Group I contained the largest number of isolates: three from PIF (HT032 to HT034), 13 from food, and the three reference strains (Fig. 1). The other food isolates, except HT026 and HT009, were assigned to group II. The sequence from HT026 was assigned to group III along with another reference sequence (AY579153), and HT009 was assigned to group IV. These observations agreed with a previous report on *E. sakazakii* heterogeneity (9).

Next, the survival of *E. sakazakii* in response to thermal stress was examined, essentially as described previously (2). In brief, approximately 10^8 *E. sakazakii* cells were grown in tryptic soy broth (TSB, Becton Dickinson) for 20 hr at 37 C, washed, and resuspended in 10 ml of sterile phosphate buffered saline (PBS, pH 7.4) to a final OD_{600} of 0.25. The suspensions were incubated at 60 C for 90 min, and 100-μl aliquots of the suspension and its serial dilutions were spread on TSA at 30-min intervals, for later quantification of the CFUs.

Representative data are shown in Fig. 2. Across the strains, there was an almost 10^4-fold divergence in thermal resistance. For example, after the 90-min incubation at 60 C, more than 10^4 CFU/ml of the three ATCC strains (29004, 29544, and BAA-894) still survived (data not shown), but two of the food strains, HT023 and HT030, formed no colonies (Fig. 2). From the thermal-resistance data, we assigned the isolates to three classes: class R, heat-resistant (more than 10^4 viable cells/ml following heat treatment); class S, heat-

sensitive (fewer than 10^2 viable cells/ml); and class M (cell survival between 10^2 and 10^4 cells/ml). We found that 8 of the 13 food isolates (61.5%) in group I and 2 of the 12 (16.7%) isolates in group II belonged to the R class (Table 1).

Given the variety in thermal resistance and genetic diversity among the *E. sakazakii* isolates, we predicted that a comparison of the gene expression profiles between heat-resistant isolates (class R) and heat-sensitive isolates (class S) might also show differences. To explore this issue, we performed a differential display PCR of *E. sakazakii* RNA obtained from representative class R (HT003, HT033) and class S (HT007, HT011) strains, using the GeneFishing DEG101 system (Seegene). Briefly, total RNA was isolated from *E. sakazakii* grown in TSB at 37 C at an OD_{600} of 0.40–0.45 and reverse-transcribed with the dT-ACP1 primer (5'-CTGTGAATGCTGCGACTACGATCCCCC(T)$_{18}$-3'). Each 50-ng aliquot of cDNA was then subjected to PCR with the following primer pairs: arbitrary ACP primers (A1 through A20) and the anchor dT-ACP2 primer (5'-CTGTGAATGCTGCGACTACGATXXX-XX(T)$_{15}$-3').

Among the differentially regulated genes that were clearly transcribed, partial genes for 16S rRNA, the heat-shock protein *groEL*, translation initiation factor *infB*, and histone-like protein *hnsB* were identified (Fig. 3A). To validate the results and to determine the relative abundance of the target sequences, two-step real-time quantitative PCR was performed using the SYBR Green Master mix in a LightCycler 480 (Roche). The primers, which were designed from the DEG sequences using Primer Express software ver.3.0 (Applied Biosystems), were as follows: *infB* (5'-GCTGCGGAAAC-GAGCAA-3' and 5'-TCCGCCTGAGCAGCTTTG-3'), 16S rRNA (5'-TCCCCTACGGTTACCTTGTTTC-3' and 5'-GCGCTTGCCACTTTGTGA-3'), *groEL* (5'-TGTTGCTGCGCCTACTTTCA-3' and 5'-AGGAAC GCGTAGCGAAACTG-3'), and *hnsB* (5'-GCAATG-GCGCGTGACTTC-3' and 5'-CGTAACGATGCGGA-ATTTCTC-3').

A real-time RT-PCR study using four each of representative heat-resistant and heat-sensitive isolates (class R—HT003, HT026, HT0031, and HT033; class S—HT007, HT011, HT023, and HT030) revealed that the high expression levels of the *groEL* and *hnsB* genes were not specific to either the R or S isolates (data not shown). Therefore, these seemed to be non-specific signals. In contrast, the *infB* gene was transcribed at higher levels by all the class R isolates than the class S isolates, in which it was either undetectable or transcribed at very low levels (Fig. 3B).

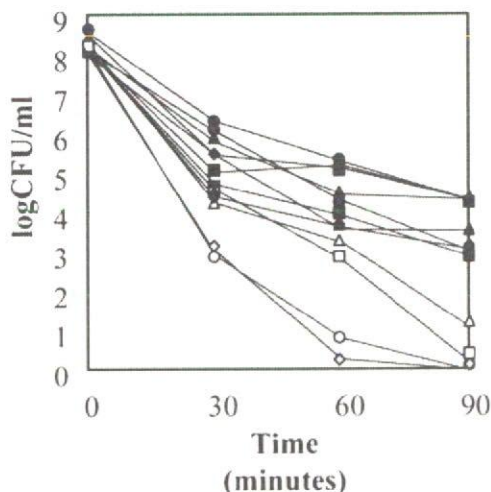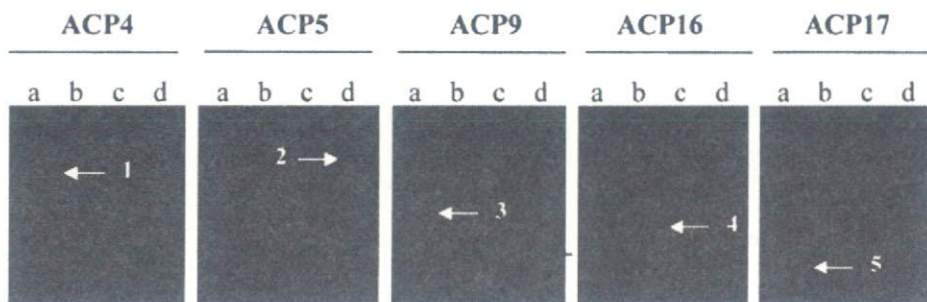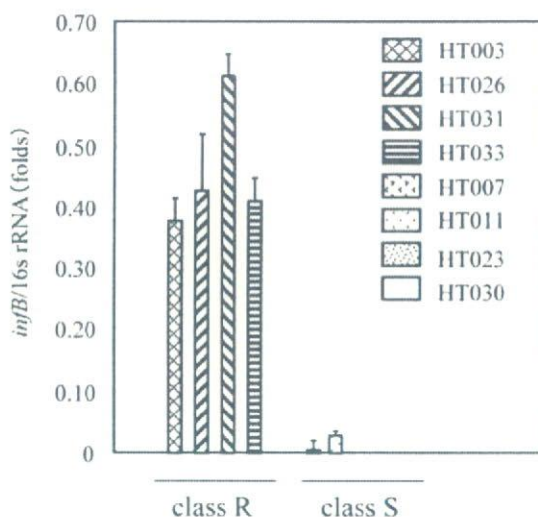PCR of the other *E. sakazakii* isolates, using the

Fig. 2. Survival of *E. sakazakii* in response to thermal stress. The graph shows survival data for representative isolates: closed symbols represent heat-resistant (class R) isolates HT003 (■), HT026 (◆), HT031 (▲), HT033 (●); half-tone (gray) symbols represent class M isolates HT008 (■), HT010 (◆), HT024 (▲), HT025 (●); open symbols denote the heat-sensitive (class S) isolates HT007 (□), HT011 (◇), HT023 (△), and HT030 (○).

A

| ACP4 | ACP5 | ACP9 | ACP16 | ACP17 |

a b c d   a b c d   a b c d   a b c d   a b c d

B

C

```
ATCC29554  536:ACAAGCTAAACGCGATGCCGCTGATAAAG-CGAAACGTGAAGCTGCGGAAACGAGCAAAGTGAGCAATCAACAAACTGACGAAGTGA-GCAAAGCTGCTCAGGCGGAAAAAGCCCGCCGC 653
ATCC29004  536:.......................................................................................................................... 653
HT033      536:.......................................................................................................................... 653
HT007      536:..................................................................................................................T....... 653
HT008      536:.......................................................................................................................... 653
HT023      536:.......................................................................................................................... 653
HT024      536:.......................................................................................................................... 653
HT028      536:.......................................................................................................................... 653
HT034      536:.......................................................................................................................T..T 653
HT011      536:.A....G..G..T..C..T..G..A....A.A...........................................C....A....C..A.............................T..T 629
HT030      536:.A.G.........T..GCTT..C..CC.....T....C...........AT..T.......................CA.CC...G..C.........T...................T..T 654
AJ002736   540:TA....G..G..T..C..G..G..A....A.A...............................G...........A.....C....A.A..C...A..................T..T 633
AJ002552   483:A....G......T..A..T..G..A....A.A...............................G...........TA...C...A.C..C.....C.................T 576
AJ002735   537:TA....G......T..A....G..A....A.A...............................C...TA...C...A.C..C.....T....GCAA...... 630
            .*..**.**.**.*.**.***.**.*...*.**............................*****...**.****..*..*.*.*...***.*....*.*.****...............**.**.
```

Fig. 3. PCR assay for characterization of thermo-tolerant *E. sakazakii*. A) ACP-PCR of *E. sakazakii* isolates. Representative *E. sakazakii* heat-resistant isolates HT003 (a) and HT033 (b) and heat-sensitive isolates HT007 (c) and HT011 (d) were examined for differential gene expression profiles using an arbitrary PCR assay. Each arrow indicates a band that was differentially expressed among the phenotypes. The numbers denote the identity of the bands (accession numbers in the parenthesis): 1 and 3. *infB* (AB265685); 2. 16S rRNA (AB265686); 4. *groEL* (AB265687); 5. *hnsB* (AB265688). ACP primers were as follows. ACP4, 5'-GTCTACCAGGCATTCGCTTCATXXXXXGCTGCTCGCG-3'; ACP5, 5'-GTCTACCAGGCATTCGCTTCA-TXXXXXAGTGCGCTCG-3'; ACP9. 5'-GTCTACCAGGCATTCGCTTCATXXXXXGATGCCGCTG-3'; ACP16, 5'-GTC-TACCAGGCATTCGCTTCATXXXXXGTCGACGGTG-3'; ACP17, 5'-GTCTACCAGGCATTCGCTTCATXXXXXCAA-GCCCACG-3'. B) Real-time PCR was used to calculate the relative expression ratio of *infB*/16S rRNA for each of four isolates in classes R and S. (C) Alignment of the *infB* sequence fragment for representative *E. sakazakii* isolates. *E. cloacae* (AJ002736), *S.* Typhimurium (AJ002552), and *K. oxytoca* (AJ002735). Nucleotides identical to ATCC29544 are shown as dots, and asterisks mean identical nucleotides among all isolates tested, respectively.

Table 2. Detection of *infB* mRNA in *E. sakazakii*

Class[a]	No. of isolates	No. of *infB* DNA +[b]	*infB* mRNA/16S rRNA (copies)[c]			
			<0.001	0.001–0.01	0.01–0.1	0.1–1.0
R	16	16	—	—	—	16
M	10	2	—	1 (HT010)	1 (HT024)	—
S	7	2	2 (HT007, 011)	—	—	—
Total	33	20	2	1	1	16

[a] The classification of heat-resistant phenotypes is shown (R, heat-resistant; M, intermediate; S, heat-sensitive).

[b] Numbers of isolates in which *infB* DNA was detected by PCR using the same primer pairs as for the real-time PCR assay.

[c] Relative gene expression values (*infB* mRNA/16S rRNA copies) are shown. Isolate names given in the parenthesis in some cases.

same *infB* primer pair as for the real-time PCR, failed to amplify *infB* DNA in several cases (Table 1), which suggested considerable genetic diversity in this region among *E. sakazakii* strains. To clarify this result, we performed PCR-based direct sequencing of the 876-bp *infB* fragment with a different primer set (5'-GCG-TAATAAACTGTAGCAGGAA-3' and 5'-CGTTCTCTTCAGCCATACGAC-3'), which was designed from the obtained sequence with the DNA walking speed up kit 2 (Seegene). PCR amplification of the 876-bp *infB* fragment could be successfully performed, and the sequence alignment showed that the target nucleotides used for the real-time PCR assay were highly variable among *E. sakazakii* isolates, and were also different from the *infB* sequences of other bacteria, such as *E. cloacae* (AJ002736), *Salmonella* Typhimurium (AJ002552), and *Klebsiella oxytoca* (AJ002735) (Fig. 3C). These findings support our interpretation of our finding that the *infB* DNA fragment could not be amplified from all the strains using our original primer set (Tables 1, 2).

