Journal of Radioanalytical and Nuclear Chemistry (2018) 318:247–257
https://doi.org/10.1007/s10967-018-6086-8

Estimation of anthropogenic organo-chlorine, bromine and iodine compounds in apolar lipid fractions of bovine milk by solid-phase extraction and neutron activation analysis (SPE–NAA)

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Received: 12 April 2018 / Published online: 14 August 2018

Abstract
Milk lipids were separated using a hexane:isopropanol mixture; then they were fractionated into apolar, medium polar and polar portions by SPE using a LC-Si column. The apolar fraction was further separated into 4 more portions depending on their polarity by another SPE method using a Florosil column. Levels of Cl, Br and I were measured by NAA. Halogens were detected in all separated fractions; but their highest levels were found in the most apolar fraction containing hydrocarbons which strongly indicates the presence of anthropogenic organohalogen compounds in milk. In addition, iodide and iodate ions in whole milk samples were measured.

Keywords Milk · Milk lipid fractionations · Organohalogen compounds · Halogen species · Iodide and iodate separation · NAA

Introduction

Organohalogen (OX) compounds can be classified as either naturally occurring or anthropogenic depending on their origin. The anthropogenic OX compounds, also known as organic pollutants (OP), are of major concern due to their toxicity, high chemical stability and persistence in the environment [1, 2]. Consequently, they are transported without chemical degradation and accumulated in the food chain [3, 4]. Ultimately, they reach human beings mainly through the foods with high fat content such as meat, fish and milk [2–5].

Twenty-six of the OX compounds are classified as persistent organic pollutants (POPs) which are regulated for their risk to human health and wildlife [2, 6]. However, the concern goes beyond these 26 POPs because many other OX compounds are both persistent and bioaccumulative [2–7] and may have potential toxicological and ecotoxicological effects. Most of the OX compounds including POPs are organochlorines (OCl) such as polychlorinated biphenyls (PCBs), followed by organobromines (OBr) like hexabromocyclododecane (HBCDD) and polybrominated diphenyl ethers (PBDE) [8], and a relatively small number of organoiodines (OI), mostly volatile compounds, such as methylene iodide and methyl iodide [9].

In the past, the determination of OX compounds has mainly focused on human milk since it is the main food for babies [9–13]. Their levels in fish and marine animals mostly in northern regions [5, 14–17] have also been measured because of their possible transport from warmer southern areas [15, 16]. Some researchers have also reported halogen content of air [18, 19], blood and serum [20, 21] and of bovine milk and yogurt [3, 4]. Canada is among the top 10 countries in the world with high bovine milk consumption. Canadians consumed 74.2, 71.8, 70.8, 69.7 and 67.0 annual liters per capita of bovine milk during 2013–2017, respectively [22]. Although there is a decreasing trend in consumption, the determination of OX

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compounds in bovine milk is still very important to investigate their potential risks and toxic effects to the consumers.

In previous studies reported by Isaac-Olive [23] and Isaac-Olive and Chatt [24], the levels of Cl, Br and I in the lipid fractions of commercially available Canadian bovine milk samples were measured by instrumental neutron activation analysis (INAA). The mass fractions in mg kg^{-1} (their relative expanded uncertainties) in bovine milk lipids were: 32 (± 8.4%), 2.65 (± 9.8%) and 0.211 (± 6.6%) for Cl, Br and I, respectively. The high concentrations of halogens, particularly Cl, in milk lipids may very well be due to some anthropogenic sources. However, neither our work [23, 24] nor any previous study, to the best of the authors' knowledge, has reported a fractionation scheme that shows the halogen profiles of milk lipids.

Milk contains various types of lipid which can be classified, for instance by their polarity, from most apolar (also called neutral lipid, mono- , di- and tri-glycerides) through less polar (glycolipids) to most polar (phospholipids). Anthropogenic OX compounds generally consist of the most apolar fraction. So, if a lipid separation scheme shows that the halogens are present in the most apolar fraction, then the hypothesis of their anthropogenic source would be more reliable. One of the objectives of the work reported here was to develop such a milk lipid fractionation scheme. In this work total lipid including OX compounds was first separated from milk samples by solvent extraction. This extract was then fractionated into three classes based on their polarity. The apolar class was further separated into 4 sub-groups also differentiated by their polarities. Both fractionation procedures were carried out using SPE instead of commonly-used adsorption chromatography. Levels of Cl, Br and I were determined in each lipid fraction by INAA.

Another issue with the high consumption of milk is related to its iodine content, in particular the type and level of the iodine species present. Previous studies from our lab [25, 26] reported the levels of total iodine, total inorganic iodine, and protein- as well as lipid-bound iodine in Canadian bovine milk samples using chemical separation followed by INAA. However, the fractionation of the total inorganic fraction into specific iodine species, such as iodide and iodate ions, has not been reported yet for the Canadian bovine milk samples. This was another objective of the work reported here and was accomplished by ion exchange chromatographic separation and quantification by INAA.

Experimental

Materials and methods

Commercially available homogenized milk (milk fat = 3.25%) was purchased from local supermarkets and kept in a refrigerator at 4 °C in its original container for no longer than a week and not beyond the expiry date. When needed, a few mL of milk was taken from the container in a pre-cleaned Pyrex beaker and allowed to reach room temperature before processing.

