# III. MICRONUTRIENTS AND TRACE ELEMENTS

## 1. NATIONAL FACILITY FOR DRIED BLOOD SPOT (DBS) TECHNOLOGY FOR VITAMIN A ESTIMATION

Vitamin A deficiency is the leading cause of blindness in children. Therefore assessment of sub - clinical form of vitamin A deficiency forms an integral part of the efforts of introducing and monitoring programs aimed at reducing vitamin A deficiency from the population. In this context a 'National facility for Dried Blood Spot (DBS) Technology for vitamin A Estimation' has been established at the Institute with the financial support of MI and MOST, India during the year 2003-2004. During the year technical services for analysis of vitamin A and validation of stability of DBS with time have been undertaken.

### Methodology

***Technical services for analysis of vitamin A***

Recently NNMB carried out the survey in eight states on the prevalence of micronutrient deficiencies in the country. To assess the sub clinical form of vitamin A deficiency DBS samples were collected from a sub-sample of 576 preschool children per State who were covered for clinical examination (N=9508 preschool children per State). Storage and analysis of the DBS samples were done at the DBS facility.

A typical set of a days analysis consisted of a blank, standard retinol, tocol for internal recovery, and about 50-60 samples.

***Stability of DBS retinol***

Overnight fasting blood samples (10 ml) were collected from 10 adult male volunteers for the study. Blood collected into heparinized vacutainers were used for preparing 50 strips of 4 spots each and stored at -20°C. Matching plasma samples were aliquoted (100µl) and stored at -20°C and analysed periodically.

### Result

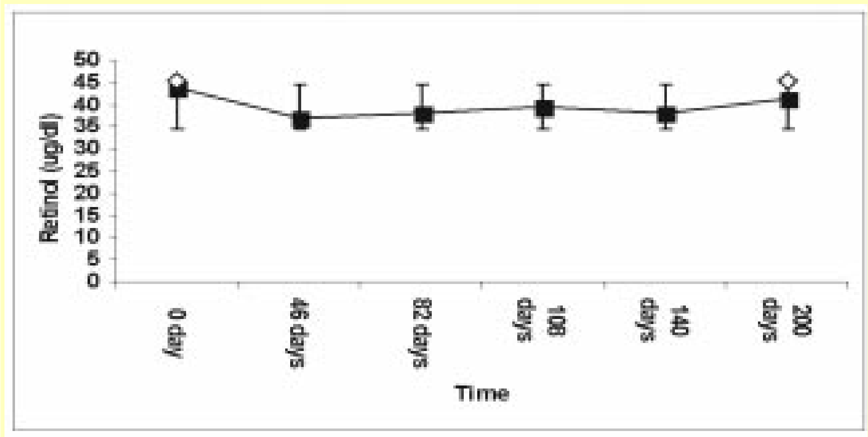
A total of 6000 samples were analyzed during the year. The period of collection and analysis varied between 90 days to 170 days. The data is being tabulated for assessing the extent of sub clinical deficiency of vitamin A by DBS method.

Stability of retinol in DBS at various time points is given in figure 7. The retinol in DBS was found to be stable and comparable to plasma retinol concentration over a period of 200 days.

### Conclusion

The facility is operational and handled about 6000 samples during the last 2 years with an estimated cost of Rs 600/- per sample, which could make the facility sustainable.

*Figure7. Stability of retinol in dried blood spot*




## 2. A COUNTRY INVESTMENT PLAN FOR FOOD FORTIFICATION IN INDIA

Considering the long-term implications of micronutrient deficiencies in the population, there is an urgent need to devise technologies aimed at controlling them. A draft proposal entitled 'Country Investment Plan (CIP)' for the provision of micronutrient fortification of food supplements to 0- 6 year children through Integrated Child Development Scheme (ICDS) in the States of Andhra Pradesh and Rajasthan was prepared and submitted to the DWCD, Government of India. The CIP was presented at the Inter-Ministerial meeting and suggested to revise the CIP.