Real-time PCR enables the rapid and sensitive detection of the causative agent from food and environment (14). *E. sakazakii* also can be detected by the genetic method with either the *rpsU-dnaG* genes of the macro-molecular synthesis (MMS) operon, or the 16–23S rRNA spacer region as targets (13, 17). Because the PCR assay detects the bacterial DNA regardless of viability, the result often did not correlate with the bacterial CFU recovered by the culture method. On the other hand, the real-time RT-PCR that targets RNA rather than DNA, not only provides a measure of cell viability but can also be used for quantitative analysis. Because the expression of the *infB* gene among *E. sakazakii* associates with the thermal tolerance as shown in this study, application of *infB* as a target for real-time RT-PCR assay should facilitate the selective detection and quantification of thermo-tolerant *E. sakazakii* in food and the environment. This could be a valuable analyti-

cal tool for identifying and tracing sources of environmental contamination in PIF processing facilities.

The *infB* gene encodes the prokaryotic translation initiation factor (IF2), a central macromolecular component of the ribosomal 70S initiation complex (12). In *E. coli*, *infB* encodes three forms of IF2 (IF2α, IF2β, and IF2γ). IF2β and IF2γ are translated in tandem from intact *infB* mRNA, and are not from post-transcriptionally truncated mRNAs (12). The IF2 family members have independent functions, suggesting there might be a difference in the functions of IF2 molecules between heat-resistant *E. sakazakii* and heat-sensitive isolates. The biological significance of the *infB* mRNA levels among the *E. sakazakii* isolates might therefore be in influencing the transcription/translation of a large number of genes. It would thus be helpful to obtain the complete genome sequence of *E. sakazakii*, to elucidate such regulation, as well as other functional roles of the stress-response mechanisms of the pathogen.

Despite finding differences in gene expression patterns in association with the differences in thermal resistance, we could not identify any clinical associations between the magnitude of stress-resistance and the severity of human disease, because *E. sakazakii* infection has not been reported in Japan. More isolates from humans need to be analyzed with these methods to better characterize the *E. sakazakii* in the food reservoir (5, 7).

References

1) Biering, G., Karlsson, S., Clark, N., Jonsdottir, K., Ludvigsson, P., and Steingrimsson, O. 1989. Three cases of neonatal meningitis caused by *Enterobacter sakazakii* in powdered milk. J. Clin. Microbiol. **27**: 2054–2056.

2) Breeuwer, P., Lardeau, A., Peterz, M., and Joosten, H.M. 2003. Desiccation and heat tolerance of *Enterobacter sakazakii*. J. Appl. Microbiol. **95**: 967–973.

3) Centers for Disease Control and Prevention. 2002. *Enterobacter sakazakii* infections associated with the use of powdered infant formula—Tennessee. 2001. MMWR Morb. Mortal. Wkly. Rep. **51**: 297–300.

4) Drudy, D., Mullane, N.R., Quinn, T., Wall, P.G., and Fanning, S. 2006. *Enterobacter sakazakii*: an emerging pathogen in powdered infant formula. Clin. Infect. Dis. **42**: 996–1002.

5) Drudy, D., O'Rourke, M., Murphy, M., Mullane, N.R., O'Mahony, R., Kelly, L., Fischer, M., Sanjaq, S., Shannon, P., Wall, P., O'Mahony, M., Whyte, P., and Fanning, S. 2006. Characterization of a collection of *Enterobacter sakazakii* isolates from environmental and food sources. Int. J. Food Microbiol. **110**: 127–134.

6) Edelson-Mammel, S.G., Porteous, M.K., and Buchanan, R.L. 2005. Survival of *Enterobacter sakazakii* in a dehydrated powdered infant formula. J. Food Prot. **68**: 1900–1902.

7) Food and Agriculture Organization, United Nations and World Health Organization. 2004. Regional Conference on Food Safety in Asia and the Pacific Annex (Seremban, Malaysia).

8) Iversen, C., and Forsythe, S. 2004. Isolation of *Enterobacter sakazakii* and other Enterobacteriaceae from powdered infant formula and related products. Food Microbiol. **21**: 771–776.

9) Iversen, C., Lancashire, L., Waddington, M., Forsythe, S., and Ball, G. 2006. Identification of *Enterobacter sakazakii* from closely related species: the use of Artificial Neural Networks in the analysis of biochemical and 16S rDNA data. BMC Microbiol. **6**: 28.

10) Iversen, C., Waddington, M., On, S.L.W., and Forsythe, S. 2004. Identification and phylogeny of *Enterobacter sakazakii* relative to *Enterobacter* and *Citrobacter* species. J. Clin. Microbiol. **42**: 5368–5370.

11) Lai, K.K. 2001. *Enterobacter sakazakii* infections among neonates, infants, children, and adults: case reports and a review of the literature. Medicine (Baltimore) **80**: 113–122.

12) Laursen, B.S., de Steffensen, S.A., Hedegaard, J., Moreno, J.M., Mortensen, K.K., and Sperling-Petersen, H.U. 2002. Structural requirements of the mRNA for intracistronic translation initiation of the enterobacterial *infB* gene. Genes Cells **7**: 901–910.

13) Liu, Y., Cai, X., Zhang, X., Gao, Q., Yang, X., Zheng, Z., Luo, M., and Huang, X. 2006. Real time PCR using *Taq*-Man and SYBR Green for detection of *Enterobacter sakazakii* in infant formula. J. Microbiol. Methods **65**: 21–31.

14) McKillip, J.L., and Drake, M. 2004. Real-time nucleic acid-based detection methods for pathogenic bacteria in food. J. Food Prot. **67**: 823–832. Review.

15) Nazarowec-White, M., and Farber, J.M. 1997. Thermal resistance of *Enterobacter sakazakii* in reconstituted dried-infant formula. Lett. Appl. Microbiol. **24**: 9–13.

16) Noriega, F.R., Kotloff, K.L., Martin, M.A., and Schwalbe, R.S. 1990. Nosocomial bacteremia caused by *Enterobacter sakazakii* and *Leuconostoc mesenteroides* resulting from extrinsic contamination of infant formula. Pediatr. Infect. Dis. J. **9**: 447–449.

17) Seo, K.H., and Brackett, R.E. 2005. Rapid, specific detection of *Enterobacter sakazakii* in infant formula using a real-time PCR assay. J. Food Prot. **68**: 59–63.

18) Simmons, B.P., Gelfand, M.S., Haas, M., Metts, L., and Ferguson, J. 1989. *Enterobacter sakazakii* infections in neonates associated with intrinsic contamination of a powdered infant formula. Infect. Control Hosp. Epidemiol. **10**: 398–401.

19) Van Acker, J., de Smet, F., Muyldermans, G., Bougatef, A., Naessens, A., and Lauwers, S. 2001. Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. J. Clin. Microbiol. **39**: 293–297.

乳児用調製粉乳中の *Enterobacter sakazakii* による感染

五十君靜信*　　朝　倉　　宏

A Brief Review of *Enterobacter sakazakii* in Powdered Infant Formula

Shizunobu Igimi* and Hiroshi Asakura

(National Institute of Health Sciences: 1-18-1 Kamiyoga,
Setagaya-ku, Tokyo 158-8501, Japan; * Corresponding author)

1. はじめに

Enterobacter sakazakii は，日本ではヒトへの感染に関し，これまであまり注目されたことはなかった．本菌が海外で乳児用調製粉乳を汚染し，乳幼児の細菌性髄膜炎に関与しているという情報を入手したとき，われわれは少々驚いた．というのは，これまで積極的に収集してきた国内で発生している新生児の細菌性髄膜炎の起因菌リストに本菌が含まれていなかったためである．平成13年度より開始された厚生労働科学研究班は，リステリア症に関する全国規模のアクティブサーベーランスを展開してきており，重症化したリステリア症の主要な病態である細菌性髄膜炎に関する情報を積極的に収集していた．乳幼児における細菌性髄膜炎の起因菌に関する情報については，髄膜炎菌研究班と協力しながら特に重点的に集めていたわけであるが，国内における *E. sakazakii* による細菌性髄膜炎の症例は全く記憶になかった．

2004年2月と2006年5月に，スイスのジュネーブのWHO本部において"乳児用調製粉乳中の *Enterobacter sakazakii* に関する FAO/WHO 合同専門家会議" が開催された．これらの会議において，*E. sakazakii* の性質，疫学，乳児用調製粉乳からの感染リスクに関する科学的な考察がされ，本菌の乳児用調製粉乳汚染は乳児の感染および疾患の原因となると結論された．そこで本稿では，本菌に関するこれまでの情報をまとめた．

2. エンテロバクター・サカザキとは

エンテロバクター属菌 (*Enterobacter* spp.) は，通性嫌気性のグラム陰性桿菌で，ヒト・動物の腸管内や環境中に広く分布している．このうち，エンテロバクター・サカザキ (*Enterobacter sakazakii*) は，形態学的特長から，かつては黄色コロニー形成 (Yellow-pigmented) *Eneterobacter cloacae* として呼称されていた[1]が，現在では別種として分類されている．健常人では本菌にさらされても不顕性で経過することがほとんどであるが，乳幼児，特に未熟児や免疫不全児，低体重出生児を中心として，敗血症や壊死性腸炎を発症することがあり，重篤な場合には髄膜炎

* 国立医薬品食品衛生研究所: 〒158-8501 東京都世田谷区上用賀 1-18-1

を併発する[2]～[18]．

本菌は，生化学性状のみでは同定できないことも多く，16S リボゾーム配列による遺伝学的分類が行われる．本菌の 16S リボゾーム RNA 配列は，*E. cloacae* と約97.0%一致するが，一方で *Citrobacter koseri* とも97.8%と高い相同性を有しており[19]，本菌の分類は今後議論の対象となりうると思われる．

本症の感染経路についてはいまだ明らかとはなっていないが，乳児用調製粉乳を介した感染例が多数報告されており，最も有力な感染源として認識されている．一方でわが国における食品の汚染実態ならびに汚染食品の喫食による発症状況については明らかとなっていない．

3. 動物・環境中における分布

E. sakazakii の動物における発症例はこれまでに報告されていない．本属菌は土壌，水，動物，汚水，ヒト糞便などから高頻度に検出される[1]が，一方，トウモロコシ，キュウリ，レモンなどといった果実・野菜からもしばしば検出される[20]．このことから，同じ腸内細菌科に属する大腸菌とは異なり，本菌は植物や環境を本来の生息域として存在していると考えられている[21]．これまでに本菌が分離された動物としては，ラット[22]やハエ[23],[24]が挙げられるが，ウシなど家畜からの検出に関する報告は少ない．一方で，ハエ (*Stomoxys calcitrans*) は重要な伝播体として関与していると推察されている．こうした節足動物が物理的に本菌を媒介することで食品汚染を引き起こし，これを喫食することで感染・発症している可能性が1つの感染経路として想定される．

4. ヒトにおける疾病

本感染症による新生児髄膜炎は1958年にイギリスで初めて発生が確認された[15]．その後，カナダ，ベルギー，デンマーク，アイスランド，ドイツ，ギリシャ，イスラエル，オランダ，スペイン，アメリカなど世界各国で，主に散発性ではあるが，敗血症，壊死性腸炎，脳膿瘍を呈した症例が報告されている．乳幼児を中心に過去に発生した主な感染事例について表1に示した．

発症数を年齢別に見ると，明らかに新生児・乳幼児の発

表 1.　E. sakazakii による乳幼児感染事例（抜粋）

発生年	国名	感染源	患者数	死亡者数	文献
1958	イギリス	不明	2	2	15
1965	デンマーク	不明	1	0	8
1979	アメリカ	不明	1	0	11
1981	アメリカ	不明	1	0	9
1983	オランダ	乳児用調製粉乳の疑い	8	0	12
1985	ギリシャ	不明	1	0	2
1986〜87	アイスランド	乳児用調製粉乳	3	1	4, 6
1987	アメリカ	不明	2	0	18
1988	アメリカ	乳児用調製粉乳	4	0	14
1990	アメリカ	ブレンダー	1	0	13
1990	アメリカ	不明	1	0	7
1995〜96	アメリカ	不明	5	3（成人のみ）	10
1998	ベルギー	乳児用調製粉乳の疑い	12	2	16
2000	アメリカ	不明	1	0	5
1999〜2000	イスラエル	乳児用調製粉乳	2	0	3
2001	アメリカ	乳児用調製粉乳	10	1	17, 44

