

SUB- AND SUPER-EQUIVALENCE METHOD
AND
ITS RADIOANALYTICAL APPLICATIONS

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1988

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CHAPTER I

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1.1.1 Sub-equivalence radioanalytical analysis

(i) Principle and classification of

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A Dissertation Submitted

for the Degree of Doctor

of Science to the University of Tsukuba

1988

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has been prepared. Before explaining the principle and characteristics of this method, a brief account of the historical background is first explained.

Radiochemical Analysis

The discovery of radioactivity gave rise to the birth of radiochemical analysis. This is one of the chemical analysis to determine the purity, quantity or concentration of a radioactive element in the sample by measuring the activity of the element.

This method was applied in the determination of ^{137}Cs , ^{90}Sr , ^{131}I and ^{125}I in the animal tissue proteins and ^{210}Po in the hot spring.

Radiometric Analysis and Radiometric Titration

Application of a radioactive radionuclide to microanalysis was performed mainly by Shrover since 1925. This method is based on the use of a radionuclide as a radioactive indicator or a tracer and determines the element indirectly by the activity measurement. This method is classified into radiometric analysis and radiometric titration.

GENERAL INTRODUCTION

Recently, a new radioanalytical method, sub- and super-equivalence method of isotope dilution analysis (SSE-IDA)¹⁾ has been proposed. Before explaining the principle and characteristics of this method, a brief aspect of the historical background is first explained.

Radiochemical Analysis

The discovery of radioactivity soon gave the birth of radiochemical analysis. This is one of the chemical analysis to determine the sorts, quantity or concentration of a radioactive element in the sample by measuring the activity of the element.

This method was applied to the determination of ^{137}Cs ²⁾, Ba ³⁾ and ^{99}Tc ⁴⁾ in the nuclear fission products and ^{218}Po ⁵⁾ in the hot spring.

Radiometric Analysis and Radiometric Titration

Application of a radioactive indicator to microanalysis was performed mainly by Ehrenberg since 1925. This method is based on the use of a radioisotope as a radioactive indicator or a tracer and determine the element indirectly by the activity measurement. This method is classified into radiometric analysis (RA) in a narrow sense and radiometric titration.

Radiometric analysis(RA) is a method to analyse inactive samples using a radioactive indicator or tracer. RA is divided broadly into the direct and indirect method. For example, an excess amount of labeled precipitant is added to the sample to precipitate the sample ion, then the activity of the precipitate is measured and the quantity of the sample ion is obtained using the standard. This is a direct method. On the other hand, an excess of the known amount of the precipitant is added to the sample. After the separation of the precipitate, activity of the excess non-reactive precipitant is measured. The quantity of the sample ion is obtained by subtraction. This is an indirect method.

The advantage of this method is that the determination is possible only by the activity measurement without weighing. RA is a rapid and simple and applied to the determination of such metal ions as K⁶⁾, U⁷⁾, Sb⁸⁾ and Tl⁹⁾.

Radiometric titration(RT) is a method to know the end point of the titration by the use of a radioisotope. For the titration, precipitation, complex formation and redox reactions are used. The authors also determined a trace amount of antimony by this method using redox reaction with KBrO₃¹⁰⁾. In many cases, the end point is more accurately recognized by the ordinary methods. When the concentration of the sample ion is quite low, determination sometimes becomes possible only by increasing the specific activity of

the sample ion or the reagent. In addition, the color or turbidity of the sample does not affect the result like in the case of the UV spectrophotometric titration.

Substoichiometric radioanalysis

Substoichiometry was proposed independently by N.Suzuki¹¹⁾, J. Ruzicka¹²⁾ and J.E Zimakov¹³⁾, and was applied to the isotope dilution analysis. Ikeda et al. applied this method to radioanalysis and determined zinc in water¹⁴⁾.

As compared the amount of the reagent with the sample ion, RA uses an excess amount and RT does equivalent, while Subst RA uses substoichiometric amount. Therefore, this method has a higher selectivity, precision and accuracy. The principle and classification of this method will be mentioned in detail in section 1.1.1

Isotope Dilution Analysis

In the above-mentioned method(RA), the sample was not isotopically diluted. Determination was performed by comparing the sample with the standard substance or under the assumption that the sample and the reagent react quantitatively. When there exist the interfering substances which have the similar property to the sample and the separation of them is difficult, a large error will be caused in the RA methods. It is especially difficult to separate

one component from a complicated mixture of organic or biological substances. Though the perfect separation of a component is very difficult, when a part of it is purely separable, the isotope dilution analysis(IDA) makes the determination possible.

The fact that the perfect separation is unnecessary is an advantage, but the weighing of the purely separated part is necessary and it seems to be disadvantageous. Weighing of a radioactive substance causes an error in the determination and is tedious.

Substoichiometric Isotope Dilution Analysis

Substoichiometric isotope dilution analysis(Subst-IDA)^{11,12,13} was proposed as a method by which determination is possible only by the activity measurement. The principle of this method is that the amount of the separated products is kept constant by using the definite but substoichiometric amount of the reagent. In this case, the reagent (more than 99 %) reacts with the sample regardless of quantity of the sample.

From the points of selectivity, accuracy and sensitivity, this method is superior to IDA, beside the unnecessary of weighing. So, it has made a large progress. The author also applied this method by using redox reaction to the determination of trace amount of antimony in a metal

(Zn^{15,16}), As¹⁷) and Sn¹⁸) and the commercial ¹²⁴Sb sample¹⁹). In this method, pre-treatment is necessary to eliminate the interfering ions in the metal.

Sub- and Super-Equivalence Method of Isotope Dilution Analysis

Subst IDA needs a strict condition that the substoichiometric and definite amount of the reaction product must be separated. In order to fulfil this condition, more than 99 % of the reagent must be consumed. In other words, the formation constant must be large enough. However, the reagent possessing such property is rather few in general.

Klas et al. proposed a new method named "sub- and super-equivalence method of isotope dilution analysis (SSE-IDA)"¹⁾, in which this strict condition is not needed. This method made the selectivity of the reagent extremely wide.

SSE-IDA is based on the idea that the quantities of the reaction and the separation from two solutions are same if the sample concentrations are same in both solution regardless of the reactivity between the sample and the reagent. This will be called hereafter "The principle of iso-concentration".

This method is relatively new, and only twenty several papers were reported in both theory and application. Klas et al., Rao et al. and the authors are main groups studying on SSE-IDA.

The comparison of the SSE method with the Subst method

seems to be an important problem, because it clarifies the usefulness of SSE method. For this purpose, the authors will show in chapter 2 the results of the experiment by SSE-RA method under the condition where Subst RA does not give satisfactory results²⁰⁾. Furthermore, SSE-IDA will be shown in chapter 3 that accurate determinations are possible even in the presence of interfering element²¹⁾. Of course, it is hard to determine by Subst IDA under such a condition. In chapter 4, SSE-IDA was applied for the determination of inactive thallium in a commercial ^{204}Tl sample²²⁾. Finally, one of the biologically important substances, DNA was determined by this method using enzymatic reaction with restriction enzyme, HindIII²³⁾. Even in such a complicated system, determination become possible by the aid of SSE-IDA.

CHAPTER 1.

PRINCIPLE, CHARACTERISTICS, CLASSIFICATION AND THE CONDITIONS NECESSARY FOR THE DETERMINATION

1.1 Substoichiometry

The principle of subst RA and subst IDA is explained here using equations. Then, the conditions necessary for the determination and the classification are discussed.

All the symbols used in the explanation of the principle are shown here.

x, y : Amounts of unknown and known samples.

$m_x, m_{iy}, m_{x+iy}, m_{kx}$

: Amounts of the product separated from $x, iy, x+iy, kx$ samples ($i=1,2,3,..i, k=1,2,3,....k$)

A : Total activity

$a_x, a_{iy}, a_{x+iy}, a_{kx}$

: Activities of $m_x, m_{iy}, m_{x+iy}, m_{kx}$.

V : Volume of the reaction solution.

M : Amount of the reagent.

1.1.1. Substoichiometric Radiometric Analysis

1) Principle and classification of Subst-RA

Conventional RA includes gravimetric analysis using the reagent of the super-equivalent amount to the sample and the volumetric analysis (e.g. titration) using the equivalent amount of the reagent. It was thought to be impossible to determine using substoichiometric quantity of the reagent in the usual chemical analysis. Radioactivity, however, made this substoichiometric analysis possible. Ikeda et al. classified this method into standard reagent method and standard sample method.¹⁴⁾

(a) Standard reagent method

To the sample solution(x mol) of a chemical species S_x to be determined, a certain amount of labeled *S (activity is A , the weight is negligible compared with x) is added. This mixture is indicated as *S_x . In this case, specific activity become A/x . To this solution, substoichiometric amount(M mol) of the reagent R is added. the reaction formula is



If the all R is reacted, the quantity of the product SR must be equal to M mol. After the separation of SR from remaining S , the activity(a_x) of SR is measured. Since the

specific activity of *S_x does not change before and after the reaction. the following equation holds.

$$A/x = a_x/M \quad (1)$$

The unknown amount of x is calculated by equation (2).

$$x = (A/a_x).M \quad (2)$$

(b) Standard sample method

The standard sample solution, namely the solution containing the known amount(y mol) of S_y , is treated in the same way as before. Putting the activity of the product(*S_yR) as a_y in equation (1), equation (3) is obtained.

$$A/y = a_y/M \quad (3)$$

From (1) and (3), A and M are eliminated and equation (4) is obtained.

$$x = (a_y/a_x).y \quad (4)$$

In this method, it is not necessary to measure the activity A of added RI and the quantity M of the reagent. By measuring the activity(a_y) of the product corresponding to the standard sample(y mol), it is possible to analyse a series of samples only by the measurement of a_x . So, this is an

excellent method for the treatment of a large number of samples.

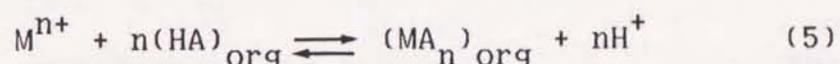
(ii) Conditions necessary for the determination by Subst-RA

It is necessary to explain about the common conditions required for Substoichiometry before referring to Subst RA.

Substoichiometry is based on the idea that the quantity of the product is constant and independent of the quantity of the analyte or the reactant if it reacts quantitatively with the substoichiometric amounts of the reagent. Therefore, the reaction between the reagent and the analyte must be completed more than 99 % for fulfilling above-mentioned strict condition.

By the way, J. Ruzicka et al. discussed theoretically the conditions when solvent extraction was applied for the substoichiometry²⁴). It is outlined as follows.

Let us consider an equilibrium reaction of eq.(5) for extraction.



Where, M^{n+} : metal ion

HA : reagent for extraction

MA_n : complex.

The equilibrium constant is given with equation (6).

$$K_{ex} = \frac{[MA_n]_{org} [H^+]^n}{[M^{n+}] [HA]_{org}^n} \quad (6)$$

For the substoichiometry, the quantity of the reagent is smaller than that of the metal ion to be determined and the reagent of more than 99 % must be consumed. From this condition, pH range is confined as follows.

$$\begin{aligned} \text{pH} > (1/n)\log(C_{\text{HA}}/n) \\ & - (1/n)\log\{C_{\text{M}} - (C_{\text{HA}}/n)(V_{\text{O}}/V)\} \\ & - (1/n)\log K_{\text{ex}} - \log 0.001C_{\text{HA}} \end{aligned} \quad (7)$$

Where,

$C_{\text{HA}}, C_{\text{M}}$: initial concentrations of the reagent and the metal ion, respectively.

V, V_{O} : volumes of aqueous and organic phases, respectively.

In order to estimate the pH range, the condition of $C_{\text{M}} = 1/2(C_{\text{HA}}/n)$ and $V = V_{\text{O}}$ is assumed. In this case,

$$\text{pH} > -(1/n) \cdot \log K_{\text{ex}} - \log 0.001C_{\text{HA}} \quad (8)$$

and when $C_{\text{HA}} = 10^{-4}\text{M}$,

$$\text{pH} > 7 - (1/n) \cdot \log K_{\text{ex}} \quad (9)$$

In the ordinary extraction system, it is more convenient to use low pH, because the precipitation by the hydrolysis is suppressed at low pH. As shown from equation (9), the larger is K_{ex} , the lower is applicable pH. Therefore, the reagent having a larger K_{ex} is more effective. This requirement is consistent with the strict condition mentioned before.

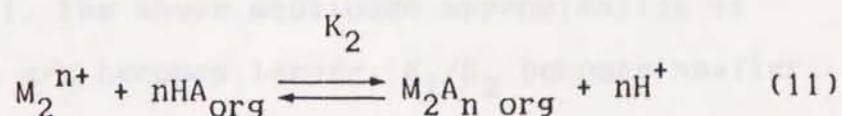
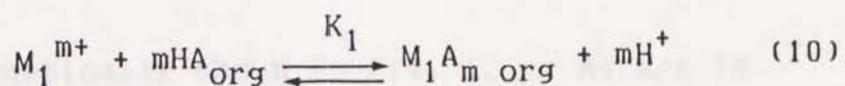
Beside the common condition mentioned in Subst method, the following conditions is required for Subst-RA. An unknown amount is calculated by comparison with the standard in the case of Subst-RA. If the sample contains interfering ions, the amounts of the interfering ions are different in the unknown and the standard samples. As the result, the amounts of the products separated are different ($m_x \neq m_y$) even though they contain the same amount of metal ions. In this case, a large error is caused. Therefore, the condition required is that the interfering ions should not be contained.

(iii) Characteristics of Subst-RA

It is necessary to explain about the common characteristics of substoichiometry before referring to those of Subst-RA.

Substoichiometry has wide selectivity of reagents, so the effect of interfering ions is more suppressed than the methods used before. This will be explained theoretically as follows.

Considering that two metal ions, M_1^{m+} and M_2^{n+} , coexist in the sample and competes in the reactions with the reagent, equation (12) is derived from two equilibrium equations (10) and (11).



$$\frac{[M_1A_m]_{org}}{[M_2A_n]_{org}} = \frac{\{ K_1 [HA]_{org}^{m-n} [M_1^{m+}] \}}{\{ K_2 [H^+]^{m-n} [M_2^{n+}] \}} \quad (12)$$

In order to make the discussion clear, the following simplification is made; n and m are equal to 1, and the initial concentrations of M_1^+ and M_2^+ are both a and the initial concentration of the reagent is b . At the equilibrium, the concentrations of M_1A and M_2A are x and y , respectively. In this case,

$$K_1 = \frac{x(x+y)}{\{(a-x)[b-(x+y)]\}} \quad (13)$$

$$K_2 = \frac{y(x+y)}{\{(a-y)[b-(x+y)]\}}. \quad (14)$$

So, equation (12) is rewritten as equation (15).

$$K_1/K_2 = \frac{x(a-y)}{[y(a-x)]} \quad (15)$$

For the quantitative separation of M_1 , we assume the condition that $x:y = 100 : 1$. Therefore, equation (15) becomes to equation (16).

$$\begin{aligned} K_1/K_2 &= 100[(a/x)-0.01]/[(a/x)-1] \\ &= 100(a/x)/[(a/x)-1] \end{aligned} \quad (16)$$

Equation (16) is graphically shown in Fig. 1. As a/x is always larger than 1, the above mentioned approximation is reasonable. When a/x becomes larger, K_1/K_2 becomes smaller.

Therefore, if the equilibrium constant of the interfering ion, K_2 is large, we must use the condition of large a/x value. As x increases with increase in b , we had better use small amount of b for obtaining a large a/x value. In other words, when the quantity of the reagent becomes smaller, the

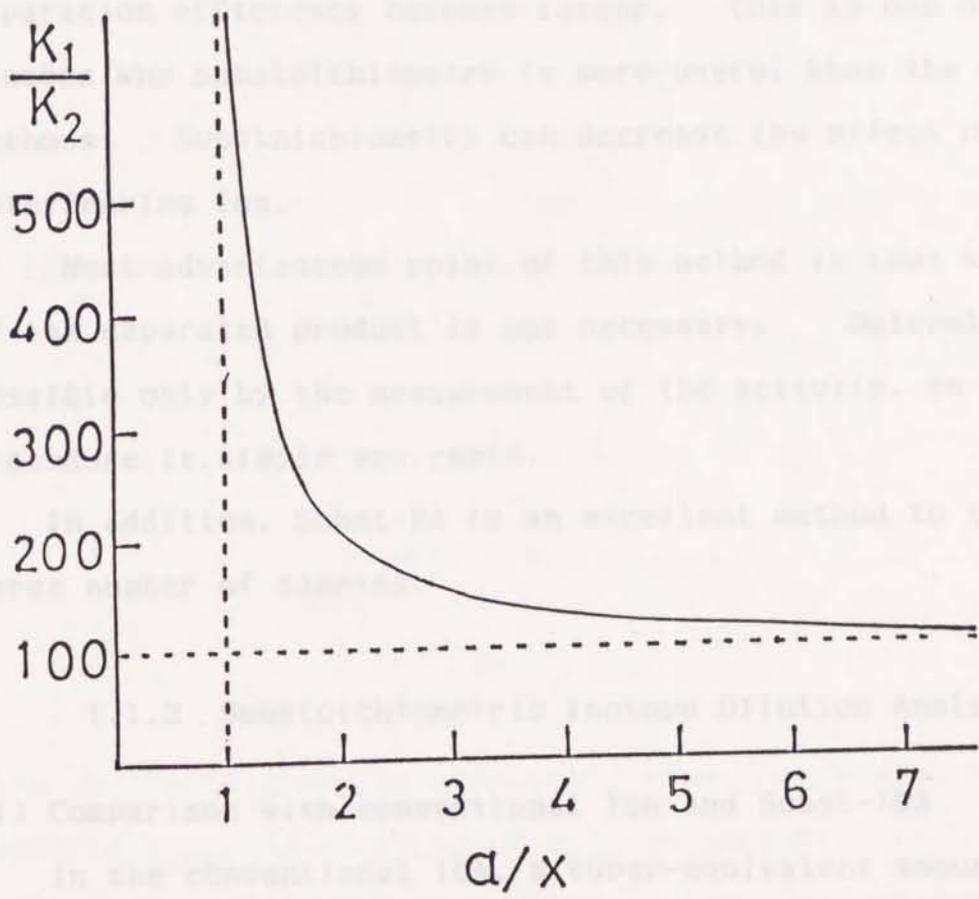


Fig. 1 Relationship between K_1/K_2 and a/x .

- x : The concentration of M_1A
- a : The original concentration of M_1
- M_1A : Complex

Therefore, if the equilibrium constant of the interfering ion, K_2 is large, we must use the condition of large a/x value. As x increases with increase in b , we had better use small amount of b for obtaining a large a/x value. In other words, when the quantity of the reagent becomes smaller, the separation efficiency becomes larger. This is one of the reasons why substoichiometry is more useful than the other methods. Substoichiometry can decrease the effect of the interfering ion.

Next advantageous point of this method is that weighing of the separated product is not necessary. Determination is possible only by the measurement of the activity, so the procedure is simple and rapid.

In addition, Subst-RA is an excellent method to treat a large number of samples.

1.1.2 Substoichiometric Isotope Dilution Analysis

(1) Comparison with conventional IDA and Subst-IDA

In the conventional IDA, a super-equivalent amount of the reagent is used, while in the Subst-IDA, a substoichiometric amount is used. It is necessary for IDA to weigh the amount and measure the activity of the separated compound. However, Subst-IDA does not need the weighing, so the procedure becomes simple and rapid and an origin of the error is eliminated. In addition, selectivity of the reagent

for separation becomes larger as explained before section (1.1.1 (iii)).

(ii) Principle of Subst-IDA

Subst-IDA has two variations, Subst direct IDA and Subst reverse IDA, like IDA. The principle of both methods are similar, so only Subst reverse IDA is explained here. an known amount of isotope (y) is added to the unknown sample (activity A, weight x). Then, substoichiometric amount(M) of the reagent is added to it and then, the product is separated. The weight and activity are m_{x+y} and a_{x+y} , respectively. As the specific activity before and after separation is unchanged, next equation is hold.

$$A/(x+y) = a_{x+y}/m_{x+y} \quad (17)$$

$$x = (A/a_{x+y}) \cdot m_{x+y} - y. \quad (18)$$

When the reagent reacts completely with the sample, M becomes equal to m_{x+y} , so weighing of m_{x+y} is unnecessary. activity (A) should be measured in advance. The unknown amount is obtained from one portion of the sample here, so this method is designated as one-point method. Next, the method combining two portions of the sample, x and x+y, is called the two-points method. It is not necessary to consider about m and A, but only the measurement of a_x and a_{x+y} is needed

by the equation (20) derived from equations (17) and (19).

$$A/x = a_x/m_x \quad (19)$$

$$x = [(a_x/a_{x+iy}) - 1].y \quad (20)$$

Furthermore, when x is diluted successively with the several known amounts of carrier (iy, i= 1,2,3,) as equation (21) shown below, x is obtained using a graph from equation (22). This method is called graphic method or multi-points method. In this case, it is also unnecessary to know m and A. The ratio of activities separated, a_x/a_{x+iy} , is plotted against the amount of carrier (iy) and according to equation (22) derived from equations (19) and (21), when is $a_x/a_{x+iy} = 2$, $x = jy$ is obtained by the way shown in Fig. 2 (Method IV).

$$A/(x+iy) = a_{x+iy}/m_{x+iy} \quad (21)$$

$$a_x/a_{x+iy} = (1/x).iy + 1 \quad (22)$$

A few methods of Subst-IDA were explained here, but many modifications were reported up to now. They were classified in the next section.