Following are the suggestions

- To include three districts from Orissa and Rajasthan
- To include 6-14 years children, adolescent girls, pregnant women and lactating mothers
- To include fortified wheat flour and DFS
- NIN to carryout zinc estimation in serum of the children
- NIN to carry out monitoring at the time of fortification
- NIN to carry out baseline diet and nutrition survey and evaluate the program at periodic intervals

### Materials and methods

The draft CIP was revised based on the suggestions of the Inter-Ministerial Committee except for inclusion of adolescent girls in CIP as they are benificaries of ICDS and MDM. Instead of Andhra Pradesh and Rajasthan the plan envisages implementation of CIP in the states of Orissa and Rajasthan for introduction of micro-nutrients-iron, iodine, vitamin A, zinc, thiamin, riboflavin, folic acid and ascorbic acid at RDA level to the beneficaries.

### Results

A draft proposal on 'Country investment plan (CIP) for micronutrient fortification of food supplements of ICDS and mid day meal in the States of Orissa and Rajasthan' was prepared and submitted to

DWCD. The estimated investment for the proposal is US$ 9.13 million (Rs 43 crores and 80 lakhs) for 3 years.

### Conclusions

Opportunities are available for investment in micronutrient fortification of food supplements of ICDS and mid day meal, which will reduce the prevalence of hidden hunger.

## 3. STABILITY OF IODINE IN DOUBLE FORTIFIED SALT (DFS): A SHORT-TERM STUDY

Iodine deficiency disorders and iron deficiency anaemia are widely prevalent and often coexist in the country. As a part of the dietary diversification, fortification of food with iodine and iron is recommended as one of the strategies to prevent and control these two deficiency disorders. The National Institute of Nutrition (NIN) developed a suitable technology for dual fortification of common salt (DFS) with iodine and iron [Food and Nutrition Bulletin, 1994; 15(1): 32-39]. The large-scale production in the factory [Food and Nutrition Bulletin, 1996; 17(1): 73-78], its bio-safety [Nutrition Research 1998; 18(1): 121-129], its ultra structure [SCANNING, The Journal of scanning Microscopies, 1998; 20(3): 271-273], and its efficacy in community [British Journal of Nutrition 2001; 85: Suppl. 2, S167-S173] have been evaluated. As per the recommendations of the ICMR Committee of Experts on DFS, the stability of DFS was evaluated under programmatic conditions in six different places in India (RMRC-Bhubaneswar, TRC-Chennai, MRC-Delhi, RMRC-Dibrugarh, NIRRH-Mumbai and GMC-Surat) under the project entitled "Operational Evaluation of the Stability of Iodine in Double fortified Salt: A Multicentric Study" during 2001-02.

The results of the study revealed that,

a) Iodized refined salt had satisfactory stability of iodine and the distribution (%) of samples with iodine content of >15 ppm was 99% at the end of 6 months.

b) Iodized ordinary salt had satisfactory stability of iodine and the distribution (%) of samples with iodine content of >15 ppm was 95% at the end of 6 months.

c) Double fortified ordinary salt had poor stability of iodine, as the iodine content was less than 7 ppm, right from the first month.

d) Double fortified refined salt had iodine content lower than expected, particularly those stored in coastal areas and the distribution (%) of samples with iodine content of >15 ppm was 60% at the end of 6 months.

e) The substantial time gap between the dates of sampling and analysis contributed to considerable variations in DFS and hence the results were not conclusive.

To resolve these, a rapid survey was undertaken under the supervision of Prof. M. G. Karmarkar, Senior Advisor, ICCIDD, New Delhi. Salt samples were randomly selected from 1 kg pouches of double fortified refined salt (DFS) from the available bags at each center for simultaneous time-controlled analysis, at ICCIDD Laboratory and NIN. These results indicated that the stability of iodine was quite satisfactory in DFS, with 76% of the samples had >15 ppm of iodine even after 22 months of fortification, which was much higher than that was reported at the end of 6 months (Table 8).

*Table 8. Distribution (%) of DFS samples having >15 ppm of iodine*

| Center | Percent* | |
|---|---|---|
| | 6 Months | 22 Months |
| Bhubaneswar | 40 | 75 |
| Chennai | 66 | 73 |
| Delhi | 77 | 74 |
| Dibrugarh | 53 | 67 |
| Mumbai | 72 | 91 |
| Surat | 56 | 77 |
| *Average* | *60* | *76* |

**Mean values of duplicates, n = 40/center.*

These results were discussed in the Scientific Advisory Committee of NIN in August 2003 and the committee recommended a short-term study to assess iodine stability under normal room conditions at NIN and the ICCIDD Laboratory by ensuring that no gap existed between sampling and analysis. The present study therefore, was carried out during 2004-05.

