## Table I. E. Sakasakii菌株に対する抗体のミクロ凝集反応

| 菌体 \ 血清希釈 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 |
|---|---|---|---|---|---|---|
| ＃1 | ++++ | ++ | ++ | + | － | － |
| ＃4 | ++++ | +++ | ++ | ± | － | － |
| ＃5 | +++ | ++ | ++ | ± | － | － |
| ＃8 | +++ | ++ | ++ | ± | ± | ± |
| ＃47-1 | ++++ | ++++ | +++ | + | ± | ± |

## Table II. E. Sakasakii菌株に対する抗体の交叉凝集活性

| 菌体 \ 血清 | ＃1 | ＃4 | ＃5 | ＃8 | ＃47-1 |
|---|---|---|---|---|---|
| ＃1 | | ± | ± | － | － |
| ＃4 | － | | － | ± | ++++ |
| ＃5 | ± | ± | | ± | － |
| ＃8 | ± | ± | － | | － |
| ＃47-1 | － | +++ | － | ± | |

Fig. 1. SDS-PAGE/ Western blottingによる各種ES菌体成分と抗体の反応性

一次抗体 #1

一次抗体 #4

一次抗体 #5

一次抗体 #8

一次抗体 #47-1
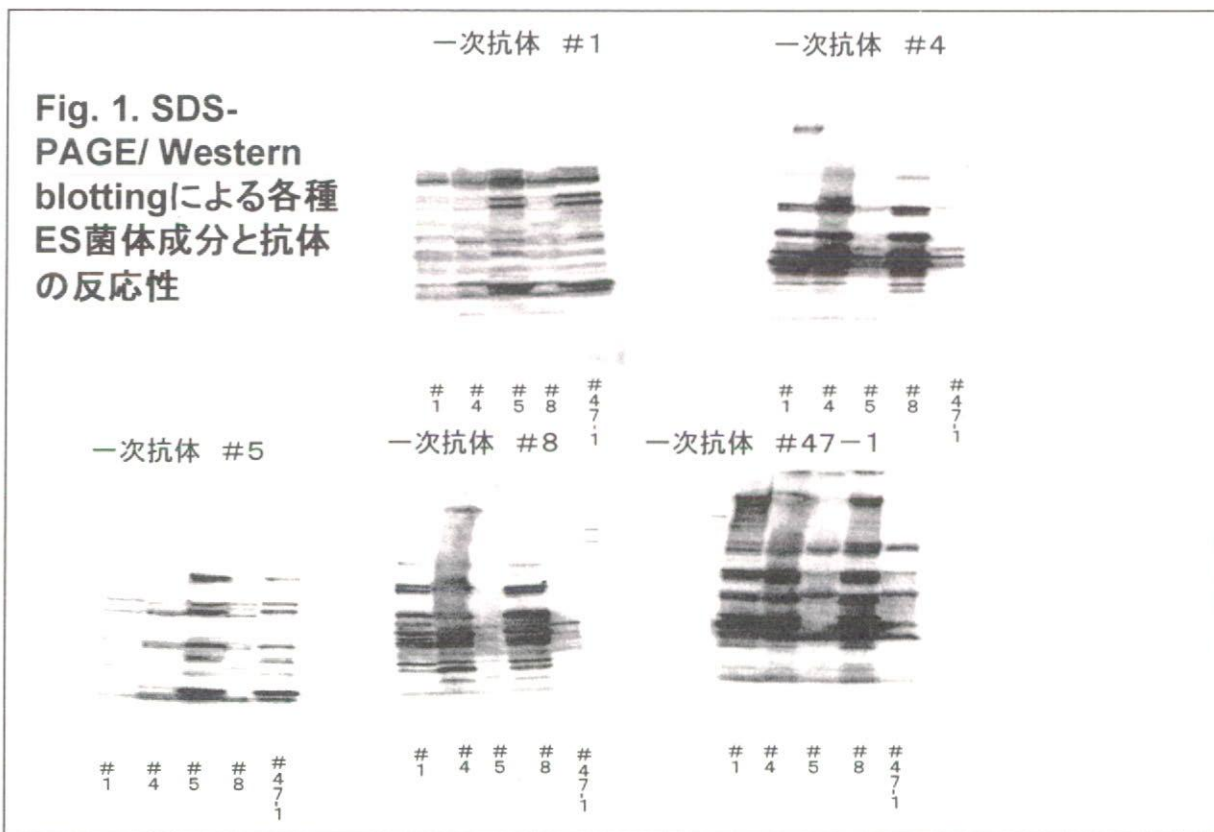


Fig. 2 ES の菌株による乾燥耐性の違い
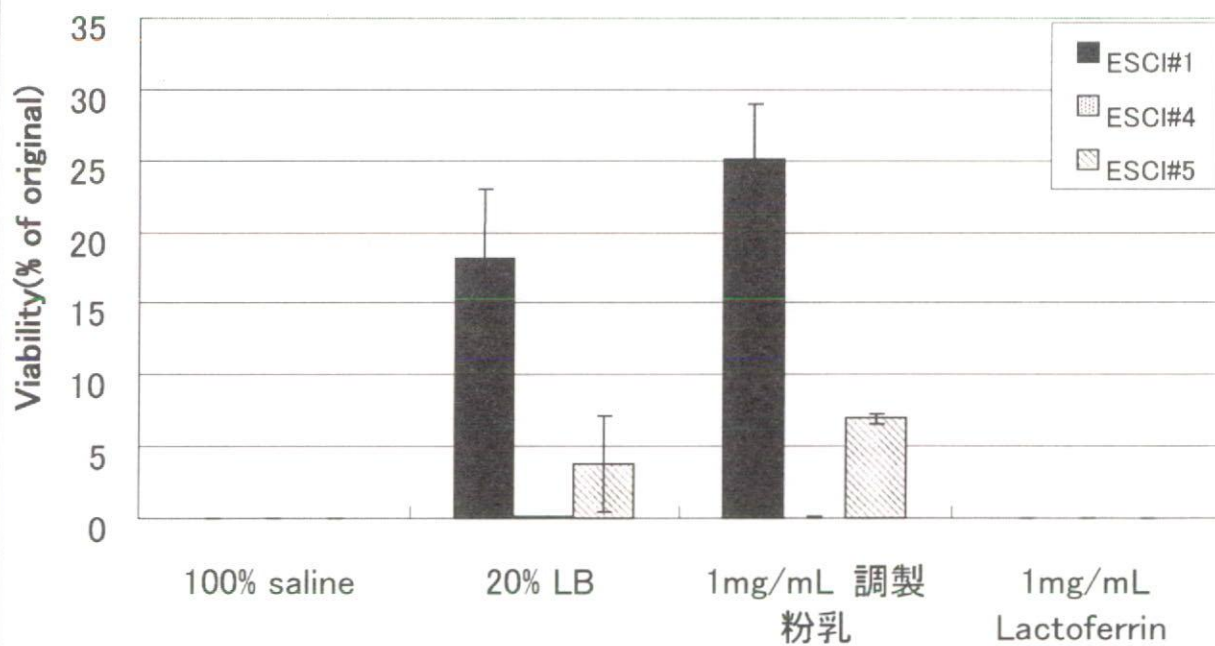
Fig. 3 ESCl#1 の LB 培地中での乾燥耐性獲得に及ぼす Lactoferrin と Apolactoferrin の影響
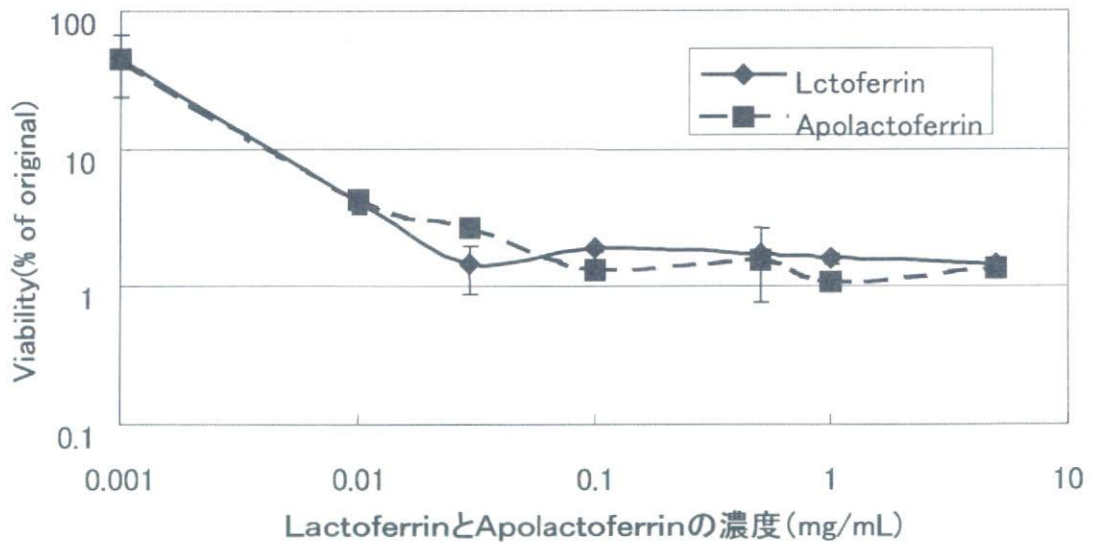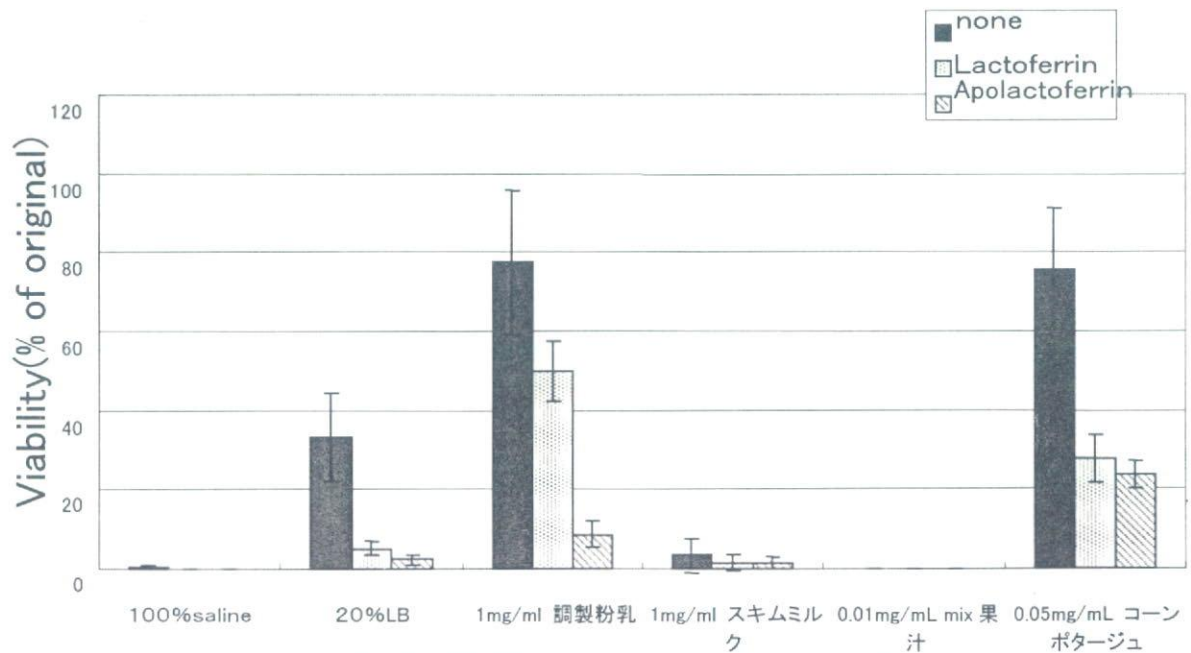