(iii) Classification of Subst-IDA

Subst method developed prominently after it was introduced into isotope dilution analysis, and various types

of Subst-IDA⁵⁰⁾ were proposed. They were classified here on the basis of the type of equations. First, they were divided into Subst-Direct-IDA, Subst-Reverse-IDA and Subst-Double-IDA. Then each of them was divided into one-point method, two-points method and multi-points or graphic method, respectively. These three methods use one, two and more than two aliquots of the sample for determination.

The classification is summarized in the Table 1. As is evident from the equations, each method has its own characteristics. Accordingly, it is better to select the most suitable method for the purpose.

Table 1 Classification of Subst-IDA

Direct Subst-IDA

One-point method;

$$A/(x+y) = a_{x+y}/M \quad (23)$$

from (23)

$$x = (A/a_{x+y}) \cdot M - y \quad (24)$$

Two-points method;

$$A/(x+y) = a_{x+y}/M \quad (23)$$

$$A/y = a_y/M \quad (3)$$

from (23) and (3)

$$x = y \cdot [(a_y/a_{x+y}) - 1] \quad (25)$$

Multi-points method or Graphic method;

Method I

$$A/(x+ly) = a_{x+ly}/M \quad (26)$$

$$A/y = a_y/M \quad (27)$$

From (26) and (27)

$$a_y/a_{x+ly} = x \cdot (1/ly) + 1 \quad (28)$$

When $a_y/a_{x+ly} = 2$, $x = jy$

Table 1 (continued)

Method II

$$A/(1x+^*y) = a_{1x+y}/M \quad (29)$$

$$\left\{ \begin{array}{l} A/^*y = a_y/M \end{array} \right. \quad (3)$$

From (29) and (3),

$$a_y/a_{1x+y} = (x/y) \cdot 1 + 1 \quad (30)$$

When $a_y/a_{1x+y} = 2$, $x = y/J$

Method III

$$1A/(x+1^*y) = a_{x+1y}/M \quad (31)$$

$$\left\{ \begin{array}{l} 1A/1^*y = a_{1y}/M \end{array} \right. \quad (32)$$

From (31) and (32),

$$a_{1y}/a_{x+1y} = 1 + (x/y) \quad (33)$$

When $a_{1y}/a_{x+1y} = 2$, $x = (2 - J) \cdot y$

Reverse Subst-IDA

One-point method;

$$A/(^*x+y) = a_{x+y}/M \quad (34)$$

from (34)

$$x = (A/a_{x+y}) \cdot M - y \quad (35)$$

Two-points method⁵²);

$$A/(^*x+y) = a_{x+y}/M \quad (34)$$

$$\left\{ \begin{array}{l} A/^*x = a_x/M \end{array} \right. \quad (1)$$

Table 1 (continued)

from (34) and (1)

$$x = [a_{x+y}/(a_x - a_{x+y})] \cdot y \quad (36)$$

Multi-points method or Graphic method;

Method IV $A/(^*x+iy) = a_{x+iy}/M \quad (37)$

$$\left\{ \begin{array}{l} A/^*x = a_x/M \quad (1) \end{array} \right.$$

From (37) and (1)

$$a_x/a_{x+iy} = (1/x) \cdot iy + 1 \quad (22)$$

$$\text{When } a_x/a_{x+iy} = 2, \quad x = jy$$

Method V $A/(^*ix+y) = a_{ix+y}/M \quad (38)$

$$\left\{ \begin{array}{l} A/^*x = a_x/M \quad (1) \end{array} \right.$$

From (38) and (1)

$$a_x/a_{ix+y} = i + y/x \quad (39)$$

$$\text{When } a_x/a_{ix+y} = 2, \quad x = y/(2 - j)$$

Method VI $iA/(i^*x+y) = a_{ix+y}/M \quad (40)$

$$\left\{ \begin{array}{l} iA/i^*x = a_{ix}/M \quad (41) \end{array} \right.$$

From (40) and (41)

Table 1 (continued)

$$a_{1x}/a_{1x+y} = (y/x) \cdot (1/1) + 1 \quad (42)$$

$$\text{When } a_{1x}/a_{1x+y} = 2, \quad x = y/J$$

Double Subst-IDA

Two-points method⁵²⁾;

$$A/(^*x+y) = a_{x+y}/M \quad (34)$$

$$\left\{ \begin{aligned} A/(^*x+2y) &= a_{x+2y}/M \end{aligned} \right. \quad (43)$$

From (34) and (43)

$$x = [a_{x+2y} \cdot 2y - a_{x+y} \cdot y] / [a_{x+y} - a_{x+2y}] \quad (44)$$

Multi-points method or Graphic method¹⁹⁾;

Method VII $A/(^*x+iy) = a_{x+iy}/M \quad (37)$

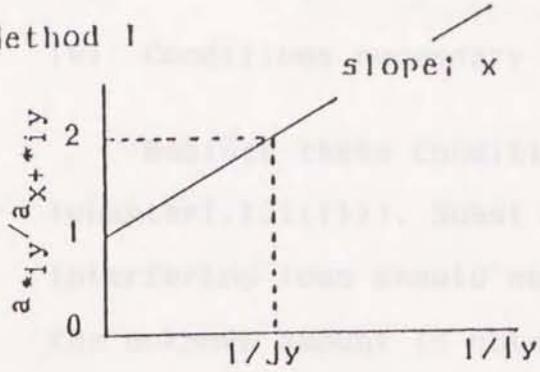
From (37)

$$iy = A \cdot M \cdot (1/a_{x+iy}) - x \quad (45)$$

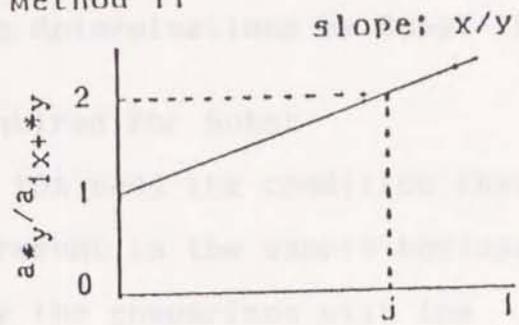
Above mentioned various types of graphic methods are illustrated in Fig. 2

Fig. 2 Various types of graphic method by Double-IDA

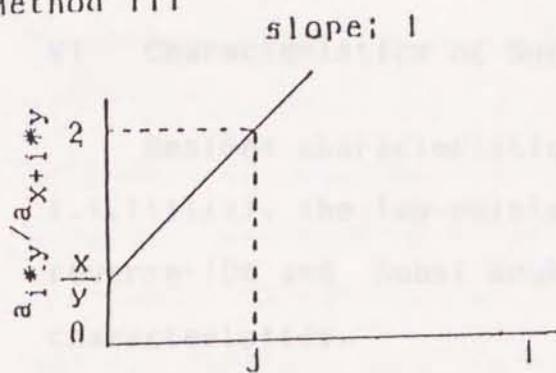
Method I



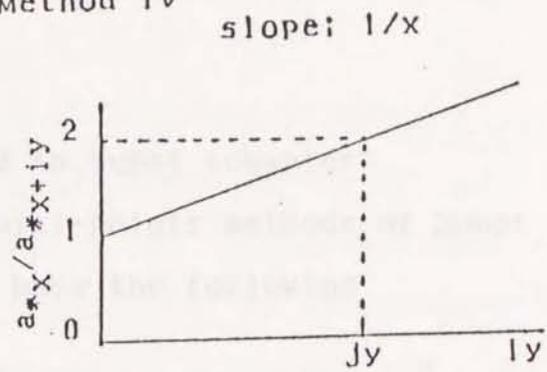
Method II



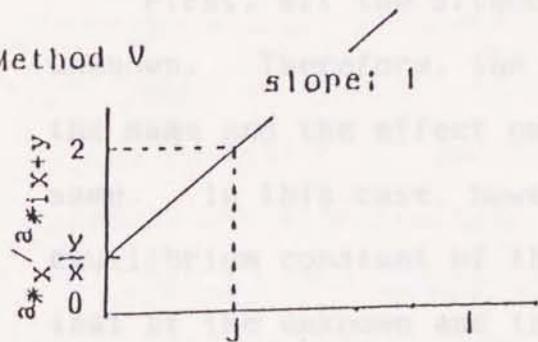
Method III



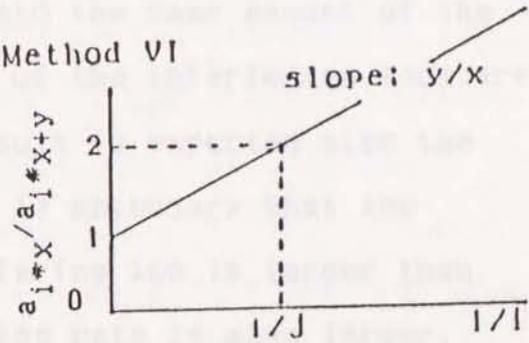
Method IV



Method V



Method VI



Method VII

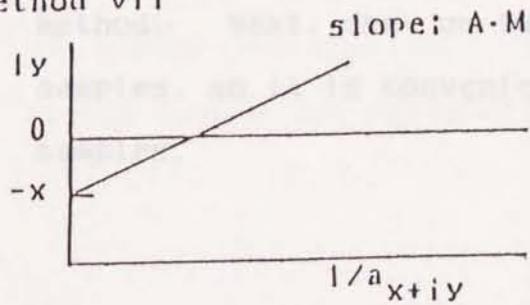


Fig. 2 Various types of graphic method by Subst-IDA

iv) Conditions necessary for the determinations by Subst-IDA

Besides these conditions required for Subst (chapter 1.1.1(ii)), Subst Direct IDA need the condition that interfering ions should not be present in the sample because the unknown amount is obtained by the comparison with the standard samples.

v) Characteristics of Subst-IDA

Besides characteristic stated in Subst (chapter 1.1.1(iii)), the two-points and multi-points methods of Subst reverse-IDA and Subst double-IDA have the following characteristics.

First, all the aliquots contain the same amount of the unknown. Therefore, the amounts of the interfering ions are the same and the effect on the result is expected also the same. In this case, however, it is necessary that the equilibrium constant of the interfering ion is larger than that of the unknown and the reaction rate is also larger. Ordinarily, the interfering ions compete with the substance to be analysed. In this case SSE-IDA will be an appropriate method. Next, one- or two-points method does not need many samples, so it is convenient to treat a large number of samples.

1.2 Sub- and super-equivalence method

SSE method was proposed in 1974 by Klas et al.¹⁾. As stated before, this method has an advantageous point that determination is possible under the condition where Subst method cannot give a correct result. The history of this method is still short, and the reports in regard to both the theory and the application are not so many. It was applied to the determination of Co²⁵⁾, VitamineB₁₂²⁶⁾, Se²⁷⁾, Tl^{28,29,30)}, Fe³¹⁾ and Zn³²⁾. On the other hand, the following theoretical reports were published; theory of complex-forming separation³³⁾, the theories in the case that the amount of the unknown is smaller than that of the reagent³⁴⁾, and the contrary case³⁵⁾, the determination of Zn and its reproducibility, accuracy, the effect of the interfering ions³⁶⁾, the theory of precipitation reaction³⁷⁾, the theory of errors of CCV Variant³¹⁾. As in the case of Subst-IDA, various types of SSE-IDA have been developed.

After the proposal of basic principle of SSE-IDA by Klas et al.¹⁾, Rao et al. proposed Reverse SSE-IDA³⁹⁾. Then, Klas et al. developed the principle of Universal SSE-IDA⁴⁰⁾. SSE method also includes NAA and IEA. In this thesis, SSE-IDA and SSE-RA are studied. The principle and the classification of SSE method are explained in sections 1.2.1 and 1.2.2.

1.2.1. Sub- and super- equivalence method of radiometric analysis

1) Principle of SSE-RA

The principle of SSE-RA is substantially identical with that of SSE-IDA¹⁾. The difference between them is that two series in SSE-IDA contain the unknown sample while in SSE-RA only one series contains the unknown and the other does the known sample.

The analytical procedure and the principle are outlined as follows :

- 1) First, unknown and known amounts of the elements to be determined (x and y_0) are labeled with the same amount of radioisotope (activity; A , the weight is negligibly small compared with x and y_0).
- 2) Next, two series of aliquots are prepared as follows. In one series, designated as the first series hereafter, each aliquot contains the same amount of the known sample (y_0). In the second series, each aliquot contains k times amount of unknown sample (kx), k is larger than 1.
- 3) An incremental known amount (iy , $i=1,2,\dots,j,\dots,i$) of non-radioactive elements is added to all the aliquots of the first series so that one (j th) of them has the same amount as the aliquots of the second series. When the amounts of each aliquot in the first series are denoted

as $Y, 2Y, \dots, jY, \dots, iY$ ($iY = y_0 + (i-1)y$), the specific activities of them are written as $A/Y, A/2Y, \dots, A/jY, \dots, A/iY$. On the other hand, each aliquot of the second series is not isotopically diluted. Therefore, the specific activity of them is equal to kA/kx .

- 4) All the aliquots in the both series are diluted to the same volume (V), then the concentration of the j th of the first series becomes the same as those of the second series.
- 5) The same amount of the reagent, which reacts with the elements to be determined, is added to all of the aliquots in the both series. The quantity of the reagent is always substoichiometric. That is to say, the quantity is smaller than iY and kx .
- 6) After the completion of the reaction, the products are isolated and the activities (a_{iY}, a_{kx}) of the isolated products (m_{iY}, m_{kx}) are measured.
- 7) As the specific activity is not changed before and after separation, the relation of the specific activity in the first series is

$$A/iY = a_{iY}/m_{iY} \quad (46)$$

Similarly, the relation in the second series is

$$kA/kx = a_{kx}/m_{kx} \quad (47)$$

- 8) From equations (46) and (47), the following equation is

derived.

$$a_{kX}/a_{iY} = (m_{kX}/m_{iY}) \cdot (1/x) \cdot iY \quad (48)$$

- 9) When the quantity of one (jth) of the first series is equal to that of the second series, ($jY = kX$), their concentrations become equal because the volumes of all the aliquots are adjusted to be equal as mentioned before. Accordingly, equation (49) is obtained.

$$jY/V = kX/V \quad (49)$$

As the extent of a chemical reaction is generally dependent on the concentration of reactants, the reagent does not always react quantitatively with the element to be determined. However, on the samples of the same concentration in both series, the quantities of the reaction and separation must be the same from the thermodynamic point of view. Therefore,

$$m_{jY} = m_{kX} \quad (50)$$

- 10) When the conditions at which equations (49) and (50) hold are fulfilled, equation (51) is derived from equation (46) and (47).

$$a_{kX}/a_{iY} = k \quad (51)$$

Substituting equation (48) by equations (49), (50) and

(51), the following equation is obtained.

$$x = iY/k \quad (52)$$

The quantity x is obtained by the graphic method shown in Fig.3. A curve is drawn by plotting a_{kX}/a_{iY} vs. iY , and x is obtained by dividing the abscissa's value (jY) corresponding to the point of $a_{kX}/a_{iY} = k$ by k .

- 11) In the case of $m_{iY} = m_{kX}$, which is the condition required for the conventional substoichiometry, equation (53) is derived

$$a_{kX}/a_{iY} = (1/x)iY \quad (53)$$

This is the limiting case of the SSE-RA, where the slope of the plotted curve is the steepest, so x is obtainable with the smallest error. Each case is shown in Fig.3.

- ii) Conditions necessary for the determination by SSE-RA

It is necessary to explain about the common conditions required for SSE method before referring to those of SSE-RA.

SSE method is based on the principle that the separated quantities are the same when two aliquots contain the same amounts of the species to be determined. Therefore, the concentration of the unknown in 2nd series (isotopically non-diluted) must be between those of two aliquots in 1st series (isotopically diluted).

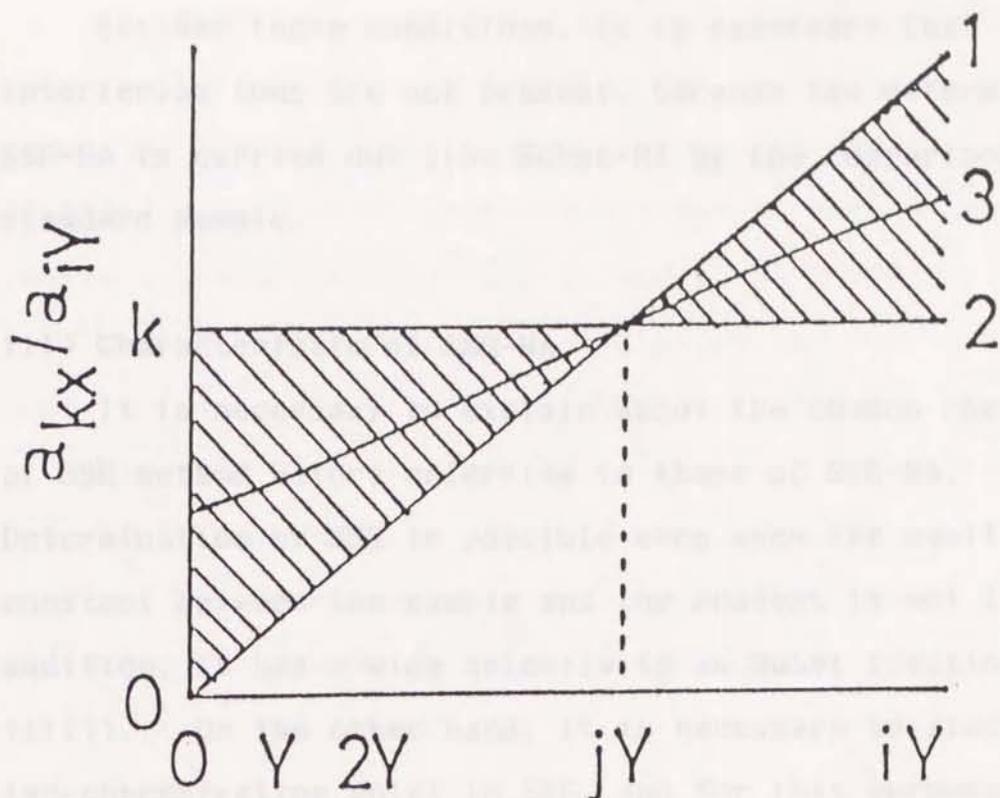


Fig. 3 Relation of a_{kx}/a_{iY} and iY

curve 1 ; Strict conditions required for the conventional substoichiometry are fulfilled (eq.53).

curve 2 ; The quantity of the reagent with respect to the element is super-equivalent, so all the element reacts and are separated. Therefore,

$$m_{iY} = iY, \quad m_{kx} = kx.$$

curve 3 ; General case where strict conditions are not fulfilled (eq. 48).

Besides these conditions, it is necessary that interfering ions are not present, because the determination by SSE-RA is carried out like Subst-RA by the comparison with the standard sample.

iii) Characteristic of SSE-RA

It is necessary to explain about the common characteristic of SSE method before referring to those of SSE-RA.

Determination by SSE is possible even when the equilibrium constant between the sample and the reagent is not large. In addition, it has a wide selectivity as Subst (section 1.1.1 (iii)). On the other hand, it is necessary to find out the iso-concentration point in SSE, and for this purpose, many samples must be prepared compared with Subst. It is troublesome and disadvantageous. However the procedure of SSE-RA is simpler than that of SSE-IDA and we can treat the many samples more rapidly.

1.2.2 Sub- and super- equivalence method of isotope dilution analysis

i) Principle of SSE-IDA

The principle of SSE-IDA is explained below together with the experimental procedure.

It is necessary to prepare two series of solutions. In the first, each solution contains the equal amount of sample(x) and the equal amount of radioisotope(A). In the second, each solution contains k times as much amount as the sample and radioisotope used in the first series. It is not essential to use duplicate analysis for the second series, but doing so will give a more reliable average value.

