COMPARISON OF LIPID EXTRACTION FROM THE FORMULA MILK BY FOUR DIFFERENT METHODS

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Introduction

Many studies concerning adverse health effects of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), dioxin-like polychlorinated biphenyls (DL-PCBs) and PCBs so far have been reported in particular on growth retardation of fetus and infants [1, 2], birth weight [3~7], neuro-developmental dysfunction [1, 2, 8~10], thyroid deficiency[1, 2, 11, 12], immune deficiency [1, 2, 13~16], reproduction and/or fertility [1, 2], diabetes [17~19] and cancer [1, 2]. In these studies, however, in order to evaluate their toxic effects, their concentrations on lipid weight basis have been employed, except for one study [12] and we have pointed out that this type of concentration is not good for their toxic and/or risk evaluations, due to the difficulties of reproducible and quantitative lipid extractions from biological samples such as the blood and breast milk [20, 21] and also, of course, from other human tissues/organs. This is very important because in order to investigate health effects and risks of toxic compounds for humans, we usually use this kind of concentration, as mentioned above. Therefore, we have inspected the quantities and qualities of lipid extraction from the blood and serum by using several extraction methods [22, 23].

In this study, we examined the qualitative and quantitative lipid extraction from formula milk (F.M.) by four methods, namely, solvent systems of acetone/n-hexane, ethanol/n-hexane, chloroform/methanol and accelerated solvent extraction (ASE) with the solvent of acetone/n-hexane, which were basically the same as the methods of Schecter and Ryan [24], Patterson et al. [25], Nygren et al. [26] and Todaka et al. [27], respectively.

Materials and Methods

F.M. named Hohoemi, which has been manufactured by Meiji Co., Ltd., Tokyo, Japan was dissolved in hot water (5.4g/40ml) and prepared each time just before use. Four extraction methods of lipid from F.M. (2g) samples are simply summarized as follows:

1) Solvent system of acetone/n-hexane (A.H.): 15ml acetone/n-hexane (2:1 v/v) was added to each sample. The sample was shaken for 30 min and then centrifuged at 2500 r.p.m. for 5 min. The upper hexane phase was transferred to a separate container and the lower aqueous-acetone phase was extracted anew with 5ml n-hexane for 30 min and centrifuged at 2500 r.p.m. for 5 min. This process was repeated once more. The combined hexane phase was washed with 10ml water three times, treated with anhydrous sodium sulfate, then concentrated, dried and weighed as lipid gravimetrically.

2) Solvent system of ethanol/n-hexane (E.H.): 3ml saturated ammonium sulfate and 12ml ethanol/ n-hexane (1:3 v/v) were added to each sample and shaken for 10 min. The upper hexane phase was transferred to a separate container and the lower aqueous-ethanol phase was extracted anew with 10ml n-hexane for 10 min and this was repeated again. The combined hexane phase was washed with 10ml water three times, treaded with anhydrous sodium sulfate, then concentrated, dried and weighed as lipid gravimetrically.

3) Solvent system of chloroform/methanol (C.M.): 15ml chloroform/methanol (1:2 v/v) was added to each sample and shaken for 30 min and then this mixture was extracted with 17.5ml chloroform/methanol/water (2:3:2 v/v) for 30 min. The upper water phase was transferred to a separate container and extracted with 12.5ml chloroform/methanol

(4:1 v/v) for 30 min and this was repeated again. The combined chloroform phase was extracted with 40ml n-hexane for 5 min and washed with 10ml water three times. The hexane phase was concentrated, dried and weighed as lipid gravimetrically.

4) Accelerated solvent extraction with acetone/n-hexane (ASE): Each sample was mixed with 4g Isolute. Mixed sample was loaded into the extraction cell of the accelerated solvent extractor and the following programmed parameters were used for the extraction of lipid: a pressure of 1500 psi and a temperature of 150°C, with a static time of 10 min, a flushing volume of 50ml, 90 sec purging, a 60% flushing volume for two cycles, and acetone/n-hexane (1:1 v/v) as the extraction solvent. The extracted hexane phase was concentrated, dried and weighed as lipid gravimetrically.

Estimates of total lipids of F.M. were calculated by summation of the individual two and/or four lipid components of F.M. by the following formulas [28].

