Application Note

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FraMiTrACR®: A Universal Tool for Single-Step Fractionation of Raw Milk Samples Before Residue Analysis

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Abstract

Residues of chlorate and perchlorate in milk products represent a major challenge in the dairy food industry. Both substances enter the dairy food chain almost exclusively as byproducts of disinfection and cleaning. As they can endanger food safety, milk samples must be routinely analyzed for chlorate and perchlorate residues. However, current methods to prepare raw milk samples for residue analysis are inefficient. In this study, we aimed to develop a faster, passive and more cost-effective method. By means of centrifugal ultrafiltration, unprocessed raw milk could be fractionated into its constituent water, fat, and protein phases, using a FraMiTrACR® spin filter. When the water phase prepared by this method was analyzed by LC-MS/MS, it was possible to achieve a detection limit as low as 0.001 mg/kg for chlorate and perchlorate residues, which is comparable to an established reference method.

Introduction

Chlorate and perchlorate are oxyanions of chlorine and are highly soluble in water. Both residues enter the food chain of dairy products when processing water treated with chlorine-containing disinfectants and chlorine-containing cleaning agents are used. The use of chlorine-free lye can also be an unexpected source of chlorate and perchlorate. These residues may be carried through the manufacturing process of the lye, leading to contamination of products that are labelled "chlorine-free". Animal feed and water can also be a natural, not insignificant source of perchlorate in milk.

Public awareness was raised when it became known that chlorate and perchlorate inhibit thyroid hormone synthesis by entering the thyroid gland.[1,2] This could be a problem for infants and young children in particular because, unlike adults, they do not have enough stored thyroid hormones to inhibit the uptake of chlorate and perchlorate into the thyroid .[1,2] Therefore, new limits for food have been established with the help of the European regulations 2020/749 for chlorate and 2020/685 for perchlorate.[3,4] In addition, the European Food Safety Authority (EFSA) has set a tolerable daily intake (TDI) for chlorate at 0.003 mg/kg and for perchlorate at 0.0003 mg/kg. Since infants consume high amounts of dairy, particularly as milk-based formula, the TDIs can easily be exceeded in this group.[5,6]

Both chlorate and perchlorate residues are often tested for using the well-established Quick Polar Pesticides (QuPPe) method. This method requires multiple sample preparation steps. First, the fat in the raw milk is removed by centrifugation. The resulting skimmed milk is then treated with acidified methanol and formic acid, and EDTA 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. Residual particles are then removed by centrifugal filtration before analysis of the final sample.[7] All these steps must be actively performed by a laboratory worker, and a reliable stock management system is required for the numerous consumables and reagents that are used. The frequent handling, manipulation and transfer steps in this method also increase the risk of sample contamination, which could contribute to erroneous results. In addition, the process is a single sample method, meaning that each sample must be prepared individually.

Some test laboratories have already attempted to simplify the preparation of raw milk samples for residue analysis. For example, Dyke *et al.* describe a preparation method that consists of a degreasing step by centrifugation, followed by ultrafiltration of the resulting skimmed milk. After 90 minutes of centrifugation, the ultrafiltrate undergoes further treatment steps, resulting in a total process time of more than two hours.[8]

In this study, we aimed to develop a fast and passive method to process raw milk samples in a single step, without the need for additives, while still enabling the detection of residues to the prescribed regulatory limits. We used FraMiTrACR® filters, which can be handled in a standard benchtop centrifuge and have been certified for the fractionation of dairy products prior to residue analysis. Our process assumes that the target analytes - chlorate and perchlorate - are completely dissolved in the water phase of milk, without interaction with the protein or fat phases. Therefore, after raw milk samples have been fractionated using FraMiTrACR®, chlorate and perchlorate can be detected directly from the water phase.