## Objectives

a. To study the stability of iodine in DFS prepared as per NIN formula and stored under room conditions over a period of six months, and

b. To study the weekly loss of iodine, if any, in DFS kept in plastic pouches and sampled frequently, simulating the domestic conditions.

## Methodology

As per the project protocol, 500 kg of double fortified refined salt (DFS), 500 kg of iodized ordinary common salt (IOS) and 500 kg of iodized refined salt (IRS) were produced by dry mixing process prescribed for DFS as well as iodized salt in the salt factory of M/s Prince International at Bhubaneswar during 29-31 March 2004 under the technical supervision of the investigators. All the raw materials were tested for their quality before the use and were found acceptable. After confirming the satisfactory iodine levels in samples (n = 20) drawn randomly from each type of salt, independently by NIN and the ICIDD laboratory, the Managing Director of the salt factory gave the colour codes to the three salts before transportation to ensure blinding. The key to the codes was handed over in a sealed envelope to the Director of NIN for safe custody, which was decoded at the end of the study.

The three types of fortified salts were packed in colour coded 0.5 kg LDPE pouches (orange/white/yellow) at the salt factory. Twenty-five pouches of each type of salt were further packed in double lined HDPE bags of respective colour. Seventeen bags of each coloured bags were transported by road during the first week of April 2004 from Bhubaneswar to NIN, Hyderabad as well as ICCIDD Laboratory, New Delhi. The consignments reached the laboratories safely during the second week of April 2004. The remaining salt bags (6 of each colour) were transported to the Orissa Unit of the National Nutrition Monitoring Bureau (NNMB) at the Regional Medical Research Centre (RMRC), Bhubaneswar. The salt bags were stored inside a room under normal local conditions at NIN, ICCIDD Laboratory and RMRC. The storage of salt bags at Bhubaneswar was intended to assess the stability of iodine under coastal environment. Sampling of salts and estimation of iodine were done simultaneously at NIN and ICCIDD Laboratory on the same pre-determined dates, to ensure uniform conditions of storage, duration and analysis. Every month one pouch was randomly picked up from each bag and the iodine content was estimated in duplicate. Loss of iodine, if any, under conditions simulating household environment was tested in a sub-sample of each type of salt during the first three months. For this purpose, 6 pouches of each salt were drawn randomly. After estimating the initial iodine content, the pouches were closed with rubber bands and stored for subsequent analysis on 7$^{th}$, 14$^{th}$, and 21$^{st}$ days of every month in the first 3 months. When the iodine content of DFS was estimated by the conventional iodometric titration using sulphuric acid, a wide variation in the iodine content of duplicate samples was observed (a) on the same day and (b) at different time points. This led to a lot of confusion in the interpretation of the results with regard to the stability of iodine in DFS. Therefore, a modified method using orthophosphoric acid was employed after a thorough validation. Both the laboratories employed the same method for iodine estimation in all the three types of fortified salts throughout the study period, to ensure uniformity.

*Procedure adopted for the estimation of iodine in the three fortified salts*

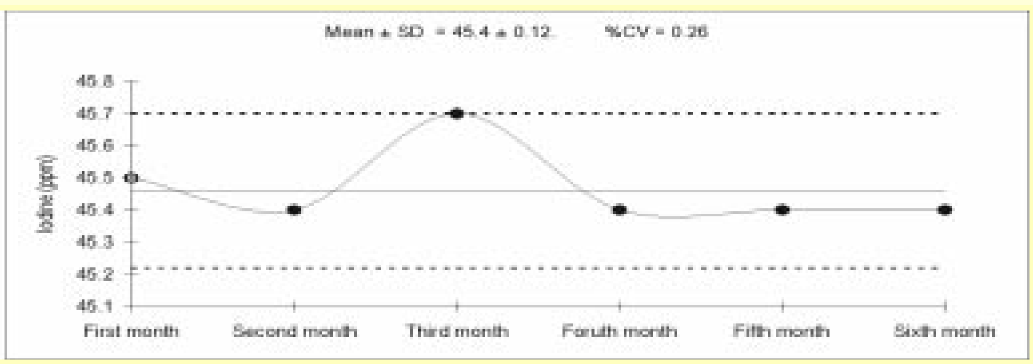
| Acid used | Procedure |
|---|---|
| $H_3PO_4$ | 10 g DFS/IS + 0.25 ml of 1% KI + 50 ml distilled water +1 ml of 4N $H_3PO_4$ Keep in dark for 10 minutes and titration with 0.005M $Na_2S_2O_3$ |