Fig. 4 ESCl#1 の乾燥耐性の獲得に及ぼす Lactoferrin と Apolactoferrin の影響

# 乳幼児の食品摂取量調査のための基礎研究

（分担研究者）　国立健康・栄養研究所国際産学連携センター　　吉池信男
（研究協力者）　国立健康・栄養研究所国際栄養プロジェクト　　三好美紀
　　　　　　　　恩賜財団母子愛育会日本子ども家庭総合研究所　堤ちはる

## 研究要旨

　乳幼児が日常的に摂取する調製粉乳、ベビーフード、その他の食品の摂取状況について、既存の調査データの解析、国民健康・栄養調査等の全国データの再解析、全国の新生児集中治療室を有する施設への調査等を行った。乳児期における詳細な摂取量データはほとんど無く、市販のベビーフードの流通状況等の情報を基に個々の食品（例：ひじき）からの曝露量等を推測する他、1歳以上のデータに限られてしまうが、国民健康・栄養調査や同様の方法を用いた調査データから摂取量分布を推定することが可能と思われた。一方、*Enterobacter sakazakii* 感染のリスクが高い低出生体重児等に対する乳児用調整粉乳の取り扱いに関しては、専門医療施設においても「乳児用調製粉乳の安全な調乳、保存及び取扱いに関するガイドライン」（2007 年、WHO）の周知・活用状況は十分とはいえず、今後の課題と考えられた。

## A．目的

　日常的な食事からの曝露評価を行うためには、乳幼児がどのような食品・食材をどの程度摂取しているかを把握することが必要となる。そこで、本分担課題では、個別的な曝露量試算を行うための基礎データの提供を主な目的とし、以下の検討を行うこととした。国民健康・栄養調査を含めて利用可能なデータを用いて、調整粉乳やベビーフード等の種類や摂取状況に関する情報に加え、その他の食品の摂取時期・摂取量に関して分布の記述等を行った。*Enterobacter sakazakii* 等の感染リスクを考えると、乳児用調整粉乳の製造過程等に着目することも重要である。そこで、医療機関における調整粉乳の調整・管理の実態及びWHO ガイドラインの認知度を把握するために、全国の新生児集中治療室(NICU)を有する施設へのアンケート調査を行った。

## B．研究方法

1) 乳幼児期における様々な食品の摂取実態の分析
① 乳児用調整粉乳の摂取量について
　中埜らが行った調査（対象：3〜18ヶ月の乳幼児

2,400 名）のデータの一部を用いて本研究の目的のための2次解析を行った。
② 離乳期における食品の摂取状況の分析
　堤らが行った全国調査（対象：5〜15ヶ月の子どもの保護者 5,199 名）のデータのうち、「児に与えたことのある食品」について、本研究の目的のための二次解析を行った。本調査で「（調査時点までに）児に与えたことがあるか？」と質問された全 97 食品の中から、主な食品グループに区分し、摂取者が多かった食品について、月齢別の分布を検討した。
③ 国民健康・栄養調査データに基づく個別食品の摂取量分布の分析
　国民健康・栄養調査は1歳以上を対象としていることから、"離乳期"の食パターンを1歳代の摂取量から類推することを目的として、2001〜04 年調査の食品摂取量データを用いて2次解析を行った(n=43,839、うち 1 歳児は 421 名)。11食品（まぐろ、ツナ缶詰、その他の魚、牛肉、牛レバー、豚肉、豚レバー、鶏肉、鶏レバー、米、ひじき）について、年齢グループごとに、一日あたりの摂取量及び体重あたりの摂取量分布を検討した。
④ 複数日の摂取量調査データに基づく、個別食品

（ひじき）の摂取量分布の分析

　19都道府県21の市町村において、平日2日と休日1日を含む連続しない3日間の食事調査を、4季節で実施した調査データを再解析した。分析対象は、1季節で3日間の食事調査を終了した1～5歳児279名（男121名、女158名）とした。各1日間の食事調査データから、ひじきの摂取量分布を詳細に解析した。

## 2）NICU全国調査

　新生児医療連絡会に加盟する全国主要NICU 202施設を対象に、郵送法による質問紙調査を行った（2008年2月実施）。調査内容は、ガイドライン等に記載されている *Enterobacter sakazakii* 等の混入に対して未熟児等のハイリスク児での感染リスクを最小限に抑えるための各種手技の実施状況、WHOガイドラインの周知・使用状況等である。

## C．研究結果

### 1）乳幼児期における様々な食品の摂取実態の分析

#### ①乳児用調整粉乳の摂取量について

調製粉乳のタイプ別に1日あたり平均摂取量は、下表の通りであった。

| 栄養法 | 乳児用ミルク | | | フォローアップミルク | | |
|---|---|---|---|---|---|---|
| | n | Mean | (SD) | n | Mean | (SD) |
| 人工栄養 | 337 | 703 | (180) | 260 | 415 | (177) |
| 混合栄養 | 136 | 336 | (224) | 44 | 197 | (146) |
| 合計 | 473 | 598 | (255) | 304 | 383 | (189) |

　使用調製粉乳のタイプによる比較では、乳児用ミルクの摂取量はばらつきがみられたが、フォローアップミルクの摂取量は8ヶ月から順次、減少傾向が示された。

#### ②離乳期における食品の摂取状況の分析

　穀類では米が最も多く、7ヶ月目までに96.4%の児に与えたことがあった。たんぱく質性食品の中では、豆腐とヨーグルトが多く、いずれも5ヶ月～8ヶ月の間の増加が大きかった。鶏卵は10ヶ月目までは黄身を中心に与えられており、その後は全卵が多かった。魚肉については、最初は白身魚と鶏肉が多い傾向がみられるが、11ヶ月以降は豚肉も多い。野菜はにんじん、かぼちゃ、ほうれん草が多く、果物はりんご、メロン、みかん、バナナの順に多かった。調味用油脂は、サラダ油、バター、マーガリンの順に多かった。

#### ③国民健康・栄養調査データに基づく個別食品の摂取量分布の分析

○まぐろ：1歳児の1日あたり粗摂取量の平均値（摂取者のみ）は他の年齢グループより低く、成人の10分の1であった。同様に、摂取者の体重あたり摂取量の平均値も低かったが、4歳～14歳より多かった。

