. The levels of both residues are often determined using the well-established quick method for the analysis of numerous highly polar pesticides (QuPPe) [8]. This method requires multiple sample preparation steps. First, the fat in the raw milk is removed, e.g., by centrifugation, or the whole milk is diluted by adjusting the water content. The resulting sample is treated with acidified methanol and formic acid, and then ethylenediaminetetraacetate (EDTA) is added to break down the protein phase and extract the residues. After a second centrifugation step, the supernatant is treated with acetonitrile and an adsorbent, and centrifuged again. The supernatant is then transferred to a centrifugal filter for a final clarification step prior to sample analysis [8]. Each of these steps must be actively performed by a laboratory worker, and require the availability of multiple consumables and reagents to be maintained via a stock management system. The frequent handling and manipulation of samples increases the risk of contamination. In addition, this process is a single sample method, meaning that each sample must be prepared individually. Some test laboratories have already attempted to simplify this raw milk sample preparation process. For example, Dyke et al. describe a method that consists of a degreasing step by centrifugation and filtration of the resulting skimmed milk using centrifugal ultrafiltration [9]. After 90 minutes of centrifugal ultrafiltration, the filtrate is further treated with

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acid and other reagents in multiple steps until the final test material is produced, with a total process time of more than two hours [9].

In this study, we aimed to develop a rapid, passive, single-step sample preparation process, without the need for additives, while still enabling the detection of residues to the prescribed regulatory limits. We used FraMiTrACR®°C/PC filters, which have been certified for the preparation of dairy products prior to residue analyses, and can be handled in a standard benchtop centrifuge. Our approach assumed that the target analytes - chlorate and perchlorate - were completely dissolved in the water phase of milk, without interaction with the protein or fat phases. Therefore, after fractionation of the milk, chlorate and perchlorate were detected directly from the water phase.

Materials and Methods

Sample preparation by fractionation: The raw milk for this study was excess material from Milchprüfring Baden-Württemberg e.V., collected as part of their routine milk quality monitoring processes. The study period was 12 weeks in order to obtain a sufficient data set. One raw milk used to determine the centrifugation time and relative centrifugal force for fractionation by FraMiTrACR® units was collected directly from the milk tank of a farm local to the laboratory. All staff involved in the study are experienced laboratory personnel with a degree in biotechnology or chemistry. FraMiTrACR®°C/PC units were filled with 5 mL of raw milk and centrifuged for 120 minutes at 4,000 g (swing out rotor) to effect fractionation into the constituent water, fat and protein phases. To determine filtration time, we quantified the filtrate volume every 15 minutes, in duplicate, using a precision balance. Fractionation of each milk sample into its three phases was observed by eye.

Analysis of the sample filtrate for chlorate and perchlorate: For the analysis of the filtrate (water phase), chromatographic separation and subsequent detection were carried out using a PerkinElmer LX50 UHPLC and QSight® 220 triple quadrupole tandem mass spectrometer. All instrument control, analysis, and data processing were performed using Simplicity™ 3Q software. For each dataset, 1 µL of filtrate was injected into the analyzer. The chlorate and perchlorate content were determined by comparing test sample filtrates against positive control standard samples (water spiked with chlorate and perchlorate). LC-MS grade acetonitrile, water and formic acid used during sample analysis were obtained from reputable reagent suppliers. Chlorate and perchlorate standards were purchased from LGC Standards or Carl Roth. Sodium perchlorate-18O4 solution used as an internal standard was purchased from HPC Standards. Analyte separation was effected with the elution gradient summarized in Table 1, at a flow rate of and column temperature of 35 °C. Solvent A was 0.5 % formic acid in water, and solvent B was 0.5 % formic acid in acetonitrile.

Table 1: Elution gradient for LC prior to injection into the mass spectrometer. Solvent A: 0.5 % formic acid in water; solvent B: 0.5 % formic acid in acetonitrile.

Step	Time (min)	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)
1	0.0	0.5	35	65
2	5	0.5	90	10
3	6.5	0.5	90	10
4	6.51	0.5	35	65
5	8	0.5	35	65

Elution gradient for liquid chromatography: To confirm comparability with the conventional sample preparation method, 16 samples were simultaneously prepared by QuPPe and analyzed by LC-MS/MS in a contracted third-party laboratory (Table 2).