In order to ensure the reliability of the results, NIN and ICCIDD Laboratory strictly adhered to the internal as well as external quality control measures. For the internal quality control, multiple analyses (20 times) for iodine content of a known reference salt and standard KIO3 (in 10g of plain non-iodized salt) were performed. The 95% confidence interval of mean iodine values were calculated along with the operating control range (Mean ± 2 SD) for preparing the Quality Control Charts. Reference salt and standard KIO3 were also analysed, whenever the iodine content of the three fortified salt samples were estimated on the pre-determined dates. For the external quality control, 10 samples each of the three fortified salts were drawn randomly at NIN and sent to the ICCIDD Laboratory. There the samples were analysed in duplicate, simultaneously by the investigators from both the centers using the same reagents. Analysis of variance was performed considering the laboratories, duration and duplicates as independent variables and iodine values as dependent variable. Percent frequency distribution according to iodine content of the three fortified salts estimated at the two laboratories was also made.

## Results

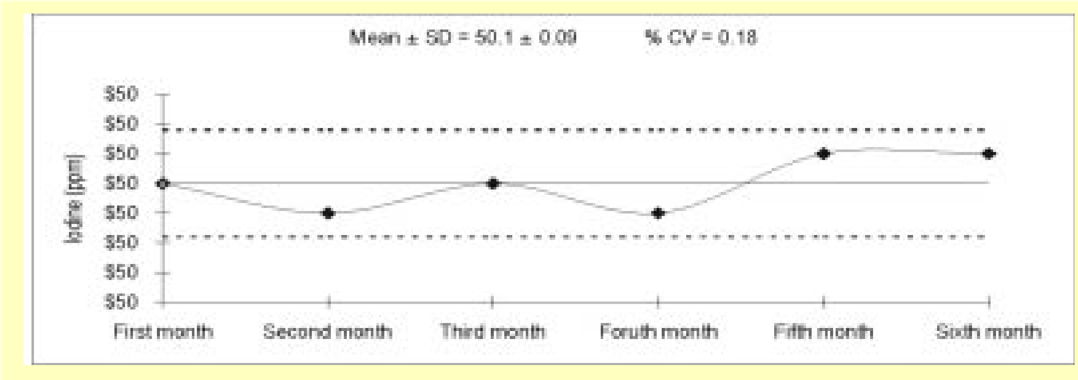
Estimation of initial iodine by the titration method using orthophosphoric acid at NIN and ICCIDD Laboratory showed that the mean iodine content was 30.0 ± 2.0 ppm in iodized ordinary salt (IOS), 42.7 ± 3.5 ppm in iodized refined salt (IRS) and 40.3 ± 3.8 ppm in DFS. The results of the internal quality control (IQC) carried out at NIN showed that the operating range of iodine content for the reference salt was 45.2 to 45.6 ppm (Figure 8) and that of the standard KIO3 was 49.9 to 50.3 ppm (Figure 9).

*Figure 8. Reference salt iodine IQC Chart at NIN**




** Similar results were obtained at the ICCIDD Laboratory*

*Figure 9. Sandard $KIO_3$ iodine IQC Chart at NIN**




** Similar results were obtained at the ICCIDD Laboratory*

The results of the external quality control (EQC) revealed good agreement between duplicate values of each laboratory as well as between the laboratories, irrespective of the type of fortified salt (Table 9). The intra-class correlation was very close to the unity ($\rho$= 0.97).

*Table 9. External Quality Control at ICCIDD laboratory**

| Laboratory | DFS | | IOS | | IRS | |
|---|---|---|---|---|---|---|
| | Iodine (ppm) | CV | Iodine (ppm) | CV | Iodine (ppm) | CV |
| ICCIDD | 42.5 ± 3.0 | 7.0 % | 35.9 ± 2.8 | 7.8 % | 46.9 ± 3.1 | 6.6 % |
| NIN | 41.5 ± 2.9 | 7.0 % | 35.1 ± 2.9 | 8.2 % | 46.1 ± 2.8 | 6.1 % |

Mean value of duplicates, n = 10/salt/Lab.
DFS: Double fortified salt; IOS: Iodized ordinary salt; IRS: Iodized refined salt.
*10 Samples of each salt were analysed simultaneously in duplicate by the investigators of the two laboratories, using the same reagents, at ICCIDD Laboratory, Delhi.