○ツナ缶：1歳児の粗摂取量は2～6歳に比べて多かったが成人の約4分の1であった。体重当たりの摂取量は7-14歳が一番多く、次いで1歳児であり、成人より多かった。

○魚（その他）：1歳児の粗摂取量は他の年齢グループより少なく、一方、体重当たりの摂取量は他の年齢グループに比べて多かった。

○牛肉：1歳児の粗摂取量は2～6歳に比べて多かったが成人の約4分の1であった。体重当たりの摂取量は他の年齢グループに比べて多かった。

○豚肉：1歳児の粗摂取量は他の年齢グループより少なく、一方、体重当たりの摂取量は他の年齢グループに比べて多かった。

○鶏肉：1歳児の粗摂取量は2～6歳に比べて多かったが成人の約3分の1であった。体重当たりの摂取量は他の年齢グループに比べて多かった。

○牛レバー、豚レバー、鶏レバー：摂取者がかなり少なく（牛レバー：2名、豚レバー：0名、鶏レバー：3名）評価が困難であった。

○米：1歳児の粗摂取量は他の年齢グループより低く、15歳以上の約3分の1であった。一方、体重当たりの摂取量は他の年齢グループに比べて多かった。

○ひじき：1歳児の粗摂取量は成人の約5分の1であるが、2-14歳より多かった。また、体重当たりの摂取量は他の年齢グループに比べて多かった。

#### ④複数日の摂取量調査データに基づく、個別食品（ひじき）の摂取量分布の分析

　3日間の食事調査のうち、ひじきを摂取していない者が大半であり、1～5歳で0日摂取者81%、1日間摂取者は13%、2日間摂取者は6%であった。そのうち、1～2歳では0日摂取者は82%、1日間摂取者は14%、2日間摂取者は4%であった。3日間ともに摂取している者はいなかった。3日間平均の摂取者におけるひじきの体重あたりの95%tile摂取量は1～5歳で1.49g、1～2歳では1.30gであった。

### 2）NICU全国調査

　102施設から回答が得られた（回答率：50.5%）。乳児用調整粉乳（PIF）の調乳に際して調乳ユニッ

トを使用している施設は約7割であった。また、PIFの調乳及び管理のためのマニュアルが未整備である施設が約3割であり、調乳担当者に *E.Sakazakii* に関する情報提供や教育を行っている施設は約半数であった（下表）。

PIF 調乳時の衛生管理の現状（複数回答）

| 設問 | 回答数 |
|---|---|
| 調乳ユニットを用いて、調乳用の湯の温度管理を行っている | 71 |
| 調乳ユニットは用いていないが、温度計を用いて湯の温度管理を行っている | 14 |
| 調乳後、終末滅菌を行っている | 53 |
| 保存前に急速冷却を行っている | 55 |
| 使用した原材料（粉乳そのもの）を検査用保存食としてサンプリングしている | 25 |
| 調乳済のミルクを検査用保存食としてサンプリングしている | 44 |
| PIFの調乳及び管理のためのマニュアルが整備されている | 70 |
| 調乳担当者に *E.Sakazakii* に関する情報提供や教育を行っている | 50 |

調乳後、冷却し冷蔵庫で保管する施設が大部分を占め（n=91）、調乳後すぐ（20分以内）に授乳しているのは9施設であった。また、冷蔵庫で保管されたミルクを児に与える前に再加温を開始するタイミングは、「30分前」が最も多く（n=68）、「15分前」が8施設であったが、一方5施設が「1時間前」と回答した。大部分の施設では、「冷蔵庫での保管期間は24時間以内」のWHO勧告に従っていたが（n=87）、冷蔵保管以外のミルクを調乳後2時間以内に廃棄している施設は3割のみであった（n=33）。調乳用の湯の温度は、約半数の施設が推奨されている70℃以上としていた（下図）。

図. 調乳用の湯の温度
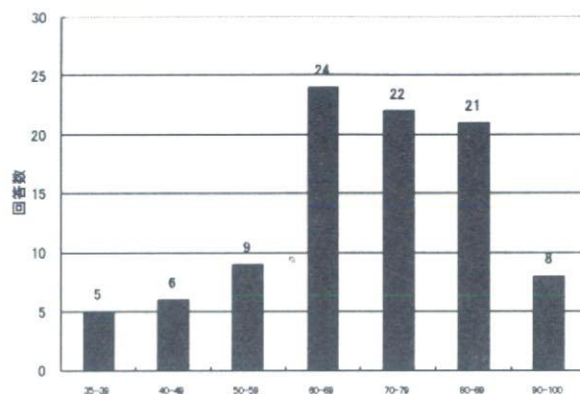


「乳児用調製粉乳の安全な調乳、保存及び取扱いに関するガイドライン」（WHO, 2007年）の周知・使用状況については、31施設が現在病院施設管理や患者指導に活用している一方で、56施設が「活用していない」又は「知らない」と回答した（下図）。

図. WHO ガイドラインの使用状況
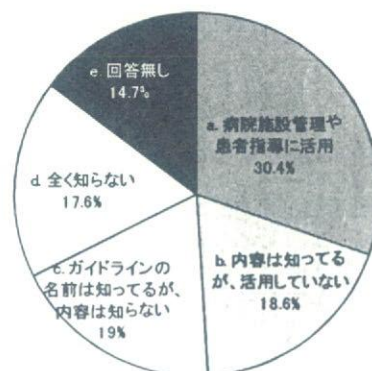


C. 考察

食品安全のための曝露評価の観点から、乳幼児期に摂取する食品として、特に *Enterobacter sakazakii* については乳児用調製粉乳、フランについてはひじきを取り上げて、利用可能なデータソースを活用して摂取量分布を詳細に検討するとともに、全国のNICUを対象とした調査を行った。

1）乳幼児期における様々な食品の摂取実態の分析

離乳期の各食品の摂取量については、量が少なく、また市販のベビーフードを利用する頻度も多いことから、個々の食材料についての量的把握は難しい。特に市販のベビーフードでは、原材料名の表示はあっても、構成量の表示や公開はされていないことがほとんどである。また、母乳の摂取量を把握するにはたい

へんな労力が必要である。

このようなことなどから、国民健康・栄養調査では、乳児期(1歳未満)を調査対象とはしておらず、限定された集団を対象とした調査においても、離乳期の食品摂取については定量的把握までされていないものがほとんどである。そこで、今回は、1歳以上のデータを用いて、離乳期(5,6ヶ月〜)の個々の食品摂取量を推定することとした。年齢(月齢)が上がるにつれて、食べる食品の種類も量も増えるという前提にたてば、1歳以上のデータを用いて推定することは、"過大側"に偏ると考えられ、食品安全対策上の曝露評価目的にはかなったことと考えた。

さらに個々の食品の中でも、ひじきについては詳細な検討を行った。ひじきは、乳幼児で不足しがちな鉄を多く含むことから、保育所等の給食やベビーフードの食材として使用されることが多い。従って、食品中からの暴露評価という観点から、集団として平均摂取量のみならず、習慣的多食者における摂取量分布を把握しておくことが必要である。そこで、国民健康・栄養調査データから得られる1日のみの摂取量分布に加えて、4季節の各3日間に行われた調査データを活用して、習慣的な摂取量分布についても検討を行った。その結果、3日のうち2日摂取している者は4%程度であり、摂取者においても3日平均の摂取量は、95%tile で 1.30g/kg/day とそれほど高くないことがわかった。従って、偏食による極端な多食がなければ、大きな問題とはならないものと推察された。

## 2)NICU全国調査

今回の対象施設の多くは栄養管理室内の調乳専用室において厳しい管理基準のもとで調乳ユニットを用いて調乳を行っていたが、PIF の調乳・管理のためのマニュアル整備および調乳担当者への *Enterobacter sakazakii* に関する情報提供がなされていない施設も多く、このために施設によって調乳に関わる品質管理・衛生環境の推奨基準にかかわる状況にばらつきがあることがわかった。WHO ガイドラインの周知・活用状況も十分とは言えず、今後の課題と言える。今回の結果から、今後、給食管理の枠組みの中での調乳の系統的なシステムづくりを強化させることが重要と考えられた。

## D．結論

乳児期における詳細な摂取量データはほとんど無く、市販のベビーフードの流通状況等の情報を基に個々の食品(例:ひじき)からの曝露量等を推測する他、1歳以上のデータに限られてしまうが、国民健康・栄養調査や同様の方法を用いた調査データから摂取量分布を推定することが可能と思われた。一方、*Enterobacter sakazakii* 感染のリスクが高い低出生体重児等に対する乳児用調整粉乳の取り扱いに関しては、専門医療施設においても「乳児用調製粉乳の安全な調乳、保存及び取扱いに関するガイドライン」(2007 年、WHO)の周知・活用状況は十分とはいえず、今後の課題と考えられた。

## E．健康危険情報

この研究において健康危険情報に該当するものはなかった。

## F．研究発表

なし

# Analysis of inorganic arsenic in foods by hydride generation-cold trap-atomic absorption spectrophotometry

Megumi Hamano Nagaoka, and Tamio Maitani

National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya, Tokyo 158-8501, Japan

## SUMMARY

The JECFA (the Joint FAO/WHO Expert Committee on Food Additives) has set a PTWI (provisional tolerable weekly intake) value of arsenic at a quantity of more toxic inorganic arsenic, since the toxicity of arsenic in foods differs greatly between inorganic arsenic and organic arsenic. To determine the inorganic arsenic contents in food samples such as seaweed, rice and water samples, a speciation analysis method by hydride generation-cold trap-atomic absorption spectrometry was applied. To extract inorganic arsenic efficiently, arsenic in foods was extracted with mixed acids (nitric acid and perchloric acid). When some water samples containing germanium as organic germanium compound at the high concentrations were applied to this system, the peak of germane was obviously detected at an earlier retention time than that of arsine in spite of the use of lamp for arsenic detection. Thus, arsine could be detected separately from germane by hydride generation-cold trap-atomic absorption spectrometry, even if both arsenic and germanium were present.