The solutions in the first series are isotopically diluted by the addition of regularly increasing amounts of carrier (iy, where $i=1,2,\dots$), so that their specific activities become $A/(x+iy)$. No carrier is added to the solutions in the second series, so their specific activities are all A/x .

All the solutions of both series are brought to the same volume and acid concentration by addition of an appropriate amount of the solvent and acid. Next, the equal substoichiometric amount of the reagent which is to react with the analyte is added to each solution in both series.

After completion of the reaction, the products are isolated and the activities(a_x, a_{x+iy}, a_{kx}) of the isolated products (masses m_x, m_{x+iy}, m_{kx}) are measured.

As the total activity of the analyte is not changed by the separation, the specific activity in the first series is written as Equation (21). Similarly, that for the second series is written as equation (47).

Hence,

$$a_{kx}/a_{x+iy} = (m_{kx}/m_{x+iy}) \cdot iy + m_{kx}/m_{x+iy}. \quad (54)$$

As the extent of chemical reaction is dependent on the concentrations of the reactants, the degree of the reaction by a fixed substoichiometric amount of reagent will depend on the concentration of the element to be determined. However, for the solution with the same total analyte concentration in both series, the degree of reaction and separation should also be the same, so in principle a solution could be prepared in the first series with j increments of carrier such that

$$m_{x+jy} = m_{kx}. \quad (55)$$

Under these conditions,

$$a_{kx}/a_{x+jy} = k. \quad (56)$$

From equations (54)-(56), we obtain equation (57).

$$x = jy/(k-1) \quad (57)$$

The quantity x is then obtained graphically as shown in Fig.11, by plotting a_{kx}/a_{x+iy} against iy and finding the abscissa value (jy) corresponding to $a_{kx}/a_{x+iy}=k$ for any value of k , and applying equation (57).

When $m_{x+iy} = m_{kx}$, which is the condition required for

conventional substoichiometry, equation (58) can be derived.

$$a_{kx} / a_{x+iy} = (1/x) \cdot iy + 1 \quad (58)$$

This is one limiting case of SSE-IDA.

ii) Classification of SSE-IDA

Various variants of SSE-IDA method proposed by Klas et al. and Rao et al. are classified here.

SSE-IDA are divided into SSE-Reverse IDA (Method I¹) and II^{this work}) and SSE-Direct-IDA (Method III⁴⁰), IV⁴⁰ and V³⁹), and explained from the following points. First, various equations obtained from the relationship between the activities of each set of aliquots in both series are shown in Table 2. Next, the principal equation is derived from them. Finally it is shown using a graph (Fig.4) and the unknown x is calculated from the iso-concentration point.

The principal equations are tabulated in Table 3. The symbols except those shown in section 1.1 are as follows :

*corresponding to radioactive species.

A/i^*y (A/i^*y , $A/2^*y$, $A/3^*y$)the different specific activity but the equal activity.

iA/i^*y (A/i^*y , $2A/2^*y$, $3A/3^*y$)the same specific activity but the different activity.

Table 2 Classification of SSE-IDA

SSE-Reverse-IDA

Method I¹⁾

$$1st; A/(^*x+ly) = a_{x+ly}/m_{x+ly} \quad (21)$$

$$2nd; kA/k^*x = a_{kx}/m_{kx} \quad (47)$$

From (21) and (47)

$$a_{kx}/a_{x+ly} = (m_{kx}/m_{x+ly}) \cdot (1/x) \cdot ly + m_{kx}/m_{x+ly} \quad (59)$$

When $kx/V = x+jy/V$,

$$m_{kx} = m_{x+jy}$$

$$a_{kx}/a_{x+ly} = k,$$

$$x = jy/(k-1) \quad (57)$$

Method II (this work)

$$1st; A/(^*ix+y) = a_{ix+y}/m_{ix+y} \quad (60)$$

$$2nd; kA/k^*x = a_{kx}/m_{kx} \quad (47)$$

From (60) and (47)

$$a_{kx}/a_{ix+y} = (m_{kx}/m_{ix+y}) \cdot (y/x) \cdot i + m_{kx}/m_{ix+y} \quad (61)$$

When $kx/V = jx+y/V$,

$$m_{kx} = m_{jx+y}$$

$$a_{kx}/a_{x+ly} = k,$$

$$x = jy/(k-1) \quad (57)$$

Table 2 (continued)

SSE-Direct-IDA

Method III⁴⁰⁾

$$\left\{ \begin{array}{l} \text{1st; } A/(ix+^*y) = a_{ix+y}/m_{ix+y} \end{array} \right. \quad (62)$$

$$\left\{ \begin{array}{l} \text{2nd; } kA/k^*y = a_{ky}/m_{ky} \end{array} \right. \quad (63)$$

From (62) and (63)

$$a_{ky}/a_{ix+y} = (m_{ky}/m_{ix+y}) \cdot (x/y) \cdot i + m_{ky}/m_{ix+y} \quad (64)$$

When $ky/V = jx/V$,

$$m_{ky} = m_{jx+y}$$

$$a_{ky}/a_{x+iy} = k,$$

$$x = (k-1) \cdot y/j \quad (65)$$

Method IV⁴⁰⁾

$$\left\{ \begin{array}{l} \text{1st; } A/(x+^*iy) = a_{x+iy}/m_{x+iy} \end{array} \right. \quad (66)$$

$$\left\{ \begin{array}{l} \text{2nd; } kA/k^*y = a_{ky}/m_{ky} \end{array} \right. \quad (63)$$

From (66) and (63)

$$a_{ky}/a_{x+iy} = (m_{ky}/m_{x+iy}) \cdot i + (m_{ky}/m_{x+iy}) \cdot (x/y) \quad (67)$$

When $ky/V = x+jy/V$,

$$m_{ky} = m_{x+jy}$$

$$a_{ky}/a_{x+iy} = k,$$

$$x = y \cdot (k-j) \quad (68)$$

Table 2 (continued)

Method V³⁹⁾ ;

$$\left\{ \begin{array}{l} \text{1st; } iA/(x+i^*y) = a_{x+iy}/m_{x+iy} \end{array} \right. \quad (69)$$

$$\left\{ \begin{array}{l} \text{2nd; } kA/k^*y = a_{ky}/m_{ky} \end{array} \right. \quad (63)$$

From (69) and (63)

$$a_{ky}/a_{x+iy} = (m_{ky}/m_{x+iy}) \cdot x \cdot (1/iy) + (m_{ky}/m_{x+iy}) \quad (70)$$

When $ky/V = x+jy/V$,

$$m_{ky} = m_{x+jy}$$

$$a_{ky}/a_{x+iy} = k,$$

$$x = jy \cdot (k-1)$$

(71)

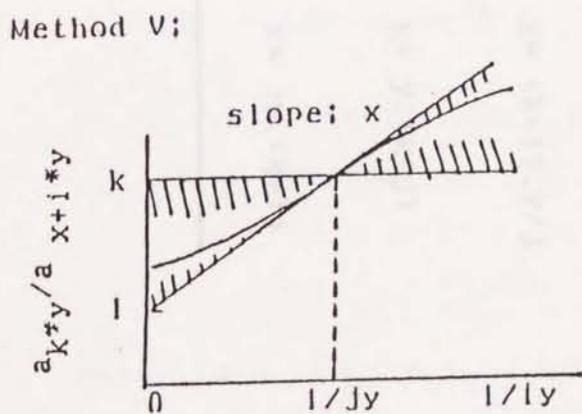
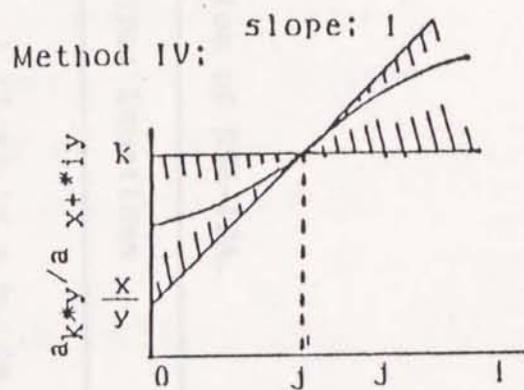
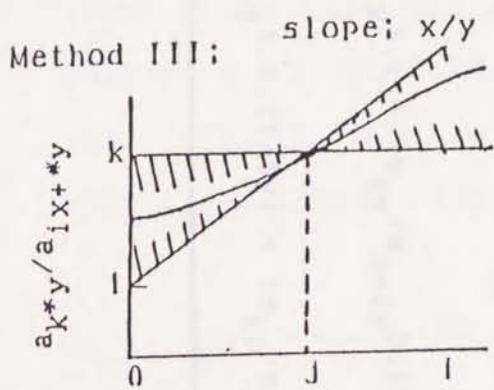
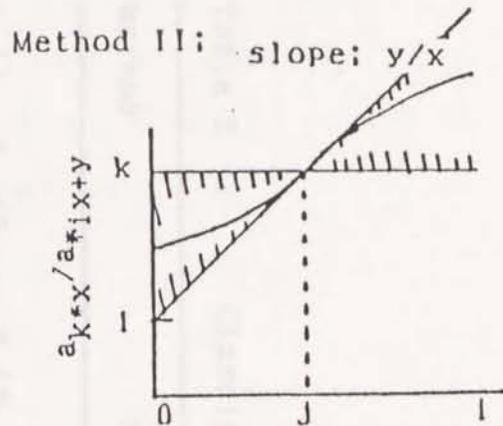
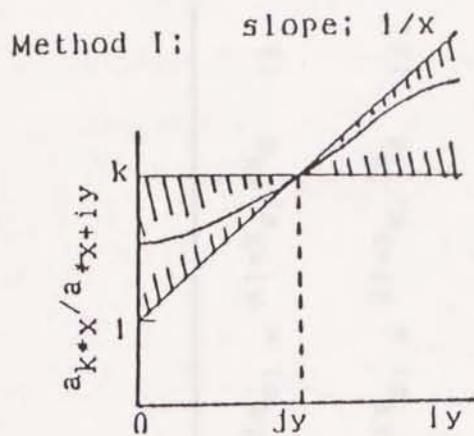


Fig. 4 Classification of SSE-IDA

Table 3 Classification of SSE-IDA.

Method	Principal Equations	The unknown
I)	$a_{kx}/a_{x+iy} = (m_{kx}/m_{x+iy}) \cdot (1/x) \cdot iy + m_{kx}/m_{x+iy}$	$x = jy/(k-1)$
II)	$a_{kx}/a_{ix+y} = (m_{kx}/m_{ix+y}) \cdot (1/x) \cdot i + m_{kx}/m_{ix+y}$	$x = jy/(k-1)$
III)	$a_{ky}/a_{ix+y} = (m_{ky}/m_{ix+y}) \cdot (x/y) \cdot i + m_{ky}/m_{ix+y}$	$x = (k-1) \cdot y/j$
IV)	$a_{ky}/a_{x+iy} = (m_{ky}/m_{x+iy}) \cdot i + (m_{ky}/m_{x+iy}) \cdot (x/y)$	$x = y \cdot (k-j)$
V)	$a_{ky}/a_{x+iy} = (m_{ky}/m_{x+iy}) \cdot x \cdot (1/iy) + (m_{ky}/m_{x+iy})$	$x = jy \cdot (k-1)$

iii) Optimum Conditions for the Determination by SSE-IDA

When the determination is carried out using SSE-IDA, optimum quantity of complex-forming reagent was devised theoretically,³³⁾ and the curves were drawn from the equation.

In the solution, the following reaction is in equilibrium.



$$K_d = [M][SR]/[MSR] \quad (73)$$

where, M, SR, MSR and K_d are metal, complex-forming reagent, complex and dissociation constant respectively. Setting (M), (SR) and (MSR) to the molar number of M, SR and MSR, respectively, equation (73) can be changed into (74).

$$K_d = [(M)-(MSR)][(SR)-(MSR)]/V(MSR) \quad (74)$$

This equation is arranged to equation (75).

$$2(MSR) = (M)+(SR)+K_d \cdot V - \{[(M)+(SR)+(K_d \cdot V)]^2 - 4(M)(SR)\}^{1/2} \quad (75)$$

By the way, the principal equation (59) of SSE-IDA is expressed as equation (76).

$$a_{kx}/a_{x+iy} = m_{kx}/m_{x+iy} (Z_i + 1) \quad (76)$$

Molar ratio (m_{kx}/m_{x+iy}) of the products separated from the both series is

$$m_{kx}/m_{x+iy} = (MSR)_{kx}/(MSR)_{x+iy}. \quad (77)$$

From equations (75), (76) and (77), equation (78) is obtained.

$$a_{kx}/a_{x+iy} = \frac{[(M)_{kx} + (SR)_{kx} + Kd.V - \{[(M)_{kx} + (SR)_{kx} + (Kd.V)]^2 - 4(M)_{kx}(SR)_{kx}\}^{1/2}](1+Z_1)}{(M)_{x+iy} + (SR)_{x+iy} + Kd.V - \{[(M)_{x+iy} + (SR)_{x+iy} + (Kd.V)]^2 - 4(M)_{x+iy}(SR)_{x+iy}\}^{1/2}} \quad (78)$$

By the way, (SR) in the both series are equal, so

$$(SR)_{kx} = (SR)_{x+iy} = (SR).$$

In addition, for the simplification of the equations, symbols are re-written as follows.

$$(M)_{kx} = k(x), \quad (M)_{x+iy} = (x) + (iy), \quad T = (SR)/(x), \\ K = Kd.V/(x).$$

The equation (78) is written as equation (79).

$$a_{kx}/a_{x+iy} = \frac{k(x) + T(x) + K(x) - \{[k(x) + T(x) + K(x)]^2 - 4k(x) \cdot T(x)\}^{1/2}(1+Z_1)}{(x) + (iy) + T(x) + K(x) - \{[(x) + (iy) + T(x) + K(x)]^2 - 4[(x) + (iy)] \cdot T(x)\}^{1/2}} \quad (79)$$

Substituting $Z_1 = (iy)/(x) = iy/x$,

the equation is simplified as equation (80).

$$\frac{a_{kx}}{a_{x+iy}} = \frac{\{k+T+K - [(k+T+K)^2 - 4kT]^{1/2}\} \cdot (Z_i + 1)}{\{1+Z_i+T+K - [(1+Z_i+T+K)^2 - 4(1+Z_i)T]^{1/2}\}} \quad (80)$$

When $K > 0$, $(T+k+K)^2 \gg 4kT$ and $(T+1+Z_i+K)^2 \gg 4T(1+Z_i)$, the Taylor development of the roots in the numerator and a denominator in the equation (80) gives equation (81).

$$\frac{a_{kx}}{a_{x+iy}} = \frac{k(T+1+Z_i+K)}{(T+k+K)} = \frac{k}{(T+k+K)} \cdot Z_i + \frac{k(T+1+K)}{(T+k+K)} \quad (81)$$

When the concentration of the reagent is low,

$$\lim_{T \rightarrow 0} \frac{k(T+1+Z_i+K)}{(T+k+K)} = \frac{k(1+Z_i+K)}{k+K} = \frac{k}{k+K} \cdot Z_i + \frac{k(1+k)}{k+K} \quad (82)$$

On the contrary, when the concentration is high,

$$\lim_{T \rightarrow \infty} \frac{k(T+1+Z_i+K)}{(T+k+K)} = k. \quad (83)$$

When $K=0$, $SR \rightarrow 0$, the theoretical curve approaches to a straight line. The slope of the line is;

when $K > 0$, slope is $k/(T+k+K)$.

$K > 0$ and $T \rightarrow 0$ slope is $k/(k+K)$.

$K > 0$ and $T \rightarrow \infty$ slope is k .

Theoretical curves are shown in Fig.5 for the various k, K, and T values. On the other hand, the curve at K=0 is shown in Fig. 6.

From the results of theoretical curves, the optimum concentration range of the reagent is as follows ;

When $K > 0$,

$$T < 1 \quad \text{or} \quad (SR) < (x)$$

when $K=0$

$$(1) \quad T < 1 + Z_i \quad \text{or} \quad (SR) < (x) + (iy)$$

$$(2) \quad \left\{ \begin{array}{l} T < 1 + Z_i \quad \text{or} \quad (SR) < (x) + (iy) \\ T < k \quad \text{or} \quad (SR) < k(x) \end{array} \right.$$

Special case,

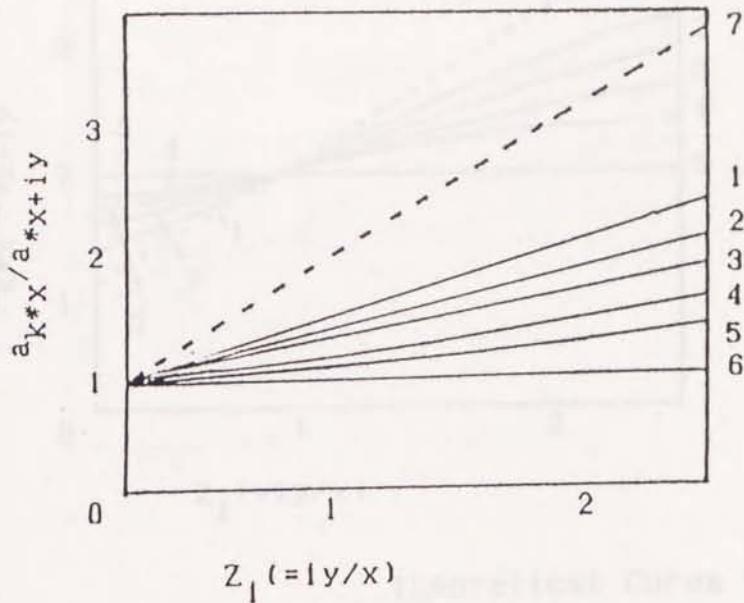
$$(3) \quad k < T < 1 + Z_i \quad \text{or} \quad k(x) < (SR) < (x) + (iy)$$

$$(4) \quad 1 + Z_i < T < k \quad \text{or} \quad (x) + (iy) < (SR) < k(x)$$

In the special cases (3) and (4), the unknown is obtained not from the isoconcentration point, but from the intercept.