Deionized distilled water (DDW) was prepared by passing distilled water through a set of cation and anion exchange columns. All solvents, namely hexane, isopropanol, acetone, methanol and diethylether, were of HPLC grade purchased from Fisher Scientific Ltd. Ultrapure ammonium iodide (SPEX, ultrapure), potassium iodate (BDH, analytical grade), potassium nitrate (BDH, analytical grade), potassium chloride (BDH, analytical grade), potassium bromide (BDH, analytical grade), hydralazine sulfate (Fluka, analytical grade), ammonium sulfate (Merck, analytical grade), concentrated ammonia solution (Seastar, ultra-pure grade), glacial acetic acid (Fisher, analytical grade), and nitric acid (Seastar, ultrapure grade) were used in this work. SPE cartridges, both LC-Si and Florosil Supelclean™ (tube bed wt. 1 g and volume 6 mL) were purchased from Sigma-Aldrich. Ion exchange resins AG (50WX8) and AG (1X8) resins were purchased from Bio-Rad.

Lipid extraction from milk

Figure 1 shows the experiments carried out for the extraction of milk lipids and its separation into different fractions. This figure also indicates the fractions irradiated for the determination of halogens by NAA. Details on the procedures in Fig. 1 are given below.

Ammonium sulfate precipitation

About 25 mL of milk were placed in a beaker and enough $(NH_4)_2SO_4$ in solid form was added to reach the concentration of about 4 mol L^{-1} in milk. Samples were centrifuged at 10,000 rpm for 30 min followed by filtration. Both solid and filtrate were subjected to solvent extraction as described below. The solid was first redissolved in a minimal amount of DDW containing 2 mL of concentrated ammonia solution and the solution was treated as such.

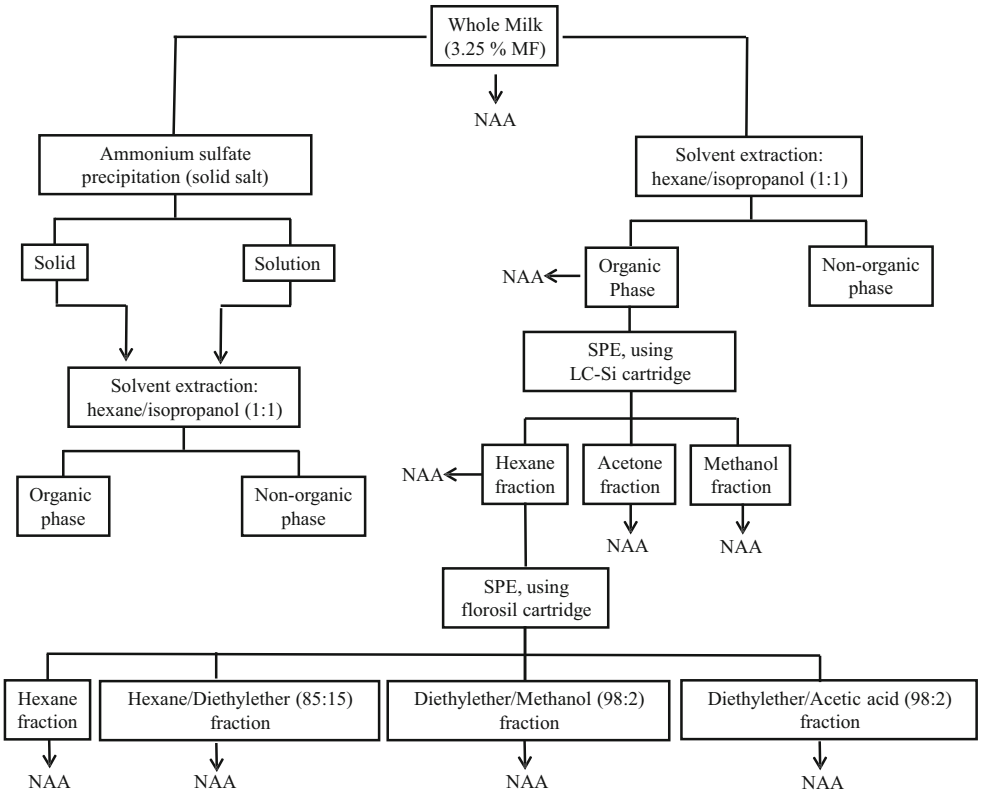

Fig. 1 Fractionation scheme for halogens bound to milk lipids

Solvent extraction

Total lipid extract was separated using a hexane:isopropanol (1:1) solvent system as previously reported [23, 24]. Briefly, 2 mL of concentrated ammonia solution were added to a sample of 25 mL of whole milk, and the milk lipids were extracted using 25 mL hexane and 25 mL isopropanol. The non-organic phase was subjected to second and third extractions with 10 mL of hexane each time. The three hexane phases were combined and washed twice using 10 mL of DDW each time. All aqueous phases resulting from the washings and the organic phase removals were also combined and extracted three times with 10 mL of hexane each time. All hexane fractions were pooled and evaporated to dryness.

