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Radosław LIZAK[1]

DEVELOPMENT AND OPTIMIZATION OF EXTRACTION PROCEDURE OF MILK FAT FOR SIMULTANEOUS DETERMINATION OF DIOXINS AND DIOXIN-LIKE COMPOUNDS

OPRACOWANIE I OPTYMALIZACJA PROCESU EKSTRAKCJI TŁUSZCZU Z MLEKA W CELU RÓWNOCZESNEGO OZNACZANIA DIOKSYN I ZWIĄZKÓW DIOKSYNOPODOBNYCH

Abstract: Polychlorinated dibenzo-*p*-dioxins, dibenzofurans and biphenyls are unintentionally produced, ubiquitous, persistent organic pollutants. Due to bioaccumulation in food chains, the main source of human exposure to the compounds is food of animal origin. European Community food safety strategy is provided through official monitoring of foodstuffs. This results in necessity of development of reliable and validated analytical method based on instrumental techniques. Milk and milk products are recognized to be good indicator of environmental exposure for persistent organic pollutants. Effective extraction of fat from complex matrix consisting of colloidally distributed sugars, vitamins, proteins and minerals is difficult. Conservative methods of fat extraction (liquid-liquid extraction) are particularly time and labour consuming; moreover require large amounts of organic solvents and laboratory space. In order to efficiently extract emulsified fat globules suspended in the aqueous phase of the milk, they have to be broken scrupulously. This condition is met with Accelerated Solvent Extraction method based on elevated pressure and temperature. Method of fat extraction based on ASE for further procedure of simultaneous determination of 35 analytes with high resolution gas chromatography-high resolution mass spectrometry from milk samples was developed. After optimisation, method was proven effective and reliable. Classical methods such as liquid-liquid extraction and Soxhlet extraction were used for comparison. Method was comprehensively validated according to EU requirements; Certified Reference Materials were used. Developed and optimised fat extraction method from milk based on Accelerated Solvent Extraction is currently routinely applied in the frame of the official monitoring of dioxins and dioxin-like compounds.

Keywords: milk, ASE, extraction, polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, polychlorinated biphenyls, HRGC-HRMS

The surveillance of dioxins and dioxin-like compounds in milk has been conducted since early 90's in many European countries. Raw milk and its products are recognized to be a fine indicator of environmental exposure for persistent organic pollutants (POPs). Ruminants are contaminated through vegetable feedstuffs, once dioxins have been absorbed by aerial deposition on the vegetation. Contaminants due to their lipophilicity and their low biodegradability bioaccumulate in cows, next they are largely excreted from the body of lactating cows by transfer to milk. Milk and milk products are one of the major contributors to the human exposure to PCDD/Fs and PCBs. Moreover, during the last decade official monitoring programmes revealed numerous dioxins crisis situations, particularly often in milk or beef meat [1-3].

Conservative fat extraction from milk products such as liquid-liquid extraction (LLE) are particularly time and labour consuming multistep methods; moreover large amounts of organic solvents and plenty of laboratory space are required. Furthermore, extraction of fat from complex matrix consisting of colloidally distributed sugars, vitamins, proteins and

[1] Department of Radiobiology, National Veterinary Research Institute, al. Partyzantów 57, 24-100 Puławy, tel. 81 889 33 60, fax 81 886 25 95, email: radoslaw.lizak@piwet.pulawy.pl

minerals is difficult. Emulsified fat globules suspended in the aqueous phase of milk, have to be broken scrupulously in order to efficiently extract lipophilic contaminants from the matrix. Accelerated Solvent Extraction (ASE) is modern extraction technique that efficiently reduces extraction effort. Functionality of ASE is based upon automation; the quintessence is to apply the liquid solvent in conditions of elevated temperature (50÷200°C) and pressure (10÷20 MPa). The pressurized solvent and elevated temperature increase the solubility of the analytes and the kinetic rate of desorption of the analytes from the matrix.

Method of fat extraction based on ASE for further procedure of simultaneous determination of polychlorinated dibenzo-*p*-dioxins, dibenzofurans and polychlorinated biphenyls with gas chromatography coupled to high resolution mass spectrometry (HRGC-HRMS) in milk samples was developed, optimized and extensively validated.