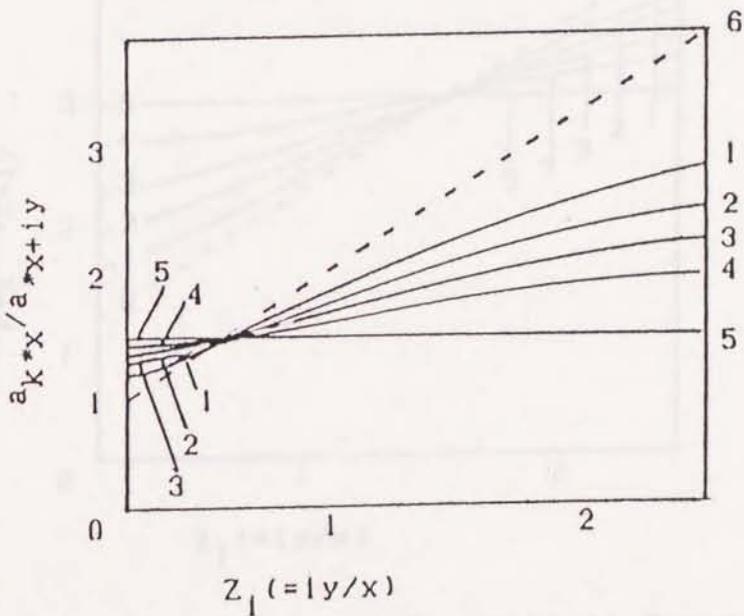
Theoretical Curve ($K > 0$)



Theoretical Curve (a)

- (1) $k = 1, K=1, SR \rightarrow 0$
- (2) $k = 1, K=1, SR=0.5x$
- (3) $k = 1, K=1, SR=x$
- (4) $k = 1, K=1, SR=2x$
- (5) $k = 1, K=1, SR=3x$
- (6) $k = 1, K=1, SR \rightarrow \infty$
- (7) $k = 1, K=0, SR < x$

Theoretical Curve (a)

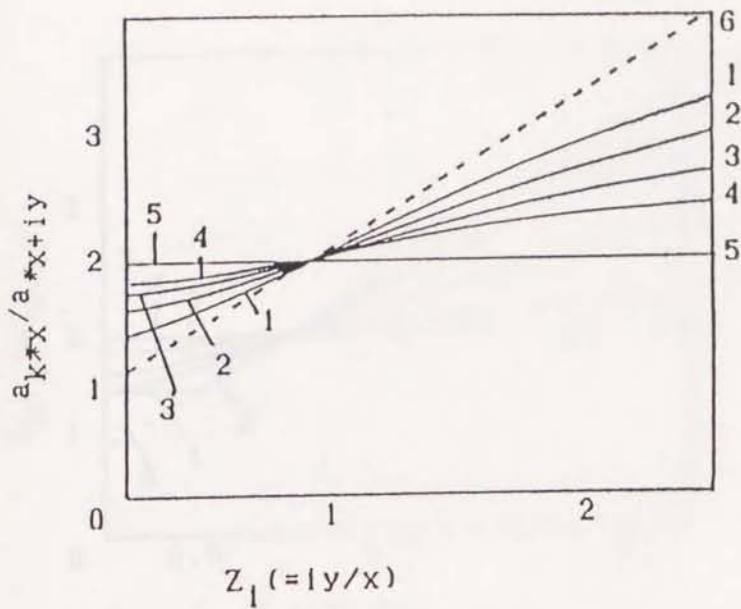


Theoretical Curves (b)

- (1) $k = 1.5, K=1, SR \rightarrow 0$
- (2) $k = 1.5, K=1, SR=0.5x$
- (3) $k = 1.5, K=1, SR=2x$
- (4) $k = 1.5, K=1, SR=3x$
- (5) $k = 1.5, K=1, SR=4x$
- (6) $k = 1.5, K=0, SR < x$

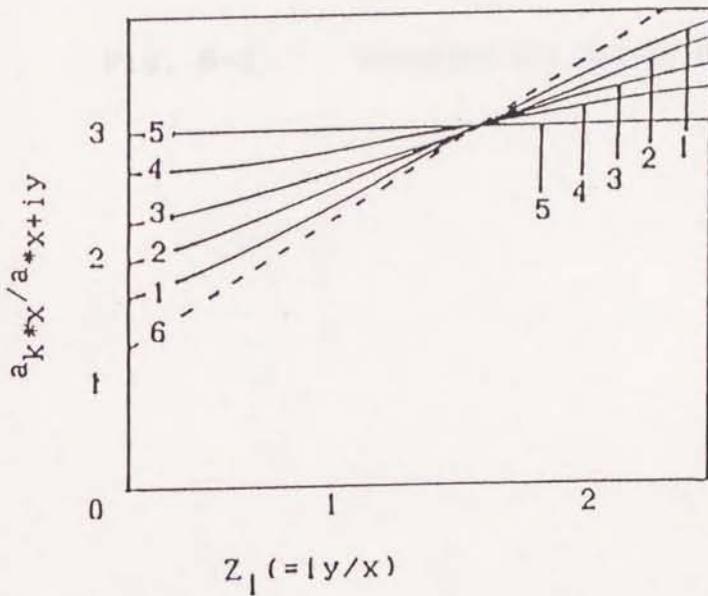
Theoretical Curves (b)

Fig. 5-1 Theoretical Curve of SSE-IDA ($K > 0$)



- (1) $k = 2, K=1, SR \rightarrow 0$
- (2) $k = 2, K=1, SR=0.5x$
- (3) $k = 2, K=1, SR=2x$
- (4) $k = 2, K=1, SR=3x$
- (5) $k = 2, K=1, SR \rightarrow \infty$
- (6) $k = 1, K=0, SR < x$

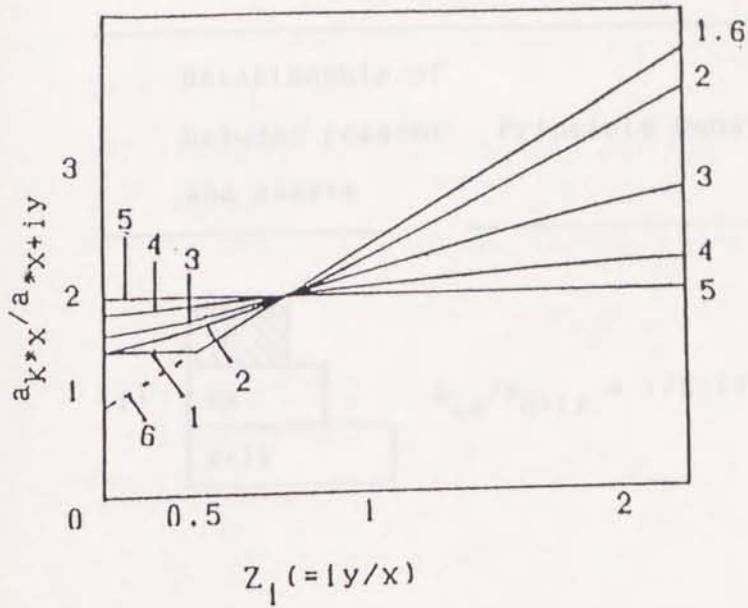
Theoretical Curve (c)



- (1) $k = 3, K=1, SR \rightarrow 0$
- (2) $k = 3, K=1, SR=0.5x$
- (3) $k = 3, K=1, SR=2x$
- (4) $k = 3, K=1, SR=3x$
- (5) $k = 3, K=1, SR \rightarrow \infty$
- (6) $k = 3, K=1, SR < x$

Theoretical Curves (d)

Fig. 5-2 Theoretical Curve of SSE-IDA ($K > 0$)

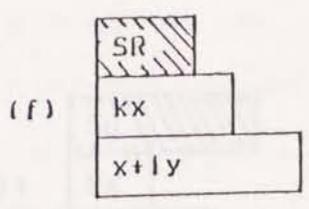


- (1) $k = 2, SR=1.5x, K=0$
- (2) $k = 2, SR=1.5x, K=0.1$
- (3) $k = 2, SR=1.5x, K=1$
- (4) $k = 2, SR=1.5x, K=10$
- (5) $k = 2, SR=1.5x, K \rightarrow \infty$
- (6) $k = 2, SR=1.5x, K=0$

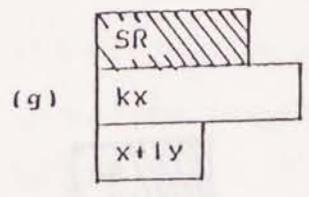
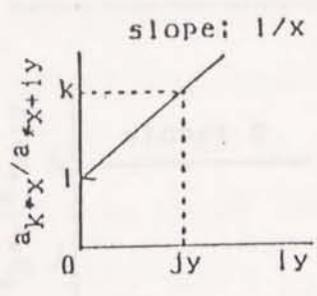
Theoretical Curves (e)

Fig. 5-3 Theoretical Curve of SSE-IDA ($K > 0$)

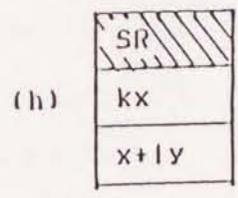
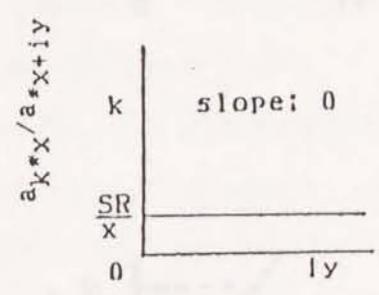
Relationship of between reagent and sample	Principle Equation	Theoretical Curve
--	--------------------	-------------------



$$a_{kx}/a_{x+ly} = 1/x \cdot ly + 1$$



$$a_{kx}/a_{x+ly} = (SR)/(x)$$



$$a_{kx}/a_{x+ly} = k$$

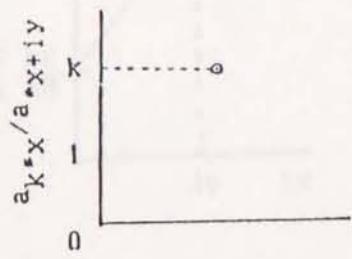
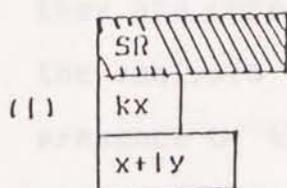


Fig. 6-1 Theoretical curve of SSE-IDA (K=0)

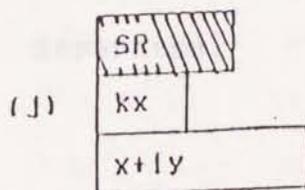
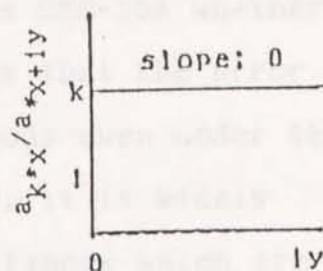
Relationship of
between reagent
and sample

Principle Equation

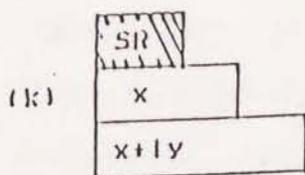
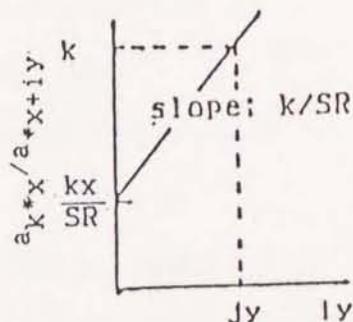
Theoretical Curve



$$a_{kx}/a_{x+ly} = k$$



$$a_{kx}/a_{x+ly} = [k/(SR)]ly + (kx)/(SR)$$



$$a_x/a_{x+ly} = (1/x)ly + 1 \quad (k=1)$$

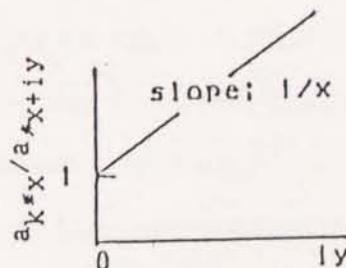


Fig. 6-2 Theoretical curve of SSE-IDA (K=0)

iv) Advantageous points of SSE-IDA

The most advantageous point of this methods is that accurate analysis is possible even when selective separation does not proceed quantitatively, in other words, equilibrium constant is not large. Second, new analytical methods or procedures are able to be checked by various SSE-IDA whether they are correct or not. The third point is that the error of the analysis is smaller than the other methods even under the presence of the interfering ions. Finally, it is widely applicable to the determination of the substances which are RI-labeled and selectively separable. For example, if a biological substance can be RI-labeled, it may be determined in a complicated biological system when it is selectively separated.

CHAPTER 2

REDOX SUB- AND SUPER-EQUIVALENCE METHOD OF RADIOMETRIC ANALYSIS

— DETERMINATION OF THE TRACE AMOUNT OF ANTIMONY —

2.1 Introduction

The principle of the sub- and super-equivalence method of isotope dilution analysis (SSE-IDA) was proposed by Klas et al.¹⁾. This method was one of the modifications and the development of the substoichiometric isotope dilution analysis (Subst-IDA). They and Rao et al. have reported theoretical study on this method and applied to the determination of cobalt²⁵⁾, vitamin B₁₂²⁶⁾, selenium²⁷⁾, thallium^{28, 29, 30)}, iron³¹⁾ and zinc³²⁾. The authors also applied the method to redox subst-IDA and examined main problems as to the determination of the trace amount of antimony by this method^{21, 41)}.

SSE-IDA has the following merits in contrast to the ordinary substoichiometry. Subst-IDA requires the strict conditions; the reaction between the species to be determined and the substoichiometric amount of the reagent must be completed. In other words, more than 99 % of the reagent must react. The product must be separated completely. However, the SSE-IDA does not need such conditions.

On the other hand, Ikeda et al.¹⁴⁾ and Grashenko et al.⁴²⁾ have reported on the substoichiometric radiometric analysis (Subst-RA). This method is more useful than ordinary radiometric analysis; for example, redox radiometric titration¹⁰⁾ in point of rapidness, simplicity, accuracy, precision and sensitivity.

Here, we developed a new method, SSE-RA, which is derived by combining the SSE-IDA with Subst-RA. It was applied to the determination of the trace amounts of antimony and fundamental problems were discussed in detail.

2.2 Experimental

2.2.1 preparation of reagents

$^{125}\text{Sb(III)}$ tracer solution⁴³⁾ : 100-300 μl of ^{125}Sb (processed unit, ca. 1 $\mu\text{Ci}/100 \mu\text{l}$ in 6 M HCl) was mixed with 10 ml of conc. HCl and refluxed for 30 minutes in order to reduce antimony(V). Thus obtained $^{125}\text{Sb(III)}$ tracer solution in 6 M HCl was always prepared just before labeling the sample solution. The labeled sample solution was stored in a brown-colored bottle and kept in a dark place in order to avoid self and photo-oxidation¹⁹⁾ of $^{125}\text{Sb(III)}$. The solution [$\text{Sb(III)}(+^{125}\text{Sb(III)})$ ca. 10 $\mu\text{g}/\text{ml}$ in 6 M HCl] was stable at the least for a week.

Sb(III) carrier solution : The solution was prepared by dissolving reagent grade Sb_2O_3 (99.999 %, Dojin Chemical Corp., Japan) in 6 M HCl. The concentration of Sb(III) was determined by KBrO_3 titration.

Oxidant : Commercially available $\text{K}_2\text{Cr}_2\text{O}_7$ solution titrated to be 0.1 N (factor; 0.995) was diluted to $(3.6 \text{ or } 3.9) \times 10^{-4}$ N.

Separating reagent : 0.05 M N-Benzoyl N-Phenylhydroxylamine (BPA) in CHCl_3 .

Unknown and known (or standard) sample solution : An appropriate amount of Sb(III) solution labeled with $^{125}\text{Sb(III)}$ was used as an unknown sample. The known sample solutions were prepared by mixing the amount of $^{125}\text{Sb(III)}$ solution with consecutively incremental amounts of non-radioactive Sb(III) solution.

2.2.2 Activity measurements

Activity of ^{125}Sb was measured on a scintillation counter (NaI(Tl), well-type), eliminating the disturbance by the activity of the daughter nuclide $^{125\text{m}}\text{Te}$.

2.2.3 Procedure

One example of the experimental procedure (Table 2 (sample No. 1)) is as follows. Two series of the solutions were prepared.

1st series ; 0.1 ml of Sb(III)(+ $^{125}\text{Sb(III)}$) solution (24.1

$\mu\text{g/ml}$ in 6 M HCl) were placed in seven brown-colored test tubes (30 ml) equipped with ground-in stoppers. Then, 0.0, 0.1, 0.2 ...and 0.6 ml of non-radioactive Sb(III) solutions (24.1 $\mu\text{g/ml}$ in 6 M HCl) were added to each of them.

2nd series : 0.2, 0.3, 0.4, 0.5, and 0.6 ml of Sb(III)(+ $^{125}\text{Sb(III)}$) solutions (24.1 $\mu\text{g/ml}$ in 6 M HCl) were placed in each of five paired test tubes.

Oxidation and separation : All the solutions in both series were adjusted to the same volume(2.2 ml) and the same acid concentration (3.3 M) by adding diluted HCl. Then, 0.1 ml of $\text{K}_2\text{Cr}_2\text{O}_7$ solutions (3.3×10^{-4} N) were added to them, and they were mixed thoroughly and were allowed to stand for 15-30 minutes in order to complete the oxidation. After that, the acid concentration was changed to 0.8-1.0 M HCl by adding diluted HCl, and unreacted Sb(III) was extracted with 3 ml of 0.05 M BPA in CHCl_3 solution. The activity of Sb(V) in the aqueous phase was measured.

2.3 Results and Discussion

2.3.1 Effect of the taken [Sb(III)] on the isolated [Sb(V)] and the result of the determination of trace amount of Antimony(III) by Redox SSE-RA.

Table 4 shows the result obtained by the above-mentioned procedure. The curves in Fig. 7 are drawn using the values in

Table 4. The results of determination were shown in Table 5 with that obtained from Table 4. There, the redox reaction between Sb(III) and $K_2Cr_2O_7$ was carried out at the acid concentrations of 1, 3 and 5 M. Table 5 shows that determination of 2-3 μg Sb(III) is possible within the accuracy of ca. 3 % in the wide acid concentration range of about 1-5 M.

When the m_{iY} and m_{kX} are constant independent of the change of iY and $m_{iY} = m_{kX}$, $iY \cdot a_{iY}$ must be constant and equal to $x \cdot a_{kX}$ as recognized from equation (46) and (47). However, as shown in Table 4, $iY \cdot a_{iY}$ and $x \cdot a_{kX}$ increase with the increase in iY and kx . This result suggests that m_{iY} and m_{kX} are not independent of the quantity of the element to be determined. The idea that the separated quantities are the same at the equal concentration point in both series is supported by the data of Table 4. For example, when $2Y=2x$ and $6Y=6x$, the relations $m_{2Y} = m_{2x}$ and $m_{6Y} = m_{6x}$ were observed. Another important result can be found in Table 5 ; precise and accurate results were obtained in spite of the varying m_{iY} and m_{kX} . This is consistent with the idea that the SSE-RA method does not require strict conditions as in the conventional substoichiometry, namely, the product must be formed and be separated constantly regardless of the initial quantity of the element.

By the way, the advantage of the SSE-IDA is that accurate

determination is possible in a wide concentration range compared with Subst-IDA, because of the unnecessary of the strict conditions⁴¹⁾. On the other hand, Subst-RA is more effective than Subst-IDA when a large number of the samples are treated.¹⁴⁾ Accordingly, SSE-RA has the merits of both the Subst-IDA and Subst RA.

Table 4. Radioactivities of the Sb(V) isolated from each aliquot

1st series ; known sample			2nd series ; unknown sample		
iY (μg)	a_{iY} (cpm)	$iY \cdot a_{iY}$ (= $A \cdot m_{iY}$)	kx (μg)	a_{kx} (cpm)	$x \cdot a_{kx}$ (= $A \cdot m_{kx}$)
$Y = 2.41$	13794	33244			
$2Y = 4.82$	6907	33292	$2x = 4.82$	13857	33395
$3Y = 7.23$	4690	33909	$3x = 7.23$	14280	34415
$4Y = 9.64$	3662	35302	$4x = 9.64$	14717	35468
$5Y = 12.05$	2985	35969	$5x = 12.05$	14968	36073
$6Y = 14.46$	2500	36150	$6x = 14.46$	14946	36020
$7Y = 16.87$	2146	36203			

The conditions are the same as for Fig. 7.

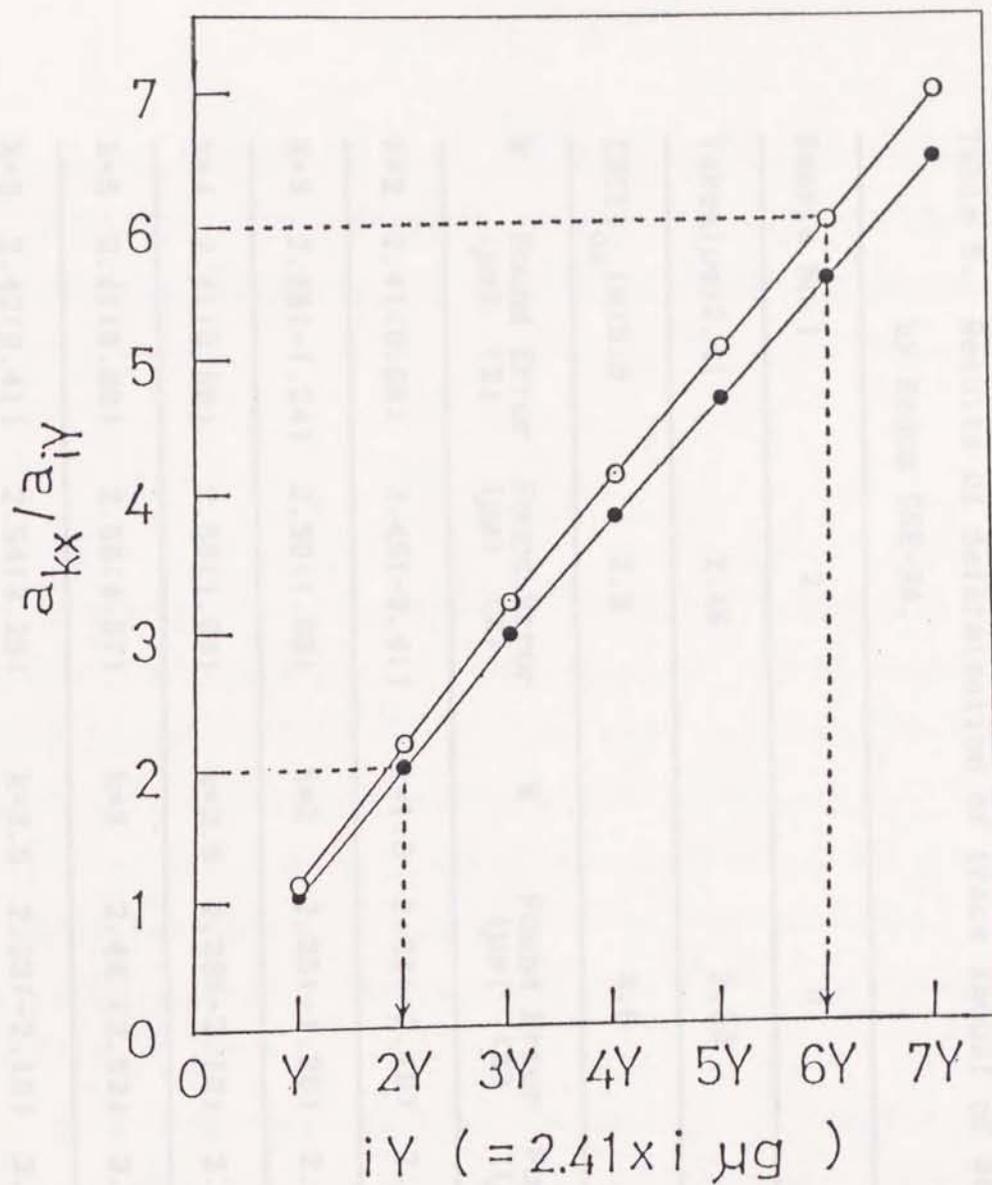


Fig. 7 An example of the determination of Sb(III) by redox SSE-RA for $k=2$ and 6.