Lipid fractionation

Lipid fractionation using LC-Si SPE cartridges

The amount total lipid extracted was first determined gravimetrically. It was then dissolved in 5 mL of hexane. About 1 mL of the solution was placed on a 6-mL LC-Si SPE cartridge previously conditioned with 2 mL hexane. Most apolar compounds were eluted by 10 mL hexane, followed by those of intermediate polarity using 15 mL acetone, and finally the more polar compounds by 10 mL methanol. These separated fractions were then dried in air and re-dissolved in 1 mL of the same solvent used for their respective elutions. Then they were passed through another LC-Si SPE cartridge for the purpose of purification using the same elution schemes. The mass of lipid in each of the purified fractions was determined gravimetrically. This SPE method, developed in our laboratory by Gonzalez-Labrada [27], is a modification of the original method developed for an open column [28].

Lipid fractionation using Florosil SPE cartridges

Various apolar fractions were combined and allowed to dry by evaporation in air for about 16 h. The dried lipids were dissolved in hexane to give a solution of about 1 g of lipid per mL. This liquid was placed on a 6-mL Florosil SPE cartridge previously conditioned with 2 mL of hexane. The sample was sequentially eluted using 15 mL hexane followed by a mixture of hexane:diethylether (85:15) v/v, 15 mL of a mixture of diethylether:methanol (98:2) v/v, and 15 mL of diethylether:acetic acid (96:4) v/v. Masses of all fractions were determined gravimetrically. Again, this SPE method originally developed for an open column [28] was modified in our laboratory by Gonzalez-Labrada [27] and used here.

Distribution and mass balance of halogens in milk lipids

Using the % of lipid mass determined by gravimetry in each fraction ($[\%\text{Lipid}]_{\text{Hex}}$, $[\%\text{Lipid}]_{\text{Acet}}$, $[\%\text{Lipid}]_{\text{Meth}}$, $[\%\text{Lipid}]_{\text{Hex:DE}}$, $[\%\text{Lipid}]_{\text{DE:Meth}}$, $[\%\text{Lipid}]_{\text{DE:AA}}$), as well as the halogen concentration [X] determined by INAA in the same fraction, the halogen recovery (X_{rec}) was calculated for both fractionation processes individually and in sequence as follows:
first fractionation process:

$$X_{rec1} = \frac{[\%\ \text{Lipid}]_{Hex1}[X]_{Hex1} + [\%\ \text{Lipid}]_{Acet}[X]_{Acet} + [\%\ \text{Lipid}]_{Meth}[X]_{Meth}}{[X]_{TotalExtract}} \quad (1)$$

second fractionation process:

$$X_{rec2} = \frac{[\%\ \text{Lipid}]_{Hex2}[X]_{Hex2} + [\%\ \text{Lipid}]_{Hex:DE}[X]_{Hex:DE} + [\%\ \text{Lipid}]_{DE:Meth}[X]_{DE:Meth} + [\%\ \text{Lipid}]_{DE:AA}[X]_{DE:AA}}{[X]_{Hex1}} \quad (2)$$

both fractionation processes in sequence:

$$X_{rec} = \frac{(X_{rec2})[X]_{Hex1}\%Lipid_{Hex1}}{[X]_{TotalExract}} \quad (3)$$

where *Hex1* and *Hex2* stands for hexane fractions from first and second fractionation processes, respectively, Acet for acetone, Meth for methanol, DE for diethylether, and AA for acetic acid.

Determination of total inorganic iodine, iodide and iodate fractions in bovine milk

Cleaning and chemical conversion of resins

The procedure employed for cleaning the resins is essentially the same as previously reported by us [26]. For the sake of completeness of this paper, the method is briefly described here. About 50 g of AG (1X8) resin in chloride form (100–200 mesh) were cleaned by soaking in 5% of NaClO solution at pH 1–2 to remove chloride ions. About 25 g of this cleaned resin was then converted to OH^- form by soaking in 25% v/v ammonia solution overnight, and another 25 g portion was converted to NO_3^- form using 25% v/v nitric acid. Both resin portions were washed with water until neutral pH was attained and then dried in air. A small sample of the dried resin was irradiated for 1 min in the inner position of the Dalhousie University SLOWPOKE-2 Reactor (DUSR) facility, allowed to decay for 1 min, and counted for 5 min. The "conversion" procedure was repeated until a constant ratio of chlorine counts/mass was obtained in the resin and the 443-keV photopeak of ^{128}I was no longer detected in the gamma-ray spectrum. About 25 g of AG (50WX8) resins in H^+ form (50–100 mesh) were converted to NH_4^+ form, in a similar way to the one explained above, by soaking in 25% v/v ammonia solution.

Separation of iodide/iodate, iodide and iodate ions by ion chromatography

Although a batch separation of inorganic iodine species was previously reported [26], a column method was tested in the present work. The AG (1X8) resin in OH^- form was packed in a polyethylene column of 4 cm length and 0.7 cm diameter; and a solution containing 0.25 μg mL^{-1} of each ion iodide and iodate (iodide/iodate) standard solution were passed through it. Five mL of DDW were passed through the column and the effluent and resin were tested for iodine according to the conditions reported in Table 1.