As per the project protocol, a draft report of the results at the end of six months was prepared and sent to the then members of the ICMR Expert Committee on DFS. The Committee concluded that there was no loss of iodine in any of the three types of fortified salts and recommended to decode and prepare the final report, as suggested by the Director-General of ICMR. Decoding of the three fortified salts revealed that orange pouches contained DFS, white pouches contained IOS and the yellow pouches contained IRS.

Regular monthly analysis of the salt samples carried out simultaneously at the two laboratories showed that the mean iodine content in DFS as well as IRS was about 40 ppm, while it was 30 ppm in IOS (Table 10). The iodine content of all the three salts estimated every month at NIN and ICCIDD Laboratory during the study period of six months was essentially similar.

*Table 10. Mean iodine content of fortified salts stored at Hyderabad and Delhi by duration*

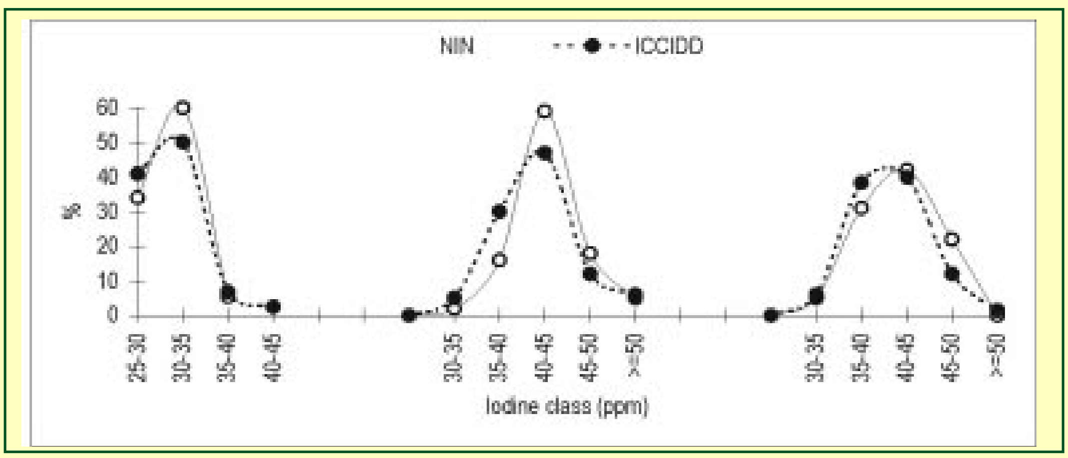
| Time | Iodine content (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | DFS | | IOS | | IRS | |
| | NIN | ICCIDD | NIN | ICCIDD | NIN | ICCIDD |
| Initial | 40.1 ± 3.9 | 40.4 ± 3.6 | 30.7 ± 1.3 | 29.7 ± 2.1 | 42.3 ± 3.7 | 43.1 ± 3.9 |
| Month 1 | 40.1 ± 4.3 | 40.1 ± 4.3 | 30.7 ± 2.7 | 33.6 ± 4.2 | 44.3 ± 4.0 | 44.5 ± 6.0 |
| Month 2 | 42.5 ± 4.7 | 42.5 ± 4.7 | 32.4 ± 1.9 | 33.9 ± 2.1 | 43.8 ± 4.9 | 45.5 ± 4.0 |
| Month 3 | 42.0 ± 4.3 | 42.0 ± 4.2 | 31.9 ± 1.2 | 31.2 ± 1.5 | 43.1 ± 3.3 | 43.0 ± 3.3 |
| Month 4 | 42.0 ± 3.6 | 39.3 ± 1.8 | 32.4 ± 3.0 | 30.8 ± 1.8* | 42.9 ± 2.5 | 40.3 ± 2.1 |
| Month 5 | 41.4 ± 3.3 | 40.4 ± 1.6 | *30.2 ± 0.9* | *28.8 ± 1.6** | *41.4 ± 3.0* | *38.2 ± 1.8** |
| Month 6 | 40.2 ± 2.1 | 39.6 ± 1.8 | *30.0 ± 1.2* | *28.2 ± 1.4** | *41.6 ± 2.3* | *37.6 ± 2.4** |

Mean value of duplicates, n = 17/salt/lab/month.
DFS: Double fortified salt; IOS: Iodized ordinary salt; IRS: Iodized refined salt.
*Between laboratories significant at $p < 0.05$.