生が多いが，成人においても発生は認められており，膣感染を起こした症例もある[25), 26)]．幼児における感染リスクは，免疫不全，未熟児，2.5 kg 以下での低体重出生などのファクターにより高まる．ただ，これらの疫学的知見については情報が乏しく，感染制御を行ううえで国内外における発生状況の把握，そして食品汚染に関する定量的汚染分析は，予防・治療へ直結することから早急に解決すべき課題である．なお，本感染症の国内における発生はいまだ確認されておらず，厚生労働科学研究費で乳幼児食品中の有害物質および病原微生物に関する研究班が立ち上がり，調査を進めているところである．

5.　病原性・発症菌数に関する知見

本菌の病原性については，いまだ明らかとなっていないが，E. cloacae ではこれまでに外毒素，エアロバクチン，ヘマグルチニンなどが主な病原因子として報告されている[27)]．また，Pagotto らは乳のみマウスを用いて食品由来 8 株，臨床患者由来 9 株および標準株（ATCC29544）について経口・腹腔内感染時の致死活性ならびにエンテロトキシン産生量を検討し，菌株間でのエンテロトキシン産生と細胞毒性（CHO, Vero, Y-1）に有意な差異があることを示している．また，経口感染による最少致死菌数は 10^8 個であったことから，新生児の最少致死菌数は非常に高く，極めて高濃度の汚染を受けた乳児用調製粉乳を長時間，増殖可能な温度域で放置するといった，衛生上の不備により本症が発生していると結論づけている[28)]．

これまでに新生児，乳幼児における E. sakazakii 感染事例は 76 症例以上報告されているが，ヒトにおける感染菌数についての知見は得られていない．Iverson らは，髄膜炎という臨床症状を判断材料として Escherichia coli O157 : H7, Listeria monocytogenes 血清型 4b, Neisseria meningitidis などの感染菌数である 10^3 個程度ではないかと考察している[29)]．

6.　その他の性質

(1)　熱抵抗性

乳児用調製粉乳は温水を用いて溶解することから，本菌の耐熱性に関して複数の検討がなされている．Enterobacter 属菌は相対的に耐熱性が高いとの報告もある[16), 30), 31)]が，加熱殺菌処理を通じて本菌が生存しているかについては疑念も提唱されている[32)〜35)]．Nazarowec-White と Farber らは，乳児用調製粉乳中での本菌の加熱抵抗性について初めて検討を行っている．彼らは食品由来 5 株と臨床由来 5 株の計 10 株を乳児用調製粉乳 1 mL あたり 10^7 個となるよう接種し，52〜60℃加熱下での D 値が 54.8 分〜2.5 分で，$10^{6 \sim 7}$ 個の殺菌には 60℃で 15〜17.5 分の加熱が必要と算出している[34)]．また，Iversen らは乳児用調製粉乳中での D 値を 54〜62℃で 16.4〜0.3 分と算出している[21)]．E. sakazakii では菌株による熱抵抗性の違いは約 20 倍にも及び，標準株（ATCC 51329）は最も熱に弱く，臨床由来株が最も熱抵抗性を示すことが知られている[33)]．本菌は代表的な乳製品汚染菌である Listeria monocytogenes に比べると熱抵抗性は強いといわれているが[36)]，他の Enterobacter 属菌とは同様のものであり，一般的な加熱殺菌処理および高温短時間殺菌（HTST, 71.2 C, 15 秒）によって十分死滅しうる[35)]．

(2)　薬剤耐性

タイでの薬剤耐性調査によると，β-ラクタム系薬剤に対する耐性インテグロンが E. sakazakii ゲノム上に座位することが報告されている[37)]．本菌はヒトの腸管内にも日和見感染菌として頻繁に通過することから，薬剤耐性の伝播源として留意する必要があると思われる．

7.　乳児用調製粉乳の汚染実態

Muytjens らは，35 か国で製造された乳児用調製粉乳計 141 検体を収集し，52.5％で腸内細菌科の細菌汚染があり，このうち 20 検体（14.2％）より E. sakazakii を検出している[38)]．Leuscher（2004）らの報告においても 58 検

体中8検体(13.8%)から E. sakazakii は検出されており[39]，本菌は乳児用調製粉乳を広く汚染していると思われる．一方で，汚染菌数については100 g あたり0.36～66個と極めて低い[38),40]．このような汚染菌数と1回に摂取する量が乳児用調製粉乳18 g に相当することから試算すると，幼児一人が哺乳時に6,000個の菌の暴露を受けるには，少なくとも14世代の増殖が粉乳もしくは調整後の液体乳中で必要となる．これを本菌の増殖時間から求めると，37℃で7時間，21℃で17.9時間，18℃で1.7日，10℃で7.9日，そして8℃では9日を要することになる[29]．しかしながら，この計算は理論値であり，乳児用ミルクがどの段階で汚染を受けているかによって大きく変化する．また，本菌の繊毛はヘマグルチニン活性と関連していることが知られているが[41]，繊毛の保有は菌株によってまちまちであるため，菌株側のファクターと相まって，容器素材なども増殖変動に大きく影響すると思われる．

　本菌は，発酵パン，発酵飲料，レタス，香辛料，米，チーズ，ミンチ肉，ソーセージ[34),42]など多岐にわたる食品から分離されている．Iversen らは乳児用調製粉乳82検体およびその他の食品404検体について調査を行い，複数の E. sakazakii を検出しているが，いずれも大腸菌群，サルモネラ属菌は検出されておらず，これらを汚染指標菌とした E. sakazakii の制御は不可能と結論づけている[42]．また，乳児用調製粉乳はその汚染実態から感染源として重要性であることは先に述べたとおりであるが，溶解調整時の二次汚染による事例も2001年に報告されている[17),43]．実際に乳児用調製粉乳自体は E. sakazakii の汚染を受けていないにもかかわらず，調製に用いたブレンダーから本菌が検出された報告もある[13]．したがって本菌の食品汚染は製造環境あるいは調製工程における二次汚染がかなりの部分を占めている可能性が高い．実際に，新生児髄膜炎症例において本菌はスプーンや洗浄スポンジから検出されている[12]．その他，水，パイプ，バイオフィルム，ビアマグに至るまで，本菌が感染にかかわる経路は多岐にわたっている[44)～47]．したがって，調整時の衛生管理も本症を予防するために重要なファクターとなる．

8. 検出法と疫学的解析法

　本菌の検出には，米国食品医薬局(FDA)の推奨する方法[48]が最初に示され普及している．先述のとおり，本菌の汚染菌数は少数であると推察されることから，サンプリング量は，わが国の食品衛生検査で一般的に適用される25グラムではなく，合計333グラムを検査対象とする最確数法(MPN 3本法)である．増菌培地には Enterobacteriaceae enrichment broth (EE 培地)を用い，選択培地である VRBG 寒天培地に直接塗抹する．疑わしいコロニーが出現した場合には少なくとも5個を釣菌し，トリプトソイ寒天培地に塗抹し，黄色のコロニーであった場合には生化学試験を実施して菌種の同定を行う．また同時に最確数法により汚染菌数を算出する．本法では複数の

類縁菌も分離され，同定には生化学性状試験によってのみ行われることから，時間・労力・コストを要する．国際的な検査法としては，ミルク製品からの検出法として IDF/ISO 法も示されている．性能の高い選択培地の開発は今後の課題である．海外のメーカーにより培地の比較試験が行われ，E. sakazakii に特異性の高い酵素基質培地(DFI 培地)も開発されている[49]．また，リアルタイム PCR 法を用いた遺伝子検出法も検討されており[50),51]，今後，食品検査への応用が期待される．パルスフィールドゲル電気泳動(PFGE)法を用いた遺伝子解析も本菌において実施されており，感染源の特定に結びつく解析手法として有用と思われる[35]．さらに，菌体タンパクの網羅的発現解析(プロテオーム解析)により，臨床患者由来株で顕著に発現するタンパク質因子も同定されている[52]．当該因子をコードする遺伝子の定量的特異検出が可能となれば，病原性のある菌株の選択的検出にも応用できるかもしれない．

9. 本菌に関する国際的動向

　乳児用調製粉乳は乳製品という特性から無菌製品ではなく，E. sakazakii による汚染リスクは常に想定される．しかしながら，発症に必要となる菌数は把握されておらず，温度管理や調理環境の衛生管理の徹底などで予防しなければならないのが現状である．乳児用調製粉乳における感染リスク低減の試みとして，アメリカでは2001年にガイドラインが発行され，施設における乳児用調製粉乳の調整，授乳方法などについて勧告している[43]が，これらは主に病院などの施設に対するものであり，家庭向けではない．2005年2月には乳児用調製粉乳中の E. sakazakii など微生物に関する FAO/WHO 合同ワークショップが開催され，E. sakazakii とサルモネラによる乳児用調製粉乳汚染は乳児の感染および疾患の原因となると結論づけられた．E. sakazakii はあらゆる年齢の小児に疾患を引き起こすが，新生児，特に早産，未熟児，免疫障害児に最もリスクが高く，HIV 陽性の母親から生まれた乳児は乳児用調製粉乳を必要とし，リスクは高い．現在の技術では粉乳の完全無菌化は不可能であり，殺菌工程以降の汚染を予防することが重要といえる．現在，Codex の乳児用調製粉乳のサルモネラに関する規格は，25グラムずつ60検体を検査し，すべて陰性であるが，E. sakazakii に関する規格については議論が続いている．E. sakazakii 汚染は低レベルであっても重大な危険因子と考えられ，予備的リスクアセスメントに基づき，保管・調合・授乳時において種々の介入手段によりリスクを効果的に減じるようにしなければならない．それらの手段として，冷凍および短期間保存による本菌の増殖抑制[14]，乳児用調製粉乳の調整段階で使用する器具・保存容器の煮沸消毒の徹底，調整後の冷蔵保存，授乳直前の電子レンジ加熱などが推奨される．その他，温水浴槽は主要な汚染源となりうることから[53]，清潔な管理を徹底する必要がある．

　乳児用調製粉乳の細菌汚染を完全に断つことは困難であ

り，上記手段などによる哺乳までの取扱い上の衛生措置を
徹底するよう，啓蒙していくことが重要である．また，ハ
イリスクな乳児については，乳児用調製粉乳を溶解すると
きに 80℃の高温で調整し，本菌を死滅させることは現状
における最善の予防策であり，その後の迅速な温度調整・
哺乳は言うまでもない．

文　　献

1) Sakazaki, R., *"Enterobacter cloacae"*, Buchanan, R. E.,
Gibbons, N. E., ed., Bergey's Manual of Determinative
Bacteriology, 8th ed. The Williams and Wilkins Co., Bal-
timore, 1974, p. 325.

2) Arseni, A., Malamou-Lada, H., Kostalos, C., Sta-I-Kou,
A., Koustia, H., A fatal case of sepsis in a premature
newborn baby associated with *Enterobacter sakazakii*
bacteremia. Acta Microbiol. Hell., 29, 402–407 (1985).

3) Bar-Oz, B., Preminger, A., Peleg, O., Block, C., Arad, I.,
Enterobacter sakazakii infection in the newborn. Acta
Paediatr., 90, 356–358 (2001).

4) Biering, G., Karlsson, S., Clark, N. C., Jonsdottir, K. E.,
Ludvigsson, P., Steingrimsson, O., Three cases of neo-
natal meningitis caused by *Enterobacter sakazakii* in
powdered milk. J. Clin. Microbiol., 27, 2054–2056
(1989).

5) Burdette, J. H., Santos, C., *Enterobacter sakazakii* brain
abscess in the neonate: the importance of neuro-
radiologic imaging. Pediatr. Radiol., 30, 33–34 (2000).

6) Clark, N. C., Hill, B. C., O'Hara, C. M., Steingrimsson, O.,
Cooksey, R. C., Epidemiologic typing of *Enterobacter
sakazakii* in two neonatal nosocomial outbreaks.
Diagn. Microbiol. Infect. Dis., 13, 467–472 (1990).