## INTRODUCTION

Since the toxicity of arsenic in foods differs greatly between inorganic and organic arsenic, the JECFA (the Joint FAO/WHO Expert Committee on Food Additives) has established a PTWI (provisional tolerable weekly intake) value of arsenic at a quantity consistent with more toxic inorganic arsenic. As a part of the project for estimating the intake of inorganic arsenic through foods and water, a speciation analysis method by hydride generation-cold trap (HG-CT) -atomic absorption spectrometry was applied to determine the inorganic arsenic contents selectively in several foods and water samples.

## MATERIALS AND METHODS

### Reagents

Standard mixed solution of methylarsonic acid, dimethylarsinic acid and trimethylarsine oxide was purchased from Wako Pure Chemical Industries, Ltd. Inc (Osaka, Japan). Nitric acid (68%) and perchloric acid (70%) of ultrapure analytical grade (TAMAPURE-AA-100) were purchased from Tama Chemical Industry (Kanagawa, Japan). Other chemicals were of reagent grade or of the highest grade available commercially.

### Equipment

Shimadzu ASA-2sp (Kyoto, Japan) was used as arsenic speciation pretreatment system for HG-CT process. Measurement principle is based on that arsenic compounds are separated accor-

ding to the boiling points of the respective arsenic hydrides. Thermo Elemental Solaar M5 (Kanagawa, Japan) was used as an atomic absorption spectrophotometer (AAS).

### Food and water samples

Food samples including infant formulae and baby foods and water samples were obtained in the Tokyo Metropolitan area and by mail order in Japan.

### Sample preparation

Foods were first heated with nitric acid, and then perchloric acid was added. Heating was continued until white fume of perchloric acid appeared to remove nitric acid. After heating, water was added to prepare the solution for analysis. Water samples were analysed without heating after filtration with 0.45-μm filter.

### Measurements

The acidic solution was applied to the ASA-2sp. Arsenic species in the solution were reduced to the respective hydrides with sodium boronhydride solution, introduced by a carrier gas (helium) to the U-tube filled with quartz wool cooled with liquid nitrogen, and then collected. Next, the U-tube was pulled out of the liquid nitrogen. Arsine, monomethylarsine, dimethylarsine and trimethylarsine were vaporized in turn, depending on their boiling points, and were introduced to the AAS for monitoring.

The optimal conditions for obtaining high sensitivity were examined for the combined ASA-2sp (Shimadzu) and AAS (Thermo Elemental) system. Several types of foods and some water samples were analyzed under the optimized conditions.


## RESULTS AND DISCUSSION

To apply this speciation method to a solid food, food containing arsenic must be converted to a solution. Moreover, to be detected, all arsenic compounds must be present as species that can be transformed into hydrides. Whether or not the organic arsenic compounds were degraded into inorganic arsenic on heating was examined using commercially available reagents, namely, methylarsonic acid, dimethylarsinic acid, trimethylarsine oxide and arsenobetaine. These organic arsenic compounds were not converted into inorganic species at the temperature below 110°C. Thus, the conditions under which organic arsenic compounds in foods did not change into inorganic species were determined.

Since the original conditions of the ASA-2sp pretreatment system were set to be optimal for the atomic absorption spectrophotometer of said corporation, the optimum conditions with the Solaar M5 spectrophotometer were studied. First, with the quartz cell of the Solaar M5 system heated electrothermally, helium gas flow rate was optimized. However, atomic absorbance was only one-third of that with the Shimadzu spectrophotometer. Moreover, the resolution of dimethylarsine and trimethylarsine was insufficient. Furthermore, the background level was not steady even when the flow rate of helium gas was changed. Since quartz cells supplied by the two companies differ in shape, the quartz cell from the Shimadzu Company could not be fitted to the Solaar M5 system.

Next, therefore, another heating method, in which a quartz cell is heated with a flame, was investigated. For this purpose, a new quartz cell suited to the Solaar M5 system was prepared. The optimum conditions for various factors were selected. Under the optimum conditions selected, the detection limit obtained with standard arsenite solution was 0.022 ppb. When this method was applied to a dry hijiki sample, the coefficient of variation for arsine was 2.6%, a satisfactory value. This method was also applied to pulverized rice, baby foods, infant formulae and water samples.

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When some water samples containing germanium as organic germanium compound at the high concentrations were applied to this system, a tiny peak of germane was obviously detected at an earlier retention time than that of arsine, in spite of the use of lamp for arsenic detection. Germanium is known to have a spectral line at 193.7 nm, which is also the wavelength of dominant spectral line of arsenic. Therefore, this method, hydride generation-cold trap-atomic absorption spectrophotometry, may be considered to be a useful technique to detect arsine and germane separately from foods and water samples containing both inorganic arsenic and germanium.

## REFERENCE

1. Nagaoka MH, Taneike Y, Akiyama H, Maitani T. Application of speciation analysis by hydride generation-cold trap-atomic absorption spectrometry to arsenic in foods. *Yakugaku Zasshi. J Pharm Soc Japan* 2005; 125 suppl.1: 69-71.

## Original

# Rapid and Improved Determination of Furan in Baby Foods and Infant Formulas by Headspace GC/MS

Izumi Yoshida[1,*], Satoshi Isagawa[1], Nobuyuki Kibune[1],
Megumi Hamano-Nagaoka[2] and Tamio Maitani[2]

[1]Japan Food Research Laboratories: 7-4-41 Asagi, Saito, Ibaraki-shi, Osaka 567-0085, Japan;
[2]National Institute of Health Sciences: 1-18-1 Kamiyoga, Setagaya-ku, Tokyo
158-8501, Japan; *Corresponding author

Furan is a 5-membered ring compound with high volatility. The U.S. Food and Drug Administration (FDA) has recently published a report on the occurrence of furan in a large number of thermally processed foods. However, the FDA's analytical method, using standard curve addition, is not suitable for high-throughput routine laboratory operations. We developed a rapid and improved method for determination of furan in foods by headspace GC/MS. Quantification was achieved by using an internal standard of $d_4$-furan and an external calibration curve of furan normalized against the internal standard. The incubation temperature for equilibration was set at 60℃ to avoid the formation of furan during analysis. The levels of furan in baby foods and infant formulas were determined with this method. Validation data showed good precision and accuracy. The LOD and LOQ were 0.2–0.5 ng/g and 0.5–2 ng/g for various food matrixes, respectively. The level of furan detected was in the range of 1.4 to 90 ng/g in baby foods and in the range of non-detectable to 36 ng/g in infant formulas.

Key words: furan; headspace; gas chromatography-mass spectrometry; baby food; infant formula

## Introduction

Furan ($C_4H_4O$) is a colorless, volatile (boiling point 31℃) and lipophilic organic compound[1]. It is considered to be a hazardous chemical and is classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans (group 2B; IARC)[2].

The U.S. Food and Drug Administration (FDA)[*1] has recently published a report on the occurrence of furan in a large number of thermally processed foods, such as canned and jarred foods, including baby foods and infant formulas. The FDA analyzed approximately 340 food samples and found furan levels ranging from non detectable to approximately 170 ng/g. The Swiss Federal Office of Public Health has presented an analytical method for the determination of furan in foodstuffs and used it to measure furan levels in a variety of foods on the Swiss market[3]. The European Food Safety Authority (EFSA)[4] has recently published a report on furan, in which they concluded that a more detailed risk assessment was required.

The primary source of furan in food was considered to be thermal degradation of carbohydrates, such as glucose, lactose and fructose[5]. Perez-Locas and Yaylayan suggested that ascorbic acid had the highest potential to produce furan on thermal treatment, followed by some sugar/amino acids mixtures[6]. Health Canada has recently reported the formation of furan *via* the oxidation of polyunsaturated fatty acids at elevated temperatures, and from the decomposition of ascorbic acid derivatives[7]. Fan has recently studied the formation of furan from sugars, ascorbic acid and organic acids under the influence of ionizing radiation and thermal treatments[8]. It was reported that the pH and concentration of sugars and ascorbic acid solution profoundly influenced furan formation during irradiation or thermal treatment.

As furan is a small, volatile molecule, headspace gas chromatography-mass spectrometry (HS-GC/MS) might be a suitable method for the determination of furan in foods. The FDA[*2] published the first version method (May 7, 2004) of determination for furan in food by

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HS-GC/MS, which involves heating the sample at 80℃, followed by sampling the headspace gas and GC/MS. Quantification was achieved through spiking an internal standard and utilizing the standard addition method. Some research groups also reported the determination of furan in foods by HS-GC/MS using an internal standard and the standard addition method[9] or using an internal standard and an external calibration curve[7, 10]. The determination of furan in foods has been recently reported using solid-phase micro-extraction (SPME) coupled with GC/MS[11]-[13].

In this study, the determination of furan in baby foods and infant formulas by HS-GC/MS was developed using a deuterium-labeled internal standard ($d_4$-furan) and external calibration curve. The standard addition method published by the FDA is complicated and not suitable for high-throughput routine laboratory operations. We used an external calibration curve to reduce the operating time. The incubation temperature for equilibration of furan between the sample and headspace was designed to avoid the formation of furan during this heating step. We examined the formation of furan during analysis. The method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy. So far, no acceptable daily intake values of furan have been established. However, baby foods and infant formulas would have higher risk than adult foods, because: (1) babies and infants may be more sensitive to furan exposure than adults, (2) babies and infants tend to consume more thermally processed foods, including infant formulas, in proportion to their body weight than adults. The levels of furan in a variety of baby foods and infant formulas were determined by this method.