The amount of taken Sb(III) was $2.41 \mu\text{g}$ and acid concentration was 3.3 M HCl at the stage of oxidation.

Table 5. Results of determination of trace amount of antimony(III)
by Redox SSE-RA.

Sample No	1	2	3	4	5	
Taken(μg)	2.41	2.46	2.38	2.38	2.38	
[HCl] _{ox} (M)	3.3	3.3	4.6	1.0	1.1	
k	Found Error (μg) (%)	Found Error (μg) (%)	k	Found Error (μg) (%)	Found Error (μg) (%)	Found Error (μg) (%)
k=2	2.41(0.00)	2.45(-0.41)	k=1.5	2.33(-2.10)	2.46(3.36)	2.42(1.68)
k=3	2.38(-1.24)	2.50(1.63)	k=2	2.35(-1.26)	2.44(2.52)	2.38(0.00)
k=4	2.41(0.00)	2.50(1.63)	k=2.5	2.33(-2.10)	2.43(2.10)	2.42(1.68)
k=5	2.41(0.00)	2.56(4.07)	k=3	2.44(2.52)	2.38(0.00)	2.40(0.84)
k=6	2.42(0.41)	2.54(3.25)	k=3.5	2.33(-2.10)	2.40(0.84)	2.41(1.26)

CHAPTER 3

COMPARISON OF THE SUB- AND SUPER-EQUIVALENCE METHOD AND SUBSTOICHIOMETRIC METHOD OF ISOTOPE DILUTION ANALYSIS FOR THE DETERMINATION OF A TRACE AMOUNT OF ANTIMONY

3.1 Introduction

Since substoichiometry^{11,12,13)} was introduced into isotope dilution analysis (IDA), various modifications of this method (Subst-IDA)^{24,44,45)} have been developed because of its higher accuracy, sensitivity and selectivity. Trace amounts of antimony in a metal were determined by the use of an oxidation-reduction reaction in substoichiometric activation analysis (redox Subst-NAA)^{46,47)} and redox Subst-IDA^{15,16,17,18)}.

Subst-IDA requires that the amounts of reagent reacted and product separated must always be constant. Ideally, more than 99 % of the reagent should be converted into the product, but even if the reagent does not react quite so quantitatively, the method can still be used provided that the amount separated is kept constant (such cases are not common).

The " sub- and super-equivalence " method of isotope dilution analysis (SSE-IDA) was therefore proposed by Klas et

al.¹⁾ This method is applicable under the conditions other than those listed above. Various types of SSE-IDA^{1,39,40)} were thought out, and theoretical studies^{33,34,35,36,37,38)} and applications to the determination of Co²⁵⁾, vitamin B₁₂²⁶⁾, Se²⁷⁾, Tl^{28,29,30)}, Fe³¹⁾, and Zn³²⁾ were reported. We also examined this method, applying Redox SSE-IDA to the determination of a trace amount of antimony⁴¹⁾.

However, no detailed analysis has been made on why (in contrast to Subst-IDA) SSE-IDA makes accurate determination possible when the basic conditions are not strictly fulfilled. We therefore examined the degree of reaction under various conditions and the effect of deviation from the required conditions, and determined antimony in the presence of an interfering species [As(III)].

3.2 Experimental

3.2.1 Reagents

¹²⁵Sb(III) tracer solution.^{43,48)} : ¹²⁵Sb (100-300 µg, ca. 1µCi/100ul in 6M hydrochloric acid) was mixed with 10 ml of concentrated hydrochloric acid and refluxed for 30 min in order to reduce antimony. This ¹²⁵Sb(III) tracer solution was always prepared just before use.

Synthetic sample solution : The solution of inactive

Sb(III) to be determined was labeled with the $^{125}\text{Sb(III)}$ tracer solution and was used as the labeled synthetic sample solution [Sb(III)+ $^{125}\text{Sb(III)}$, 24.1 $\mu\text{g/ml}$ in 6M hydrochloric acid]. It was stored in a brown bottle and kept in the dark to avoid self- and photo-oxidation⁴³⁾ of $^{125}\text{Sb(III)}$. This solution was stable for at least a week.

Sb(III) carrier solution : Prepared by dissolving reagent grade Sb_2O_3 (99.999% purity) in 6 M hydrochloric acid, and standardized by bromate titration.

Oxidant : Commercially available 0.1N potassium dichromate, suitably diluted.

Separation reagent : A 0.05M solution of N-benzoyl-N-phenylhydroxylamine(BPA) in chloroform⁴⁹⁾ was used.

3.2.2 Activity measurements

The activity of ^{125}Sb was measured with a scintillation counter, NaI(Tl), well-type, to eliminate interference by the activity of the daughter nuclide $^{125\text{m}}\text{Te}$ ($t_{1/2} = 58$ days, 35 and 110 keV).

3.2.3 Procedure

1st series : Aliquots (0.1 ml) of the synthetic sample solution [Sb(III)+ $^{125}\text{Sb(III)}$, 24.1 $\mu\text{g/ml}$ in 6M hydrochloric acid], were placed in seven brown test-tubes equipped with ground-in stoppers, and 0.0, 0.1,0.6 ml of carrier

solution [inactive Sb(III) solution, 24.1 $\mu\text{g/ml}$ in 6M hydrochloric acid] were added. Then 1.1, 1.0,0.5 ml of 6M hydrochloric acid were added so that all the solutions had the same volume and acidity.

2nd series : Pairs of 0.2, 0.3, 0.4, 0.5 and 0.6 ml portions (i.e., $k=2, 3, 4, 5, 6$) of the synthetic sample solution were placed in test-tubes of the type used for the first series.

All the solutions in both series were adjusted to the same volume and acid concentration by addition of 2.0 ml of 1.8M hydrochloric acid.

Oxidation and separation : Exactly 0.1 ml of $6 \times 10^{-4} \text{N}$ potassium dichromate was added to each tube, to give a total volume of 3.3 ml and acidity of 3.27M. The solutions were mixed and then stood for 15-30 min to complete oxidation. The acid concentration was then reduced to 1.0M by adding 8.0 ml of 0.001M hydrochloric acid, and the unreacted antimony was extracted with 3.0 ml of 0.05M BPA solution in chloroform.

The activity of antimony(V) in each aqueous phase was then measured.

3.3 Results and Discussion

The amounts of reagent and product separated must be strictly controlled in Subst-IDA, so the concentration ranges of the reagents, interfering elements and the species to be

determined are narrow, and large errors are caused by any deviation from the permitted range. In contrast, these limitations do not apply to SSE-IDA. We therefore examined the effect of the concentrations of the interfering element As(III), of hydrochloric acid and the reactant Sb(III) in order to define a set of the conditions under which Subst-IDA would not be practicable, but SSE-IDA would. Determination of the amount of the product (m) is usually difficult, but it is possible here to calculate m by means of the equation for Subst-IDA, since the activities before separation (A) and after separation (a), the quantity of Sb(III) to be determined (x) and that of non-radioactive carrier (iy) added to x can be measured.

3.3.1 Effect of the concentration of hydrochloric acid

The relationship between the amount of Sb(V) produced and the acid concentration was examined under the conditions shown in Fig. 8. The curves in Fig. 8 show that the amount separated decreases with increasing C_{HCl} and the difference from the expected value becomes remarkable. This suggests that some of dichromate may react with hydrochloric acid or that the equilibrium constant of the redox reaction between Sb(III) and $\text{K}_2\text{Cr}_2\text{O}_7$ becomes smaller with increasing acidity. In either case, the amount of Sb(III) oxidized would decrease.

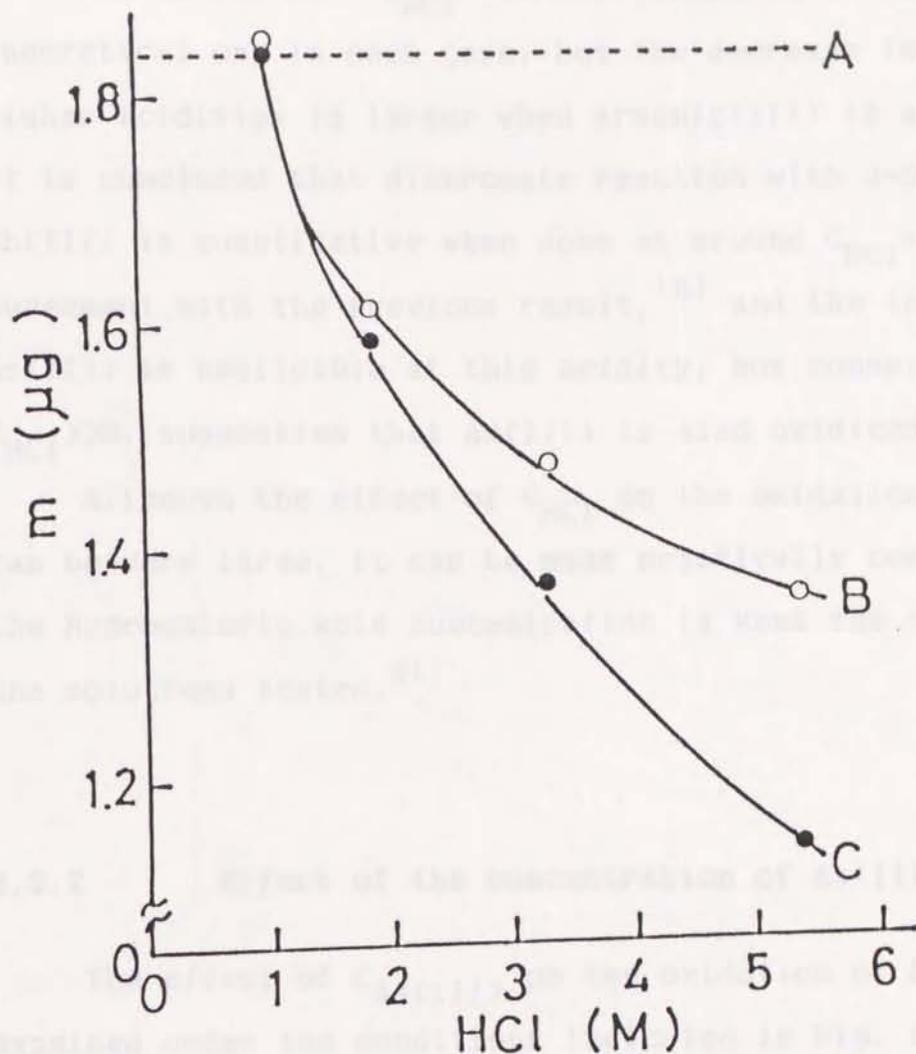


Fig. 8. Effect of $[HCl]_{ox}$ on the amount of Sb(V) separated.

A; Theoretical line, B; Sb(III) $8 \times 10^{-2} \mu eq$, C; Sb(III) + As(III) $8 \times 10^{-2} \mu eq + 4 \times 10^{-1} \mu eq$. $Vol_{ox} = 8.5, 4.5, 2.5$ and 1.5 ml for $[HCl]_{ox} = 1, 2, 3$ and $5N$ respectively. $K_2Cr_2O_7$ $3 \times 10^{-2} \mu eq$, $[HCl]_{ex} = 0.8N$, $Vol_{ex} = 10.5$ ml. $[HCl]_{ox}$ and Vol_{ox} are the acid concentration and total volume respectively after the addition of $K_2Cr_2O_7$; $[HCl]_{ex}$ and Vol_{ex} are the acid concentration and total volume respectively for the extraction stage.

The values near $C_{\text{HCl}} = 1\text{M}$ are almost equal to the theoretical one in each case, but the decrease in oxidation at higher acidities is larger when arsenic(III) is also present. It is concluded that dichromate reaction with 3~5 μg of Sb(III) is quantitative when done at around $C_{\text{HCl}} = 1\text{M}$, in agreement with the previous result,¹⁸⁾ and the interference of As(III) is negligible at this acidity, but conspicuous at $C_{\text{HCl}} > 2\text{M}$, suggesting that As(III) is also oxidized.

Although the effect of C_{HCl} on the oxidation of Sb(III) can be very large, it can be made practically constant when the hydrochloric acid concentration is kept the same for all the solutions tested.⁴¹⁾

3.3.2 Effect of the concentration of As(III)

The effect of $C_{\text{As(III)}}$ on the oxidation of Sb(III) was examined under the conditions indicated in Fig. 9.

In 1M hydrochloric acid medium, As(III) does not interfere with the oxidation of Sb(III) over the range tested. However, at higher acid concentration, As(III) takes part in the oxidation and its effect becomes larger with increasing $C_{\text{As(III)}}$ at constant $C_{\text{Sb(III)}}$ and C_{HCl} . On the other hand, when $C_{\text{As(III)}}/C_{\text{Sb(III)}}$ and C_{HCl} are kept constant, its effect becomes smaller with increasing $C_{\text{Sb(III)}}$.

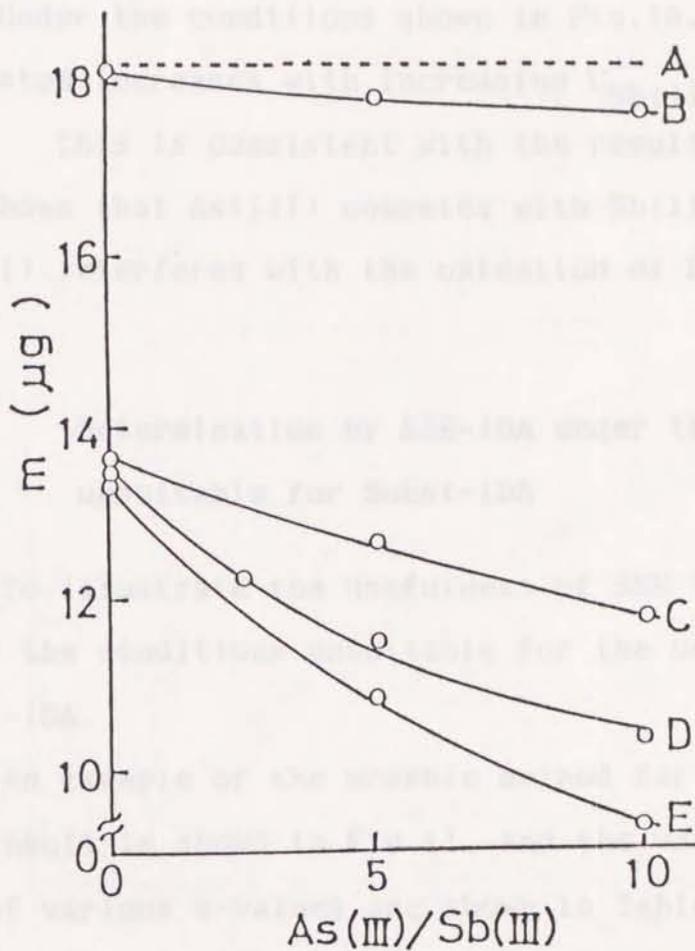


Fig.9. Effect of the molar ratio, As(III)/Sb(III), on the amount of Sb(V) separated

A: Theoretical line.

	Sb(III) µeq	[HCl] _{ox} M	Vol _{ox} ml	[HCl] _{ex} M	Vol _{ex} ml
B;	4×10^{-2}	1.0	8.5	0.8	10.5
C;	12×10^{-2}	5.4	1.5	0.8	10.5
D;	8×10^{-2}	5.4	2.0	0.9	11.8
E;	4×10^{-2}	5.4	1.5	0.8	10.5

$K_2Cr_2O_7 = 3 \times 10^{-2} \mu\text{eq.}$

3.3.3 Effect of the concentration of Sb(III)

Under the conditions shown in Fig.10, the amount of Sb(V) separated increases with increasing $C_{\text{Sb(III)}}$ at constant C_{HCl} . This is consistent with the results shown in Fig. 9, and shows that As(III) competes with Sb(III). As the result, As(III) interferes with the oxidation of Sb(III).

3.3.4 Determination by SSE-IDA under the conditions unsuitable for Subst-IDA

To illustrate the usefulness of SSE-IDA, it was tested under the conditions unsuitable for the use of conventional Subst-IDA.

An example of the graphic method for calculating the result is shown in Fig.11, and the values obtained by the use of various k-values are shown in Table 6. Table 7 shows the values calculated by means of various equations^{19,50,51,52)} for Subst-IDA, from the data obtained for each aliquot in the first series in this experiment, assuming that the amounts of Sb(V) separated are equal to the theoretical quantities. As expected, a large error is introduced in the Subst-IDA results, whereas SSE-IDA permits accurate determination within the expected error.

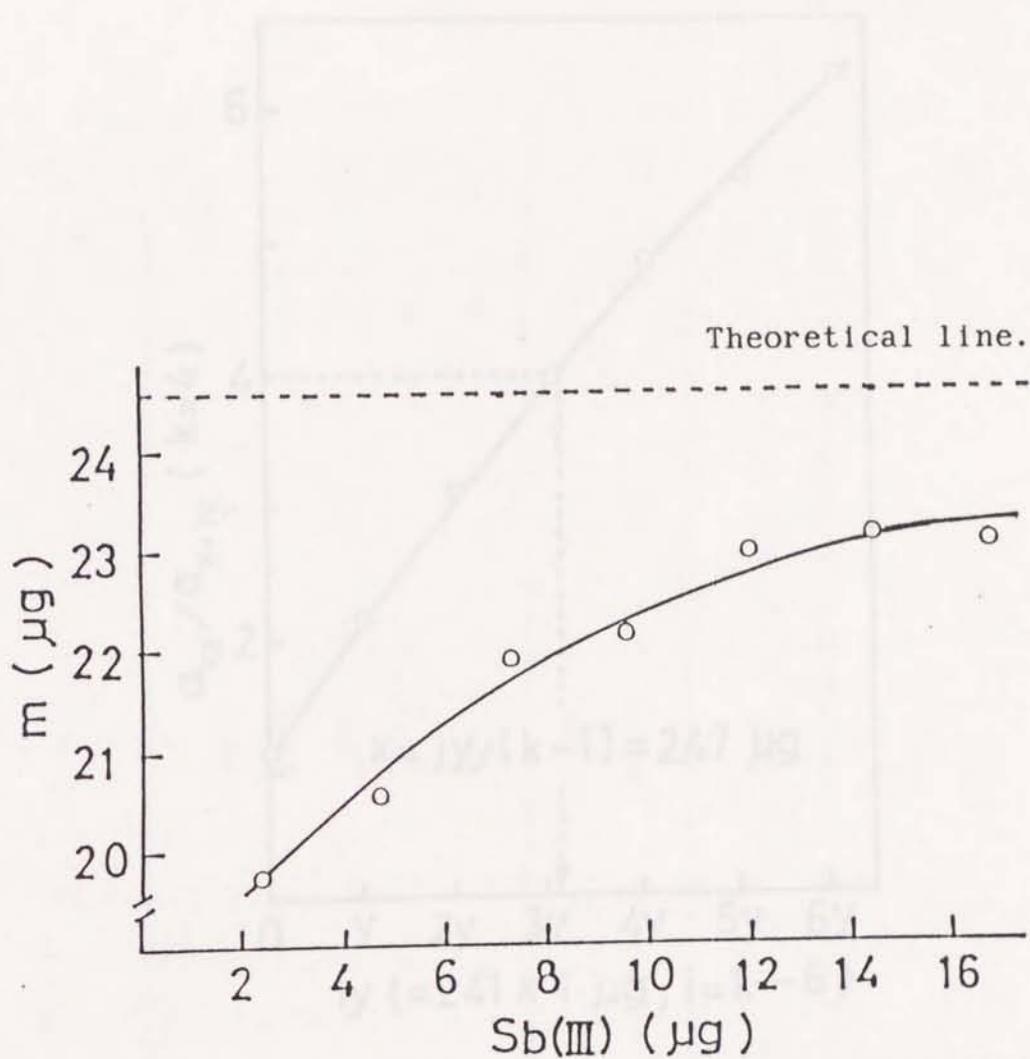


Fig. 10. Effect of amount of Sb(III) on amount of Sb(V) separated.