The affinities of the AG (1X8) resin for the ions involved in this separation are $I^- > NO_3^- > IO_3^- > OH^-$ as reported by us [26]. When the resin is in OH^- form, both iodide and iodate ions are retained, and this was already investigated by us [26]. In this case, it was expected that if the AG (1X8) resin is in NO_3^- form would retain iodide while iodate would pass through the column. To test this hypothesis, 10 mL of a 0.5 μg mL^{-1} of iodide, 10 mL of a 0.5 μg mL^{-1} of iodate, and 10 mL of a solution containing 0.25 μg mL^{-1} of each ion (iodide/iodate) were passed through three different polyethylene columns of 4 cm length and 0.7 cm diameter, respectively. Columns were packed with purified AG (1X8) resin in NO_3^- form. The effluents and washings of each column were collected in 10 mL portions. In each case, fraction 1 was the effluent from the initially loaded sample. The columns were then washed with 50 mL of DDW (fractions 2–6). An additional 40 mL of 0.1 M potassium nitrate solution was used to wash them (fractions 7–10), followed by a final wash with 20 mL DDW (fraction 11–12). Under these conditions no iodate should be present on the columns. Effluent (0.50 mL) and the resin were carefully collected and irradiated according to the conditions given in Table 1. This

Table 1 Experimental conditions used for halogen determination by INAA

Sample analyzed	Halogen determined	INAA technique used	T_i–t_d–t_c (min)
Homogenized milk	Cl, Br, I	INAA-AC	5–2–10
Lipids and lipid fractions	Cl, Br, I	INAA-Conv	10–2–10
Effluent and solutions from AG (1X8) columns or batch separations	I	PC-INAA-AC	5–2–10
AG (1X8) resins in OH^- or NO_3^- forms	I	EINAA-Conv	5–2–10
Solutions from AG (50X8) batch separations	I	INAA-AC	5–2–10

separation method is based on the procedure previously reported by Hou et al. [29].

For each column, all fractions were combined, and 5 g of hydrazine sulfate were added to reduce iodate to iodide ions. This solution was then passed through a fresh ion exchange column packed with AG (1X8) resin in NO_3^- form. The column was then washed with 50 mL of DDW as described above, and all collected fractions and the resin were irradiated to determine their iodine content according to the conditions reported in Table 1.

Determination of anionic iodine, cationic iodine, iodide and iodate content of milk

Figure 2 shows the experiments carried out for the determination of inorganic iodine species in milk. To separate all anionic iodine species from milk, 10 mL of a milk sample were first passed through a column containing the AG (1X8) resin in OH^- form. The column was then washed with 5 mL of DDW. To separate iodide and iodate fractions, another 10 mL of the same milk were passed through a different column containing the same resin in the NO_3^- form. The column was washed with 50 mL of DDW; and 5 g of hydrazine sulfate were added to the effluent. The sample was then passed through a third column containing the AG (1X8) resin in NO_3^- form and it was also washed out with 50 mL of DDW. All three resins from the three columns were carefully collected and irradiated for the determination of iodine content according to the conditions given in Table 1.

Finally, to study the possible presence of cationic species of iodine in milk, 25 mL of a milk sample was first subjected to a batch separation on 0.5 g of AG (1X8) resin in OH^- form followed by a batch separation using 0.5 g of the cationic resin AG (50WX8) in NH_4^+ form. The experiment was also carried out in the reverse order, i.e. AG (50WX8) resin first followed by AG (1X8) resin to observe any possible change of species that could have occurred during the separation process. After 30 min of batch separations, samples were filtered, and the solutions