7) Gallagher, P. G., Ball, W. S., Cerebral infarctions due to
CNS infection with *Enterobacter sakazakii*. Pediatr.
Radiol., 21, 135–136 (1991).

8) Joker, R. N., Norholm, T., Siboni, K. E., A case of neo-
natal meningitis caused by a yellow *Enterobacter*.
Danish Med. Bull., 12, 128–130 (1965).

9) Kleiman, M. B., Allen, S. D., Neal, P., Reynolds, J.,
Meningoencephalitis and compartmentalization of the
cerebral ventricles caused by *Enterobacter sakazakii*. J.
Clin. Microbiol., 14, 352–354 (1981).

10) Lai, K. K., *Enterobacter sakazakii* infections among neo-
nates, infants, children, and adults: case reports and a
review of the literature. Med. Baltimore, 80, 113–122
(2001).

11) Monroe, P. W., Tift, W. L., Bacteremia associated with
Enterobacter sakazakii (yellow, pigmented *Enterobacter
cloacae*). J. Clin. Microbiol., 10, 850–851 (1979).

12) Muytjens, H. L., Zanen, H. C., Sonderkamp, H. J.,
Kollee, L. A., Wachsmuth, I. K., Farmer, J. J. 3rd, Analy-
sis of eight cases of neonatal meningitis and sepsis
due to *Enterobacter sakazakii*. J. Clin. Microbiol., 18,
115–20 (1983).

13) Noriega, F. R., Kotloff, K. L., Martin, M. A., Schwalbe,
R. S., Nosocomial bacteremia caused by *Enterobacter
sakazakii* and *Leuconostoc mesenteroides* resulting from
extrinsic contamination of infant formula. Pediatr.

14) Simmons, B. P., Gelfand, M. S., Haas, M., Metts, L.,
Ferguson, J., *Enterobacter sakazakii* infections in neo-
nates associated with intrinsic contamination of a pow-
dered infant formula. Infect. Control Hosp. Epidemiol.,
10, 398–401 (1989).

15) Urmenyi, A. M. C., Franklin, A. W., Neonatal death
from pigmented coliform infection. Lancet, 1, 313–315
(1961).

16) van Acker, J., de Smet, F., Muyldermans, G., Bougatef,
A., Naessens, A., Lauwers, S., Outbreak of necrotizing
enterocolitis associated with *Enterobacter sakazakii* in
powdered milk formula. J. Clin. Microbiol., 39, 293–
297 (2001).

17) Weir, E., Powdered infant formula and fatal infection
with *Enterobacter sakazakii*. CMAJ, 166, 1570 (2002).

18) Willis, J., Robinson, J. E., *Enterobacter sakazakii* meningi-
tis in neonates. Pediatr. Infect. Dis. J., 7, 196–199
(1988).

19) Iversen, C., Waddington, M., On, S. L., Forsythe, S., Iden-
tification and phylogeny of *Enterobacter sakazakii* rela-
tive to *Enterobacter* and *Citrobacter* species. J. Clin.
Microbiol., 42, 5368–5370 (2004).

20) Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F.,
Kloepper, J. W., Bacterial endophytes in agricultural
crops. Can. J. Microbiol., 43, 895–914 (1997).

21) Iversen, C., Lane, M., Forsythe, S. J., The growth
profile, thermotolerance and biofilm formation of
Enterobacter sakazakii grown in infant formula milk.
Lett. Appl. Microbiol., 38, 378–82 (2004).

22) Gakuya, F. M., Kyule, M. N., Gathura, P. B., Kariuki, S.,
Antimicrobial resistance of bacterial organisms
isolated from rats. East Afr. Med. J., 78, 646–649
(2001).

23) Hamilton, J. V., Lehane, M. J., Braig, H. R., Isolation of
Enterobacter sakazakii from midgut of *Stomoxys
calcitrans*. Emerg. Infect. Dis., 9, 1355–1356 (2003).

24) Kuzina, L. V., Peloquin, J. J., Vacek, D. C., Miller, T. A.,
Isolation and identification of bacteria associated with
adult laboratory Mexican fruit flies, *Anastrepha ludens*
(Diptera: Tephritidae). Curr. Microbiol., 42, 290–294
(2001).

25) Hawkins, R. E., Lissner, C. R., Sanford, J. P., *Entero-
bacter sakazakii* bacteremia in an adult. South Med. J.,
84, 793–795 (1991).

26) Ongradi, J., Vaginal infection by *Enterobacter sakazakii*.
Sex Transm. Infect. 78, 467 (2002).

27) Keller, R., Pedroso, M. Z., Ritchmann, R., Silva, R. M., Oc-
currence of virulence-associated properties in *Entero-
bacter cloacae*. Infect. Immun., 66, 645–649 (1998).

28) Pagotto, F. J., Nazarowec-White, M., Bidawid, S.,
Farber, J. M., *Enterobacter sakazakii*: infectivity and en-
terotoxin production *in vitro* and *in vivo*. J. Food Prot.,
66, 370–375 (2003).

29) Iversen, C., Forsythe, S., Risk profile of *Enterobacter
sakazakii*, an emergent pathogen associated with
infant milk formula. Trends Food. Sci. Technol., 14,
443–454 (2003).

Infect. Dis., 9, 447–449 (1990).

30) Buchanan, L. R., *E. sakazakii* resistance-thermal and other. 2003. U.S. Food and Drug Administration, Food Advisory Committee Mtg., March 18–19, Washington, DC.

31) Nazarowec-White, M., Farber, J. M. Thermal resistance of *Enterobacter sakazakii* in reconstituted dried-infant formula. Lett. Appl. Microbiol., 24, 9–13 (1997).

32) Breeuwer, P., Lardeau, A., Peterz, M., Joosten, H. M., Desiccation and heat tolerance of *Enterobacter sakazakii*. J. Appl. Microbiol., 95, 967–973 (2003).

33) Edelson-Mammel, S. G., Buchanan, R. L., Thermal inactivation of *Enterobacter sakazakii* in rehydrated infant formula. J. Food. Prot., 67, 60–63 (2004).

34) Nazarowec-White, M., Farber, J. M., *Enterobacter sakazakii*: a review. Int. J. Food Microbiol., 34, 103–113 (1997).

35) Nazarowec-White, M., Farber, J. M., Phenotypic and genotypic typing of food and clinical isolates of *Enterobacter sakazakii*. J. Med. Microbiol., 48, 559–567 (1999).

36) Piyasena, P., Liou, S., McKellar, R. C. Predictive modeling of inactivation of *Listeria* spp. in bovine milk during HTST pasteurization. Int. J. Food Microbiol., 39, 167–173 (1998).

37) Girlich, D., Poirel, L., Leelaporn, A., Karim, A., Tribuddharat, C., Fennewald, M., Nordmann, P., Molecular epidemiology of the integron-located VEB-1 extended-spectrum beta-lactamase *in* nosocomial enterobacterial isolates in Bangkok, Thailand. J. Clin. Microbiol., 39, 175–182 (2001).

38) Muytjens, H. L., Roelofs-Willemse, H., Jaspar, G. H., Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. J. Clin. Microbiol., 26, 743–746 (1988).

39) Leuscher, R. G. K., Baird, F., Donald, B., Cox, L. J., A medium for the presumptive detection of *Enterobacter sakazakii* in infant formula. Food Microbiol., 21, 527–533 (2004).

40) Nazarowec-White, M., Farber, J. M., Incidence, survival, and growth of *Enterobacter sakazakii* in infant formula. J. Food Prot., 60, 226–230 (1997).

41) Adegbola, R. A., Old, D. C., Fimbrial haemagglutinins in *Enterobacter* species. J. Gen. Microbiol., 129, 2175–2180 (1983).

42) Iversen, C., Forsythe, S., Isolation of *Enterobacter sakazakii* and other Enterobacteriaceae from powdered formula milk and related products. Food Microbiol., 21, 771–777 (2004).

43) Himelright, I., Harris, E., Lorch, V., Anderson, M., *Enterobacter sakazakii* infections associated with the use of powdered infant formula-Tennessee, 2001. J. Am. Med. Assoc., 287, 2204–2205 (2002).

44) Al-Hadithi, H. T., Al-Edani, T. A. A., A comparative study on the antibiotic susceptibility of six species of faecal *Enterobacter* isolated from aquatic and clinical sources. Dirasat (Pure Appl. Sci.), 22B, 35–41 (1995).

45) Bartolucci, L., Pariani, A., Westfall, F., Gardini, F., Guerzoni, M. E., Interaction by microbiological processes between water, biofilm and pipe material in water distribution systems. A proposed method for determining bacterial colonization in drinking water pipe networks. Water Supply, 14, 457–463 (1996).

46) Oliver, E. D., Atypical, non-lactose fermenting isolates shown to be total coliforms by the α-galactosidase reaction. Proc.Water Qual. Technol. Conf., 225–231 (1997).

47) Schindler, P. R., Metz, H., Coliform bacteria in rinsed beer mugs- identification with the API 20 E system and resistance behavior. Offentliche Gesundheitswesen, 52, 592–597 (1990).

48) U. S. Food and Drug Administration. Center for Food Safety and Applied Nutrition, July 2002. Isolation and Enumeration of *Enterobacter sakazakii* from Dehydrated Powdered Infant Formula. Available at: http://www.cfsan.fda.gov/‾comm/mmesakaz.html

49) Iversen, C., Druggan, P., and Forsythe, S., A selective differential medium for *Enterobacter sakazakii*, a preliminary study Int. J. Food Microbiol., 96, 133–139 (2004).

50) Liu, Y., Cai, X., Zhang, X., Gao, Q., Yang, X., Zheng, Z., Luo, M., Huang, X., Real time PCR using TaqMan and SYBR Green for detection of *Enterobacter sakazakii* in infant formula. J. Microbiol. Methods., 65, 1–31 (2006).

51) Seo, K. H., Brackett, R. E., Rapid, specific detection of *Enterobacter sakazakii* in infant formula using a real-time PCR assay. J. Food Prot., 68, 59–63 (2005).

52) Williams, T. L., Monday, S. R., Edelson-Mammel, S., Buchanan, R., Musser, S. M., A top-down proteomics approach for differentiating thermal resistant strains of *Enterobacter sakazakii*. Proteomics, 5, 4161–4169 (2005).

53) Muytjens, H. L., Kollee, L. A., *Enterobacter sakazakii* meningitis in neonates: causative role of formula? Pediatr. Infect. Dis. J., 9, 372–373 (1990).

Original

Nitric Acid-based Partial-digestion Method for Selective Determination of Inorganic Arsenic in Hijiki and Application to Soaked Hijiki

Megumi Hamano-Nagaoka[1],*, Ken'ichi Hanaoka[2], Masakatsu Usui[2], Tsutomu Nishimura[3] and Tamio Maitani[1]

[1]National Institute of Health Sciences: 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; [2]Department of Food Science and Technology, National Fisheries University: 2-7-1 Nagata-honmachi, Shimonoseki 759-6595, Japan; [3]Japan Food Research Laboratories: 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025, Japan; * Corresponding author

Because there is a great difference between the toxicity of inorganic arsenic (As) and organic As in food, the JECFA has set a PTWI value for inorganic As (iAs) rather than for total As. The difference in As toxicity makes it necessary to extract iAs completely from food samples for toxicological analysis, but complete extraction of As from most foods including seaweed has not been achieved to date. We developed a partial-digestion method that uses nitric acid as a solvent in order to extract almost all arsenicals from the solid matrix of hijiki (*Hizikia fusiforme*, a brown alga) samples. In this method, organic As species were not converted into iAs. HPLC/ICP-MS was then used to determine the concentration of iAs. Total As was measured by hydride generation-atomic absorption spectrometry. The adopted conditions for 0.1 g of ground fine powder sample were: 2 mL of 0.3 mol/L nitric acid; heating, 80℃ for 1 hr. Intra-laboratory validation of the method showed good precision and accuracy. The repeatability and intermediate precision for iAs were 1.5% and 1.5%, respectively. The LOD and LOQ for iAs were 0.14 and 0.46 mg/kg dry weight, respectively. Recovery studies performed by spiking 0.5 mg/kg dry weight as the LOQ level and by spiking 3 mg/kg dry weight as the iAs concentration of an un-spiked hijiki sample showed good accuracy. The method was applied to hijiki samples after a water soaking process and a water soaking and simmering process. The results suggested that the As concentration in hijiki after both processes was lower than that before the treatments and that the water soaking and simmering process reduced the iAs concentration much more effectively than the water soaking process.