Materials and methods

Two fat extraction techniques were comprehensively compared: classical liquid-liquid extraction and Accelerated Solvent Extraction (Tab. 1). Extraction in Soxhlet apparatus was additionally used as a reference technique. Prior to all extractions the mixtures of ^{13}C labelled solutions comprised of 35 analogues of contaminants analysed were added.

Liquid-liquid extraction. Raw milk (30 g) was transferred to 1000 ml separatory funnel; 4 cm^3 of saturated solution of KOH was added in order to digest the fat content; 200 cm^3 of anhydrous ethanol and 100 cm^3 of diethyl ether were added in order to denature the proteins; fat was extracted twice vigorously for 5 min with 140 cm^3 of *n*-pentane. Inorganic phase was discarded, while organic phase dehydrated with saturated solution of Na_2SO_4, twice. Finally organic phase was left with 50 g of Na_2SO_4 for final dehydration.

Soxhlet extraction. Extraction was performed with toluene and dichloromethane/*n*-hexane (1/1, v/v), both in triplicates. Freeze-dried milk (5 g) was extracted for 6 h in 200 ml Soxhlet extractors (Bibby Sterlin, Great Britain) at the speed of 6 siphons per h.

Accelerated Solvent Extraction. 200 g of milk sample was frozen (~20°C, 6 h). The water was eliminated in laboratory freeze-drier (1 mbar, –40°C) (Alpha 1-4 LSC, Martin Christ, Germany). Sample (5 g) was mixed with diatomaceous earth (4/1, w/w) (Hydromatrix, Varian, USA) for better dispersion. ASE 300 (Dionex, USA) with 34 cm^3 cells was used, filled up to 85÷90%. Pressure for all experiments was set to 10 MPa.

Consecutive parameters of ASE were optimized: solvents (or its mixtures), temperature (50÷150ºC), number of static cycles (1-4), time (1÷5 min) and flush volume (60÷120%). Dichlorometane, *n*-hexane, methanol and their mixtures were used. Extraction time and extract volume were measured. Final validation experiments were performed with *n*-hexane/dichloromethane/methanol (5/2/1, v/v/v) [4], pressure of 10 MPa, temperature of 100°C, within 3 extraction cycles of 2 min each and with a flush volume of 80%.

Further analysis was performed according to the method based on isotope dilution mass spectrometry (IDMS) with gas chromatography coupled to high resolution mass spectrometry. Samples were defatted on acidic silica columns and further purified and fractionated on Florisil® and Carbopack C. PCDD/Fs, non-*orto*-PCBs and mono-*orto*-PCBs fractions were analysed with HRGC-HRMS (MAT 95XP, Thermo Scientific, Germany) on a DB-5MS (60m, J&W Scientific, USA). Expanded uncertainty was estimated at the level

of interest and was established below 20% for WHO-TEQs (Toxic Equivalency Factors of World Health Organization).

Validation study. ASE and LLE with subsequent PCDD/Fs and PCBs quantification were comprehensively validated according to EU standards [5]. Certified Reference Materials (IRMM, Geel, Belgium) were used to determine trueness [6, 7].

Table 1

Comparison of method validation results of Accelerated Solvent Extraction and liquid-liquid extraction

Extraction method	**ASE**	**Liquid-liquid**
Levels of contaminants	[pg/g fat]	
WHO-PCDD/F-TEQ	1.7 / 3.4 / 8.3	1.8 / 3.6 / 9.1
WHO-PCDD/F-PCB-TEQ	2.9 / 6.4 / 12.3	2.9 / 6.6 / 13.1
LOQ	[pg/g fat]	
WHO-PCDD/F-TEQ	0.14	0.29
WHO-PCDD/F-PCB-TEQ	0.19	0.33
Within-laboratory reproducibility, C.V.	[%]	
WHO-PCDD/F-TEQ	3.1	5.1
WHO-PCB-TEQ	3.6	6.5
Repeatability, C.V.	[%]	
WHO-PCDD/F-TEQ	1.8÷6.9	4.0÷6.2
WHO-PCDD/F-PCB-TEQ	2.8÷3.7	2.9÷6.5
Recovery	[%]	
TeCDD/Fs, PeCDD/Fs, HxCDD/Fs	60.1÷15.2	51.7÷106.7
HpCDD/Fs, OCDD/Fs	55.9÷107.4	59.7÷118.9
dl-PCBs	50.8÷118.3	42.7÷124.8
Within-laboratory reproducibility of fat extraction, C.V.	[%]	
	4.5	6.5
Repeatability of fat extraction, C.V.	[%]	
	1.4÷4.7	3.6÷9.0

Results

The fat extraction efficiency obtained with Soxhlet apparatus for BCR-607 was 26.9% for toluene (n = 3) and 26.5% for dichloromethane/*n*-hexane (1/1, v/v) (n = 3), whilst 26.8% for liquid-liquid extraction (n = 20).