Table 2: LC-MS/MS data from the comparability test for samples prepared by the FraMiTrACR® and QuPPe methods. Samples were spiked with chlorate and perchlorate. Analysis of samples prepared by the QuPPE method was performed in a third-party laboratory.

	Chlorate (mg/kg)		Perchlorate (mg/kg)	
Sample no.	FraMiTrACR® method	QuPPe method	FraMiTrACR® method	QuPPe method
1	0.01	0.011		
2	0.011	0.01		
3	0.01	0.009		
4	0.22	0.167		
5	0	0		
6	0	0		
7	0.036	0.033		
8	0.029	0.032		
9			0.039	0.033
10			0.032	0.028
11			0.007	0.007
12			0.003	0.004
13			0.024	0.02
14			0.017	0.011
15			0.009	0.004
16			0.004	0.002

Results and Discussion

FraMiTrACR®°C/PC fractionates milk samples quickly, in a single step: The aim of this study was to reduce process time to a minimum and avoid all unnecessary steps for the preparation of milk samples prior to detection of chlorate and perchlorate residues. By using the FraMiTrACR®°C/PC we could obtain a filtrate sample for analysis, without the need for degreasing or homogenization steps, in a maximum process time of 30 minutes. For a 5 mL sample, average filtrate volumes of 1.4 and 3.9 mL were obtained after centrifugation for 15 and 60 minutes, respectively (Figure 1 A). Fractionation of the sample into phases was observed in the FraMiTrACR®°C/PC units (Figure 1 C), with a yellow-white fatty phase and turbid aqueous phase in the retentate, and a clear aqueous phase in the filtrate. Due to the small pore sizes of the ultrafiltration membrane, it could be assumed that the aqueous phase in the filtrate contained substantially less protein than the aqueous phase in the retentate.

Overall, we reduced sample preparation to one step. For comparison, the QuPPe method requires 7 sample preparation steps. The QuPPe method requires approximately 28 minutes active working time to prepare 16 samples for analysis. In addition, there are 55 minutes of passive working time attributed to centrifugation and incubation times, resulting in a total process time of 83 minutes. In contrast, the method we present here enables passive sample preparation by centrifugation in 30 minutes. For routine analyses, there is also the possibility to reduce this time further, since centrifugation for 15 minutes already yields sufficient material (approx. 1.5 mL) (Figure 1 A). Another 9 minutes are added in active working time, making the total process

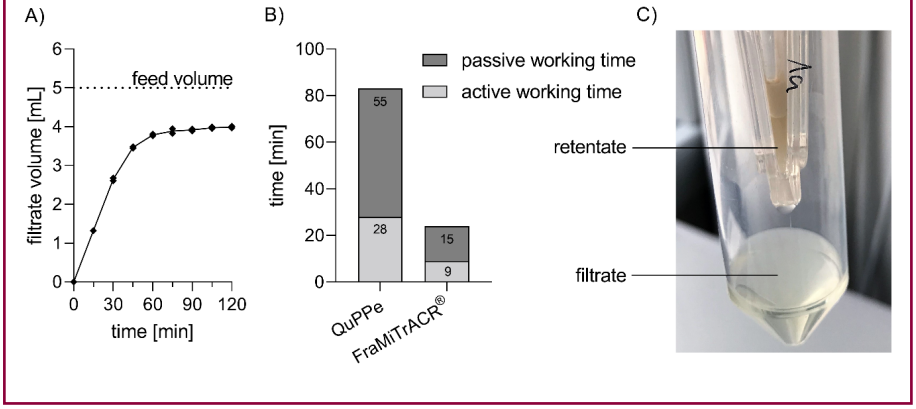

Figure 1: 5 mL of raw milk sample was fractionated using a FraMiTrACR®°C/PC unit, by centrifugation at 4,000 g (swing-out rotor, n = 2). The filtrate volume was monitored over time, gravimetrically (A). Fractionation of 16 samples by FraMiTrACR®°C/PC was compared against the established QuPPe method in terms of active and passive time (B). Sample preparation by FraMiTrACR®°C/PC enabled fractionation of milk samples into three phases. Fat and protein phases formed predominantly in the retentate, while the aqueous water phase, including chlorate and perchlorate analytes, passed into the filtrate (C).

with FraMiTrACR®°C/PC units as little as 24 minutes (Figure 1 B).