Two components: $TL = 2.27\,TC + TG + 0.623$

Four components: $TL = 1.677\,(TC - FC) + FC + TG + PL$

Where TL is total lipid, TC is total cholesterol, FC is free cholesterol, TG is triglyceride and PL is phospholipids, all expressed in unit of mg/dl. Concentrations of TC, FC, TG and PL of F.M. and also of the lipid extracted by each of the four methods, which was resolved in 2ml of isopropanol, were measured using standard enzymatic and automatic clinical procedures on a Hitachi 7600-300s for TC and TG and on a BM 9130 for FC and PL in CRC Inc., Fukuoka, Japan. Concentrations of TL, TC, FC, TG and PL in F.M. and in the lipids extracted by the four methods were statistically examined by Student's T test.

Results and Discussion

Respective concentrations of TC, FC, TG and PL in F.M. were 10, 6.9, 3094 and 24 mg/dl and 98.7% of TL was attributable to TG. As indicated in Figs. 1 and 2, concentrations of TG and TC in the lipids extracted by A.H. and E.H. were a little bit higher than those in F.M.. As also shown in Fig.2, concentrations of PL in the lipids extracted by the four methods were much lower than that in F.M. and they were only 5 to 21% of the concentration in F.M.. Concentrations of FC in the lipids extracted by the four methods were 10 to 28% higher than that in F.M.. Accordingly, in the lipids extracted by the four methods TG were 99.2 to 99.4% of the TL, which were higher than that in F.M..

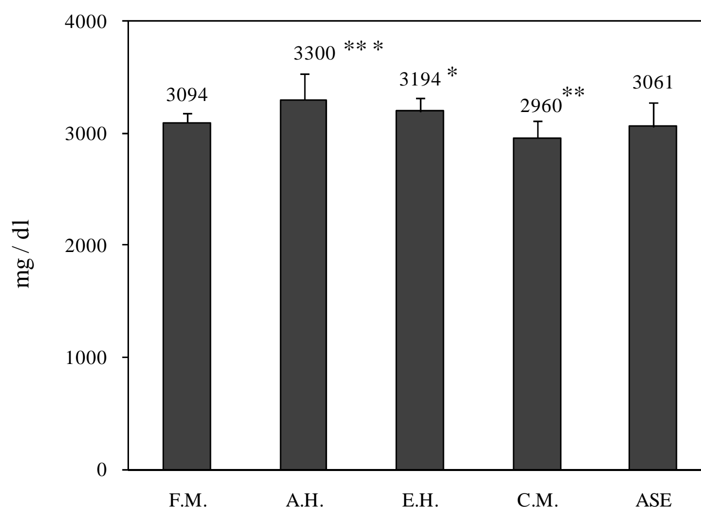

Fig.1. Comparison in concentrations of triglyceride

*: $p<0.02$, **: $p<0.01$, ***: $p<0.001$

In case of the serum, a concentration of PL in the lipid extracted by C.M. was the highest in these four methods and estimated concentrations of TL calculated by four components were also the highest [23].

In case of F.M., however, due to the higher concentrations of TG in the lipids extracted by A.H. and E.H. than in F.M., their estimated concentrations of TL calculated by two and four components were also higher than those of F.M. and the estimated concentrations of TL calculated by these two methods were the lowest in the lipid extracted by C.M.. These results probably indicate that the extractability of lipid differ by the biological samples. Among the four methods in this study, E.H. seemed the best method. However, even E.H. was not good enough for the quantitative lipid extraction. So we have to investigate new method in order to establish the reproducible and quantitative extraction of lipid.

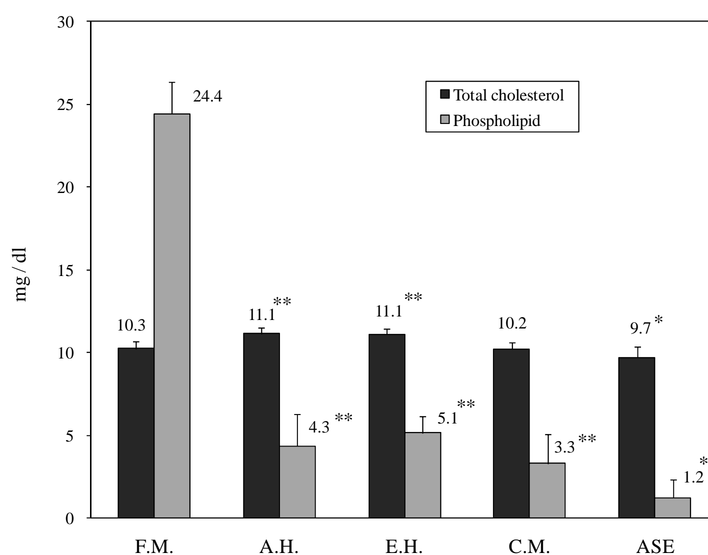

Fig.2. Comparison in concentrations of total cholesterol and phospholipid
*: p=0.01, **: p<0.001

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