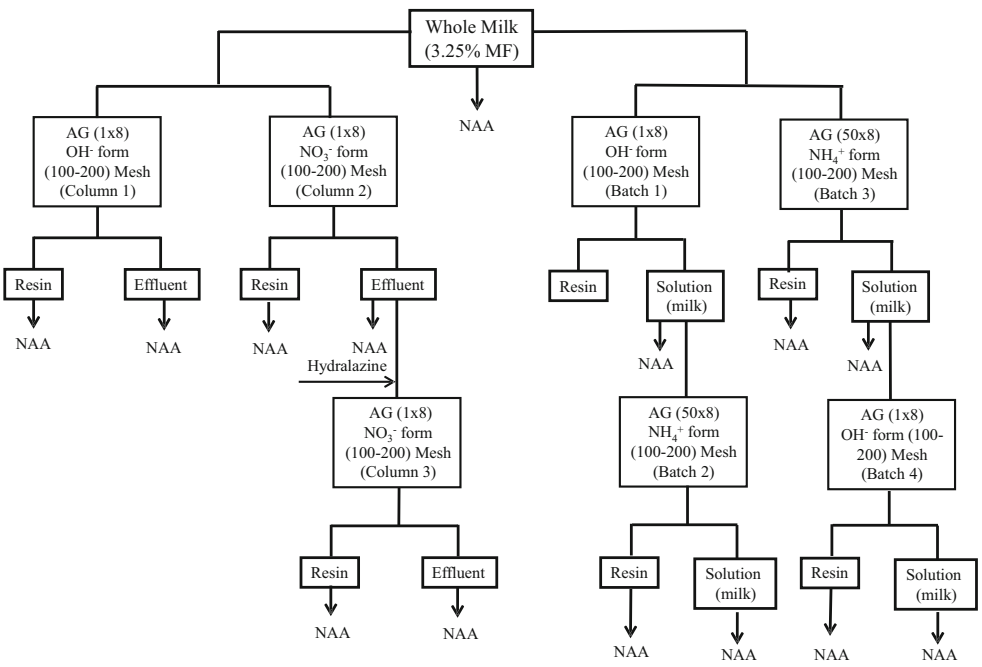

Fig. 2 Scheme for the separation of anionic and cationic iodine fractions in milk

were analyzed by a pseudo-cyclic INAA (PC-INAA) method, using 3 cycles of irradiation-decay-counting, for the quantification of iodine under the conditions shown in Table 1.

Halogen determination by INAA

All dried lipid fractions as well as total lipid extracts were dissolved in 1 mL of hexane, and 0.5 mL of it was transferred using a positive displacement pipette to small polyethylene irradiation vials which were previously cleaned with 4 mol L^{-1} HNO_3. About 0.5 mL of milk sample and effluents collected from AG (1X8) columns as well as AG (1X8) resin samples were also placed in the same type of polyethylene vials.

All samples were irradiated at the DUSR facility at a neutron flux of 2.5×10^{11} cm^{-2} s^{-1}. The stability, homogeneity and reproducibility of the DUSR neutron flux were previously reported [30–32]. The levels of halogens were measured by four different INAA methods, namely conventional INAA using conventional gamma-ray spectrometry (INAA-Conv), epithermal INAA (EINAA) in a cadmium-shielded site in conjunction with conventional gamma-ray spectrometry, conventional INAA coupled to anti-coincidence (INAA-AC) gamma-ray spectrometry, and pseudo-cyclic INAA using anti-coincidence (PC-INAA-AC) gamma-ray spectrometry. The details of these methods have been described in previous publications [24–26].

The conventional Ge(Li) detection system consisted of a Canberra 60 cm^3 Ge(Li) detector with a D-SPEC plus pulse height analyser. The system had a peak-to-Compton ratio of 35:1, an efficiency of 9.5% and a resolution of 1.88 keV at the 1332.5 keV photopeak of ^{60}Co. The anti-coincidence (AC) gamma-ray spectrometry system consisted of an EG&G ORTEC HPGe p-type coaxial detector with a relative efficiency of 25%, a resolution (FWHM) of 1.72 keV at the 1332.5-keV photopeak of ^{60}Co and coupled to an ORTEC D-SPEC$^+$ pulse height analyzer. The guard detector was a $10'' \times 10''$ NaI(Tl) annulus with 5 photomultiplier tubes (PMT) and a $3'' \times 3''$ NaI(Tl) plug with one PMT. The peak-to-Compton ratio, at the 662-keV photopeak of ^{137}Cs, of this system was 582:1. The set-up and performance of the AC gamma-ray spectrometry system was studied in detail by Zhang [33]. Halogens were quantified using the following radionuclides and corresponding photopeaks: ^{38}Cl ($t_{1/2}$ = 37.4 min, 1642 keV), ^{80}Br ($t_{1/2}$ = 17.68 min, 617 keV) and ^{128}I ($t_{1/2}$ = 25 min, 443 keV). The experimental conditions employed are summarized in Table 1. Halogen levels were quantified by the relative method using freshly prepared standard solutions containing 100, 10 and 1 μg mL^{-1} of Cl, Br and I, respectively. The accuracy and precision of the INAA methods used were very good and reported previously [23–26].

Results and discussion

Lipid extraction from milk

Although milk lipids have been previously separated in one step directly from milk samples by our group [23–26], in the present work the two-step separation was investigated. Firstly, a precipitation using ammonium sulfate salt in solid form was carried out followed by the solvent extraction (second step), as indicated in Fig. 1. Secondly, the lipid extraction was done in one step. The yields of these two procedures are shown in Table 2.

In our previous work, ammonium sulfate was added to milk samples as a saturated solution (3.93 mol L^{-1}) [25]. In the present work it was added in solid form to avoid increasing the sample volume. However, the resulting precipitate did not appear to be homogeneous due to the presence of the undissolved salt. Therefore, the precipitate was washed with DDW to remove the excess of the salt. Proteins were found in the precipitate (proteins were assessed by Bradford method using casein as standard, data not shown here but given in [23]). The filtrate (solution remaining after the ammonium sulfate precipitation) was subjected to solvent extraction (as shown in Fig. 1) and it was found to be free of lipids.

This means that the lipids coprecipitated with proteins when ammonium sulfate was added. To further separate the lipids, the precipitate was re-dissolved in a volume of water identical to the initial sample volume (25 mL). Two mL of concentrated ammonia solution were added, and the lipid extraction was carried out as described above. Lipids were extracted in the organic phase. The results of the lipid yields obtained by the two methods employed are shown in Table 2.