$[HCl]_{ox} = 3.3M$; $Vol_{ox} = 4.4 ml$,

$[HCl]_{ex} = 0.7M$; $Vol_{ex} = 19.4 ml$

$K_2Cr_2O_7 = 4 \times 10^{-2} \mu eq.$

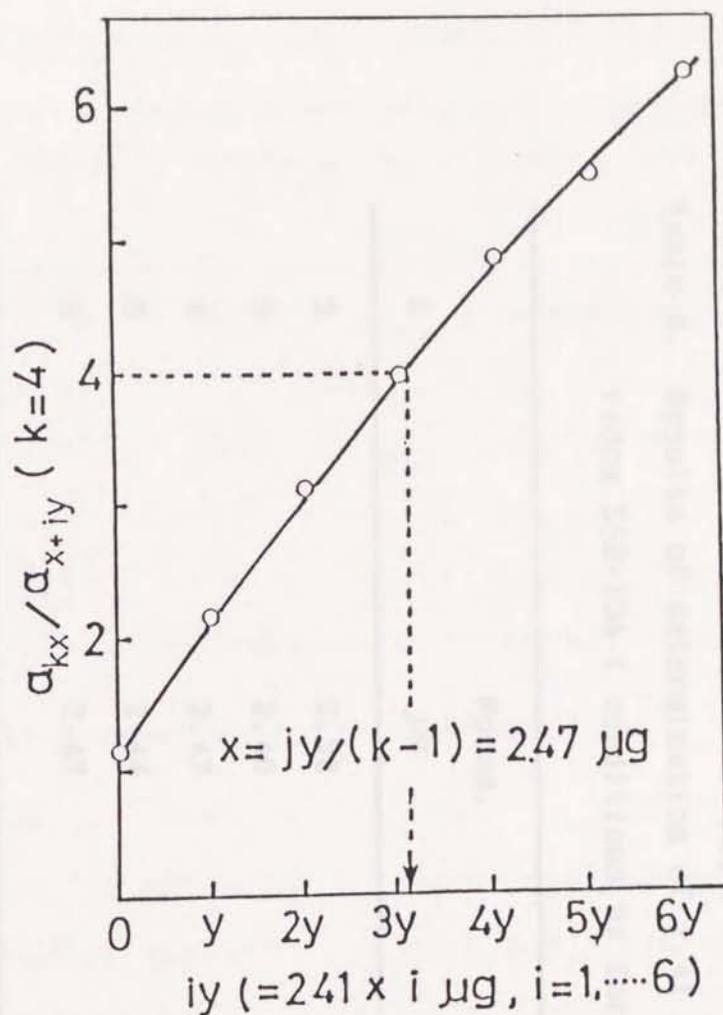


Fig. 11. An example of the determination of Sb(III) in the presence of an interfering element [As(III)] by Redox SSE-IDA.

0.2 ml of [Sb(III) (+ $^{125}\text{Sb(III)}$) 12.04 μg (total) +
 As(III) 7.49 μg l/ml solution in 6M HCl was taken.
 $[\text{HCl}]_{\text{ox}} = 3.3\text{M}$; $\text{Vol}_{\text{ox}} = 3.3 \text{ ml}$; $\text{K}_2\text{Cr}_2\text{O}_7 = 6 \times 10^{-2} \mu\text{eq}$
 $[\text{HCl}]_{\text{ex}} = 0.9\text{M}$; $\text{Vol}_{\text{ex}} = 11.3 \text{ ml}$.

Table 6. Results of determination of 2.41 μg of Sb(III) by redox SSE-IDA (conditions as for Fig. 11)

k	Found, μg	Error, %
2	2.32	-3.7
3	2.40	-0.4
4	2.47	2.5
5	2.44	1.2
6	2.47	2.5

Table 7. Results of determination of 2.41 μg of antimony(III) by Redox Subst-IDA (conditions as for Fig. 11)

Equation Subst-IDA	Found, μg	Error, %	Data used (see Table 8)
I	8.29	244	a_{x+y} , $A=14690$ cpm, $^*m_{\text{theory}}=3.65 \mu\text{g}$
II	2.82	17	a_{x+y} , a_x
III	2.89	20	a_{x+y} , a_{x+2y} , a_x
IV	2.81	17	a_{x+2y} , a_{x+3y}
V	3.13	30	a_{x+y} , a_{x+2y} , a_{x+3y} , a_x

The following equations of Subst-IDA were used.

Reverse Subst-IDA

- I One-point method $x = (m_{x+y})(A/a_{x+y}) - y$
 II Two-point method⁵¹⁾ $x = y \cdot a_{x+y} / (a_x - a_{x+y})$
 III Graphic method $a_x / a_{x+ly} = (1/x) \cdot ly + 1$

Reverse Subst Double-IDA

- IV Two-point method⁵²⁾ $x = (2y \cdot a_{x+2y} - y \cdot a_{x+y}) / (a_{x+y} - a_{x+2y})$
 V Graphic method¹⁹⁾ $ly = (m_{x+ly}) \cdot (A/a_{x+ly}) - x$

Only the values giving the best result for antimony found were used from Table 8.

$^*m_{\text{theory}}$ = weight of Sb equivalent to dichromate added.

The amounts separated were measured in order to confirm the reason given for the difference between the two sets of results. The amounts, m_i , separated under the conditions for Fig.11, were calculated on the basis of the activities (Table 8) by the use of equations (21) and (47). These values are plotted in Fig.12 against the total amount of Sb(III) in each aliquot in both series. The curve shows that m_{x+iy} and m_{kx} increase with increasing $x+iy$ and kx as shown in Fig. 10, and that the strict conditions for Subst-IDA are no longer fulfilled. At the same concentrations in both series, $x+iy = 2x$, $x+5y = 6x$, however, the amounts separated are equal over the whole range. This result accords with the principle of SSE-IDA and ensures the accurate determination under unsuitable conditions for Subst-IDA.

SSE-IDA obviously has many advantages over Subst-IDA and is a promising method for determination of trace amounts of metals in various substances.

Table 8. Activities of Sb(V) isolated from each aliquot
 (A = 14690 cpm ; conditions as for Fig. 11)

Amount of Sb(III) x + iy, (μg)	Activities of separated Sb(V)	
	1st series a _{x+iy} , (cpm)	2nd series a _x , (cpm)
x = 2.41	a _x = 9291	
x + y = 4.82	a _{x+y} = 5011	a _{2x} = 10227
x + 2y = 7.23	a _{x+2y} = 3489	a _{3x} = 10508
x + 3y = 9.64	a _{x+3y} = 2726	a _{4x} = 10672
x + 4y = 12.05	a _{x+4y} = 2284	a _{5x} = 11337
x + 5y = 14.46	a _{x+5y} = 1968	a _{6x} = 11632
x + 6y = 16.87	a _{x+6y} = 1736	

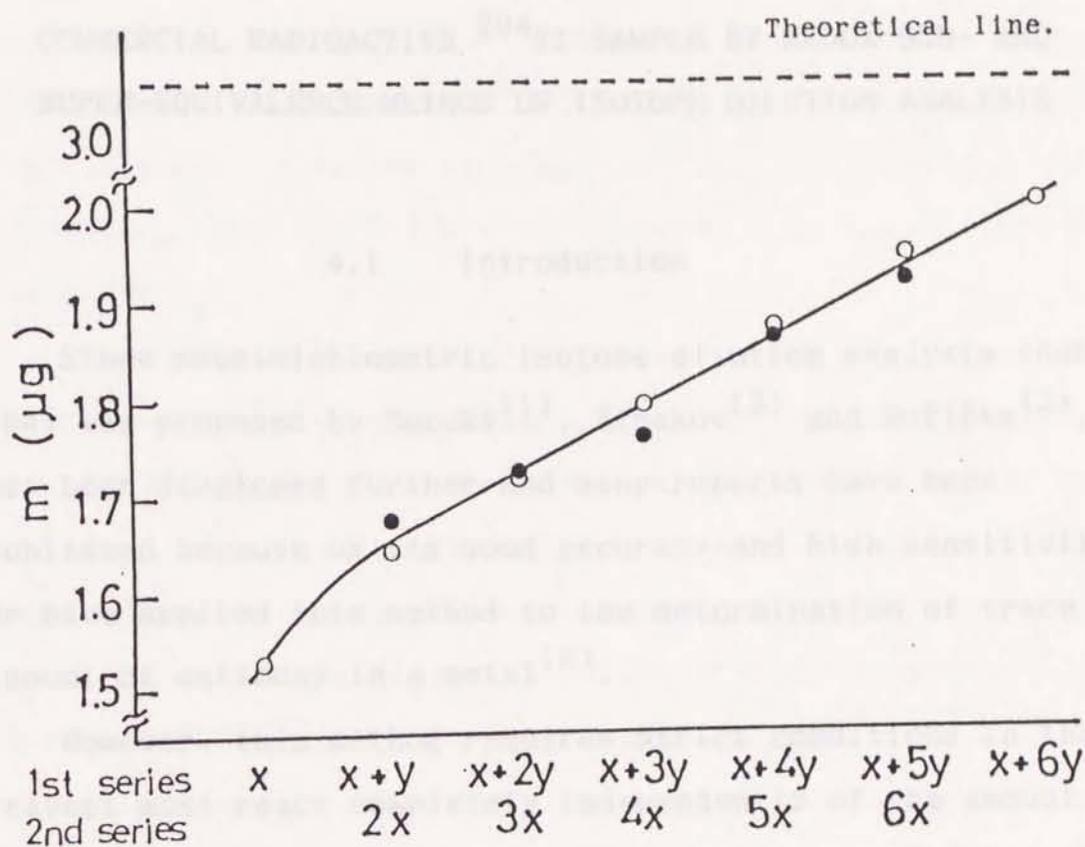


Fig. 12. Relationship between the amount of Sb(V) separated, m , and the total amount of Sb(III) in each aliquot in both series.

Sb(III) taken ($=x$) = $2.41 \mu\text{g}$; $1y = 2.41 \times 1 \mu\text{g}$,
 $l = 1, 2, \dots, 6$. The conditions are the same as for
 Fig. 11.

CHAPTER 4

DETERMINATION OF THE TRACE AMOUNT OF THALLIUM IN THE COMMERCIAL RADIOACTIVE ^{204}Tl SAMPLE BY REDOX SUB- AND SUPER-EQUIVALENCE METHOD OF ISOTOPE DILUTION ANALYSIS

4.1 Introduction

Since substoichiometric isotope dilution analysis (Subst-IDA) was proposed by Suzuki¹¹⁾, Zimakov¹³⁾ and Ružička¹²⁾, it has been developed further and many reports have been published because of its good accuracy and high sensitivity. We have applied this method to the determination of trace amount of antimony in a metal¹⁶⁾.

However, this method requires strict conditions in that the reagent must react completely independently of the amount of reactants and the product must be separated completely. These conditions are very severe and generally unfulfilled.

Klas et al. subsequently proposed a new method named the "sub- and super-equivalence method of isotope dilution analysis (SSE-IDA),"¹⁾ where these conditions were not required.

Both the theory and applications of this method have since been developed,⁵³⁾ but a comparison with the other methods was not carried out to demonstrate its advantages. Therefore, author demonstrated that the sensitive and accurate

determinations were possible over a wider range than that of the Subst-IDA when interfering ions are present in the sample solution²¹⁾.

In this report, author determined trace amounts of thallium in a commercial ^{204}Tl sample and examined the factors that affect oxidation and separation. In addition, data obtained using Subst-IDA were compared with those obtained with SSE-IDA.

For safety reasons it is desirable that the volume of radioactive waste water remaining after the experiments is as small as possible, so it is important to minimize the total volume of the reaction solution. Author tried to carry out the determination using a volume about 15 or 20 times smaller than usual, and the results indicated that the determinations were possible under such conditions.

4.2 Experimental

4.2.1 Preparation of the sample and the reagents

The sample to be analysed ; 200 μl of radioactive ^{204}Tl [88 mCi/g of Tl, 41 mCi/ml of $\text{Tl}(\text{NO}_3)_3$ in 3.7 M HNO_3] purchased from JAERI (Japan Atomic Energy Research Institute), was diluted to 10 ml with 2.4 M HNO_3 . This solution was named Orig.1. Ten-fold dilution of Orig.1 solution with 0.5 M HClO_4 gave Orig.2. A 4 ml of Orig.2 solution

was placed in conical beaker and 260 μ l of 0.06 % H_2SO_3 were added to effect complete reduction of Tl(III). The mixture was boiled on a hot-plate in order to accelerate the reaction and to eliminate the excess H_2SO_3 by the evaporation. This solution obtained was diluted with 0.5 M HClO_4 to a suitable concentration.

Stock carrier solution :

- i) Tl(III) carrier solution : Prepared by dissolving $\text{TlCl}_3 \cdot 4\text{H}_2\text{O}$ in 4 M HCl and standardized by EDTA titration.
- ii) Tl(I) carrier solution : Prepared by dissolving TlNO_3 (99.0 %), (Wako Pure Chemical Industries) in 2.4 M HNO_3 and diluting with 0.5 M HClO_4 in the same way as the sample. The concentration of Tl(I) was determined by the redox titration with KBrO_3 .

Stock Tracer solution :

- i) $^{204}\text{Tl(III)}$ tracer solution : Orig.2 solution was used as the ^{204}Tl tracer solution. On storage for several months, about 10-20 % of Tl(III) was reduced naturally, so the Tl(I) stock solution was oxidized by addition of KBrO_3 or concentrated HClO_4 just before use.
- ii) $^{204}\text{Tl(I)}$ tracer solution : The $^{204}\text{Tl(III)}$ tracer stock solution was reduced by the same method as for the sample.

Oxidizing Reagent : KBrO_3 dried at 130°C for 1h was used for the preparation of 0.100 N KBrO_3 solution.

Separating reagent : A 20% solution of tributyl phosphate (TBP) in benzene was used for the separation of Tl(III) from Tl(I) .

Diluent for carrier and tracer solutions : A 0.5 M HClO_4 - 2.4 M HNO_3 (9+1) mixed acid solution was used.

All reagents rest were of special grade.

4.2.2 Activity measurement

Activity of ^{204}Tl was measured with an end-window G.M.counter.

4.2.3 Procedure

Two series of solution were prepared.

1st series : Aliquots (0.1 ml) of the sample solution, $\text{Tl(I)} + ^{204}\text{Tl(I)}$, $20.7 \mu\text{g/ml}$ in ($[\text{H}^+] = 0.8 \text{ M}$, $[\text{ClO}_4^-] = 0.5 \text{ M}$, $[\text{NO}_3^-] = 0.2 \text{ M}$), were placed in six 1.5 ml polycarbonate micro test-tubes equipped with stoppers. Then 0.1 ml of various Tl(I) carrier solutions, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0 $\mu\text{g/ml}$ in ($[\text{H}^+] = 0.8 \text{ M}$, $[\text{ClO}_4^-] = 0.5 \text{ M}$, $[\text{NO}_3^-] = 0.2 \text{ M}$) were added.

2nd series : Pairs of 0.1 ml portions of the variously concentrated sample solutions, $[\text{Tl(I)} + ^{204}\text{Tl(I)}] = 20.7 \times 2 \mu\text{g/ml}$, $20.7 \times 3 \mu\text{g/ml}$ in ($[\text{H}^+] = 0.8 \text{ M}$, $[\text{ClO}_4^-] = 0.5 \text{ M}$, $[\text{NO}_3^-] = 0.2 \text{ M}$), were placed in the test tubes of the same type as used for the

first series.

All the aliquots in both series were adjusted to the same volume and same acid concentration by addition of 0.2 ml of mixed acid solution (0.1 ml of 4 M HCl + 0.1 ml of 5 M HClO₄) and then homogenized by shaking using a micro test-tube mixer; the drops adhering to the stoppers and the walls of the tube were collected by centrifugation. The same operations were carried out in the following experiments using micro-tubes.

Oxidation and separation : A 0.1 ml of 1.64×10^{-4} M KBrO₃ solution was added to each aliquot ; the total volume was 0.5 ml and the ion concentrations were [H⁺]=2.2 M, [Cl⁻]=1.0 M, [ClO₄⁻]=1.2 M and [NO₃⁻]=0.05 M. After the oxidation, thallium(III) was extracted with 0.5 ml of 20 % TBP in benzene. The activity of thallium(III) in each organic phase was then measured with a GM counter.

4.3 Results and Discussion

4.3.1 Suitable solvents for the oxidation and the separation

It has been reported that Tl(III) is separable from Tl(I) in the mixed chloride-sulfate solution by the extraction with TBP⁵⁴). therefore, we first examined how the pH values of HCl, HClO₄, and (HCl+HClO₄) solutions affect the efficiency of the separation. It was found that Tl(III) at concentrations

of several tens of $\mu\text{g/ml}$ was completely separated from all the aqueous Tl(I) solution over a wide $[\text{H}^+]$ range with 20 % TBP solution in benzene.

However, insoluble TlCl was precipitated because of its low solubility in HCl solution with increasing concentration of the Tl(I) . This precipitation results in changes of concentration and it becomes difficult to obtain reliable data. Hence this HCl solution is not suitable as the reaction solution.

Next, we examined the redox reaction in HClO_4 solution. As shown in Fig.13, oxidation with KBrO_3 was almost inhibited in 1~3 M HClO_4 solution. On the other hand, it was found that Tl(I) is oxidized undesirably by concentrated HClO_4 solution. Therefore, HClO_4 solution is also unsuitable as the reaction solution.

Then, we examined the behavior of Tl(I) in the HCl-HClO_4 mixed solution. As the result, TlCl was not precipitated, so the oxidation and the separation were carried out in the HCl-HClO_4 solution.

4.3.2 Effect of $[\text{Tl(I)}]$ on the precipitation ratio

Author examined the effect of $[\text{Tl(I)}]$ on the precipitation ratio of TlCl in HCl-HClO_4 solution. Under the conditions shown in Fig.14, the precipitation ratio was about 2 % when

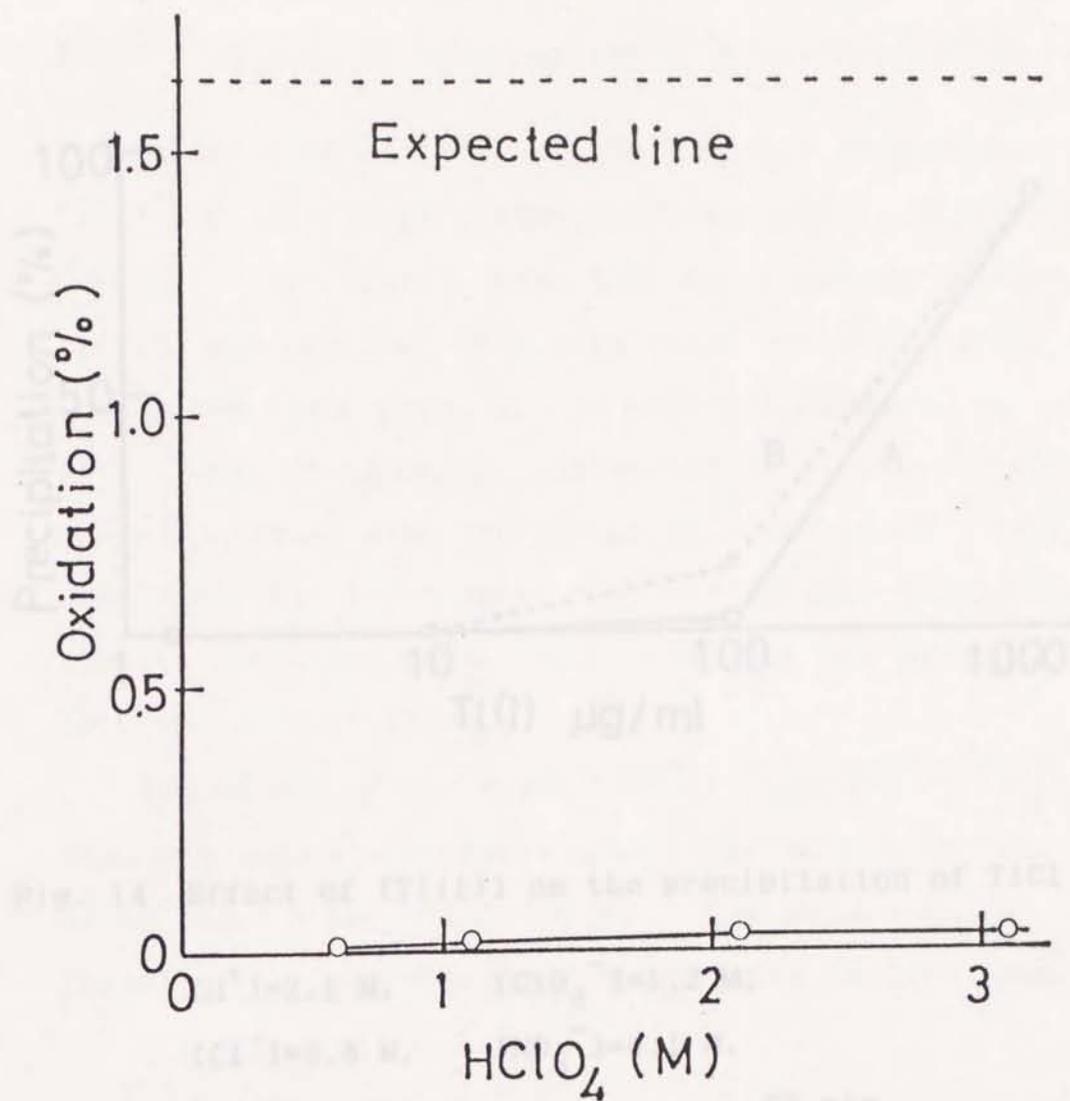


Fig. 13 Effect of $[\text{HClO}_4]$ on the oxidation of Tl(III) in the HClO_4 system.