Key words: arsenic; inorganic arsenic; hijiki; HPLC/ICP-MS; partial digestion; nitric acid; soaking

Introduction

Since inorganic arsenic (As) in food is much more toxic than organic As[1),2)], the JECFA (the Joint FAO/WHO Expert Committee on Food Additives) has established a provisional tolerable weekly intake (PTWI) level in terms of the value of inorganic As (iAs) (0.015 mg/kg bw/week). In July 2004, the Food Standards Agency of the UK issued a warning advising the public not to eat the seaweed *Hizikia fusiforme* (hijiki), because it contained a high level of iAs[3)].

One of the requirements for the risk assessment of food containing As is a quantitative or nearly quantitative determination method of arsenicals that does not alter the original chemical species. Moreover, quantitative extraction is also essential, because extraction procedures may selectively remove non-toxic species from food and/or leave the toxic species unextracted within the solid matrix.

Since the 1970s, the speciation of As in seafood has attracted great attention, because As poisoning *via* seafood has not been observed in spite of the high As content. Various analytical methods have been used for speciation, and it become feasible to determine iAs and methylated As species separately[4)-6)].

Sonication in combination with methanol/water (or methanol alone) as an extraction solvent has been used in an attempt to quantitatively extract arsenicals from seafood[7)]. However, since methanol is an inadequate solvent for iAs extraction[8),9)], water alone has also been used as the solvent to extract iAs because of its high polarity[10),11)]. Moreover, attempts to utilize enzyme-assisted extraction have been reported for seafood[12),13)]. Quantitative extraction from some foods has been achieved, but it has been difficult to achieve thorough extraction from samples of seaweed, including hijiki[14)]. Accelerated solvent extraction (ASE) is a relatively new extraction technique that makes it possible to optimize

solvent mixtures, pressure applied, temperature, and time to achieve a more quantitative extraction[14]-[17]. However, As extraction from some foods is incomplete even with ASE[9].

To achieve complete extraction, a partial-digestion method may be effective. However, an alkali-digestion method was considered to be inadequate for the extraction of iAs, because of low recovery rates*1. This paper reports a partial acid-digestion method. Nitric acid was used to completely extract iAs from a hijiki sample matrix under conditions such that organic As species were not converted to iAs. This method was coupled with HPLC/ICP-MS to determine As species including iAs. The developed method was applied to soaked hijiki samples.

Materials and Methods

Reagents

Sodium arsenate (Na_2HAsO_4), sodium arsenite ($NaAsO_2$), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and trimethylarsine oxide (TMAO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Arsenobetaine (AB) was obtained from Trichemical Laboratory (Yamanashi, Japan). Ultra-pure analytical grade nitric acid (68%) and tetramethylammonium hydroxide (TMAH, 20%) (TAMAPURE-AA-100) were purchased from Tama Chemical Industry (Kanagawa, Japan). Other chemicals were of reagent grade or of the highest grade commercially available. All laboratory ware used was immersed in approximately 2 mol/L HNO_3 at least overnight and rinsed with ultra-pure water prepared with a Milli-Q SP Reagent Water System (Millipore, Bedford, MA) to avoid contamination with various ions. Water of this grade was used throughout the experiment.

Hijiki

Hijiki specimens in the form of dried food were obtained from supermarkets in the Tokyo Metropolitan area and by mail order in Japan, and were freeze-dried. In the water soaking process or the water soaking and simmering process, they were freeze-dried after the process. Before analysis, all samples were carefully ground into a fine powder sample with a grinder.

Water soaking process and water soaking and simmering process

(1) Water soaking process: The hijiki sample was soaked in a 40 volumes of water at room temperature for 30 min, and the swollen hijiki sample was then rinsed twice with 40 volumes of water.

(2) Water soaking and simmering process: After the above-described soaking process, a swollen hijiki sample having a wet weight of 10 g was simmered at

---

*1 Miyashita M. *et al.* "Speciation analysis of arsenicals in animal feed by hydride generation-cold trap-atomic absorption spectrometry". Proceedings of '04 Tsukuba Seminar. Tsukuba, Japan, 2004-7-1/2. Discussion Group for Plasma Spectrochemistry in Japan, 2004, p. 37-45.

100°C in 200 mL of water for 30 min, and then rinsed twice with 200 mL of water.

Extraction procedure for hijiki

A ground hijiki powder sample (0.1 g dry weight) was mixed with 2 mL of nitric acid solution in a 10 mL capped high density polyethylene (HDPE) centrifuge tube. The mixture was heated on an aluminum heat block. After cooling, TMAH solution was added to the mixture to adjust the pH to 3.0, and the solution obtained was diluted to 50 mL with water. Finally, the solution was diluted with an appropriate volume of 0.1 mol/L nitric acid, and passed through a 0.45 μm filter prior to analysis. The established conditions for the extraction of arsenicals in hijiki coupled with HPLC/ICP-MS were: hijiki sample size, 0.1 g dry weight (finely ground); extraction solution, 0.3 mol/L nitric acid solution; extraction temperature, 80°C; extraction time, 1 hr.

Determination of iAs by HPLC/ICP-MS

The HPLC apparatus (Agilent 1200 Series, Agilent Technologies, Tokyo, Japan) was equipped with a column (CAPCELL PAK C_{18} MG, 4.6 mm i.d.×250 mm, Shiseido Ltd., Tokyo, Japan). The solvent (pH 3.0), composed of 10 mmol/L sodium 1-butanesulfonate, 4 mol/L TMAH, 4 mmol/L malonic acid, and 0.05% methanol, was used for isocratic elution at a flow rate of 0.75 mL/min. The sample solution (20 μL) was applied to the column and eluted at room temperature. The retention time did not change at this temperature in this study.

As an ICP-MS, an Agilent 7500ce ICP-MS system (Agilent Technologies) was used. The analytical conditions were: RF power, 1.6 kW; plasma gas, 15 L/min; carrier gas, 0.70 L/min; auxiliary gas, 0.90 L/min; makeup gas, 0.43 L/min. Signal ion monitoring at m/z 75 and a sampling rate of 1 Hz were used to collect the chromatographic data.

Total As analysis

A ground hijiki fine powder sample (0.1-1 g dry weight) was weighed, transferred to a Kjeldahl flask, and heated with nitric acid (10 mL). Then, 5 mL of sulfuric acid was added to the flask, and heating was continued until white fumes of sulfuric acid appeared. Hydrogen peroxide (2 mL, 30%) was added to the flask, and heating was continued. After cooling, 15 mL of saturated ammonium oxalate solution was added to the flask, and heating was continued. A 5 mL volume of potassium iodide (40%, w/w) was added, and after allowing the solution to stand for 30 min, 5 mL of ascorbic acid (10%, w/w) was added, and, finally, water was added to adjust the volume to 50 mL. The total amount of As was measured by hydride generation-atomic absorption spectrometry (HG-AAS) (Varian Spectra AA 220 with VGA-77, Varian Japan, Tokyo).

Extraction efficiency

Extraction efficiency (extraction [%]) was evaluated as the ratio of extracted As content to total As content.

The As concentration of each extract solution was determined by ICP-MS after dilution with 0.1 mol/L nitric acid solution, unless otherwise noted.

Microwave oven

The As concentration of each extract solution was measured by ICP-MS after further digestion with a microwave oven (Multiwave 3000, Microwave Sample Preparation System, Perkin-Elmer Japan, Tokyo). The digestion procedure consisted of the following steps: 5 min at 300 W, 10 min at 300–800 W, 20 min at 800 W. The digested solutions were applied to the ICP-MS system.

Results and Discussion

Speciation analysis of standard arsenicals and a partially digested hijiki sample by HPLC/ICP-MS

The HPLC/ICP-MS system was used to analyze six standard arsenic compounds, arsenate, arsenite, MMA, DMA, AB, and TMAO. The concentration of each standard was 10 ng As/mL. As shown in Fig. 1, they were separately detected within 10 min.

Figure 2 shows a typical HPLC/ICP-MS chromatogram for a hijiki sample treated by the partial acid-digestion method. The hijiki sample was treated as described below. An unknown peak was detected at the retention time of 7.8 min. The peak area decreased with increasing heating time in the presence of nitric acid and the peak area of DMA gradually increased concomitantly, which suggested that the peak is that of arsenosugar (see also Table 1-1). The broad peak of As (V) seemed to be due to a matrix effect. No change of

Fig. 2. HPLC/ICP-MS chromatogram of a partially acid-digested hijiki sample

A hijiki powder sample (0.1 g dry weight) was mixed with 2 mL of conc. nitric acid. The mixture was heated at 80℃ for 1 hr.

the peak area of iAs(V) was observed during heating (data not shown). No peak was observed between 11 and 14 min (data not shown).

Effect of extraction temperature

Table 1-1 and Table 1-2 show the results of a comparison of extraction temperatures. Ground hijiki fine powder (0.1 g dry weight) was mixed with 2 mL of conc. nitric acid (68%), and the mixture was heated for 1 hr at 60℃, 80℃, 100℃, or 120℃ on an aluminum heat block or allowed to stand for 1 hr at room temperature. In Table 1-1, the As concentration was determined after filtration and further digestion with a microwave oven. The extraction efficiency (%) of As showed the highest

Table 1-1. Effect of heating temperature on the extraction efficiency

Temperature (℃)	Extract total As[1] (mg/kg dry weight)	Extraction[2] (%)
Room temp.	106	92
60	108	94
80	116	100
100	114	99
120	110	95

Data are the means of duplicate analyses.
[1] The As concentration of each extract solution was measured by ICP-MS after further digestion with a microwave oven.
[2] The extraction efficiency (%) was calculated as the ratio of extracted As content to total As content. The total As concentration was determined to be 115.6 mg/kg dry weight.
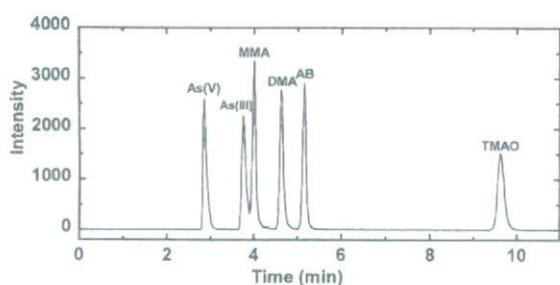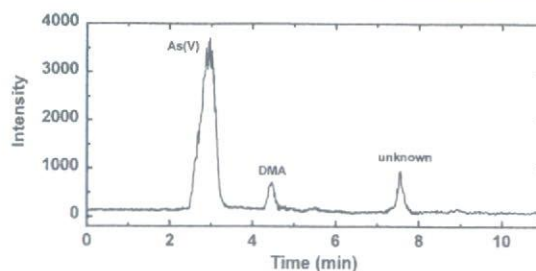

Fig. 1. HPLC/ICP-MS chromatogram of six standard As compounds

Sample injection volume, 20 μL; concentration of each standard As compound, 10 ng As/mL.

Table 1-2.   Effect of heating temperature on the chemical forms of As

Temperature (℃)	Concentration (mg/kg dry weight)				
	As(V)	As(III)	iAs (As(V)+As(III))	MMA	DMA
Room temp.	88.3	N.D.	88.3	N.D.	1.0
60	91.8	N.D.	91.8	N.D.	2.8
80	99.1	N.D.	99.1	N.D.	6.7
100	97.5	N.D.	97.5	N.D.	10.2
120	95.7	N.D.	95.7	N.D.	20.4

Data are the mean of duplicate analyses.
N.D.: Not detected.

value at 80℃.

When the chemical forms of As in the extract were studied, the highest iAs concentration was also observed at 80℃, though it remained almost constant at high temperatures. DMA was detected at room temperature[18] and increased with temperature due to the degradation of arsenosugar(s) as mentioned above in relation to Fig. 2. MMA was not detected at any temperature. Since iAs concentration did not increase with the increase of DMA concentration and MMA was not detected, it was considered that the detected iAs was originally present as iAs in the hijiki sample. In a separate experiment, it was shown that DMA is not converted to iAs below 120℃ even in the presence of conc. nitric acid[19]. Consequently, it was concluded that 80℃ was the best extraction temperature to determine iAs in this method.