Methods

Sample Preparation by Fractionation

FraMiTrACR® filters were filled with 5 mL of raw milk. In a centrifuge with swing out rotor, 16x samples were fractionated into their constituent water, fat and protein phases by centrifugation for 120 minutes at 4,000 *g*. To determine the speed of filtration, we measured the filtrate volume gravimetrically every 15 minutes, in duplicate, using a precision balance. Fractionation of the raw milk into its three phases was visible by eye.

Sample Preparation by the QuPPE Method

For comparison with conventional methods, the same raw milk samples were prepared for residue analysis by an independent contract laboratory using the QuPPe method.

LC-MS/MS Analysis for Chlorate and Perchlorate Residues

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used for residue analysis of samples following both preparation methods. 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 the filtrate was injected into the analyzer. The chlorate and perchlorate content were determined by comparison of native to standard (spiked control) samples.

Results

FraMiTrACR® Fractionates Milk Samples Quickly and in a Single Step

The aim of our method development was to minimize the time needed to prepare samples for chlorate and perchlorate analysis by avoiding all unnecessary sample manipulation and process steps. By using FraMiTrACR® filters, we could obtain a filtrate sample for analysis, without the need for degreasing or homogenization steps, in a maximum process time of 30 minutes at 20 °C. For a 5 mL sample, average filtrate volumes of 1.4 and 3.9 mL were obtained after 15 and 60 minutes of centrifugation, respectively (Figure 1A).

Different phases were observed after fractionation (Figure 1B). A white-yellow fat phase and a turbid, protein-containing aqueous phase were visible in the retentate, while a clear aqueous phase was found in the filtrate. Due to the small pore size of the membrane in FraMiTrACR® filters, it could be assumed that the filtrate contained substantially less protein than the aqueous phase in the retentate.

In comparison to the QuPPe method that requires at least seven sample preparation steps, our method involves single-step fractionation of raw milk to prepare each sample for residue analysis. The QuPPe method requires approximately 28 minutes of active working time to perform the steps needed to obtain 16 samples for analysis. In addition, there are 55 minutes of passive working time during centrifugation and incubation steps, resulting in a total of 83 minutes' sample preparation time.

In contrast, our fractionation method enables passive sample preparation with a single, 30-minute centrifugation step. For routine testing, there is also the possibility to reduce the centrifugation time to 15 minutes, which already produces sufficient material (approx. 1.5 mL filtrate) for residue analysis. Another 9 minutes are added in active working time, making the total process time with FraMiTrACR® as little as 24 minutes (Figure 1C).