ASE extractions with dichlorometan, *n*-hexane and methanol and their mixtures (1/1, v/v) led to lower extraction yields in comparison with reference techniques. Denaturized proteins instead of fat were obtained when methanol and its mixtures (1/1, v/v) were used; *n*-hexane, dichlorometane and their mixtures were insufficiently effective. When a mixture of *n*-hexane/dichloromethane/methanol (60/25/15, v/v/v) was used, the extraction efficiency was comparable to Soxhlet extraction and surpassed liquid-liquid extraction. Temperature of process was not significant parameter; efficiency increased of 5% with temperature increase from 50 to 100ºC. However during extraction in temperatures exceeding 100 to 150ºC, milk fat was accompanied by not-fatty fraction; share of non-fatty fraction within extract increased with temperature. Insufficient extraction was obtained within one extraction cycle, independently to its length (1÷5 min). There was no significant distinction in extraction yields between 2 to 5 extraction cycles. Extraction phase of one minute was not sufficient, however there was not difference between extraction cycles from

2 to 5 min. Flush volume of 60, 80, 100 and 120% was applied and 80% was found as most proficient.

After optimization of ASE method validation was performed for both techniques with naturally contaminated samples at the level of interest ranging from 50 to 200% of respective maximum levels for PCDD/Fs and the sum of PCDD/Fs and dl-PCBs (Tab. 2) [8]. Within-laboratory reproducibility of WHO-TEQs and fat extraction efficiency was estimated with 4 series of BCR-607 each in six replicates. Repeatability was calculated at each of three levels of analytes (n = 6). The fat extraction efficiency for BCR-607 (n = 24) was 28.9%. All values of within-laboratory reproducibility and repeatability were far below required value of 15%. Recovery of analytes was in the required range of 60÷120% for TeCDD/Fs to HxCDD/Fs. Method was proved sensitive with limits of quantifications for both extraction types far below required levels (0.6 and 1.2 pg of WHO-PCDD/F-TEQ and WHO-PCDD/F-PCB-TEQ, respectively) [5, 8], however LOQ values for LLE were much lower.

Trueness of PCDD/Fs estimated with BCR-532 and BCR-607 was usually below ±5% for both ASE and LLE, complying with EU requirement (< 20%) (Tab. 2) [5]. Trueness of some PCDD/Fs with high values of uncertainty (~40÷100%) ranged up to 36%.

Table 2

Trueness of Certified Reference Materials analysed (BCR-532, BCR-607)

CRM	BCR-532 (n = 6)			BCR-607 (n = 24)		
Extraction method	**[pg/g]***	**LLE [%]**	**ASE [%]**	**[pg/g]***	**LLE [%]**	**ASE [%]**
2.3.7.8-TCDD	0.10 ± 0.02	–10.45	1.69	0.25 ± 0.03	–0.87	4.32
1.2.3.7.8-PeCDD	0.29 ± 0.06	–9.07	–7.42	0.79 ± 0.04	–0.36	–2.93
1.2.3.4.7.8-HxCDD	0.17 ± 0.04	–6.00	–7.77	0.42 ± 0.07	–6.17	–5.69
1.2.3.6.7.8-HxCDD	0.43 ± 0.09	–1.75	–5.31	0.98 ± 0.11	–6.55	–4.19
1.2.3.7.8.9-HxCDD	0.16 ± 0.04	–8.32	–15.41	0.34 ± 0.05	–7.15	–2.86
2.3.7.8-TCDF	0.16 ± 0.08	–24.02	–31.87	0.05 ± 0.03	–22.00	–35.56
1.2.3.7.8-PeCDF	0.10 ± 0.10	–15.22	–16.93	0.05 ± 0.01	–0.84	10.10
2.3.4.7.8-PeCDF	0.67 ± 0.13	2.14	–4.06	1.81 ± 0.13	–1.98	–5.52
1.2.3.4.7.8-HxCDF	0.34 ± 0.07	–3.78	–7.73	0.94 ± 0.04	–2.74	–6.87
1.2.3.6.7.8-HxCDF	0.34 ± 0.07	–3.12	–7.06	1.01 ± 0.09	–1.04	3.87
1.2.3.7.8.9-HxCDF	0.39 ± 0.08	1.50	8.87	1.07 ± 0.05	–0.59	0.01
WHO-PCDD/F-TEQ	3.22	–4.83	6.48	9.02	–2.88	0.00