Effect of nitric acid concentration of the extraction solution

When conc. nitric acid is added to a sample to extract As compounds, the solution may suddenly boil, and neutralization is required to prevent damage to the HPLC column. Therefore, different acid concentrations were tested to identify the minimum nitric acid concentration that could be used without reducing the extraction efficiency, and the results are shown in Table 2. Ground hijiki fine powder (0.1 g dry weight) was mixed with 2 mL of 0.1, 0.15, 0.3, 1, 3 mol/L or conc. nitric acid solution, water alone, and 2 mol/L trifluoroacetic acid (TFA) in a 10 mL capped HDPE centrifuge tube, and the solutions were heated for 1 hr at 80℃. The iAs concentration and extraction efficiency were almost the same at the concentrations of 0.15 mol/L and above. The As (III) concentration of hijiki was low as previously reported[11]. The DMA concentration increased at higher nitric acid concentrations.

Reasonably good extraction efficiency (76%) was obtained with water alone. Since the hijiki samples in this experiment were carefully milled with a grinder, water seemed to easily penetrate the sample matrix.

To extract As from rice, 2 mol/L TFA and heating at

100℃ for 6 hr were adopted[17]. In our study, the sample was treated with 2 mol/L TFA at 80℃ for 1 hr. The iAs concentration was similar to that obtained when nitric acid solution was used. Interestingly, however, As(III) concentration was increased and As(V) concentration was decreased, compared with the nitric acid treatment. The recoveries of As(V) ranged from 30–35% in the case of rice samples[17]. These results may suggest the conversion of As(V) to As(III) when 2 mol/L TFA was used. On the other hand, As(III) was not detected when nitric acid of 1 mol/L and higher concentration was used. Moreover, the As(III) concentration ranged from 1.5–2.8 mg/kg/dry weight when 0.1–0.3 mol/L nitric acid was used. Care should be taken when selecting the extraction solvent and/or concentration, if separate detection of As(III) and As(V) is required.

Based on the results described above, we chose 0.3 mol/L as the nitric acid concentration of the extraction solution to use in this method. In this study, the conditions were optimized for 0.1 g dry weight of ground fine powder sample.

Effect of extraction time

The results for the comparison of extraction times are shown in Table 3. A milled hijiki powder sample (0.1 g dry weight) was mixed with 2 mL of 0.3 mol/L nitric acid solution in a 10 mL capped HDPE centrifuge tube and heated for 15, 45, 60, or 90 min at 80℃. The extraction efficiency (%) of As showed the highest value when heating was done for 60 min. Thus, an extraction time of 60 min was adopted for use in the method. Interestingly, As(III) concentration ranged from 1.3 to 2.7 mg/kg dry weight during the longer heating period. The quantification of As(III) concentration was considered to be difficult even when nitric acid of 0.3 mol/L concentration was used.

Method validation (single-laboratory validation of the method for extraction of iAs from hijiki samples)

The method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), preci-

Table 2. Effect of the HNO₃ concentration of the extraction solution on extraction efficiency

Extraction solution	Concentration (mg/kg dry weight)						Extraction[2] (%)
	As(V)	As(III)	iAs (As(V)+As(III))	MMA	DMA	Extract total As[1]	
0.10 mol/L HNO₃	77.5	1.5	79.0	N.D.	1.5	95.9	79.9
0.15 mol/L HNO₃	88.1	2.6	90.7	N.D.	1.6	117	97.5
0.30 mol/L HNO₃	84.9	2.8	87.7	N.D.	1.5	118	98.7
1.0 mol/L HNO₃	85.9	N.D.	85.9	N.D.	1.6	119	99.3
3.0 mol/L HNO₃	89.1	N.D.	89.1	N.D.	2.6	123	102
conc. HNO₃	89.1	N.D.	89.1	N.D.	9.0	117	97.7
Water alone	66.5	1.0	67.5	N.D.	1.2	91.3	76.1
2 mol/L TFA	74.6	14.3	88.9	N.D.	1.5	113	94.0

Data are the means of duplicate analyses.

N.D.: Not detected.

[1] The As concentration of each extract solution was measured by ICP-MS only after dilution with 0.1 mol/L nitric acid solution.

[2] Extraction efficiency (%) was calculated as the ratio of extracted As content to total As content. The total As concentration was determined to be 120.0 mg/kg dry weight.

Table 3. Effect of extraction time on extraction efficiency

Extraction time (min)	Concentration (mg/kg dry weight)						Extraction[2] (%)
	As(V)	As(III)	iAs (As(V)+As(III))	MMA	DMA	Extract total As[1]	
15	71.9	1.3	73.2	N.D.	1.2	107.5	93.0
45	81.0	1.8	82.8	N.D.	1.6	110.6	95.7
60	84.9	2.4	87.3	N.D.	1.6	113.4	98.1
90	83.0	2.7	85.7	N.D.	1.2	106.6	92.2

Data are the means of duplicate analyses.
N.D.: Not detected.
[1] The As concentration of each extract solution was measured by ICP-MS after dilution with 0.1 mol/L nitric acid solution.
[2] Extraction efficiency (%) was calculated as the ratio of the As concentration of each extract solution to the total As concentration.

sion, and accuracy. Since the JECFA set the PTWI value of As as the value of iAs, the concentration of iAs was evaluated. iAs was quantified by using a standard curve that was established by using iAs standards at concentrations of 1, 2, 5, 10, and 20 ng As/mL; the plot showed good linearity ($r=0.9998$).

The LOD and LOQ was set at 3 and 10 times the standard deviation of the values obtained from 10 replicate analyses, respectively. The analyses were performed on a hijiki sample prepared by means of the water soaking and simmering process in order to decrease the iAs concentration. The iAs concentration was determined to be 2.92 ± 0.046 mg/kg dry weight (mean$\pm$S.D. ($n=10$)). The LOD and LOQ values of iAs were 0.14 mg/kg dry weight and 0.46 mg/kg dry weight, respectively.

The recovery studies were performed by spiking 0.5 mg/kg dry weight as the LOQ level and 3 mg/kg dry weight as the iAs concentration of an un-spiked hijiki sample (iAs: 2.92 ± 0.046 mg/kg dry weight) to evaluate accuracy. Either As(V) or As(III) was added to the sample as iAs. Since the PTWI was set for iAs, the sum of the iAs(V) and iAs(III) recovered is shown in Table 4. Recovery of iAs at 0.5 mg/kg and 3 mg/kg ranged from 93.1% to 106.7%. Recovery studies for MMA and DMA were also performed by spiking 2.5 mg MMA/kg dry weight and 2.5 mg DMA/kg dry weight, respectively. The hijiki sample originally contained DMA of 1.51 mg/kg dry weight, but did not contain any MMA. Recovery of MMA and DMA was 92.8% and 99.8%,

Table 4. Recovery of As species from hijiki samples

	Spiked species	Spiked level (mg/kg dry weight)	n	Recovery (%)[1]
iAs (As(V)+As(III))	As(V)	0.50	5	93.1±8.2
		3.0	5	100.6±4.0
	As(III)	0.50	5	100.7±5.7
		3.0	5	106.7±1.5
MMA	MMA	2.5	3	92.8±2.8
DMA	DMA	2.5	3	99.8±3.8

[1] Percent recovery studies were performed on a hijiki sample (iAs, 2.92 mg/kg; MMA, not detected; DMA, 1.51 mg/kg) by five replicate analyses for iAs and by triplicate analyses for MMA and DMA.

respectively. Since the recovery of both MMA and DMA was almost 100%, essentially no conversion of organic As to iAs occurred under the conditions used in the method.

To evaluate the precision of the method, the repeatability and intermediate precision for iAs were obtained by performing two replicate analyses on five different days. The analyses were performed on a hijiki sample prepared by the water soaking and simmering method (iAs, 7.47 mg/kg dry weight; DMA, 0.169 mg/kg dry weight). The repeatability and intermediate precision for iAs obtained were 1.5% and 1.5%, respectively. The repeatability and intermediate precision for DMA were 7.9% and 8.4%, respectively.

These results showed that the method is efficient in terms of accuracy and precision.

Speciation analysis of hijiki samples after water soaking process and water soaking and simmering process

The concentration of As in hijiki has been reported to be reduced by a water soaking procedure, depending on temperature[11), 20]. In these papers, however, the extraction efficiency was insufficient and the As content was not evaluated as iAs, but as total As. Moreover, it has not been clarified whether the non-extracted As in hijiki samples was iAs or not. In this study, both the water soaking process and the water soaking and simmering process were used in preparing cooked hijiki samples and the established method was applied to hijiki samples after both processes in order to clarify the retained iAs concentration.

Typical HPLC/ICP-MS chromatograms of a hijiki sample after the water soaking process (Fig. 3(a)) and after the water soaking and simmering process (Fig. 3(b)) are shown in Fig. 3. The water soaking and simmering process greatly reduced the iAs concentration. The retention time of the unknown peak in Fig. 3 was different from that in Fig. 2. The pH of the sample solution may affect the retention time of the arsenosugar-derived peak.

The results of the measurements of iAs and total As in hijiki are given in Table 5. When the method was applied to untreated hijiki samples, the concentrations of iAs and total As detected were in the 64.5 to 104.5 mg/kg dry weight range, and 92.0 to 131.5 mg/kg dry

weight range, respectively. After the water soaking process, the concentrations of iAs and total As detected were in the 32.3 to 64.9 mg/kg dry weight range, and 55.0 to 84.1 mg/kg dry weight range, respectively. The percentages of iAs and total As retained were 38.4–62.1% and 52.6–64.0%, respectively. Thus, the water soaking process reduced the concentrations of both iAs and total As by half.

The water soaking and simmering process profoundly reduced the concentrations of iAs and total As to
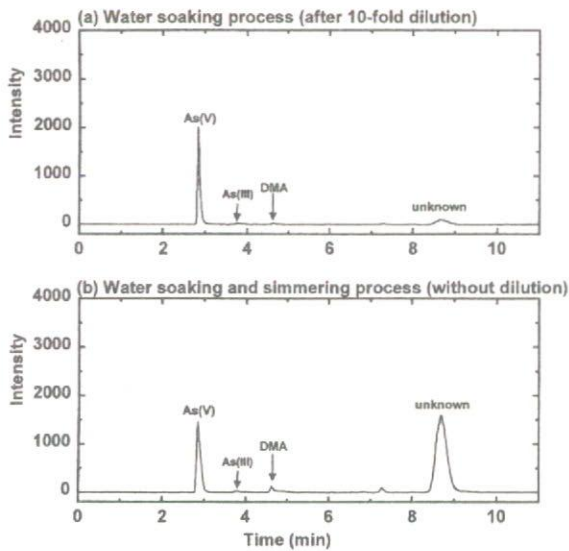

Fig. 3. HPLC/ICP-MS chromatograms for cooked hijiki samples

(a) hijiki sample after the water soaking process. The extract solution was diluted 10 times with 0.1 mol/L nitric acid solution. The iAs concentration was determined to be 52.6 mg/kg dry weight; (b) hijiki sample after the water soaking and simmering process. The iAs concentration was determined to be 5.2 mg/kg dry weight.

3.7–15.5 mg/kg dry weight and 26.1–36.4 mg/kg dry weight, respectively. The percentages of iAs and total As retained were 4.3–20.0% and 21.3–34.9%, respectively. The retained percentage of iAs was lower than that of total As. Thus the water soaking and simmering process was more effective in removing iAs, rather than total As.

It is especially noteworthy that the iAs concentration in hijiki samples A, B, and C, prepared by the water soaking and simmering process, was reduced to 1/10 compared to the concentration after the water soaking process. These results suggest that the water soaking and simmering process, the traditional Japanese cooking style for hijiki, is effective in removing iAs rather than total As, from the hijiki sample matrix.