$\text{Tl(I)} = 4.05 \mu\text{g}$, $\text{KBrO}_3 = 1.6 \times 10^{-2} \mu\text{eq}$, $[\text{H}^+] = 2.1 \text{ M}$
 $[\text{NO}_3^-] = 8 \times 10^{-2} \text{ M}$, $\text{Vol.}_{\text{aq+org}} = 1.0 \text{ ml}$

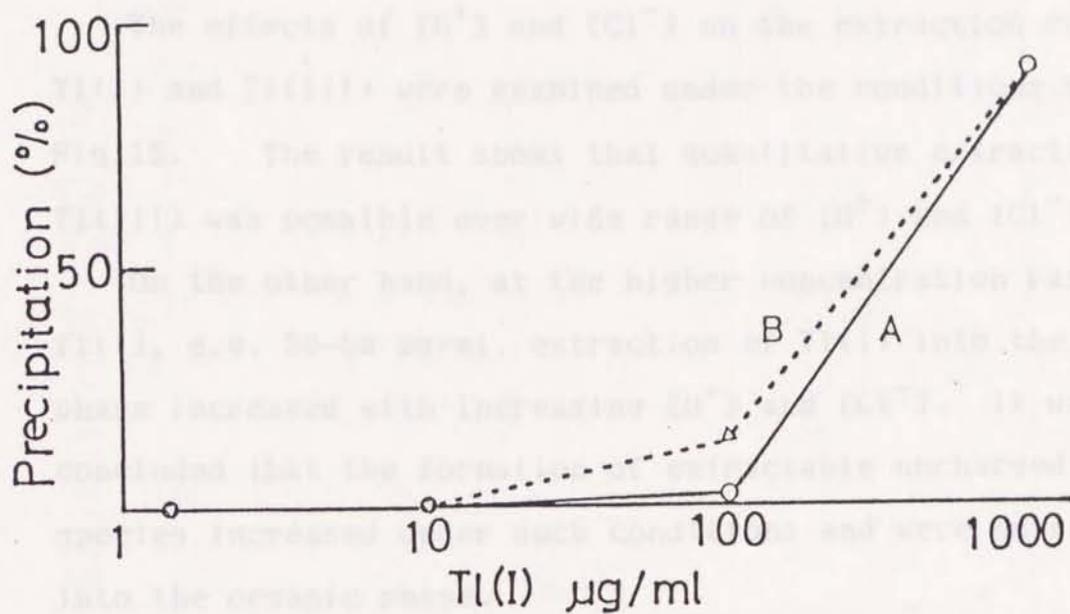


Fig. 14 Effect of [Tl(I)] on the precipitation of TlCl

$[H^+] = 2.1 \text{ M}$, $[ClO_4^-] = 1.2 \text{ M}$,

$[Cl^-] = 0.8 \text{ M}$, $[NO_3^-] = 0.1 \text{ M}$,

Standing time ; A ——— 30 min,

B - - - - - 1 day

[Tl(I)] was below 100 $\mu\text{g/ml}$. Such low values will not seriously affect on the analysis using SSE-IDA.

4.3.3 Effect of $[\text{H}^+]$ and $[\text{Cl}^-]$ on the extraction ratio

The effects of $[\text{H}^+]$ and $[\text{Cl}^-]$ on the extraction ratio of Tl(I) and Tl(III) were examined under the conditions shown in Fig.15. The result shows that quantitative extraction of Tl(III) was possible over wide range of $[\text{H}^+]$ and $[\text{Cl}^-]$.

On the other hand, at the higher concentration range of Tl(I), e.g. 30~50 $\mu\text{g/ml}$, extraction of Tl(I) into the organic phase increased with increasing $[\text{H}^+]$ and $[\text{Cl}^-]$. It was concluded that the formation of extractable uncharged TlCl species increased under such conditions and were extractable into the organic phase.

The extent of the undesirable extraction of Tl(I) was less than 2 % when the extraction was carried out at concentrations of $[\text{H}^+]=2.5 \text{ M}$ and $[\text{Cl}^-]=1.0 \text{ M}$. The separation of Tl(I) and Tl(III) from each other was almost quantitative here.

4.3.4 Effect of extraction time

As shown in Fig.16, extraction was completed in 1 min.

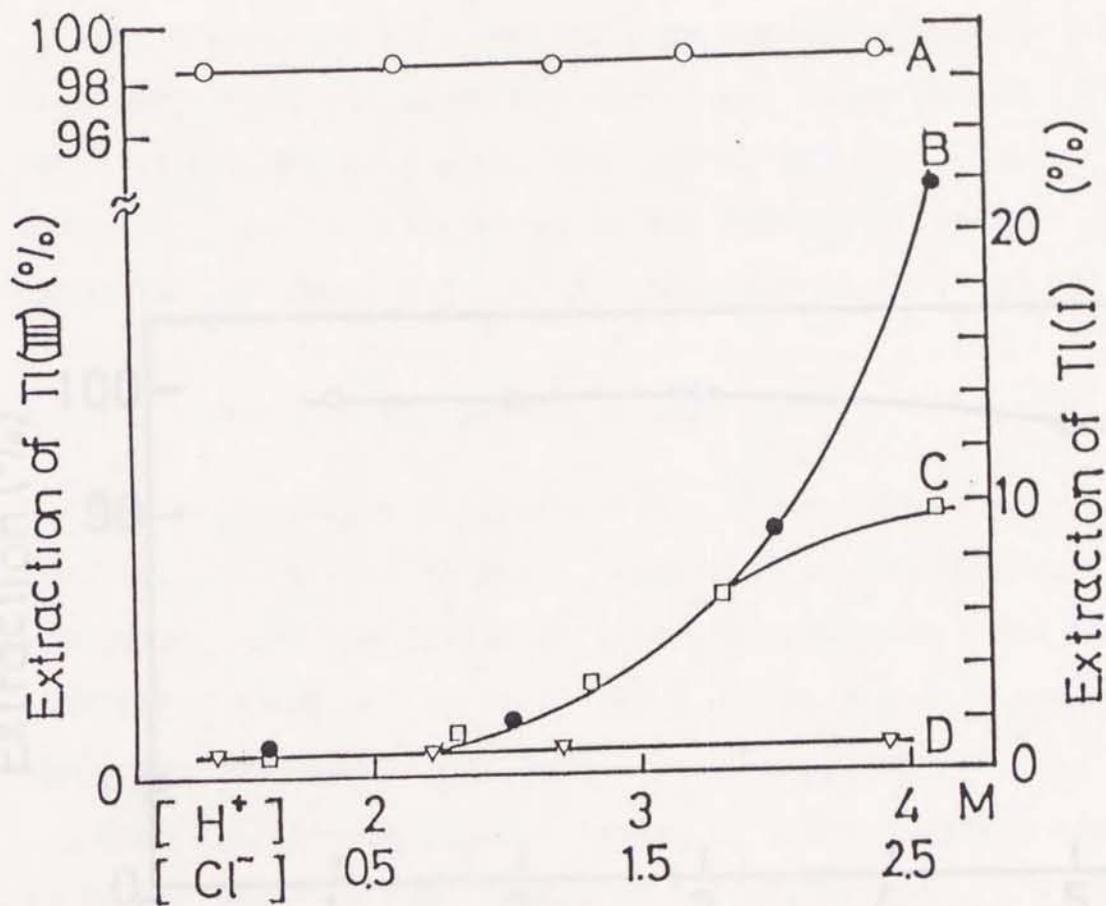


Fig.15 Effect of $[H^+]$ and $[Cl^-]$ on the extraction of $Tl(III)$ and $Tl(I)$.

A ; $[^*Tl(III)] = 26.0 \mu g/ml$,

B ; $[^*Tl(I)] = 48.7 \mu g/ml$,

C ; $[^*Tl(I)] = 26.8 \mu g/ml + [Tl(III)] = 26.0 \mu g/ml$

D ; $[^*Tl(I)] = 2.7 \mu g/ml + [Tl(III)] = 2.6 \mu g/ml$

$[NO_3^-] = 7 \times 10^{-2} M$, $[ClO_4] = 1.3 M$, Vol. aq. + org. = 0.8 ml

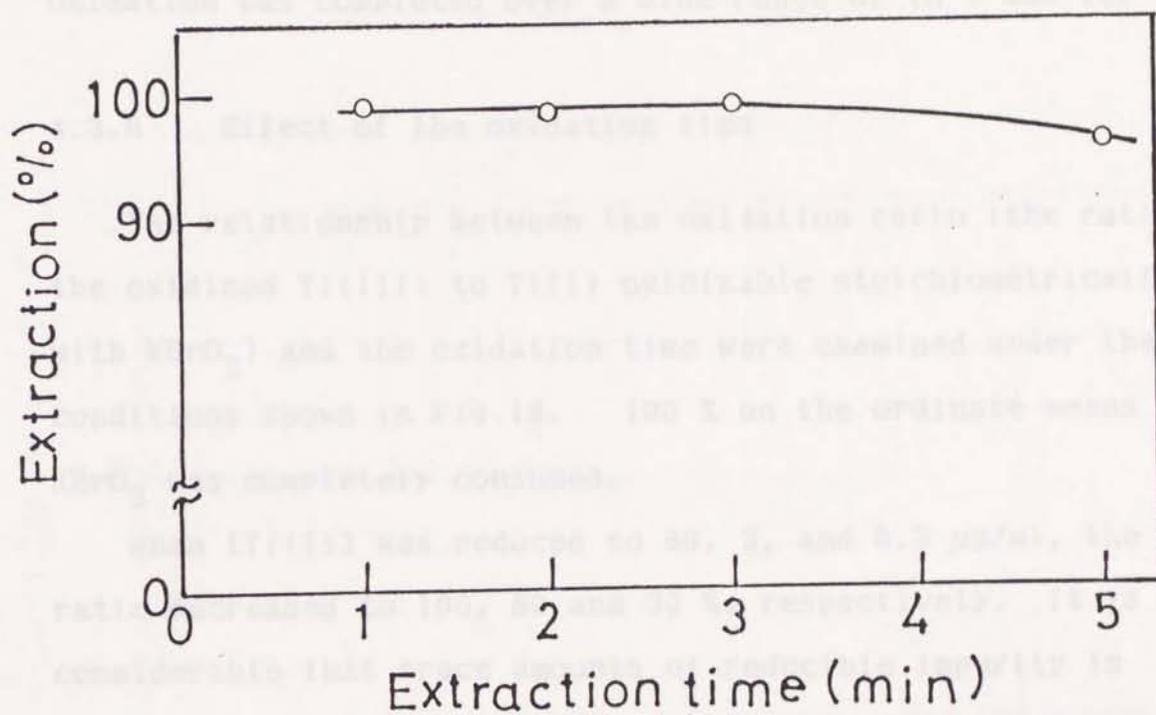


Fig.16 Effect of the extraction time of Tl(III) on the extraction ratio.

$[Tl(III)] = 21.8 \mu\text{g/ml}$, $[H^+] = 2.9 \text{ M}$, $[ClO_4^-] = 1.5 \text{ M}$,
 $[Cl^-] = 1.4 \text{ M}$, $[NO_3^-] = 6 \times 10^{-3} \text{ M}$, $\text{Vol.}_{\text{aq.}+\text{org.}} = 0.8 \text{ ml}$

4.3.5 Effect of $[H^+]$ and $[Cl^-]$ on the oxidation ratio of Tl(I)

The effects of $[H^+]$ and $[Cl^-]$ on the oxidation ratio of Tl(I) were examined under the conditions shown in Fig. 17; 100 % on the ordinate means that $KBrO_3$ was completely consumed. At $[Tl(I)] = 80 \mu g/ml$ and $[KBrO_3]/[Tl(I)] = 0.4$, oxidation was completed over a wide range of $[H^+]$ and $[Cl^-]$.

4.3.6 Effect of the oxidation time

The relationship between the oxidation ratio (the ratio of the oxidized Tl(III) to Tl(I) oxidizable stoichiometrically with $KBrO_3$) and the oxidation time were examined under the conditions shown in Fig. 18. 100 % on the ordinate means that $KBrO_3$ was completely consumed.

When $[Tl(I)]$ was reduced to 80, 3, and $0.3 \mu g/ml$, the ratio decreased to 100, 80 and 30 %, respectively. It is considerable that trace amounts of reducible impurity is present in the solution and it consumes $KBrO_3$. This impurity will compete with Tl(I) in consuming $KBrO_3$. Therefore, when $[Tl(I)]$ decreases, the proportion of the consumption by the impurity increases and results in a decrease in the oxidation ratio of Tl(I).

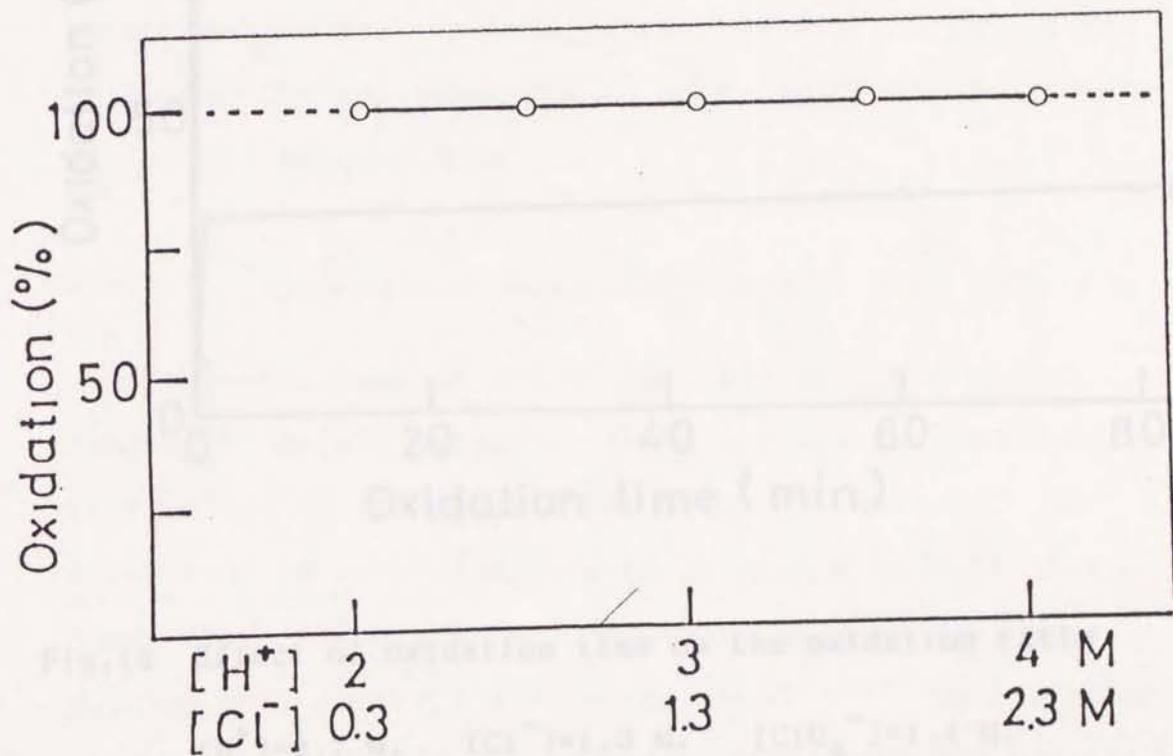


Fig.17 Effect of $[H^+]$ and $[Cl^-]$ on the oxidation ratio

$[Tl(I)] = 78 \mu\text{g/ml}$, $KBrO_3 = 0.4 \mu\text{eq}$, $[ClO_4^-] = 1.7 \text{ M}$
 $[NO_3^-] = 3 \times 10^{-3} \text{ M}$, $\text{Vol. aq. + org.} = 0.8 \text{ ml}$

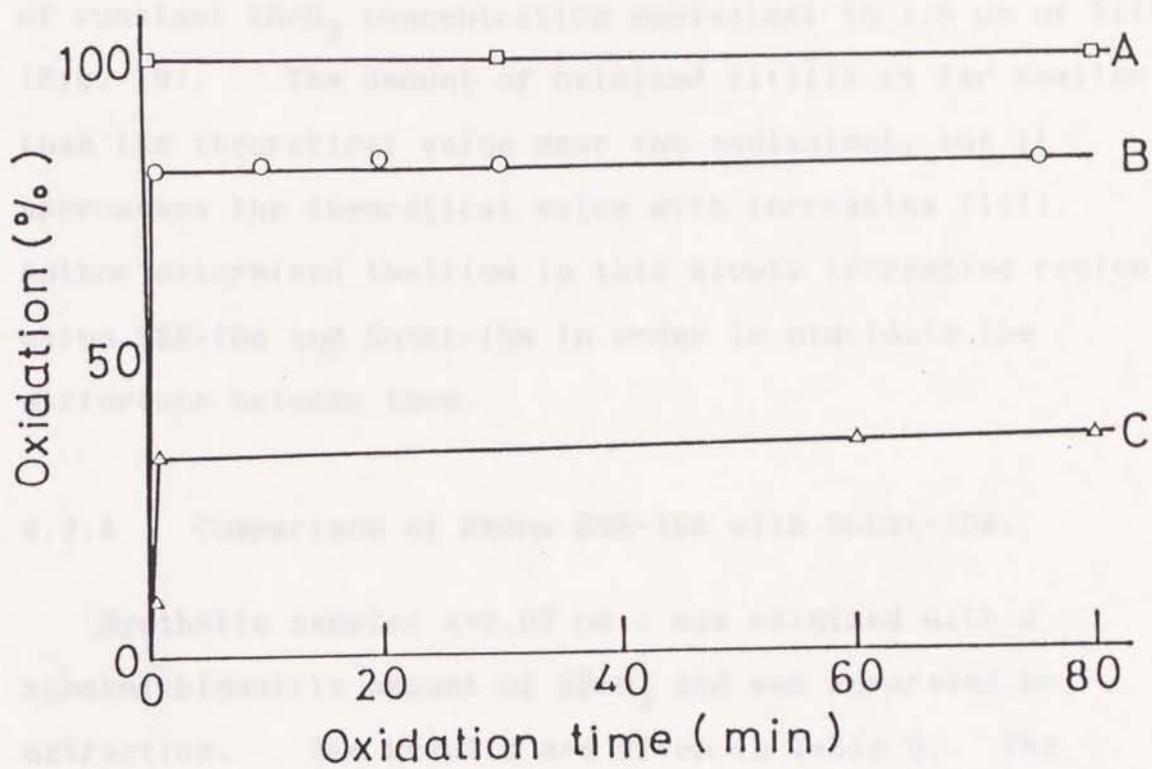


Fig.18 Effect of oxidation time on the oxidation ratio

$[H^+] = 2.7 \text{ M}$, $[Cl^-] = 1.3 \text{ M}$, $[ClO_4^-] = 1.4 \text{ M}$,

$[NO_3^-] = 6 \times 10^{-2} \text{ M}$, Vol. aq. + org. = 0.8 ml

A ; $Tl(I) = 0.29 \mu\text{eq}$, $KBrO_3 = 0.12 \mu\text{eq}$, $KBrO_3/Tl(I) = 0.41$

B ; $Tl(I) = 1.0 \times 10^{-2} \mu\text{eq}$, $KBrO_3 = 8 \times 10^{-3} \mu\text{eq}$, $KBrO_3/Tl(I) = 0.8$

C ; $Tl(I) = 1.5 \times 10^{-3} \mu\text{eq}$, $KBrO_3 = 1.6 \times 10^{-3} \mu\text{eq}$, $KBrO_3/Tl(I) = 1$

4.3.7 The relationship between Tl(III) oxidized and Tl(I) used

On the basis of the above arguments, the effect of $[Tl(I)]$ on the amount of oxidized Tl(III) was examined in the presence of constant $KBrO_3$ concentration equivalent to $1.6 \mu g$ of Tl(I) (Fig. 19). The amount of oxidized Tl(III) is far smaller than the theoretical value near the equivalent, but it approaches the theoretical value with increasing Tl(I). Author determined thallium in this slowly increasing region using SSE-IDA and Subst-IDA in order to elucidate the difference between them.