Once the precipitate formed after the addition of ammonium sulfate, samples were centrifuged and filtered. The differences in yields shown in Table 2 are statistically significant ($p < 0.05$), and the main reason is that during the filtration process the solid is not quantitatively recovered prior to solvent extraction. The fact that all lipids cannot be recovered when protein precipitation is carried out first is not a problem, since the lipid concentration can be determined by an indirect method [24]. In the case where the lipids were extracted first from the milk sample, proteins remained in the aqueous phase (proteins were assessed by Bradford method using casein as standard, data not shown here but given in [23]). Both lipid separating methods are good and the selection of one over the other very much depends on the purpose. Ammonium sulfate precipitation is a very easy and quick method if the

Table 2 Lipid content of milk before and after protein precipitation

Lipid content (%) after protein precipitation and solvent extraction (two-step separation)	Lipid content (%) by direct solvent extraction of milk (one step separation)
2.52 ± 0.07	3.13 ± 0.06

Table 3 Halogen content of bovine milk lipids fractionated according to their polarity

Halogen mass fractions of the lipid extract of bovine milk (mg kg^{-1})				
Cl: 32 ± 1	Br: 2.65 ± 0.04		I: 0.21 ± 0.02	
Mass of lipids in the extract of bovine milk (%)				
Apolar (hexane): 96 ± 1[b]	Medium polarity (acetone): 3.6 ± 0. 6	Polar (methanol): 0.18 ± 0.07	Lipid recovery 100 ± 1	
Halogen mass fractions (mg kg^{-1}) in various lipid fractions				
	Apolar (hexane)	Medium polarity (acetone)	Polar (methanol)	Halogen recovery (%)*
Cl	30 ± 1[a]	40 ± 1	652 ± 7	98 ± 5
Br	2.54 ± 0.09	6.795 ± 0.004	9.71 ± 0.04	102 ± 4
I	0.206 ± 0.002	0.64 ± 0.03	0.71 ± 0.05	106 ± 10
Halogen mass distribution (μg) in 1 g of milk lipids				
	Apolar (hexane)	Medium polarity (acetone)	Polar (methanol)	
Cl	29 ± 1[c]	1.4 ± 0.2	1.2 ± 0.5	
Br	2.44 ± 0.09	0.24 ± 0.04	0.017 ± 0.007	
I	0.198 ± 0.003	0.023 ± 0.004	0.00130.0010	

[c] = ([a]) × ([b]); other values were similarly calculated

*Calculated using Eq. (1)

objective is to separate the organic and inorganic fractions of the elements. Since the objective of this work was to fractionate the lipid extracts, the direct extraction from milk samples in one step using a mixture of hexane:isopropanol (1:1) was chosen because it gave a higher yield.

Lipid fractionation on LC-Si solid-phase extraction cartridge

The results of the first lipid fractionation applied to the total lipid extract as well as the halogen determination in lipid extract and fractions are shown in Table 3. The total extract was fractionated into three different groups differentiated by their polarities from low to high. The recovery of total lipid was 99.8% and those of halogens using Eq. (1) were also very high indicating the reliability of the SPE method developed.

Most of the lipid mass was eluted in the apolar fraction with hexane which is expected to include simple (or neutral) lipids. Glycolipids should be present in the medium polar class eluted with acetone and phospholipids in the polar one eluted with methanol, as mentioned before. Although the identification of the individual lipids present in each fraction was not carried out, it has been reported that neutral lipids and polar lipids represent about 98 and 0.2%, respectively, of the total bovine milk fat [34]. The excellent agreement between the lipid distribution of milk obtained in this work and that reported in the literature demonstrates that the SPE method developed here is a suitable procedure for lipid fractionation.

Table 3 also shows similar behavior by the three halogens studied, namely highest halogen concentration in the polar fraction followed by the medium polar and apolar fractions. Chlorine is the more concentrated halogen in all three fractions, followed by bromine and iodine. The halogen recoveries were quantitative in all three cases, following Eq. (1). Finally, Table 3 also shows the halogen distribution by mass. More than 90% of halogen mass are in the apolar fraction for the three halogens studied.

Lipid fractionation on Florosil solid-phase extraction cartridge

The results of the second lipid fractionation process applied to the apolar fraction obtained from the first fractionation method are shown in Table 4. The apolar fraction from the first fractionation step was separated into four different groups differentiated by their polarities, as well. The recovery values in this second lipid fractionation were

Table 4 Halogen content of the apolar lipid fractions after the second fractionation