Acknowledgments

References

1) Shiomi, K. "Arsenic in marine organisms: chemical forms and toxicological aspects". Arsenic in the Environment, part II: Human Health and Ecosystem Effects. Nriagu, J. O., ed. New York, USA, John Wiley & Sons, 1994, p. 261–282. (ISBN 0471304360)
2) Shiomi, K. The chemical form, toxicity, and metabolism of the arsenicals in marine seaweed. Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Jpn.), 33, 1–10 (1992).
3) FSA report and recommendation: Agency advises against eating Hijiki seaweed Wednesday, 28 July 2004, Ref: R938-28.
4) Braman, R. S., Foreback, C. C. Methylated forms of As in the environment. Science, 182, 1247–1249 (1973).
5) Lunde, G. Separation and analysis of organic-bound and inorganic As in marine organisms. J. Sci. Food Agric., 24, 1021–1027 (1973).
6) Oygard, J. K., Lundebye, A. K., Julshamn, K. Determination of inorganic As in marine food samples by hydro-

Table 5. iAs concentration, total As concentration and ratio of the retained iAs concentration to the original total As concentration

Sample	Treatment process	Concentration (mg/kg dry weight)		%[1]	
		iAs	T. As	iAs	T. As
Hijiki A	None	96.6	123	100	100
	Water soaking	52.6	72.7	54.4	59.3
	Water soaking & simmering	5.2	26.1	5.4	21.3
Hijiki B	None	64.5	92.0	100	100
	Water soaking	32.3	55.0	50.1	59.8
	Water soaking & simmering	3.7	26.3	5.7	28.6
Hijiki C	None	91.2	112	100	100
	Water soaking	35.0	58.7	38.4	52.6
	Water soaking 6 simmering	3.9	29.3	4.3	26.3
Hijiki D	None	71.9	104.3	100	100
	Water soaking	39.7	61.5	55.2	59.0
	Water soaking & simmering	14.4	36.4	20.0	34.9
Hijiki E	None	104.5	131.5	100	100
	Water soaking	64.9	84.1	62.1	64.0
	Water soaking & simmering	15.5	35.5	14.8	27.0

Data are the means of duplicate analyses.

[1] Ratio of the retained iAs concentration to the original total As concentration.

chloric acid distillation and flow-injection hydride-generation atomic absorption spectrometry. J. AOAC Int., **82**, 1217–1223 (1999).

7) Shibata, Y., Morita, M. Exchange of comments on identification and quantitation of As species in a dogfish muscle reference material for trace elements. Anal. Chem., **61**, 2116–2118 (1989).

8) Edmonds, J. S., Shibata, Y., Prince, R. I. T., Francesconi, K. A., Morita, M. As compounds in tissues of the leatherback turtle, *Dermochelys coriacea*. J. Mar. Biol. Assoc. UK, **74**, 463–466 (1994).

9) Francesconi, K. A., Kuehnelt, D. Determination of As species: a critical review of methods and applications, 2000–2003. Analyst, **129**, 373–395 (2004).

10) Raab, A., Fecher, P., Feldmann, J. Determination of As in algae—results of an interlaboratory trial: Determination of As species in the water-soluble fraction. Microchim. Acta, **151**, 153–166 (2005).

11) Ichikawa, S., Kamoshida, M., Hanaoka, K., Nagaoka-Hamano, M., Maitani, T., Kaise, T. Decrease of As in edible brown algae *Hijikia fusiforme* by the cooking process. Appl. Organomet. Chem., **20**, 585–590 (2006).

12) Branch, S., Ebdon, L., O'Neill, P. Determination of As species in fish by directly coupled high-performance liquid chromatography-inductively coupled plasma mass spectromtry. J. Anal. At. Spectrom., **9**, 33–37 (1994).

13) Milstein, L. S., Essader, A., Murrell, C., Pellizzari, E. D., Fernando, R. A., Raymer, J. H., Akinbo, O. Sample preparation, extraction efficiency, and determination of six As species present in food composites. J. Agric. Food Chem.,

51, 4180–4184 (2003).

14) McKiernan, J. W., Creed, J. T., Brockhoff, C. A., Caruso, J. A., Lorenzana, R. M. A comparison of automated and traditional methods for the extraction of arsenicals from fish. J. Anal. At. Spectrom., **14**, 607–613 (1999).

15) Gallagher, P A., Murray, S., Wei, X., Schwegel, C. A., Creed, J. T. An evaluation of sample dispersion media used with accelerated solvent extraction for the extraction and recovery of arsenicals from LFB and DORM-2. J. Anal. At. Spectrom., **17**, 581–586 (2002).

16) Buldini, P. L., Cavalli, S., Trifirò, A. State-of-the-art ion chromatographic determination of inorganic ions in food. J. Chromatogr. A, **789**, 529–548 (1997).

17) Heitkemper, D. T., Vela, N. P., Stewart, K. R., Westphal, C. S. Determination of total and speciated As in rice by ion chromatography and inductively coupled plasma mass spectrometry. J. Anal. At. Spectrom., **16**, 299–306 (2001).

18) Maitani, T., Uchiyama, S., Saito, Y. Hydride generation-flame atomic absorption spectrophotometry as an arsenic detector for high-performance liquid chromatography. J. Chromatogr., **391**, 161–168 (1987).

19) Nagaoka, M. H., Maitani, T. Analysis of inorganic arsenic in foods by hydride generation-cold trap-atomic absorption spectrophotometry. Metal Ions in Biology and Medicine, **9**, 75–77 (2006).

20) Hanaoka, K., Yoshida, K., Tamano, M., Kuroiwa, T., Kaise, T., Maeda, S. Arsenic in the prepared edible brown alga, *Hizikia fusiforme*. Appl. Organomet. Chem., **15**, 561–565 (2001).

Original

Evaluation of a Nitric Acid-based Partial-digestion Method for Selective Determination of Inorganic Arsenic in Rice

Megumi Hamano-Nagaoka[1,*], Tsutomu Nishimura[2], Rieko Matsuda[1]
and Tamio Maitani[1]

[1]National Institute of Health Sciences: 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan;
[2]Japan Food Research Laboratories: 11-10 Nagayama 6-chome, Tama-shi,
Tokyo 206-0025, Japan; *Corresponding author

Arsenic (As) uptake in human occurs via the food chain mainly. The Joint FAO/WHO Expert Committee on Food Additives has established the provisional tolerable weekly intake level for As as an inorganic As (iAs) value, because iAs in food is much more toxic than organic As. In this study, we studied an acid based partial-digestion method for the complete extraction of arsenicals from rice. HPLC/ICP-MS was used to determine the concentration of iAs selectively. The conditions adopted to extract arsenicals from a 0.5 g of finely ground rice sample were addition of 2 mL of 0.15 mol/L nitric acid and heating at 80℃ for 2 hr. The LOD and LOQ for iAs were 0.0024 and 0.0079 mg/kg dry weight, respectively. Recovery studies showed good accuracy. When the method was applied to ten short-grain brown rice samples, the iAs concentrations were 0.108–0.227 mg/kg dry weight and the total As concentrations were 0.118–0.260 mg/kg dry weight. Although dimethylarsinic acid was also detected in most samples, the percentage of iAs content in total As content was 62.2–96.3%. Thus, iAs was the principal As species in the short-grain brown rice samples tested.

Key words: arsenic; inorganic arsenic; rice; HPLC/ICP-MS; partial digestion; nitric acid

Introduction

Uptake of arsenic (As) in human mainly occurs via the food chain, and, in special cases, also via occupational exposure[1)-3)]. Since the inorganic arsenic (iAs) in food is much more toxic than the organic As[4),5)], the JECFA (the Joint FAO/WHO Expert Committee on Food Additives) has established a provisional tolerable weekly intake (PTWI) level as an value (0.015 mg/kg bw/week).

The major amount of ingested As comes from fish, shellfish, and seaweed, in which most of As compounds are fortunately non-toxic arsenobetaine (AB) or arsenosugars[6)]. In contrast to the seafoods, the rice plant bio-accumulates more toxic iAs species, arsenate and arsenite[7),8)]. Therefore, the contribution of rice to the total intake of iAs may be considerably high. For example, when a person weighing 50 kg consumes 150 g of brown rice containing As at the level of 0.16 mg/kg dry weight*[1] per day, the As intake is calculated to be 0.0034 mg total As/kg bw/week. If all As compounds in rice are present as iAs, this intake corresponds to 23% of the PTWI value. Consequently, the speciation of As in rice is very important from the viewpoint of risk-assessment of As for the Japanese population.

One of the requirements for assessing the risk of consuming food containing As is a quantitative or nearly quantitative method for determining arsenicals without transformation of the original chemical species. The main problems encountered have been low recovery and oxidation/reduction between As(III) and As(V)[9)]. Quantitative extraction is also essential, because the extraction procedure may selectively extract non-toxic species from food and/or leave toxic species unextracted within the solid matrix. Arsenic speciation demands mild extraction so as to maintain species integrity. Although speciation studies have been widely performed for As in marine products, the speciation studies of As in rice are not numerous. In those studies, methanol, water, a methanol/water mixture, and tri-fluoroacetic acid (TFA) have been used as solvents to extract As species from rice samples[10)-15)]. Although quantitative extraction of As from some foods has been achieved with these solvents, it has been difficult to achieve thorough extraction from rice samples. Attempts to utilize an accelerated solvent extraction technique[1,10,16,17)] or enzymatic ultrasonic probe[18)] have been reported for rice, but As extraction from rice samples remains difficult[6),19)].

In our preceding report, the nitric acid-based partial-digestion method was assessed as a mean of achieving complete As extraction from hijiki samples[20)]. In this paper, therefore, the partial acid-digestion method was

---

*[1] http://www.maff.go.jp/syohi_anzen/profiles/arsenic.pdf. Annex.

applied for rice, to completely extract iAs from rice samples. Nitric acid was used under conditions such that organic As species were not converted to iAs. This method was coupled with HPLC/ICP-MS to determine As species including iAs separately.

Materials and Methods

Reagents

Sodium arsenate (Na_2HAsO_4), sodium arsenite ($NaAsO_2$), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and trimethylarsine oxide (TMAO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). AB was obtained from Trichemical Laboratory (Yamanashi, Japan). Ultra-pure analytical grade nitric acid (68%) and tetramethylammonium hydroxide (TMAH, 20%) (TAMAPURE-AA-100) were purchased from Tama Chemical Industry (Kanagawa, Japan). Other chemicals were of reagent grade or of the highest grade available commercially. All laboratory glass ware and plastic ware used were immersed in approximately 2 mol/L HNO_3 at least overnight and rinsed with ultra-pure water prepared with a Milli-Q SP Reagent Water System (Millipore, Bedford, MA) to avoid contamination with various ions. Water of this grade was used throughout the experiment.

Rice

Short-grain brown rice samples were obtained from supermarkets in the Tokyo Metropolitan area and by mail order in Japan. Before analysis, all samples were carefully ground into fine powders with a grinder (Micron Milcer, Iwatani Int. Corp., Tokyo, Japan) for 1 min four times at intervals of 3 min.

Extraction procedure for rice

A ground rice powder sample (0.5 g dry weight) was mixed with 2 mL of nitric acid (0.15 mol/L) in a 10 mL capped high-density polyethylene (HDPE) centrifuge tube, and the mixture was heated on an aluminum heat block at 80℃ for 2 hr. The solution obtained was diluted to 10 mL with water and passed through a 0.45 μm filter prior to analysis.

Determination of iAs by HPLC/ICP-MS

The HPLC apparatus (Agilent 1200 Series, Agilent Technologies, Tokyo, Japan) was equipped with an HPLC column (CAPCELL PAK C_{18} MG, 4.6 mm i.d.× 250 mm, Shiseido Ltd., Tokyo, Japan). The solvent, a solution (pH 3.0) composed of 10 mmol/L sodium 1-butanesulfonate, 4 mmol/L TMAH, 4 mmol/L malonic acid, and 0.05% methanol, was used for isocratic elution at a flow rate of 0.75 mL/min. The sample solution (20 μL) was loaded onto the column and eluted at room temperature. In this study, the retention time did not change at this temperature.

The Agilent 7500 ce ICP-MS system (Agilent Technologies) was used. The analytical conditions were: RF power, 1.6 kW; plasma gas, 15 L/min; carrier gas, 0.70 L/min; auxiliary gas, 0.90 L/min; makeup gas, 0.43 L/

min. Signal ion monitoring at m/z 75 and a sampling rate of 1 Hz were used to collect the chromatographic data.