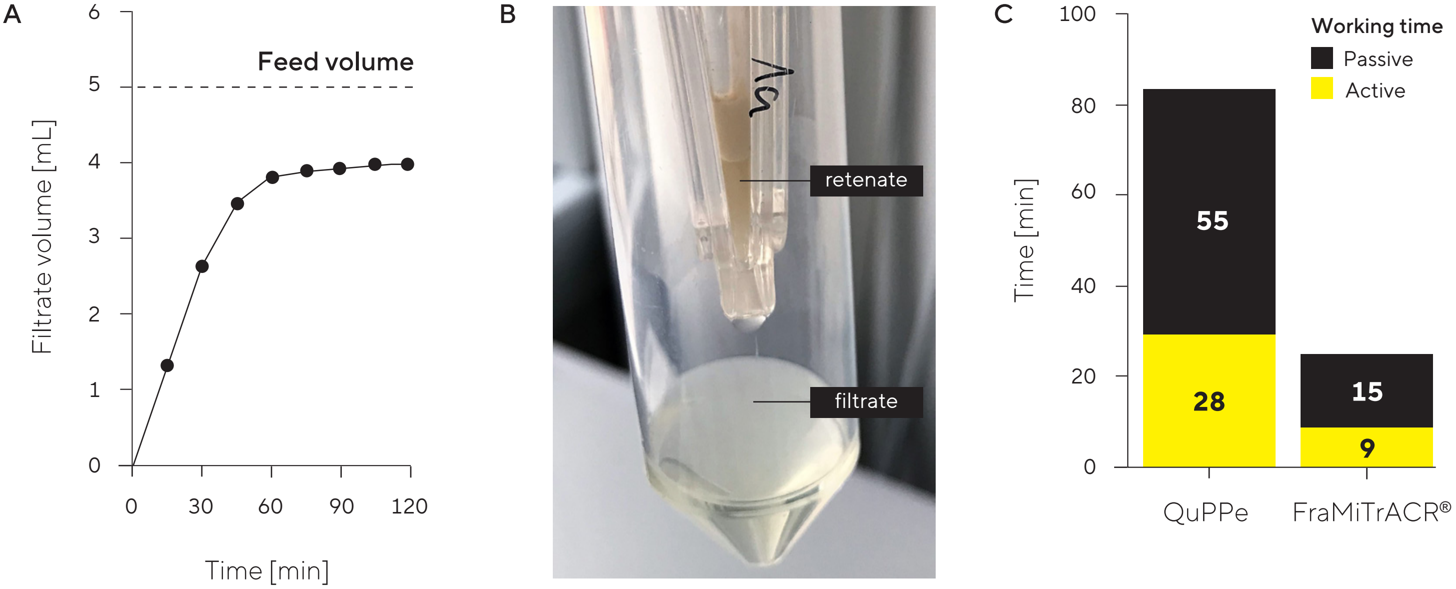

Figure 1: *A 5 mL raw milk sample was fractionated by centrifugal filtration with a FraMiTrACR® unit at 4,000 g (swing-out rotor, n=2). The filtrate volume was measured gravimetrically at 15-minute intervals (A). Sample preparation by FraMiTrACR® enabled fractionation of milk samples into three phases. Fat and protein phases formed in the retentate, while the chlorate- and perchlorate-containing water phase occurred in the filtrate (B). Active and passive working time were determined for the preparation of 16 raw milk samples by fractionation with FraMiTrACR® or by the established QuPPe method (C).*

FraMiTrACR® Facilitates Reliable Detection of Chlorate and Perchlorate

In the water phase prepared by fractionation with FraMiTrACR®, a 0.005 mg/kg limit of quantification and 0.001 mg/kg limit of detection (LOD) could be achieved for chlorate and perchlorate in the LC-MS/MS analysis. Since the LOD was comparable to the QuPPe reference method for both residues (Table 1), our method is a valuable approach to reliably detect chlorate and perchlorate in milk.

Sample no.	FraMiTrACR® method	QuPPe method
Chlorate (mg/kg)		
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
Perchlorate (mg/kg)		
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

Table 1: *Each raw milk sample was prepared by our FraMiTrACR® method or by the QuPPe reference method. Chlorate and perchlorate content was determined for each sample by LC-MS/MS.*

Summary

Our method enables fractionation of raw milk just-in-time for residue analysis. We have shown that it is possible to detect analytes directly from the water phase of milk without further preparation or the use of additives. Our results following LC-MS/MS analysis were comparable to those obtained using the QuPPe reference method, demonstrating that our fractionation method can be applied even when low detection limits are required.

We have demonstrated that our method offers several advantages. Samples are prepared 71% faster than by the QuPPe method, in a single-step process that is mostly passive and removes the need for additional reagents and consumables. This contributes to lower personnel and operating costs, as well as reduced stock management effort. The risk of sample contamination is also mitigated, thanks to minimal sample manipulation.

Fractionation by FraMiTrACR® opens new possibilities for residue and contaminant determination in dairy products. We intend to test the suitability of our method for sample preparation before residue testing for other analytes in future studies.

The results from this study have been peer reviewed and published in Milk Science International (76) 2023 P. 24-27; ISSN 2567-9538; https://openjournals.hs-hannover.de/milkscience/issue/view/63.[9]

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Abbreviations

EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
LC-MS/MS	Liquid chromatography with tandem mass spectroscopy
QuPPe	Quick Polar Pesticides
TDI	Tolerable Daily Intake
UHPLC	Ultra High Performance Liquid Chromatography

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