Analysis of variance revealed that there was no significant difference between duplicate samples. Post-hoc analysis indicated minor differences in iodine content between the laboratories at some points mainly in IOS and IS. Since the external quality control showed good agreement between the laboratories and the frequency distributions of the iodine values were identical, the minor differences noted between laboratories have no practical relevance (Figure 10).

Even these small differences could be attributed to the inherent nature of the raw salts or distribution of iodine. However, the percentage of salt samples having >15 ppm of iodine was 100% in all the three salts over the study period of 6 months.

*Figure 10. Frequency (%) distribution of fortified salts according to iodine content during the study period of 6 months*




*IOS: Iodized ordinary salt; IRS: Iodized refined salt; DFS: Double fortified salt.*

Table 11 shows the mean iodine content of DFS, IOS and IRS stored at Bhubaneswar and tested at NIN and ICCIDD Laboratory. The mean iodine content of DFS and IS was about 40 ppm while that of IOS was 30 ppm, throughout the study period. No differences of practical relevance were observed in the iodine content.

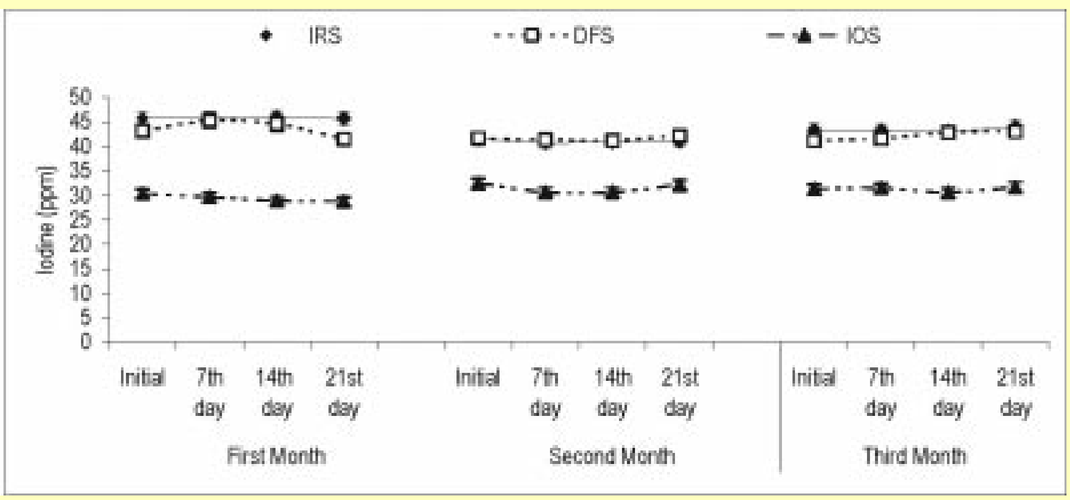
*Table 11. Mean iodine content of fortified salts stored at Bhubaneswar by duration*

| Time | Iodine content (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | DFS | | IOS | | IRS | |
| | NIN | ICCIDD | NIN | ICCIDD | NIN | ICCIDD |
| Initial | 40.1 ± 3.9 | 40.4 ± 3.6 | 30.7 ± 1.3 | 29.7 ± 2.1 | 42.3 ± 3.7 | 43.1 ± 3.9 |
| Month 1 | 40.2 ± 1.4 | 43.5 ± 3.0 | 33.6 ± 1.0 | 34.0 ± 0.8 | 41.2 ± 1.1 | 42.3 ± 1.5 |
| Month 2 | 40.9 ± 1.3 | 41.6 ± 1.0 | 32.8 ± 1.4 | 31.9 ± 1.4 | 41.4 ± 2.1 | 41.3 ± 3.4 |
| Month 3 | 44.4 ± 3.3 | 40.2 ± 0.7 | 30.8 ± 1.0 | 30.3 ± 0.2 | 42.5 ± 5.6 | 38.5 ± 2.2 |
| Month 4 | *44.4 ± 3.3* | *41.3 ± 1.1** | 32.0 ± 0.3 | 31.4 ± 0.8 | *43.2 ± 5.0* | *40.2 ± 1.0** |
| Month 5 | 40.7 ± 0.6 | 39.8 ± 0.9 | *28.7 ± 2.6* | *30.7 ± 0.9** | *44.0 ± 0.6* | *40.0 ± 1.6** |
| Month 6 | 40.7 ± 0.9 | 40.4 ± 0.8 | 29.8 ± 0.8 | 29.5 ± 0.8 | 40.5 ± 1.1 | 40.0 ± 0.8 |

Mean value of duplicates, n = 6/salt/lab/time point.
DFS: Double fortified salt; IOS: Iodized ordinary salt; IS: Iodized refined salt.
*Between laboratories significant at $p < 0.05$.

