A SIMPLIFIED METHOD BASED ON ACID DIGESTION FOR DETERMINATION OF DIOXIN-LIKE COMPOUNDS IN MILK-BASED PRODUCTS

Shen HT[1,2,3], Han JL[1], Xu QY[1], Liu HM[1]

1, Zhejiang Provincial Center for Disease Control and Prevention, 3399 Binsheng Road, Hangzhou, 310051, China
2, Chinese Center for Disease Control and Prevention, 7 Panjiayuan Nanli, Beijing, 100021, China
3, China National Center for Food Safety Risk Assessment, 7 Panjiayuan Nanli, Beijing, 100021, China

Introduction

Milk powder is an important type of dairy product because of its wide spread consumption, especially by infants and children. After the 2008 melamine crisis in China, many studies have been carried out around the world, especially in China, to investigate illegal additives and other pollutants, including dioxin-like compounds, in infant formula, other milk-based products, and to estimate the health effects of these additives.

Because POPs are lipophilic chemicals, results for lipid-rich samples, e.g., egg, meat, fish and dairy products, have often been reported on the basis of lipid weight. For these kinds of matrices, sample extraction, including lipid determination, is usually the first step. Classical methods for determining the lipid content in dairy products include acid digestion, the Rose-Gottlieb method, Babcock method and Gerber method. The Rose-Gottlieb method for example, has been recommended internationally (by ISO/FAO/WHO) as the standard procedure for determining the lipid content in dairy products. In the field of POPs analysis, however, traditional or more convenient methods, like liquid-liquid extraction (LLE), column extraction, Soxhlet extraction, supercritical fluid extraction and accelerated solvent extraction (ASE) (after freeze-drying of liquid samples), are more frequently adopted. As part of a plan enacted in 2012 by the Chinese government to monitor national food contaminants, we have measured over 300 dairy products and other foods of animal origin for POPs analysis. We found that the lipid content of measured infant formula and fresh milk accorded well with the marked value when using LLE, Soxhlet extraction or ASE. For other kinds of milk powder, however, the measured values were much lower than the marked values despite recoveries of spiked, labeled standards being nearly 80% or higher. Roams et al. reported that these conventional extraction methods are not always effective.[1] The isotope dilution method is used most often for dioxin-like POPs analysis. This method is based on the assumption that spiked, labeled compounds behave similarly to endogenous samples during all analysis steps. Following this logic, the greater the recovery of surrogated compounds, the more accurate the results of POPs analysis should be. On the other hand, this assumption is unsuitable if the endogenous and spiked compounds behave differently. During the spray-drying process, the milk fat is encapsulated by a protein-lactose matrix (so called "encapsulated fat"). Different milk protein products differ widely in their ability to encapsulate liquid oils, with the larger protein aggregates, i.e. sodium caseinate, calcium caseinate and casein micelles, being more efficient encapsulates than whey proteins.[2] Hence, only part of the fat (so called "free fat") can be extracted by organic solvents. Soxhlet extraction works well to extract free fat and spiked, labeled standards (high recovery obtained), but not encapsulated fat. This difference can cause POP levels in these kinds of milk powder to be overestimated. Therefore, accurate determination of lipid content is critical to quantify target chemical concentrations.

The primary aim of the present work was to develop an accurate method utilizing acid digestion to thoroughly extract lipid components in milk powder. Although this lipid extraction approach was not new, it was expected to be practical for POPs analysis, especially for analyzing non-infant formula milk powder.

Materials and methods

Standards and reagents: Calibration standard solutions (PCDD/Fs: EPA-1613CVS, CS1-CS5; PCBs: 68A-CVS, CS0.2-CS5), 13C-labeled surrogate standards (PCDD/Fs: EPA-1613 LCS; PCBs: WP-LCS, EC9605-SS), and injection standards (PCDD/Fs: EPA-1613 ISS; PCBs: EC9605-RS) were obtained from Wellington Laboratories (Guelph, Canada). All solvents were distilled-in-glass grade and purchased from Caledon Laboratories Ltd. (Ontario, Canada). Sodium sulfates, silica gel, aluminum oxide and other chemicals were purchased as standard grade from Sigma Aldrich (Milwaukee, USA).

Sample collection and pretreatment: For the present study, we purchased 35 brands of infant formula, 29 brands of non-infant formula milk powder (full fat milk powder, sweet milk powder and some nutrient-strengthened milk powder, etc.) and 30 brands of fresh milk from a market and measured their lipid contents by Soxhlet extraction and acid digestion liquid–liquid extraction (LLE). For Soxhlet extraction, solid samples were extracted directly, and liquid or semi-liquid samples were freeze dried over 24 h to produce a solid powder prior to the extraction. For LLE, both solid and liquid samples were treated by acid digestion prior to the extraction.