Halogen mass fractions of the apolar lipids after first fractionation (mg kg^{-1})					
Chlorine: 30 ± 1		Bromine: 2.54 ± 0.09		Iodine: 0.206 ± 0.002	
Mass of lipids in the apolar extract after the second fractionation (%)					
Hexane	Hexane:DE (85:15)	DE:Meth (98:2)	DE:AA (96:4)		Lipid recovery
95.0 ± 0.3[e]	2.4 ± 0. 2	0.32 ± 0.03	0.53 ± 0.06		8 ± 0.4
Halogen mass fractions in various lipid fractions after the second fractionation (mg kg^{-1})					
	Hexane	Hexane:DE (85:15)	DE:Meth (98:2)	DE:AA (96:4)	Halogen recovery*
Cl	26 ± 1[d]	16.2 ± 0.4	757 ± 2	408 ± 16	99 ± 5
Br	1.5 ± 0.2	13.2 ± 0.2	52 ± 5	46.4 ± 0.4	85 ± 8
I	0.065 ± 0.007	2.2 ± 0.2	11.1 ± 0.8	10.7 ± 0.7	101 ± 6
Halogen mass distribution (μg) in 1 g of milk lipid after the second fractionation					
	Hexane	Hexane:DE (85:15)	DE:Meth	DE:AA (96:4)	Halogen recovery[+]
Cl	23.7 ± 0.9[f]	0.37 ± 0.03	2.3 ± 0.2	2.1 ± 0.3	89 ± 4
Br	1.4 ± 0.2	0.30 ± 0.03	0.16 ± 0.2	0.24 ± 0.03	79 ± 7
I	0.059 ± 0.007	0.051 ± 0.005	0.034 ± 0.004	0.054 ± 0.007	94 ± 11

[f] = ([d]) × ([e]) × ([b]), b value taken from Table 3; other values were respectively calculated

*Calculated using Eq. (2)

[+]Calculated using Eq. (3)

higher than 98%. Table 4 shows that most lipids are contained in the first eluted fraction (hexane fraction).

It is expected that free fatty acid, di- and mono-glycerides will be eluted in the fourth, third and second fractions, respectively, although their identification was not carried out in this work. First fraction should contain hydrocarbon compounds. If milk contains any of the toxic anthropogenic organohalogen compounds, they are expected to be in the hexane (first) fraction.

The diethylether:methanol (98:2) fraction, contained the highest halogen concentrations (Table 4), followed by the diethylether:acetic acid (96:4) fraction. In the case of Br and I, hexane:diethylether (85:15) fraction is the third most concentrated fraction and the least concentrated fraction is the most apolar one (hexane). In the case of Cl, however, the third and least concentrated fraction are reversed in relation to Br and I. The third most chlorinated fraction is the hexane one and the less chlorinated is the hexane:diethylether (85:15) fraction.

Table 4 also shows the halogen mass distribution (μg) after the second fractionation process relative to 1 g of milk lipid. The most apolar fraction contained 23.7, 1.4 and 0.059 μg of Cl, Br and I, respectively, which could be of anthropogenic nature. These values represent 74% (23.7/32), 53% (1.4/2.65) and 28% (0.059/0.21) of the total halogen content in milk lipids for chlorine, bromine and iodine, respectively. These results strongly suggest the presence of anthropogenic organohalogen compounds in milk lipids, as it is known that anthropogenic organochlorine compounds are more abundant than organobromine and organoiodine compounds, which is also true for human milk [35, 36]. This same pattern was found with the halogen concentration in the hexane fraction. The low content of iodine in this fraction agrees with the fact that most extractable organoiodine (EOI) compounds are volatile and of natural origin (not anthropogenic). These EOI compounds mostly come from iodine solutions for disinfecting cow teats, iodized supplements in foodstuff such as lipiodol, and ethylenediamine dihydroiodide (EDDI) to prevent iodine deficiency [37].

Total inorganic iodine, iodide and iodate fractions in bovine milk

The determination of organohalogen compounds is one portion of a larger project designed for the determination of iodine species in bovine milk. In our previous work [24–26], levels of total iodine, total organically bound and total inorganically bound iodine fractions were determined. The organic fraction was also separated to lipid and protein fractions. In the present work lipid fraction was being further fractionated for estimating the presence of organohalogen compounds including organo-iodine. Following the initial project, and the rationale followed in our work, the separation of the inorganic species is given here as shown in Fig. 2.

Iodine levels in bovine milk from Canada have been reported to be in the range of 0.4–0.5 μg mL^{-1}, about 90% of which is inorganic iodine [24–26]. For this reason, the separation method was evaluated here with a standard

Table 5 Separation of iodide and iodate ions in milk samples using column separation

Milk (n = 3)	Mass fraction (μg mL^{-1})	Column represented in Fig. 2
Total iodine	0.457 ± 0.008	
Total anionic species[a]	0.403 ± 0.003	Column 1: AG 1X8 (OH^-)
Iodide[b]	0.335 ± 0.003	Column 2: AG 1X8 (NO_3^-)
Iodate[c]	0.061 ± 0.006	Column 3: AG 1X8 (NO_3^-) after iodate reduction
Recovery* (%)	98 ± 2	

*Calculated using $^{(b+c)}/^{a}$

Table 6 Comparison between two batch separation methods

Ion exchange separation of charged iodine species in milk	Organically bound iodine mass fraction (μg mL^{-1})
AG (1X8)–AG (50WX8)	0.041 ± 0.04
AG (50WX8)–AG (1X8)	0.044 ± 0.03

solution of 0.5 μg mL^{-1} of total iodine containing 0.25 μg mL^{-1} of each of iodide and iodate ions. This solution was first passed through a AG (1X8) resin in OH^- form (100–200 mesh); and no iodine peak was detected in the effluent from it when analyzed by INAA-AC. This indicated that both iodine species were completely retained on the resin. This conclusion was further confirmed by irradiating the resin where 100% of the added iodide/iodate was found. As expected, anionic iodine can be retained on a small column (4 cm length and 0.7 cm width) containing AG (1X8) resin in OH^- form, like that in the batch separation previously reported [25], but within a shorter time.