## Materials and Methods

### Reagents and samples

Furan 99＋% [CAS No. 110-00-9] was supplied by Sigma-Aldrich (St Louis, MO). Furan-$d_4$ 99.5 atom% D [CAS No. 6142-90-1] was supplied by C/D/N Isotopes (Quebec, Canada). Methanol (for RP/PCB anal.) was obtained from Wako Pure Chemical Industries (Osaka, Japan), and sodium chloride was from Kanto Chemical (Tokyo, Japan). L-Ascorbic acid was supplied by Sigma-Aldrich. Ferrous sulfate heptahydrate was supplied by Wako Pure Chemical Industries. Water was obtained from a Millipore MilliQ Gradient A10 purification system (Bellerica, MA).

All samples were purchased from local retail stores.

### Apparatus

The following equipment was used: Agilent Model 6890N/5973N gas chromatograph/mass spectrometer equipped with an Agilent Model G1888 Headspace Sampler (Palo Alto, CA); TC-WAX capillary column, 60 m×0.25 mm i.d. (0.25 $\mu$m, GL Sciences, Tokyo, Japan); head space crimp vials, 20 mL (Agilent); aluminum crimp caps (Agilent); septums, PTFE/BYTL (Agilent).

### HS-GC/MS operating condition

Headspace vials were equilibrated at 60, 70 and 80℃ for 15, 30 and 60 min with vigorous shaking to identify optimum equilibration conditions. For determination of furan in baby foods and formulas, headspace vials were equilibrated at 60℃ for 30 min. After sample equilibration, the vials were pressurized with helium gas (15 psi) for 0.3 min. Volatile gas was filled into the 3 mL loop for 2 sec, equilibrated for 0.3 min and injected for 2 min into a capillary column in 40 : 1 split mode. The loop and needle were held at 100℃, and the transfer line at 130℃. The carrier gas was helium at a constant flow of 1.0 mL/min.

The GC/MS injection port was held at 200℃. The temperature program for the capillary column was started at 40℃, and raised after 10 min at 15℃ per min to 200℃. The temperature was maintained at 200℃ for 5 min. The mass spectrometer was operated in electron-ionization mode with a source temperature of 230℃ and an ionization voltage of 70 eV. Furan was detected by selected ion monitoring of the major ion at $m/z$ 68 and confirmed by monitoring of the ion at $m/z$ 39. The internal standard (furan-$d_4$) was detected by monitoring the equivalent ion at $m/z$ 72.

### Standards

Stock solutions (2.5 mg/mL) of furan and furan-$d_4$ were prepared in methanol according to the procedure described by the US FDA[*2]. Working solution of furan (0.5 or 5 $\mu$g/mL) was prepared in water by dilution of the stock solution. Working solution of $d_4$-furan (5 $\mu$g/mL) was prepared in water according to the same procedure.

Calibration standards (1, 2.5, 5, 10, 25, 50, 100, 250, 500 and 1,000 ng) were prepared by injecting 2–200 $\mu$L working solution of furan (0.5 or 5 $\mu$g/mL) and 10 $\mu$L working solution of $d_4$-furan (5 $\mu$g/mL) through the septums of 20 mL sealed headspace vials containing 10 mL water and 4 g sodium chloride, respectively.

### Sample preparation and determination of furan

Samples were chilled in a refrigerator for a minimum of 4 h. Samples that were not homogenous were homogenized using a chilled homogenizer for a maximum duration of 1 min. The samples, 1–2 g of semi-solid/solid samples or 5–10 g of liquid samples, were weighed into chilled 20 mL headspace vials containing 4 g sodium chloride. Chilled water was added into each vial to adjust the total volume of sample solution to 10 mL. To avoid loss of furan, the vials were kept chilled in an ice bath and they were immediately sealed. The procedure was conducted as quickly as possible. Internal standard, 10 $\mu$L of $d_4$-furan solution (5 $\mu$g/mL), was injected through the septum of each vial. For recovery experiments, aliquots of furan standard solution (5 $\mu$g/mL) were additionally spiked through the septums of the sealed vials. The vials were shaken vigorously and equilibrated at room temperature for at least 3 h before analysis by HS-GC/MS.

Quantification was achieved using the internal standard of $d_4$-furan and an external calibration curve of furan normalized against the internal standard.

## Results and Discussion

### Formation of furan during incubation for equilibration

The first version HS-GC/MS method (May 7, 2004) reported by the US FDA[*2] was conducted after equilibration at 80℃. In the preliminary study, the level of furan in infant formula was analyzed in triplicate, with different sample weights (1.0, 1.5 and 2.0 g), using an incubation period of 80℃ for equilibration. The results varied, and the furan level was found to be 1.6 and 2.3 ng/g at the sample weights of 1.0 and 2.0 g, respectively. Between 1.0 and 2.0 g sample weight, the furan level increased by 0.7 ng/g. There is a possibility that furan formation would occur during the equilibration at 80℃. The formation of products during HS-GS/MS analysis can occur increasingly with an increase in the amount of substance with potential for the formation. Therefore, it was concluded that the amount of furan formed during equilibration at 80℃ increased with increase in the sample weight. The infant formulas are fortified with various nutrient compositions, such as minerals and vitamins. These compounds might influence the formation of furan during analysis.

The furan levels of infant formula and powdered creamer were analyzed using the equilibration condition of 80℃ for 15, 30 or 60 min. The powdered creamer is not fortified with minerals and vitamins. The furan level increased with increasing incubation time in infant formula, while no such increase was observed in powdered creamer (Fig. 1). This result indicates that the formation of furan occurred in infant formula during the incubation at 80℃.

The changes of furan levels in infant formulas equilibrated at 60, 70 and 80℃ are shown in Fig. 2. At 80℃, the furan level increased as the equilibration time increased. However, no increase of the furan level was observed in the incubation at 60℃, when the equilibra-
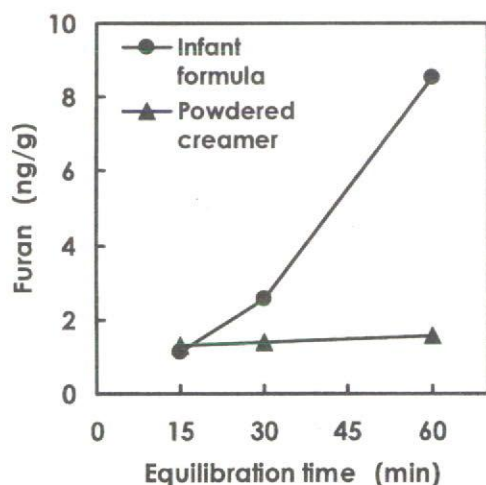


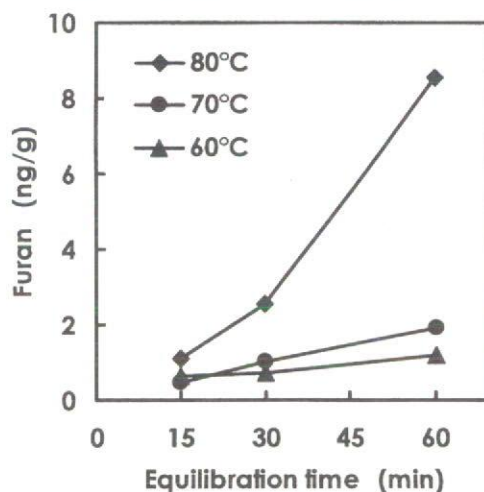Fig. 2.   Changes of furan levels in infant formulas during equilibration at 60, 70 and 80℃

tion time was increased from 15 to 30 min. The furan level increased slightly at 60℃ after incubation for 60 min. Therefore, the equilibration condition was set at 60℃ for 30 min.

We further examined the components in infant formulas that influenced the formation of furan during the equilibration. Furan was formed upon thermal treatment *via* the oxidation of polyunsaturated fatty acids and the degradation of ascorbic acids[7]. Infant formulas contain polyunsaturated fatty acids, iron and ascorbic acid. As ascorbic acid is known to possess both anti- and pro-oxidant effects, some authors have suggested that dose-dependent lipid peroxidation occurs in the presence of iron[14, 15]. They indicated that the pro-oxidant effect of ascorbic acid was related to the ability of ascorbic acid to promote the formation of a Fe(II) : Fe (III) complex required for iron-catalyzed lipid peroxidation. Almaas *et al.* has also reported that ascorbic acid enhances hydroxyl radical formation and lipid peroxidation in iron-fortified infant formulas[16].