Determination of total As

A finely ground powdered rice sample (0.1–1 g dry weight) was weighed, transferred to a Kjeldahl flask, and heated with nitric acid (10 mL). Then, 5 mL of sulfuric acid was added to the flask, and heating was continued until white fumes of sulfuric acid appeared. Hydrogen peroxide (2 mL, 30%) was added to the flask, and heating was continued. After cooling, 15 mL of saturated ammonium oxalate solution was added to the flask, and heating was continued. A 5 mL volume of potassium iodide solution (40%) was added, and after the solution had been allowed to stand for 30 min, a 5 mL volume of ascorbic acid solution (10%) was added. Finally, water was added to adjust the volume to 50 mL. The total amount of As was measured by hydride generation-atomic absorption spectrometry (HG-AAS) (Varian Spectra AA220 with VGA-77, Varian Japan, Tokyo).

Extraction efficiency

Extraction efficiency (extraction [%]) was evaluated as the ratio of extracted As content to total As content. The As concentration of each extract solution was determined by ICP-MS after dilution with 0.1 mol/L nitric acid solution, unless otherwise noted.

Results and Discussion

Speciation analysis of standard arsenicals and a partially digested rice sample by HPLC/ICP-MS

The HPLC/ICP-MS system was used to analyze six standard As compounds, i.e., arsenate, arsenite, MMA, DMA, AB, and TMAO. The concentration of each standard was 5 ng As/mL. As shown in Fig. 1, they were separately detected within 10 min.

Figure 2 shows typical HPLC/ICP-MS chromatograms for two rice samples treated according to the established partial acid-digestion method (see below). The samples shown in Figs. 2(a) and 2(b) are representatives of samples containing a low concentration (total As: 0.04 mg/kg dry weight) and high concentration
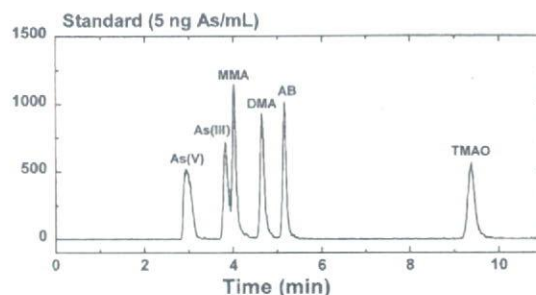

Fig. 1.   HPLC/ICP-MS chromatogram of six standard As compounds

Sample injection volume, 20 μL; concentration of each standard As compound, 5 ng As/mL.
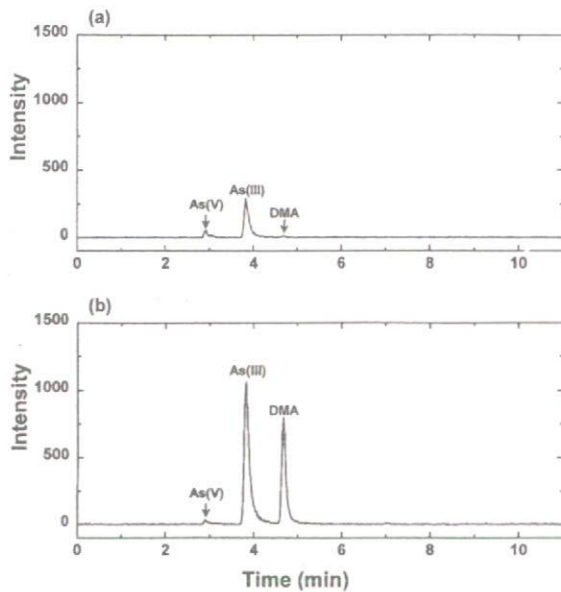
Fig. 2. HPLC/ICP-MS chromatograms for partially acid-digested rice samples

Each powdered rice sample (0.5 g dry weight) was mixed with 2 mL of 0.15 mol/L nitric acid, and the mixture was heated at 80℃ for 2 hr. The total As concentrations of the samples (a) and (b) were determined to be 0.04 mg/kg dry weight and 0.26 mg/kg dry weight, respectively.

(total As: 0.26 mg/kg dry weight) of total As, respectively. MMA was not detected in either sample, but DMA was detected.

Concentration of nitric acid as extraction solution and extraction time

Our preceding study on hijiki samples[20] suggested that organic As species were not converted to iAs at 80℃ and that complete extraction of As was possible by selecting optimal extraction conditions. Accordingly, a temperature of 80℃ was also adopted in this study, and several factors were optimized as described below.

First, various acid concentrations were tested to identify the lowest nitric acid concentration that could be used without reducing the extraction efficiency. The results are shown in Table 1-1. A powdered rice sample (0.5 g dry weight) was mixed with 2 mL of 0.075, 0.10, 0.15, 0.30, and 1.0 mol/L nitric acid solutions, and 2.0 mol/L trifluoroacetic acid (TFA) in 10 mL capped HDPE centrifuge tubes, and the solutions were heated for 2 hr at 80℃. The iAs concentration and extraction efficiency were almost the same at the 0.10 mol/L concentration and above. The DMA concentration did not increase at the higher nitric acid concentrations. Since the pH of the solution for analysis was 2–3 in the case of 0.15 mol/L nitric acid and adjustment of the pH was unnecessary, the 0.15 mol/L concentration was adopted.

Addition of 2.0 mol/L TFA and heating at 100℃ for 6 hr had been adopted to extract As from rice[17], whereas in our study the sample was treated with 2.0 mol/L TFA and heated at 80℃ for 2 hr. Black insoluble matter remained after the TFA treatment. Moreover, compared to the nitric acid treatment, the As(III) and As(V) concentrations were higher and lower, respectively. Furthermore, the pH of the solutions for analysis was 1. Therefore, TFA was not used in this study.

Next, the extraction time was varied (Table 1-2). A milled rice powder sample (0.5 g dry weight) was mixed with 2 mL of 0.15 mol/L nitric acid solution in a 10 mL capped HDPE centrifuge tube and heated for 0.5, 1, 2, 4, 6, or 8 hr at 80℃. Since the extraction efficiency (%) of As was almost the same from 1 hr to 4 hr, the extraction time was fixed on 2 hr.

Nitric acid is generally regarded as an oxidative acid. However, As(III) was detected even in the case of 1.0 mol/L nitric acid. This might suggest the presence of reductive compound(s) in brown rice.

Based on the results described above, a 0.15 mol/L nitric acid concentration and 2 hr extraction time were adopted.

Method validation

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

Table 1-1. Effect of nitric acid concentration on extraction efficiency

Extraction solution	Concentration (mg/kg dry weight)						Extraction[2] (%)
	As(V)	As(III)	iAs (As(V)+As(III))	MMA	DMA	Total As extracted[1]	
0.075 mol/L HNO₃	N.D.	0.079	0.079	N.D.	0.013	0.120	87.6
0.10 mol/L HNO₃	0.013	0.106	0.119	N.D.	0.022	0.127	92.7
0.15 mol/L HNO₃	0.026	0.105	0.120	N.D.	0.025	0.129	94.2
0.30 mol/L HNO₃	0.031	0.091	0.122	N.D.	0.026	0.126	92.0
1.0 mol/L HNO₃	0.019	0.101	0.120	N.D.	0.023	0.127	92.7
2.0 mol/L TFA	0.009	0.114	0.123	N.D.	0.021	0.125	91.2

Data are means of duplicate analyses.
N.D.: Not detected.
[1] The As concentration of each extract solution was measured by ICP-MS only after dilution with 0.1 mol/L nitric acid solution.
[2] Extraction efficiency (%) was calculated as the ratio of extracted As content to the total As content. The total As concentration was determined to be 0.137±0.005 mg/kg dry weight (n=5).

Table 1-2.　Effect of extraction time on extraction efficiency

Extraction time (min)	Concentration (mg/kg dry weight)						Extraction[2] (%)
	As(V)	As(III)	iAs (As(V)+As(III))	MMA	DMA	Extract total As[1]	
0.5	0.015	0.174	0.189	N.D.	0.016	0.215	91.1
1	0.031	0.177	0.208	N.D.	0.018	0.235	99.6
2	0.032	0.189	0.221	N.D.	0.019	0.239	101.3
4	0.030	0.182	0.212	N.D.	0.018	0.234	99.2
6	0.043	0.177	0.220	N.D.	0.018	0.232	98.3
8	0.049	0.157	0.206	N.D.	0.017	0.226	95.8

Data are means of duplicate analyses.

N.D.: Not detected.

[1] The As concentration of each extract solution was measured by ICP-MS only after dilution with 0.1 mol/L nitric acid solution.

[2] Extraction efficiency was calculated as the ratio of extracted As content to the total As content.　The total As concentration was determined to be 0.236 ± 0.004 mg/kg dry weight ($n=5$).

iAs (as As(V) or As(III)) was quantified by using a linear calibration plot ($r=0.9998$) that was established by using standard iAs solutions having As concentrations of 0.5, 1, 5, 10, and 20 ng/mL.

The LOD and LOQ were set at 3 times and 10 times, respectively, the standard deviation (S.D.) of the value obtained from 10 replicate analyses.　The analyses were performed on a rice sample whose iAs concentration was determined to be 0.0435 ± 0.0008 mg/kg dry weight (mean$\pm$S.D. ($n=10$)).　The LOD and LOQ values of iAs were 0.0024 mg/kg dry weight and 0.0079 mg/kg dry weight, respectively.

Accuracy was evaluated by calculating recovery in five replicate analyses.　The recovery studies were performed by spiking 0.01 mg/kg dry weight as the LOQ level and 0.08 mg/kg dry weight as twice the iAs concentration of an un-spiked rice sample (iAs: 0.0435 ± 0.00079 mg/kg dry weight, MMA: not detected, DMA: not detected).　Either As(V) or As(III) was added to the sample.　Since the JECFA set the PTWI of As as the value of iAs, the sum of iAs (V) and iAs(III) recovered is given in Table 2.　Recovery of iAs at 0.01 mg/kg and 0.08 mg/kg dry weight ranged from 82.2% to 106.1%.　Although a slightly low recovery rate was observed in the spiking at the LOQ level, recovery in the spiking at

Table 2.　Recovery of As species from finely powdered brown rice samples

Spiked species	Spiked level (mg/kg dry weight)	n	Recovery (%)[1]
As(V)	0.01	5	96.7 ± 6.3[2]
	0.08	5	102.4 ± 3.1[2]
As(III)	0.01	5	82.2 ± 7.6[2]
	0.08	5	106.1 ± 1.3[2]
MMA	0.01	5	92.9 ± 1.7
	0.08	5	92.3 ± 9.0
DMA	0.01	5	101.5 ± 3.8
	0.08	5	100.2 ± 1.2

[1] Percent recovery studies were performed on a rice sample (iAs, 0.0435 ± 0.0008 mg/kg dry weight).

[2] Recovery as total iAs was given.

the sample level was satisfactory.　Recovery studies for MMA and DMA were also performed by spiking 0.01 mg/kg and 0.08 mg/kg dry weight.　The recovery of MMA and DMA was 92.3% to 101.5%.　The method was also applied to polished rice.　When iAs was added to polished rice at the final concentration of 0.22 mg/kg dry weight, the recovery of iAs ranged from 101% to 105% (data not shown), suggesting that the method can be applied to both brown rice and polished rice.

Precision was evaluated by calculating the coefficient

Table 3.　The concentrations of iAs, MMA, DMA, and total As and the ratio of iAs content to the total As content

Sample	Concentration (mg/kg dry weight)						%
	As(V)	As(III)	iAs (As(V)+As(III))	MMA	DMA	Total As	iAs/Total As
1	N.D.	0.146	0.146	N.D.	0.015	0.169	86.6
2	N.D.	0.162	0.162	N.D.	0.082	0.260	62.2
3	N.D.	0.108	0.108	N.D.	0.011	0.118	91.5
4	0.010	0.171	0.176	N.D.	0.054	0.222	79.2
5	N.D.	0.199	0.199	N.D.	0.022	0.247	80.6
6	N.D.	0.114	0.114	N.D.	0.010	0.125	90.8
7	N.D.	0.117	0.117	N.D.	N.D.	0.122	96.3
8	0.012	0.221	0.227	N.D.	0.042	0.252	89.9
9	0.010	0.210	0.215	N.D.	0.012	0.229	93.9
10	N.D.	0.174	0.174	N.D.	0.022	0.199	87.4

Data are means of duplicate analyses.

N.D.: Not detected.