The separation of iodide and iodate ions was based on the method reported by Hou and coworkers [29]; but it was slightly modified in our work. In their method, iodide/iodate were passed through a column loaded with an anionic exchange resin in NO_3^- form; the iodide ions were retained while the iodate ions were eluted and then reduced to iodide ions by potassium bisulfite. A slight modification was made in our case in which hydrazine sulfate was used as the reducing reagent instead of potassium bisulfite. Hydrazine sulfate was previously used in our laboratory in 1999 by Rao and Chatt for reducing iodate into iodide ions in NIST SRM-1549 Non-fat Milk Powder with very good results [38, 39].

For testing the method described above, three columns packed with AG (1X8) resin in NO_3^- form were used. In the first column (Column A), where only the iodide solution was passed through, no iodine was found in any of the fractions collected indicating that all the iodide ions were retained on the column. In the second column (Column B) iodate solution was passed through and all iodate ($\sim$ 99%) ions were found in the first 5 fractions (fractions of 10 mL DDW each) indicating that further washings were not required, and that iodate is not retained in the column packed with AG (1X8) resin in NO_3^- form. In the trial run (Column C) containing the iodide/iodate solution, the elution profile was identical to that obtained previously for column 2 indicating that the iodate was eluted from the column while the iodide was retained, and 50 mL of DDW were enough to quantitatively elute all the iodate ions from the resin. Iodate ions contained in the effluent were reduced to iodide with 5 g of hydralazine and passed through another column (Column D) packed with AG (1X8) resin in NO_3^- form. No iodine signal was found in any fraction from washings indicating that all the iodate ions were reduced to iodide ions and they were quantitatively retained on the resin. In this fashion the separation of iodide/iodate was easily accomplished.

This method was then applied to real milk samples, but in this case only 3 columns are required. Column 1 for retaining all anionic species including iodide and iodate, column 2 for retaining iodide ions, and column 3 for retaining iodate ions once they were reduced to iodide with hydralazine. The average of three results is presented in Table 5. This method is reproducible, relatively simple and fast. The INAA-AC method is highly suitable because the resin can be directly irradiated. Anionic iodine species accounted for 88% of total iodine in milk which agrees with other values reported in the literature [25, 26, 29, 40, 41]. Iodide ion, as expected, was the most abundant iodine species in milk (73%).

Table 6 shows the results obtained when milk samples were subjected to two batch extraction procedures. In one procedure a milk sample was first passed through a AG (1X8) resin in OH^- form (Batch 1) followed by a AG (50WX8) resin in NH_4^+ form (Batch 2). In the second procedure, the order of columns was reversed: NH_4^+ form (Batch 3) followed by OH^- form (Batch 4). The final concentration of iodine in milk represents the neutral iodine species which is mostly organically bound iodine species.

The iodine concentration found after the two sequential batch separations (Table 6) is not statistically different from that found in milk after the extraction with AG (1X8) (Batch 1) resin. In a similar manner, the iodine concentration found in milk after the extraction with AG (50WX8) (Batch 3) is statistically no different than the iodine concentration in the initial milk. Therefore, there is no indication at all about the presence of cationic iodine species in milk.

Then total iodine in milk is 10 times higher than organically bound iodine. This result agrees with other values previously reported by us [25, 26] and is coherent with other authors who reported that inorganic iodine species are about 90% of total iodine in milk [29, 40, 41]. The results given in Table 6 also indicate that there was no interconversion of one species into another during the batch separation.

Conclusions

A method was developed to separate various lipid fractions in milk based on their polarity. Total lipids were first extracted and fractionated into three groups. The most apolar fraction was subjected to a second separation process which yielded 4 more fractions differentiated again by their polarity. The highest halogen levels relative to the total halogen in milk lipids were found in the most apolar fraction, namely 74, 53 and 28% for Cl, Br and I, respectively. These results along with the fact that this fraction contains neutral lipids strongly suggest the presence of anthropogenic organohalogen compounds in milk lipids. The structural identification of these halogen containing molecules is needed and perhaps can be studied in future. A method for the separation and quantification of inorganic iodine species in milk was studied and iodide ion was found to be the most abundant iodine species (73%) followed by iodate (15%). There was no indication of the presence of cationic iodine species in bovine milk. The INAA methods used were invaluable for the determination of low levels of halogens with high precision and accuracy within a short time.

Acknowledgements The authors would like to acknowledge the assistance of Dr. J. Holzbecher and Blaine Zwicker of the DUSR facility for irradiations. The financial assistance from NSERC Canada in the form of Discovery and Infrastructure Grants (AC), the Dalhousie University Faculty of Graduate Studies for a scholarship (K.I-O.), and the International Atomic Energy Agency (IAEA) Visiting Scholarship granted to T.T. Kyaw are also acknowledged.

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