Weekly analysis of sub-samples of the three fortified salts stored under household storage conditions, during the first 3 months of the study period revealed no significant change in the iodine content (Figure 11).

*Figure 11. Weekly variation of iodine content in fortified salts during the first 3 months (Pooled for centers)*

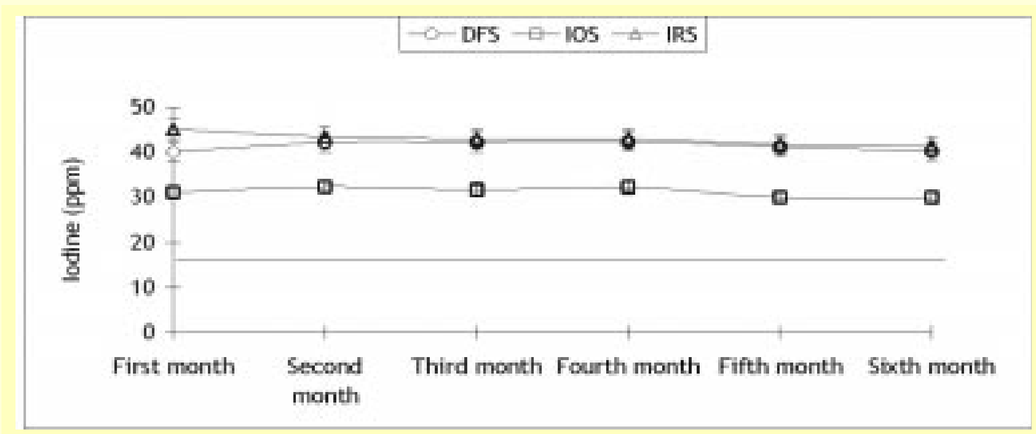



*Mean value of duplicates, n = 6/salt/Lab/Time point.*
*IRS: Iodized refined salt; DFS: Double fortified salt; IOS: Iodized ordinary salt.*

The present study has reconfirmed that large-scale production of DFS and iodized salts by dry mixing, packing and long distance transportation is feasible. There was a difference in the initial iodine content of different salts though the amount of $KIO_3$ used was same. This could be due to nature of the raw salts used. The modified method was found to be suitable for iodine estimation in all the types of salts. The IOS, IRS and DFS stored at New Delhi, Hyderabad and Bhubaneswar showed excellent iodine stability by retaining more or less the initial iodine content (30-40 ppm) at all the time points, throughout the study period. All the three fortified salts retained >15 ppm of iodine throughout the period of 6 months. The coastal environmental conditions at Bhubaneswar did not affect the stability of iodine in all the three fortified salts, indicating that the poor stability noted at Bhubaneswar in the earlier multicentric stability study on DFS appears to be due to the inadequacies in the method of estimation of iodine. The salt pouches stored and handled under simulated household conditions did not show any loss of iodine in all the three fortified salts. The conventional iodine estimation using $H_2SO_4$ is not suitable as it yields inconsistent levels of iodine in DFS. Monitoring the iodine content by the modified method using $H_3PO_4$ demonstrated good stability of iodine in DFS.

The study, thus, revealed that over a period of 6 months, the stability of iodine (Figure 12) was consistently good in DFS, IOS and IRS.

*Fig 12. Mean iodine content (ppm) of fortified salts by duration (Pooled for centers)*




*DFS: Double fortified salt; IOS: Iodized ordinary salt; IRS: Iodized refined salt*

## Conclusions

1. The results conclusively proved that iodine in double fortified refined salt prepared according to NIN formula has very good stability with very little loss of iodine in six months.
2. This study also demonstrated that a modified method is essential for consistent and accurate iodine estimation in DFS.

These results are in consonance with those of the positive and beneficial effects of DFS observed earlier.