Acid digestion and sample purification: Milk powder (4g) was spiked with ^{13}C-labeled surrogate standards (1613LCS 1ng, EC9605-SS 1ng and WP-LCS 1ng), then dissolved thoroughly with 20 mL of deionized water at 40–50°C (for milk, this step was omitted). An aliquot of 20 mL hydrochloric acid (37%, w/w) was added and the solution was then quantitatively transferred into a 250 mL separating funnel. The funnel was placed on an 80°C water bath shaker and shaken at 150 rev min^{-1} for at least 1 h to release milk lipid. The hydrolyzate solution was cooled to room temperature, 40 mL anhydrous alcohol and 80 mL hexane/dichloromethane (1:1, v/v) were added, and then the solution was shaken again for 30 min to extract milk lipid. This step was repeated twice, and the organic phase was combined. The extract was vacuum concentrated to 3-5 mL and placed in a fume hood overnight to dry. The flask plus lipid was then weighed to calculate the lipid weight gravimetrically. The sample purification procedure was similar to that in our previous work.[3]

Results and discussion

Milk and milk-based products were first extracted by Soxhlet extraction and acid digestion LLE respectively; then lipids were weighted and compared with marked (label) values to assess their lipid extraction efficiency. The results are summarized in Table 1. For infant formula, lipid contents (%) determined by direct Soxhlet extraction (19.6±2.0) and by LLE (20.9±1.2) were close to marked values (21.8±1.3), and the differences in efficiency between these two methods were not significant (*t*-test, $P>0.05$). Similar results were also observed when fresh milk and breast milk were tested. For other kinds of milk powder, except infant formula, however, lipid content (%) determined by direct Soxhlet extraction (0.9±0.3) was much lower than the marked value (18.9±5.5) (Table 1, line 2). Kim et al. reported that during the spray-drying process, the milk fat is encapsulated by a protein-lactose matrix (so called "encapsulated fat"), and only part of the fat can be extracted by organic solvents (so called "free fat").The encapsulated fat could then be extracted after milk powder was re-dissolved in warm water and centrifuged.[4] In this study, to avoid interference from plastic centrifuge tubes, we re-dissolved milk powder in warm water, kept it in an ultrasonic water bath for 15 min, and then freeze dried it to recreate a powder for Soxhlet extraction. Unfortunately, the measured lipid content (%) was only slightly increased to 1.4±0.5 (Table 1, line 2 b), but still significantly ($P<0.05$) lower than the marked value (18.9±5.5). On the contrary, results from acid digestion plus LLE showed that the measured lipid content value (18.8±6.0) was very close to the marked value (18.9±5.5). We also observed mean recoveries of spiked, isotope-labeled internal standards (Table 1 column 4, 8) for both Soxhlet extraction and LLE, which ranged from 67% to 77%. No significant differences ($P>0.05$) in mean recoveries were observed between the two extraction methods. This implied that spiked chemical recovery was not a suitable extraction efficiency indicator for non-infant formula milk powder. If POP levels are calculated on the basis of improperly extracted lipids, the results would be overestimated.

In general, lipid content measured by acid digestion plus LLE accorded well with the marked values for all types of dairy products. Soxhlet extraction was also applicable for lipid determination, except for non-infant formula. This difference may be due to different manufacturing processes. Common milk powder (non-infant formula) is made from concentrated animal milk (usually cow milk), in which caseins (molecular weight: ~60,000 to 350,000 Da) contribute 80% or more to the protein content.[5] This kind of protein can be assimilated by adults, but not by infants (0-1 year old). To mimic human breast milk, infant formula manufacturers usually eliminate some caseins and saturated fatty acids, but increase the proportion of whey protein (molecular weight: ~15,000 Da) and polyunsaturated fatty acids (vegetable oil). In infant formula, the ratio of whey protein to casein

is usually adjusted to approximately 60:40,[5] which approximates that of breast milk. During the spray-drying process, the milk lipid is encapsulated by protein. Larger protein aggregates, like caseins, are more prone to encapsulate milk lipid than whey proteins.[2] Thus, lipid in infant formula, which contains more whey protein than non-infant milk powder, was more easily extracted via direct Soxhlet extraction. We also tried accelerated solvent extraction (ASE) for non-infant formula. The lipid extraction efficiency of ASE was very close to that of Soxhlet extraction (data not shown).

Table 1. Comparison of lipid extraction efficiency between Soxhlet and acid digestion LLE

		Soxhlet without acid digestion				Liquid–liquid extraction after acid digestion			
	Line	Lipid (%) Measured value (mean±SD)	Lipid (%) Marked Value (mean±SD)	Average Rec. of surrogate std. (%)	Sample loaded (g)	Lipid (%) Measured value (mean±SD)	Lipid (%) Marked Value (mean±SD)	Average Rec. of surrogate std. (%)	Sample loaded (g)
1	Infant formula(n=35)	19.6±2.0	21.8±1.3	77±14	10	20.9±1.2	21.8±1.3	74±10	4
2	Non-infant milk powder(n=29)	0.9±0.3 **a** 1.4±0.5 **b**	18.9±5.5	73±14 72±15	10	18.8±6.0	18.9±5.5	71±12	4
3	Fresh cow milk(n=30)	3.0±0.4*	3.3±0.3	70±13	50	3.1±0.5	3.3±0.3	67±13	20
4	Breast milk **d**	3.14	3.19±0.4 **c**	75±12	43.8	NA	3.19±0.4 **c**	NA	NA
5	Breast milk **e**	3.5	3.4±0.39 **c**	69±16	39.5	3.5	3.4±0.39 **c**	70±12	10.5

*: freeze dried prior to Soxhlet extraction; **a**:direct Soxhlet extract, n=14; **b**:re-dissolved in ultrasonic warm water bath , then freeze dried, n=15; **c**:consensus value; **d**:results of Interlaboratory comparison on POPs in Food 2006, **e**: results of Interlaboratory comparison on POPs in Food 2010, more details see final report at http://www.fhi.no/eway/default.aspx?pid=239&trg=Content_6503&Main_6157=6246:0:25,5498&MainContent_6246=6503:0:25,5508&Content_6503=6259:87046:25,5508:0:6250:97:::0:0

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