To investigate furan formation during the equilibration, ascorbic acid and/or iron were added to powdered creamer that contains polyunsaturated fatty acids. Most commercial infant formulas contain 0.5 mg/g ascorbic acid and 0.06 mg/g iron. Ascorbic acid and iron were added at these concentrations or two-fold greater concentrations. Formation of furan during equilibration is shown in Table 1. When only ascorbic acid was added to powdered creamers, furan formation was hardly observed during equilibration at 80℃ for 30 min. Powdered creamer in the presence of added iron at the concentration of 0.12 mg/g, *i.e.*, twice the amount present in most infant formulas, produced a small amount of furan during the equilibration at 80℃. When both ascorbic acid and iron were added to powdered creamers at the two concentrations, the furan levels increased 1.5 and 2.2 times, respectively, compared to those of powdered creamer alone after incubation at 80℃ for 30 min. No furan formation was observed during the equilibration at 80℃ in water containing



Fig. 1.   Changes of furan levels in infant formulas and powdered creamers during equilibration at 80℃

**Table 1.**   Formation of furan during equilibration of powdered creamers with added ascorbic acid and iron[a]

| Reagent | Addition levels of reagent (mg/g) | Furan concentration (ng/g) | |
|---------|------------------------------------|-----------------------------|---|
| | | Equilibration at 60℃ for 30 min | Equilibration at 80℃ for 30 min |
| (No addition) | — | 1.0 | 1.2 |
| Ascorbic acid | 0.5 | 1.1 | 1.2 |
| | 1.0 | 1.1 | 1.3 |
| Iron | 0.06 | 1.1 | 1.3 |
| | 0.12 | 1.1 | 1.4 |
| Ascorbic acid+Iron | 0.5+0.06 | 1.1 | 1.8 |
| | 0.5+0.12 | 1.2 | 2.1 |
| | 1.0+0.06 | 1.2 | 2.1 |
| | 1.0+0.12 | 1.3 | 2.6 |

[a] Experiments were conducted with 2 g of powdered creamers after addition of ascorbic acid and/or iron.

both ascorbic acid and iron (data not shown). These results showed that the formation of furan during the equilibration at 80℃ for 30 min was likely to have occurred *via* lipid peroxidation catalyzed by ascorbic acid and iron[14)-16)].

Furan formation was not observed during the equilibration at 60℃ for 30 min in the powdered creamers to which both ascorbic acid and iron were added at the same concentrations as those present in most infant formulas (0.5 mg/g ascorbic acid and 0.06 mg/g iron, respectively). Even the addition of both ascorbic acid and iron to powdered creamers at twice the concentration present in most infant formulas caused an increase of only 1.3 times in the furan level compared with that of the powdered creamer alone.

More recently, the FDA has published the third version method (October 27, 2006) for the determination of furan in foods[*2]. The headspace oven temperature was reduced from 80℃ to 60℃ to prevent furan formation during analysis. Method ruggedness testing[*3] showed that low-level furan formation can occur in a few relatively high fat foods when test portions are equilibrated at 80℃ for 30 minutes. In addition, the FDA showed that a longer equilibration time at 60℃ did not affect the amount of furan in peanut butter and infant formula. For potato chips, the amount of furan increased with thermal equilibration times greater than 30 minutes. They suggested that this effect might be the result of the high concentration of polyunsaturated fatty acids in potato chips.

*Method validation*

The analytical method developed by the FDA is a standard addition method. This method is complicated and time-consuming. We developed a method that is more suitable for routine laboratory operations. The levels of furan in baby foods and infant formulas were determined using the internal standard $d_4$-furan and an external calibration curve of furan normalized against

the internal standard. In headspace analysis, the composition of the sample matrix can affect the amount of furan that goes into the headspace. The effect of the sample matrix should be reduced in the case of determination with an external standard method. The concentration of the sample matrix was diluted by adding 10 mL of water to 1–2 g of solid sample. In addition, 4 g of sodium chloride was added to the vial. Adding an inorganic salt to aqueous samples increases the concentration of furan in the headspace by making it less soluble in the sample matrix. Using this procedure, the recoveries of the internal standard $d_4$-furan were more than at least 40% in various sample matrixes. The proposed method permitted the determination of furan levels with good precision without using the standard addition method.

The method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy.

The linearity of the calibration curve was evaluated from 1 to 1,000 ng in the headspace vials by plotting the peak height ratio of furan/$d_4$-furan. The calibration data are shown in Table 2. Good linearity ($R^2=0.9999$) was obtained.

The LOD and LOQ were evaluated according to the procedure proposed by International Union of Pure and Applied Chemistry (IUPAC)[17)] and the US Environmental Protection Agency (EPA)[18)], based on the constant error model described by Currie[19)]. The LOD is defined as the minimum concentration of an analyte that can be reliably detected or differentiated from the background for a given matrix by a given analytical method. The LOQ is defined as the minimum concentration of an analyte that can be reliably quantified with a certain degree of reliability within a given matrix by a given analytical method. The LOD is determined from the analysis of at least seven replicate samples containing the target analyte at an estimate of the detection limit. The LOD is calculated as:

$$\mathrm{LOD} = 2t_{(n-1, 1-\alpha=0.95)}S$$

where:

$t_{(n-1, 1-\alpha=0.95)}$ = the Student's *t*-value appropriate for a 95% confidence level with $n-1$ de-

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*3 US Food and Drug Administration. (October 27, 2006) Method ruggedness testing, determination of furan in foods. Available from: http://www.cfsan.fda.gov/~dms/furan2.html

**Table 2.** Method validation data

| | Parameter | Values | | |
|---|---|---|---|---|
| Linearity | Range (ng) | 1, 2.5, 5, 10, 25, 50, 100, 250, 500 and 1,000 ng | | |
| | Equation | $y=0.0209x+0.0024$ | | |
| | $R^2$ | 0.9999 | | |
| Limit of detection; LOD (ng/g) | | | | |
| | Baby food (semi-solid/solid) | 0.5 | | |
| | Baby food (liquid) | 0.2 | | |
| | Infant formula | 0.4 | | |
| Limit of quantification; LOQ (ng/g) | | | | |
| | Baby food (semi-solid/solid) | 2 | | |
| | Baby food (liquid) | 0.5 | | |
| | Infant formula | 1 | | |
| Precision[a] | CV (%) | | | |
| | Baby food | 4.2 | | |
| | Infant formula | 4.0 | | |
| Accuracy | Spiked level (ng/g), Percent recovery[b] (%), CV (%) | | | |
| | Baby food | 25, | 91.8, | 2.7 |
| | | 50, | 93.8, | 2.4 |
| | | 125, | 96.4, | 0.8 |
| | Infant formula | 5, | 102, | 9.6 |
| | | 10, | 101, | 2.2 |
| | | 25, | 101, | 1.0 |

[a] Precision studies were performed with baby foods (furan; 37 ng/g) and infant formulas (furan; 5 ng/g) in replicate ($n=7$), respectively.

[b] Percent recovery studies were performed with baby foods (furan; 25 ng/g) and infant formulas (furan; 5 ng/g) in triplicate, respectively.

grees of freedom,

$s=$ the standard deviation of the replicate analyses.

The LOQ was set at 3 times LOD. The LOD and LOQ were determined in three types of sample matrixes. The analyses were performed with cheese dessert, orange juice and milk-based infant milk for baby food (semi-solid/solid), baby food (liquid) and infant formula, respectively. Figure 3 shows the SIM chromatogram of orange juice. No interference was encountered in the extracted ion chromatograms of $m/z$ 68 and $m/z$ 39. The LOD and LOQ values are shown in Table 2. The LOD values were 0.5, 0.2 and 0.4 ng/g and the LOQ values were 2, 0.5 and 1 ng/g for baby food (semi-solid/solid), baby food (liquid) and infant formula, respectively.

The precision was evaluated by calculating the coefficient of variation (CV) based on seven replicates with two different sample matrixes, vegetables and chicken (baby food) (furan; 37 ng/g) and milk-based infant formula (furan; 5 ng/g). The CV was 4.2 and 4.0% in baby food and infant formula, respectively (Table 2). The data showed good reproducibility.

The accuracy was evaluated by calculating the recoveries from two different sample matrixes, vegetables and fish (baby food) and milk-based infant formula, in triplicate. The recovery studies were performed by spiking 1-, 2- and 5-fold concentrations of furan detected in each sample matrix. The recovery was calculated as the percent ratio between the detected and

spiked concentrations. The recoveries at 25, 50 and 125 ng/g were 91.8, 93.8 and 96.4% in baby food, respectively (Table 2). The recoveries at 5, 10 and 25 ng/g were 102, 101 and 101% in infant formula, respectively
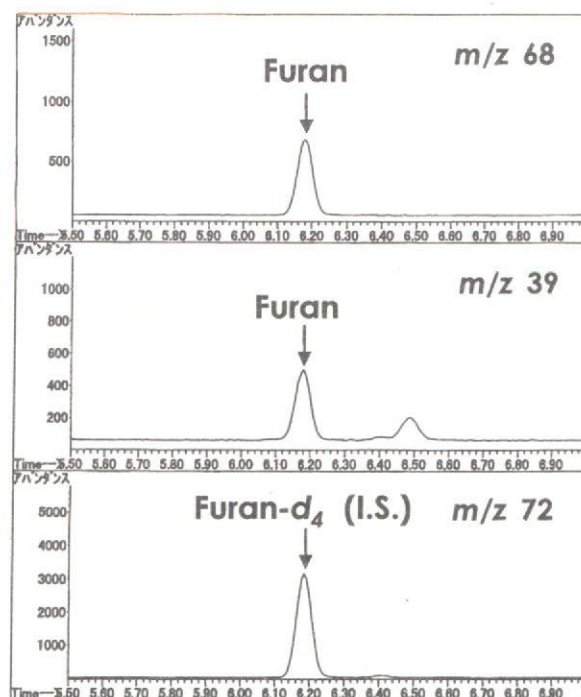


Fig. 3. Typical GC/MS SIM chromatogram of a headspace sample of orange juice (furan; 1.5 ng/g)

(Table 2).