FraMiTrACR®°C/PC facilitates accurate results and low limit of detection: To validate our method, and as a basis for accreditation by the Deutsche Akkreditierungsstelle GmbH, we tested 10 samples prepared from raw milk, or whole or skimmed milk reconstituted from powder. Each sample was analyzed at five different concentrations. All standards used in testing were certified and traceable reference materials, according to the National Institute for Standards and Technology. Each analyzed sample was tested twice to confirm accuracy and reproducibility. The limit of quantification (LoQ) and detection (LoD) and measurement uncertainty (MU) were established according to DIN ISO 11352:2013 and the guidelines for determining LoQ and LoD according to G. Lieck [10,11]. In the water phase following fractionation by FraMiTrACR®°C/PC units, a LoQ of 0.001 mg/kg with an expanded MU of 39.3% and LoD of 0.0005 mg/kg for perchlorate could be achieved. For chlorate, a LoQ of 0.010 mg/kg with an expanded MU of 62.8% and LoD of 0.005 mg/kg could be achieved. Since the MU is laboratory-specific, no general comparisons to the QuPPe method can be made. However, according to the guidelines "Analytical quality control and method validation procedures for pesticide residues analysis in food and feed - SANTE/2021/11312" the measurement uncertainty should not be higher than 50% if the maximum residue value is exceeded [12]. Therefore, we evaluated the MU for chlorate and perchlorate at the maximum residue limit (MRL) for chlorate (0.1 mg/kg in raw milk). Since there is no MRL defined for perchlorate, we also chose 0.1 mg/kg as the MRL for our study. We found that the MU for chlorate and perchlorate at 0.1 mg/kg was 19.1% and 8%, respectively, confirming that our process could meet the aforementioned guidelines for these residues. The difference in measurement uncertainties between the target substances can possibly be explained by the assumption that chlorate is a very small polar molecule that is more difficult to analyze, ideally requiring a highly sensitive column. In comparison, perchlorate is larger than chlorate and is more stable, due to the higher degree of oxidation. With our method, it is possible, depending on the analytical equipment available, to fractionate raw milk just-in-time for analysis. It should be noted that results following LC-MS/MS analyses were comparable, regardless of the sample preparation method used (FraMiTrACR®°C/PC or QuPPe). Following both methods, the limit of quantification was 0.01 mg/kg for chlorate and 0.001 mg/kg for perchlorate. The 16 analyzed samples, which prepared by the FraMiTrACR® and QuPPe methods, showed comparable results in relation to the above discussed MU (Table 2).

In additional experiments, samples prepared by our method were also successfully tested using ion chromatography, using conductivity measurements for the quantification of chlorate and perchlorate. The limits of quantification and detection from this technique were comparable to those achieved by LC-MS/MS (data not shown).

Conclusion

The method for analytical sample preparation by FraMiTrACR®°C/PC units described here shows that it is possible to quantify defined analytes directly from the water phase of milk in a single step. The advantages of this method are that milk samples can be prepared quickly, passively and without the use of additives. This leads not only to a reduction in personnel costs by minimizing active working time of laboratory staff, but also to savings in operating resources and stock management. The risk of contamination is also mitigated, since each sample is contained within a sealed FraMiTrACR unit for the majority of the processing time, and treatment with additives is avoided.

FraMiTrACR® units open up new possibilities for residue and contaminant quantification in dairy products, and we will continue to investigate additional analytes with our method.

Compliance with Ethical Standards

The authors declare the following interests which may be considered as potential competing interests: Jan-Michael Steils and Christian Baumgartner are employed by pureMilk analytical, Klaus Schoene, Harald Thenmaier and John Cashman are employed by Sartorius, and Maren Lang and Melina Kraus are employed by Milchprüfring Baden-Wurttemberg. However, these affiliations do not alter the authors' adherence to the scientific policies on sharing study results, data and materials.

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