* - certified value ± uncertainty of measurement

Discussion

During routine work, liquid-liquid extraction of milk fat was found labour and time consuming. Vast amounts of organic solvents used and excessive laboratory space requirements led to search of other solutions. Fat from various foodstuffs is routinely extracted with ASE after desiccating the matrix with diatomaceous earth, sodium or magnesium sulphate. One of the pitfalls of chemical desiccation with diatomaceous earth is limited volume of sample, resulting in inadequate limits of detection. The other group of chemical desiccants is even more hazardous, when used in elevated temperatures with polar solvents might be harmful for ASE systems. More sophisticated dehydration solution applies physical desiccation of sample by means of freeze-drying was applied. Dehydration

of the sample was an important factor, allowing for solvent penetration through dispersion with diatomaceous earth.

Extraction mixture was the major operational parameter of ASE. Mixture of solvents *n*-hexane/dichloromethane/methanol (5/2/1, v/v/v) was found selective and efficient for extraction of saturated and unsaturated milk fats as well as not-hydrolysed phospholipids. Other tested solvents or mixtures led to non-selective extraction with proteins and sugars next to fat. Temperature was another significant parameter. Effective extraction was obtained with low temperature of 100ºC, what can be explained with low melting point of milk fat, consisting in 98% of fatty acids and triglycerides. Whilst ASE extraction of POPs are usually performed in high temperatures (150÷200°C), there is potential of obtaining coextractives of fat when milk would be ASE extracted in such temperatures; in such conditions sugars were extracted. Efficient extraction of fat is critical step of POPs analysis, because concentrations of PCDD/Fs and PCBs in foodstuffs are expressed per gram of fat. Presence of coextractives might be confusing and severe misjudgement of analytical results might happen. Time and number of extraction cycles were of minor importance for extraction efficiency; however optimization allowed to finish the process in 16 min (3 cycles of 2 min each).

ASE and LLE methods were comprehensively validated according to EU requirements [5] and proven fully reliable. Generally, validation parameters were satisfactory for both methodologies. In terms of analytes recoveries ASE was found slightly more consistent. Within-laboratory reproducibility and repeatability of both respective WHO-TEQ values as well as fat extraction was significantly better for automatic technique. Considerable distinction between LOQs for ASE technique in comparison to LLE might be explained with increase of background noise due to high temperature and pressure for the former technique.

Only few automated methods of milk extraction for further analysis of POPs are described. This method was developed and optimised for simultaneous analysis of 7 PCDDs, 10 PCDFs, 12 dl-PCBs and 6 ndl-PCBs with GC-ID-HRMS. Further, the method may be applied to other emerging environmental contaminants eg hexachlorobenzene, mixed bromo-chlorinated dibenzo-*p*-dioxins and furans, organochlorine pesticides, polybrominated biphenyls, polybrominated diphenyl ethers, polybrominated dibenzo-*p*-dioxins and furans, polychlorinated naphtalenes, polycyclic aromatic hydrocarbons and toxaphens. The most time and labour consuming step of liquid-liquid extraction was successfully replaced with the automated process. Satisfactory results of validation make the method very attractive for the routine analysis of divers POPs.

Conclusions

1. Procedure of fat extraction from milk for subsequent multianalyte determination of PCDDs, PCDFs, dl-PCB and ndl-PCBs was developed and optimised. Method may be adapted to the other lipophilic environmental contaminants.
2. Automated method of fat extraction based on Accelerated Solvent Extraction with subsequent analysis of 35 dioxins, furans and polychlorinated biphenyls with high resolution mass spectrometry with isotope dilution was comprehensively validated according to European Community standards. Certified reference materials were used.

Measurement uncertainty was estimated. Finally, method was accredited according to ISO EN-PN 17025:2005.