These results showed the efficiency of this method in terms of precision and accuracy without using the standard addition method.

*Analysis of baby foods and infant formulas*

The EFSA reported that analyses on 273 baby foods showed levels of furan ranging from non detectable to 112 ng/g[4]. For a consumption of 234 g/day of commercial baby food, these data indicate a furan exposure of <0.03 to 3.5 μg/kg b.w./day (assuming a body weight of 7.5 kg of a 6 month baby).

The furan levels in baby foods and infant formulas on the Japanese market were analyzed according to this described method. The concentrations of furan are shown in Table 3.

The level of furan detected was in the range of 1.4 to 90 ng/g in baby foods. The highest level was found in a fish-containing baby food packed in a retort pouch. The levels of furan in dried juice and vegetable soups, not processed in cans, jars or retort pouches, but subjected to other heat processing such as spray-drying, were 25 and 29 ng/g, respectively. The furan formation

could occur during the manufacture of these products.

The furan levels of infant formulas ranged from nondetectable to 36 ng/g. The general milk-based infant formulas, including iron-fortified milk-based infant formulas, showed <1 ng/g furan levels. The special infant formulas, *i.e.* formulas for infants with milk protein allergy, contained relatively higher concentrations of furan levels than the general ones. The protein in the formula for infants with milk protein allergy has been hydrolyzed. During spray-drying and/or the hydrolysis process in the production of this infant formula, furan might be formed.

### Acknowledgments

### References

1) National Toxicology Program (NTP), Ninth Report on Carcinogenesis, Furan CAS No. 110-00-9 (Carcinogenicity), 1993.

2) International Agency for Research on Cancer, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 63, 1995.

3) Reinhard, H., Sager, F., Zimmermann, H., Zoller, O., Furan in foods on the Swiss market—method and results. Mitt. Lebensm. Hyg., **95**, 532–535 (2004).

4) European Food Safety Authority, Report of the scientific panel on contaminants in the food chain on provisional findings on furan in food. EFSA J., **137**, 1–20 (2004).

5) Maga, J. A., Furans in foods. CRC Crit. Rev. Food Sci. Nutr., **11**, 355–400 (1979).

6) Perez, Locas, C., Yaylayan, V. A., Origin and mechanistic pathways of formation of the parent furan—A food toxicant. J. Agric. Food Chem., **52**, 6830–6836 (2004).

7) Becalski, A., Seaman, S., Furan precursors in food: A model study and development of a simple headspace method for determination of furan. J. AOAC Int., **88**, 102–106 (2005).

8) Fan, X., Formation of furan from carbohydrates and ascorbic acid following exposure to ionizing radiation and thermal processing. J. Agric. Food Chem., **53**, 7826–7831 (2005).

9) Senyuva, H. Z., Gökmen, V., Analysis of furan in foods. Is headspace sampling a fit-for-purpose technique?. Food Addit. Contam., **22**, 1198–1202 (2005).

10) Hasnip, S., Crews, C., Castle, L., Some factors affecting the formation of furan in heated foods. Food Addit. Contam., **23**, 219–227 (2006).

11) Goldmann, T., Périsset, A., Scanlan, F., Stadler, R. H., Rapid determination of furan in heated foodstuffs by isotope dilution solid phase micro-extraction-gas chromatography-mass spectrometry (SPME-GC-MS). Analyst, **130**, 878–883 (2005).

12) Ho, I.-P., Yoo, S.-J., Tefera, S., Determination of furan levels in coffee using automated solid-phase micro-extraction and gas chromatography/mass spectrometry. J. AOAC Int., **88**, 574–576 (2005).

13) Bianchi, F., Careri, M., Mangia, A., Musci, M., Development and validation of a solid phase micro-extraction-gas chromatography-mass spectrometry method for the determination of furan in baby-food. J. Chromatogr. A, **1102**, 268–272 (2006).

Table 3. Furan levels in baby foods and infant formulas

| Samples | Furan (ng/g) |
| --- | --- |
| **Baby foods** | |
| Apple juice, 30%[a] | 1.4 |
| Roasted barley tea[a] | 3.1 |
| Dried prune juice[b] | 25 |
| Dried vegetable soup[b] | 29 |
| Apple squash[c] | 2 |
| Pumpkin and Sweet potato[c] | 25 |
| Sweet potato[c] | 12 |
| Vegetables and Fish A[d] | 90 |
| Vegetables and Fish B[d] | 13 |
| Vegetables and Pork[d] | 30 |
| Vegetables and Beef[c] | 41 |
| Vegetables and Chicken[d] | 25 |
| Vegetables, Tofu and Chicken[d] | 14 |
| Vegetables, Rice and Fish A[d] | 18 |
| Vegetables, Rice and Fish B[d] | 5 |
| **Infant formulas** | |
| Milk-based A[e] | N.D.[f] |
| Milk-based B[e] | N.D.[f] |
| Milk-based C[e] | <1 |
| Milk-based D[e] | <1 |
| Milk-based, iron-fortified A[e] | N.D.[f] |
| Milk-based, iron-fortified B[e] | <1 |
| Milk-based, iron-fortified C[e] | <1 |
| Milk-based, iron-fortified D[e] | <1 |
| Milk-based, lactose free[e] | 5 |
| Milk-based, for milk allergy[e] | 36 |
| Soy-based[e] | 2 |

[a] packed in a PET bottle
[b] packed in a foil-sealed plastic bag
[c] packed in a glass jar
[d] packed in a retort pouch
[e] packed in a can
[f] Not detected (<LOD)

14) Miller, D. M., Aust, S. D., Studies of ascorbate-dependent, iron-catalyzed lipid peroxidation. Arch. Biochem. Biophys., 15, 113–119 (1989).

15) Minotti, G., Aust, S. D., Redox cycling of iron and lipid peroxidation. Lipids, 27, 219–226 (1992).

16) Almaas, R., Rootwelt, T., Øyasæter, S., Saugstad, O. D., Ascorbic acid enhances hydroxyl radical formation in iron-fortified infant cereals and infant formulas. Eur. J. Pediatr., 156, 488–492 (1997).

17) Currie, L. A., Nomenclature in evaluation of analytical methods including detection and quantification capabilities (IUPAC Recommendations 1995). Pure Appl. Chem., 67, 1699–1723 (1995).

18) US EPA. 40 CFR Part 136; Guidelines establishing test procedures for the analysis of pollutants; Procedures for detection and quantification. Federal Register, 68, 11770–11790 (2003).

19) Currie, L. A., Limits for qualitative detection and quantitative determination. Anal. Chem., 40, 586–593 (1968).

# Genetic Characterization of Thermal Tolerance in *Enterobacter sakazakii*

**Hiroshi Asakura[1], Tomoko Morita-Ishihara[1,2], Shigeki Yamamoto[1], and Shizunobu Igimi\*[1]**

[1]*Division of Biomedical Food Research, National Institute of Health Sciences, Setagaya-ku, Tokyo 158–8501, Japan, and* [2]*Division of Bacteriology, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162–8640, Japan*

*Abstract: Enterobacter sakazakii* **is an opportunistic pathogen that causes meningitis and necrotizing enterocolitis in neonates. Here we characterized the thermal tolerance of *E. sakazakii* isolates obtained from powdered infant formula and other food products in Japan. Isolates were categorized into three classes according to their thermal tolerance, and differential gene expression analysis showed that the heat-resistant clones expressed a higher level of *infB* (which encodes a translation initiation factor), than did the heat-sensitive isolates. Gene expression and DNA polymorphism analyses suggested that this gene target might be useful to unequivocally detect and identify heat-resistant clones, permitting epidemiological surveillance for this pathogen.**

*Enterobacter sakazakii* is a gram-negative pathogen that causes meningitis, septicaemia, and necrotizing enterocolitis in neonates and infants. Premature and low-birth-weight infants and those younger than 28 days are at greater risk than older ones for this infection (4). The mortality rate for *Enterobacter*-associated meningitis is 33%–80% (11). Most children who survive develop severe neurological sequelae, which can include seizures, brain abscesses, hydrocephalus, and developmental delay.

Several *E. sakazakii* outbreaks have occurred in hospital units for newborns and have been strongly associated with contaminated powdered infant formula (PIF) (1, 3). The *E. sakazakii* contamination of PIF can be intrinsic or extrinsic. With intrinsic contamination, the organism is introduced into the PIF during the manufacturing process (18). Extrinsic contamination may result from the use of contaminated utensils, such as blenders and spoons, in the preparation of PIF, and bacteria have been isolated from such utensils in the past (16).

Although the bacteria experience and survive some thermal stress with both transmission routes (either during manufacture, or when the PIF is reconstituted with hot water), the atypically large number of *Enterobacter* species that can be found in PIF has been attributed to the relatively high thermal resistance of some of them

(6, 15, 19). This implies that the thermal tolerance capacity of the bacteria is an important risk factor for human infection, with important implications for food hygiene.

Between June and October 2006, we collected 30 *E. sakazakii*-like isolates from food products, including PIF, in Japan, in the process of food inspection in which about 2,000 products were targeted (see Table 1). For this study, we performed biochemical and phylogenetic analyses, to uncover the relationships among these isolates. We also performed a comparative gene-expression analysis, to characterize the thermal resistance of these isolates genetically, and we identified a candidate gene for the heat-resistant phenotype in *E. sakazakii*. The genetic association of variants of this gene with thermal tolerance is discussed below.