3. The most profound parameters of effective milk extraction with Accelerated Solvent Extraction were solvent mixture and temperature.
4. The method based on Accelerated Solvent Extraction is currently routinely applied in the National Laboratory for Dioxins and PCBs in Feed and Food in the frame of official surveillance program.

References

[1] EC Regulation No. 1883/2006 of 19 December 2006. Off. J. Europ. Union L 364/32.
[2] Borrello S., Brambilla G., Candela L., Diletti G., Gallo P. et al: Organohalogen Comp., 2008, **70**, 891-893.
[3] Malisch R.: Chemosphere, 2000, **40**, 1041-1053.
[4] Marchand P., Vénisseau A., Brosseaud A., Gadé-Hildevert C., Antignac J.P. et al: Organohalogen Comp., 2008, **70**, 906-909.
[5] Anonymous: Determination of fat in dried milk products using Accelerated Solvent Extraction. Dionex, Salt Lake City 2000.
[6] Maier E.A., van Cleuvenbergen R., Kramer G.N., Tuinstra L.G. and Pauwels J.: Fresenius J. Anal. Chem., 1995, **352**, 179-183.
[7] Tuinstra L.G., Startin J.R., Maier E.A. and Kramer G.N.: Fresenius J. Anal. Chem., 1997, **359**, 222-229.
[8] EC Regulation No. 1881/2006 of 19. Off. J. Europ. Union 2006, L 364/5.

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Zakład Radiobiologii, Państwowy Instytut Weterynaryjny - Państwowy Instytut Badawczy

Abstrakt: Polichlorowane dibenzo-*p*-dioksyny, dibenzofurany i bifenyle są wszechobecnymi, trwałymi zanieczyszczeniami organicznymi. Z powodu bioakumulacji w łańcuchach troficznych głównym źródłem narażenia człowieka na dioksyny jest spożywanie żywności zwierzęcego pochodzenia. Bezpieczeństwo żywności zapewnianie jest we Wspólnocie Europejskiej (WE) w drodze kontroli urzędowej. W celu zapewnienia jej prawidłowego funkcjonowania WE zobowiązuje laboratoria do posługiwanie się wiarygodnymi i zwalidowanymi metodami analitycznymi wykorzystującymi techniki instrumentalne. Surowe mleko i jego produkty są uznawane za dobry wskaźnik zanieczyszczenia środowiska trwałymi związkami organicznymi. Metody ekstrakcji tłuszczu z mleka obarczone są licznymi wadami, ponieważ złożona matryca zawiera również koloidy cukrów, witamin, białek i minerałów. Tradycyjne metody ekstrakcji (ekstrakcja techniką ciecz-ciecz) są wyjątkowo czaso- i pracochłonne; dodatkowo zużywają duże ilości rozpuszczalników organicznych i wymagają znacznej przestrzeni laboratoryjnej. Podstawowym warunkiem wydajnej ekstrakcji tłuszczu z mleka jest efektywne rozbicie zemulgowanych drobin tłuszczu zawieszonych w fazie wodnej. Ten warunek w pełni spełnia technika przyśpieszonej ekstrakcji za pomocą rozpuszczalników dzięki zastosowaniu podwyższonych ciśnienia oraz temperatury. Opracowano metodę ekstrakcji tłuszczu z próbek mleka z wykorzystaniem techniki przyśpieszonej ekstrakcji za pomocą rozpuszczalników w celu dalszego równoczesnego oznaczania 35 toksycznych związków techniką chromatografii gazowej sprzężonej ze spektrometrią mas wysokiej rozdzielczości. Za metody odniesienia przyjęto ekstrakcję techniką ciecz-ciecz oraz ekstrakcję w aparacie Soxhleta. Przygotowaną metodę poddano walidacji z wykorzystaniem m.in. certyfikowanych materiałów odniesienia. Opracowana i zoptymalizowana metoda ekstrakcji tłuszczu z mleka z wykorzystaniem techniki przyśpieszonej ekstrakcji za pomocą rozpuszczalników jest obecnie rutynowo wykorzystywana w urzędowej kontroli próbek na obecność dioksyn, furanów i polichlorowanych bifenyli.

Słowa kluczowe: mleko, ASE, ekstrakcja, polichlorowane dibenzo-*p*-dioksyny, polichlorowane dibenzofurany, polichlorowane bifenyle, HRGC-HRMS