Of the 33 *E. sakazakii* isolates used throughout this study (Table 1), 30 were food isolates (from 23 kinds of food products), and three were ATCC strains (29004, 29544, and BAA-894) that were included as controls in all the experiments. All isolates were biochemically identified as *Enterobacter* species with the API20E test kit (BioMérieux, France), following the manufacturer's instructions. Additionally, since some *E. sakazakii* strains could not be differentiated from closely related species, such as *E. cloacae*, by the biochemical characterization alone (9), genetic identification was also per-

\*Address correspondence to Dr. Shizunobu Igimi, Division of Biomedical Food Research, National Institute of Health Sciences, Kamiyoga 1–18–1, Setagaya-ku, Tokyo 158–8501, Japan. Fax: +81–3–3700–9246. E-mail: igimi@nihs.go.jp

*Abbreviations*: DEG, differentially expressed gene; PIF, powdered infant formula; RT-PCR, reverse transcription-PCR; UPGMA, unweighted pair group method with arithmetic mean.

Table 1. Summary of *E. sakazakii* isolates used in this study

| Strain | Source | API20E | 16S[b] | Class[c] | infB DNA (65-bp)[d] |
|--------|--------|--------|--------|----------|--------------------|
| ATCC29004 | Human | 3305373 | I | R | + |
| ATCC29544 | Human | 3305373 | I | R | + |
| ATCC BAA-894 | Human | 3305173 | I | R | + |
| HT003 | Yam | 3205373 | I | R | + |
| HT004 | Agar powder | 3305173 | I | R | + |
| HT006 | Chondroitin sulfate | 3345173 | I | R | + |
| HT016 | Radish sprouts | 3305773 | I | R | + |
| HT017 | Barley leaves | 3305373 | I | R | + |
| HT018 | Agar powder | 3207373 | I | R | + |
| HT019 | Soy bean protein | 3207373 | I | R | + |
| HT022 | Mushroom powder | 3305373 | I | R | + |
| HT032 | PIF[a] | 3305173 | I | R | + |
| HT033 | PIF[a] | 3305373 | I | R | + |
| HT010 | Red pepper | 3305373 | I | M | + |
| HT024 | Garlic | 3207373 | I | M | + |
| HT008 | Wheat flour | 3347173 | I | M | — |
| HT034 | PIF[a] | 3305173 | I | M | — |
| HT007 | Rapeseed | 3304373 | I | S | + |
| HT028 | Tuna/corn salad | 3347173 | I | S | — |
| HT012 | Liquid enzyme | 3207373 | II | R | + |
| HT031 | Powdered squid | 3145173 | II | R | + |
| HT013 | Powdered gluten | 3207373 | II | M | — |
| HT014 | Mulberry leaves | 3207173 | II | M | — |
| HT015 | Rice calcium | 3205173 | II | M | — |
| HT025 | Buckwheat flour | 3305173 | II | M | — |
| HT029 | Buckwheat flour | 3306773 | II | M | — |
| HT002 | Rice | 3207173 | II | S | — |
| HT023 | Gluten | 3205173 | II | S | — |
| HT027 | Sandwich | 3205173 | II | S | — |
| HT011 | Natto kinase | 3307773 | II | S | + |
| HT030 | Rice | 3307773 | II | S | — |
| HT026 | Packed rice | 3345773 | III | R | + |
| HT009 | Frozen bread | 3005373 | IV | M | — |

[a] PIF, powdered infant formula (final product).

[b] Shows the phylogenetic group from the 16S rRNA sequence comparison (Fig. 1).

[c] Thermal tolerance of *E. sakazakii* was assigned according to the survival numbers after heating at 60 C for 90 min; class R (resistant), class M (intermediate), and class S (sensitive).

[d] The detection of *infB* fragment is indicated, which was detected by PCR using primer pairs (5'-GCT-GCGGAAACGAGCAA-3' and 5'-TCCGCCTGAGCAGCTTTG-3'). The program for amplification was 30 cycles of amplification consisting of denaturing at 94 C for 30 sec, annealing at 62 C for 30 sec and extension at 72 C for 30 sec.

formed by PCR, with the 16S rRNA sequence as the template. In brief, a 528-bp fragment of cDNA was amplified from the total DNA of each isolate with the following primer pair: 5'-TGGAGAGTTTGATCCTG-GCTCAG-3' and 5'-TACCGCGGCTGCTGGCAC-3' (8). The PCR products were cycle-sequenced with the BigDye terminator on an ABI310 sequencer (Applied Biosystems), and the sequences obtained were deposited in the DNA Data Bank of Japan (DDBJ) as accession numbers AB274273 through AB274302, and

AB292182.

The food isolates showed more than 98.17% sequence identity in their 16S rRNA gene to that in *E. sakazakii* type strain ATCC29544 (AB004746), supporting the results of the biochemical test. We next generated a phylogenetic tree from the 16S rRNA sequences, by the unweighted pair group method with arithmetic mean (UPGMA) (Fig. 1). The phylogenetic tree indicated 4 groups of most-closely related isolates, which turned out to share 98.36% sequence identity.
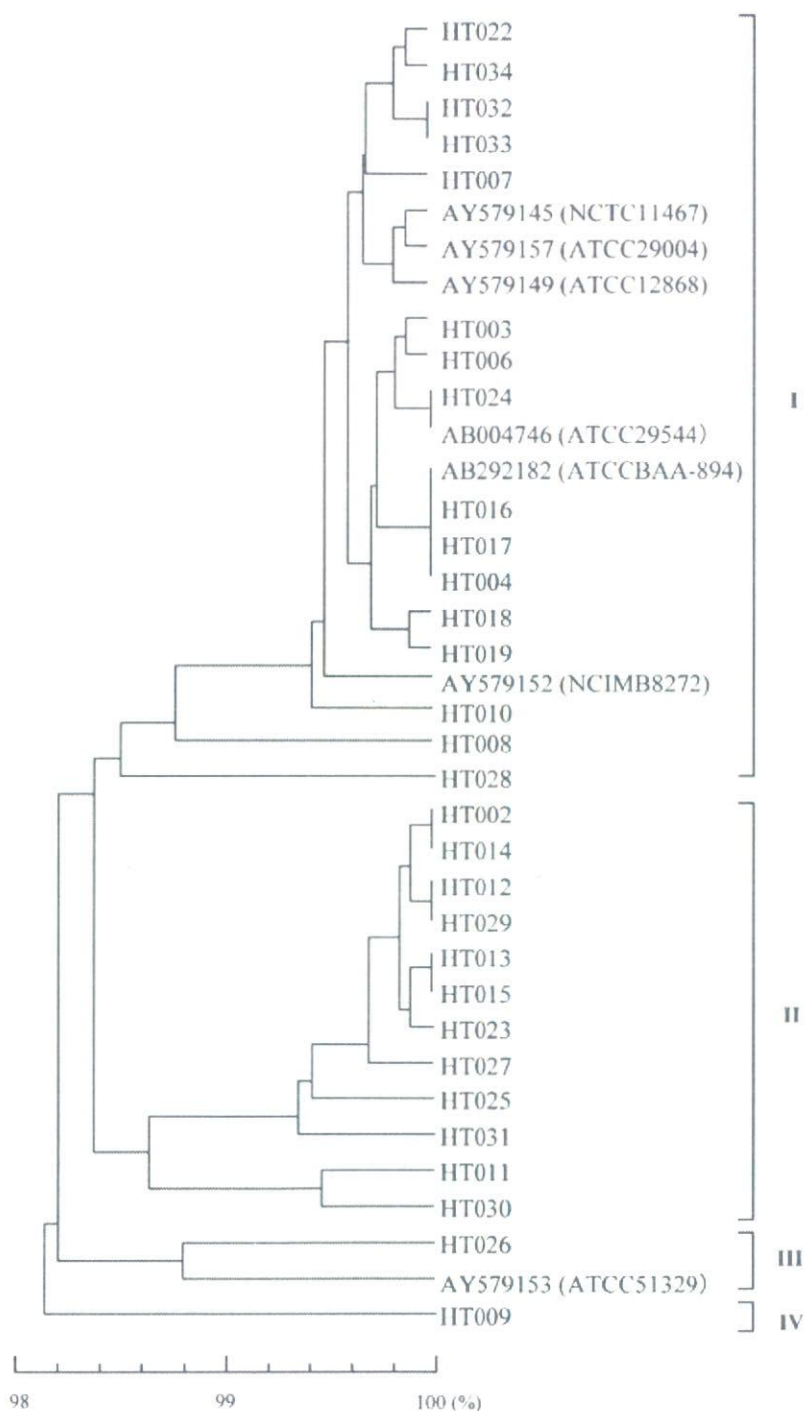
Fig. 1. Dendrogram analysis of *E. sakazakii* isolates. Phylogenetic tree of the 16S rRNA gene sequence from *E. sakazakii* strains obtained in this study and constructed by the UPGMA method, in comparison with sequences deposited in the DDBJ database (strain names are given in parentheses). The scale bar represents the percentage of similarity. Information about the reference sequences (AY579145, AY579149, AY579153) can be obtained from the reference (10).