4.3.8 Comparison of Redox SSE-IDA with Subst-IDA.

Synthetic sample ($x=2.07 \mu g$) was oxidized with a substoichiometric amount of $KBrO_3$ and was separated by extraction. The results are given in Table 9. The activities of Tl(III) separated from each aliquot in the first series progressively decreases because they were diluted with carrier ($i y=1\sim 6 \mu g, i=1\sim 6$). On the other hand, the aliquots in the second series were not diluted with the carrier, so their activities remained unchanged. It seems that the values increased with increasing k .

Using these data of Tl(III) separated from the two series, a synthetic sample was analysed using graphic methods.

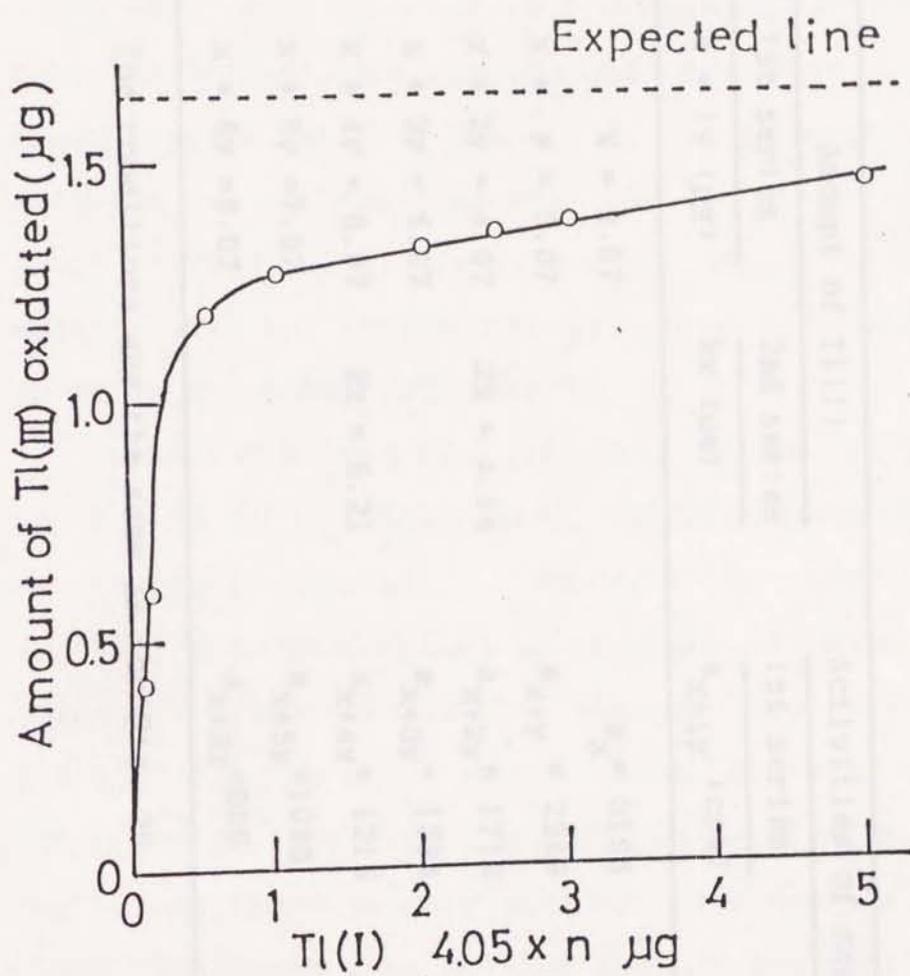


Fig. 19 Effect of amount of Tl(I) on Tl(III) oxidized.

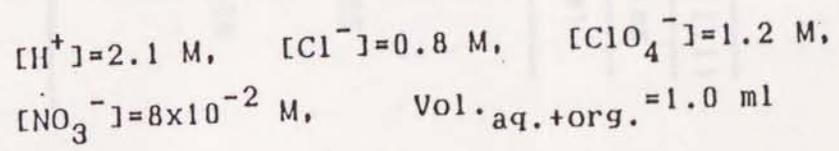


Table 9 Activities of Tl(III) isolated from two series of solutions.

Amount of Tl(I)		Activities of separated Tl(III)	
Ist series	2nd series	1st series	2nd series
$x + iy$ (μg)	kx (μg)	a_{x+iy} (cpm)	a_{kx} (cpm)
$x = 2.07$		$a_x = 3165$	
$x + y = 3.07$		$a_{x+y} = 2249$	
$x + 2y = 4.07$	$2x = 4.14$	$a_{x+2y} = 1713$	$a_{2x} = 3365$
$x + 3y = 5.07$		$a_{x+3y} = 1394$	
$x + 4y = 6.07$	$3x = 6.21$	$a_{x+4y} = 1216$	$a_{3x} = 3538$
$x + 5y = 7.07$		$a_{x+5y} = 1028$	
$x + 6y = 8.07$		$a_{x+6y} = 906$	

The conditions are the same as for Fig. 20

The results are shown in Fig. 20 and Table 10. The error of the values determined by SSE-IDA was always below 2 % and the results agreed well with the theoretical value.

On the other hand, the values obtained by Subst-IDA ($k=1$) had a much large error of 13.5 %, one of the reasons being that the analysis was carried out at a $[\text{KBrO}_3]/[\text{Tl(I)}]$ concentration ratio of 0.8. As can be seen from Fig. 19, oxidation is not complete near the equivalent concentration point ($[\text{KBrO}_3]/[\text{Tl(I)}]=1$) and the amount of the product is smaller than the expected value, which must cause a large error in the Subst-IDA results.

When $k=2$ or $k=3$ with SSE-IDA, the ratio $[\text{KBrO}_3]/[\text{Tl(I)}]$ is 0.4 or 0.27, respectively, so KBrO_3 considered is taken to be completely consumed. Hence, it is expected that Subst-IDA will also give values as accurate as those obtained by SSE-IDA. Therefore, author assigned two aliquots ($X_1 = x+2y$, $X_2 = x+3y$) in the first series as the unknown, where $[\text{KBrO}_3]/[\text{Tl(I)}] = 0.41$ and 0.33 , respectively, and determined the values from the data obtained by Subst-IDA. In this case, the errors were smaller than in the case of $[\text{KBrO}_3]/[\text{Tl(I)}]=0.8$, but they were still over 10 %.

Accordingly, these results really confirmed that the accurate determination by SSE-IDA was possible without the strict conditions of complete oxidation and separation that are necessary in the case of Subst-IDA.

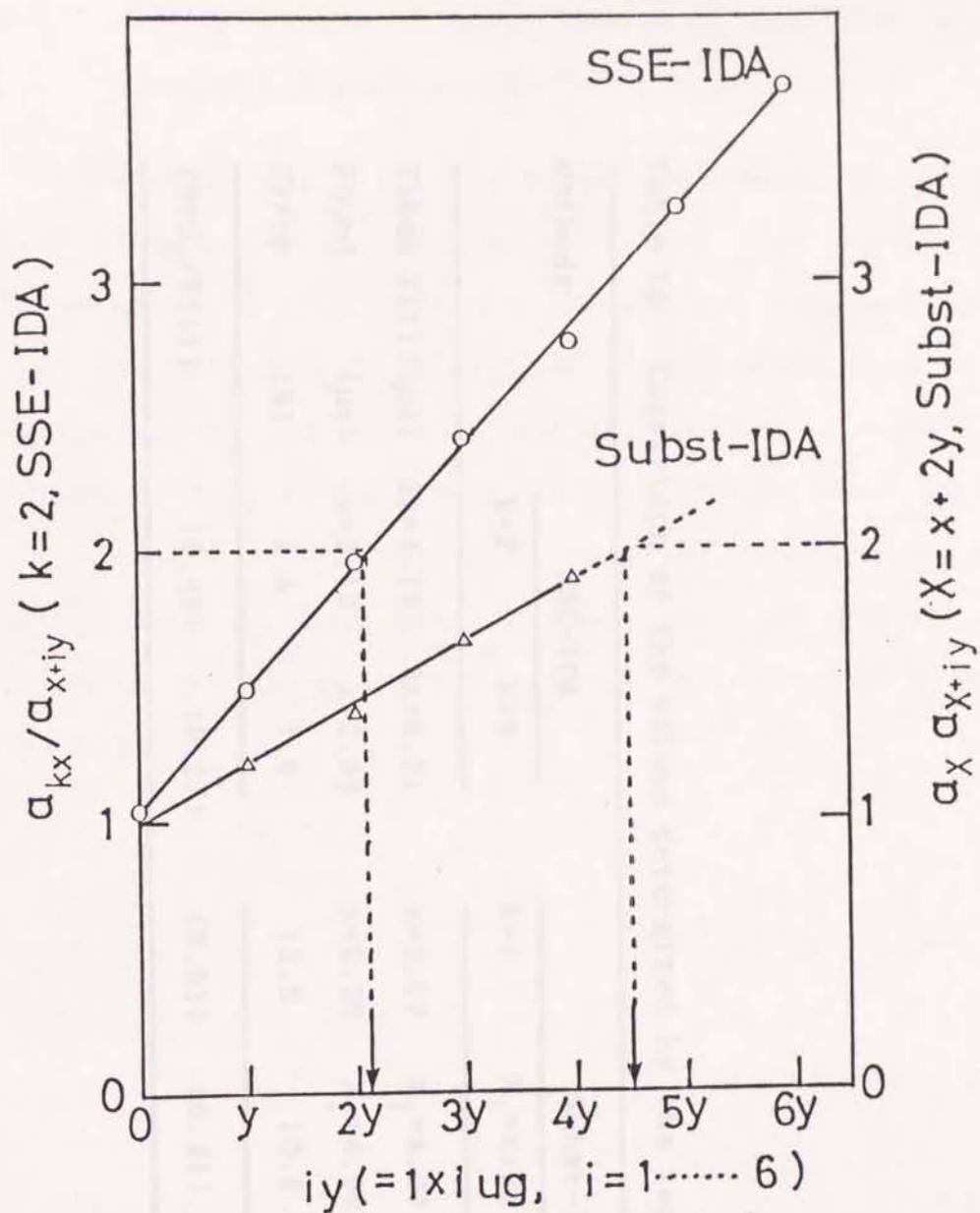


Fig.20 An example of determination of Tl(I) by SSE-IDA and Subst-IDA.

$[H^+] = 2.2 \text{ M}$, $[Cl^-] = 1.0 \text{ M}$, $[ClO_4^-] = 1.2 \text{ M}$, $[NO_3^-] = 5 \times 10^{-2} \text{ M}$,
 $KBrO_3 = 1.64 \times 10^{-2} \text{ } \mu\text{eq}$, $\text{Vol.}_{\text{aq.}+\text{org.}} = 1.0 \text{ ml}$

SSE-IDA ; Taken ($2.07 \text{ } \mu\text{g}$), Found ($2.10 \text{ } \mu\text{g}$), Error (1.4 %)

Subst-IDA ; Taken ($4.07 \text{ } \mu\text{g}$), Found ($4.50 \text{ } \mu\text{g}$), Error (10.6 %)

Table 10 Comparison of the values determined by the two method

Methods	SSE-IDA		Subst-IDA		
	k=2	k=3	k=1	$X_1=x+2y$	$X_2=x+3y$
Taken Tl(I) (μg)	$2x=4.14$	$3x=6.21$	$x=2.07$	$X_1=4.07$	$X_2=5.07$
Found (μg)	$x=2.10$	$x=2.09$	$x=2.35$	$X_1=4.50$	$X_2=5.60$
Error (%)	1.4	1.0	13.5	10.6	10.5
$\text{KBrO}_3/\text{Tl(I)}$	(0.40)	(0.27)	(0.81)	(0.41)	(0.33)

4.3.9 Determination of trace amount of thallium in commercial radioactive ^{204}Tl samples

As shown in Table 11, the value determined by the SSE-IDA was in agreement with the one calculated from specification with an error of less than 3 % (except for sample No.1).

It has been demonstrated that the determination of thallium by SSE-IDA was possible with errors of less than 2 % under conditions where Subst-IDA gave errors above 10 %.

In addition, the volume of solution required was of the order of 15 ~ 20 times smaller than usual and did not cause additional errors, which will reduce the radioactive waste water problem.

Table 11 Determination of the trace amount of thallium in the commercial ^{204}Tl samples prepared at JAERI

Sample No.	1	2	3	4	5	6
Taken (μg)	0.95	1.43	1.90	1.95	2.43	2.90
Found (μg)	1.02	1.45	1.95	1.91	2.41	2.96
Error (%)	7.4	1.4	2.6	2.1	0.8	2.1

Conditions are the same as for Fig. 20

DETERMINATION OF DNA BY SUB- AND SUPER-EQUIVALENCE
METHOD OF ISOTOPE DILUTION ANALYSIS USING ENZYME REACTION

5.1 Introduction

Since substoichiometry^{11, 12, 13)} was introduced into isotope dilution analysis (IDA), this method has been developed because of its accuracy, sensitivity and simple and quick procedure.

Author have determined trace amounts of antimony in some metal (Zn, As, Sn) using redox substoichiometric neutron activation analysis (Redox Subst-NAA)⁴⁷⁾ and isotope dilution analysis (Redox Subst-IDA)¹⁶⁾. However, a preliminary process is acquired for the elimination of the interfering substances which compete with the element to be analysed. This seems to be quite disadvantageous. For the improvement of this defect, sub- and super-equivalence method of isotope dilution analysis (SSE-IDA)¹⁾ was proposed, where the elimination was unnecessary. This method is so new that a small number of reports were published hitherto both in the theory³⁸⁾ and in the application³²⁾.

Author compared this method²¹⁾ with substoichiometry in order to clarify the advantage of this method. In this

report. SSE-IDA was first applied for the determination of a biological substance, DNA. Today, spectrophotometry⁵⁵⁾, densitometry after electrophoresis⁵⁶⁾ and fluorimetry⁵⁷⁾ are useful for the determination of DNA. Author can propose a new method for this purpose.

5.2 Principle

Plasmid DNA extracted from E. coli is the mixture of the two forms. One is covalently closed circular form (CCC-form ca.80%) and the other is open circular form (OC-form ca.20%). Author designate this mixture as native DNA hereafter.

By the reaction of restriction enzyme, HindIII, native DNA is cut at the certain position recognized by the enzyme into the linear form DNA (L-form) shown in Fig. 21. Author show here that the determination of DNA by SSE-IDA using this enzyme reaction is possible. The principle of this method and the procedure are explained as follows.

Two series of solutions are prepared.

1st series : Equi-volume (V_1 μ l) of native*DNA sample solutions (x μ g/ V_1 μ l) labeled with the activity (A dpm) are taken in five microtubes, then progressively concentrated non-labeled native DNA (iy μ g/ V_2 μ l) carrier solutions of a definite volume (V_2) are added to them. (* means the labelled atom.)

Specific activity : A dpm/($x+iy$) μ g

[DNA] : ($x+iy$) μ g/(V_1+V_2) μ l

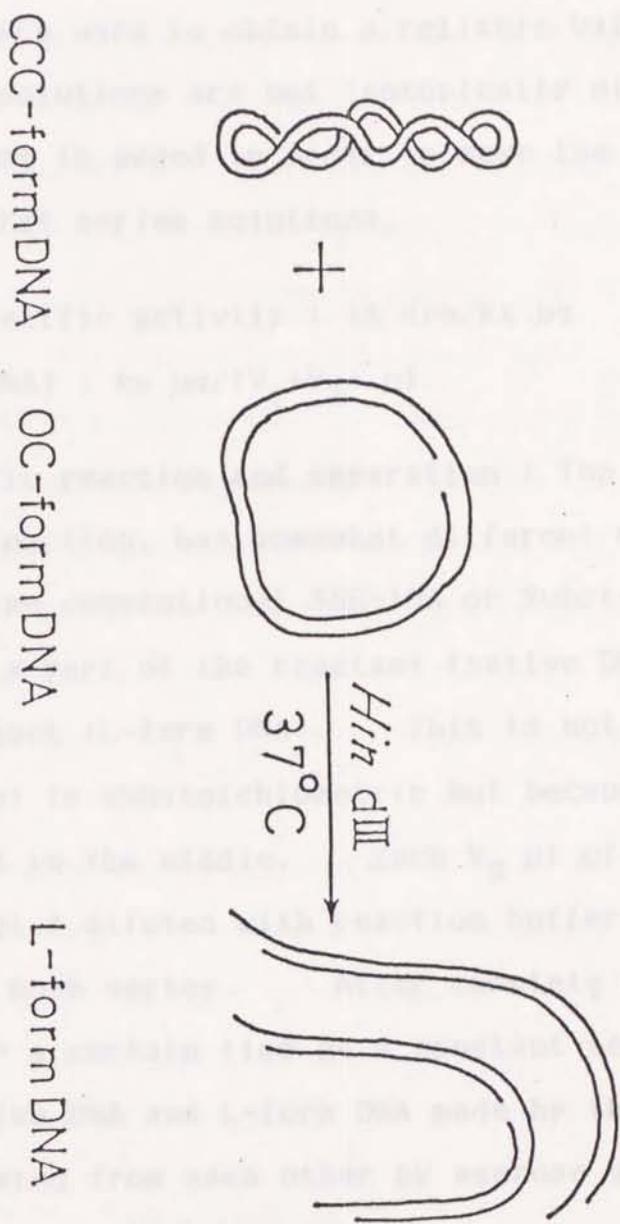


Fig. 21 Reaction scheme of the DNA scission with *Hind* dIII

2nd series : Each V_1 μl of k times concentrated native $^*\text{DNA}$ (kx $\mu\text{g}/V_1$ μl) sample solution is taken in three tubes. Here, three tubes were used to obtain a reliable value by averaging. These solutions are not isotopically diluted, but each V_2 μl of solvent is added in order to make the same condition as the first series solutions.

Specific activity : kA dpm/ kx μg

[DNA] : kx $\mu\text{g}/(V_1+V_2)$ μl

Substoichiometric reaction and separation : The term, substoichiometric reaction, has somewhat different meaning from that used in the conventional SSE-IDA or Subst-IDA. It means here that a part of the reactant (native DNA) is changed to the product (L-form DNA). This is not because the reagent (enzyme) is substoichiometric but because the reaction is stopped in the middle. Each V_3 μl of the enzyme solution (MU/V_3 μl) diluted with reaction buffer was added to each aliquot of both series. After complete mixing, they were stood for a certain time at a constant temperature. The mixture of native DNA and L-form DNA made by the strand scission was separated from each other by agarose gel-electrophoresis⁵⁶⁾.

Representing the activities of L-form DNA (m_{x+iy} , m_{ky}) separated from each aliquot of both series as a_{x+iy} and a_{kx} , equations (17) and (47) hold because the specific activity

does not change before and after the reaction. From equations (17) and (47), equation (54) is obtained.

Generally speaking, the quantities of the product and the separated matter depend on the concentration of the reactant. So, m_{x+iy} is not constant. However, if the quantity in an aliquot of the second series becomes equal to that of an aliquot of the first one, the same degree of the reaction must proceed. In this case, equation (55) must hold. As the result, equation (56) is obtained. From equations (54), (55) and (56), equation (57) is obtained. Author can calculate the unknown x by the graphic method as shown in Fig. 25. By plotting a_{kx}/a_{x+iy} on the ordinate against iy on the abscissa and finding the abscissa value (jy) of the crossing point of this curve and the line $a_{kx}/a_{x+iy} = k$, x is calculated from equation (57).

As mentioned before, enzyme reaction does not attain the thermodynamic equilibrium. However, it is stopped at the same time, so the same amounts of L-form DNA is obtained when the quantities of the native DNA are same.

5.3 Experimental

5.3.1 Preparation of the sample and the reagent

Native *DNA to be analysed : Plasmid DNA (pUC18)^{59,60} was labeled by growing E. coli (JM105) in a medium containing

³H-thymidine. Crude plasmid was then extracted and was purified by gel-electrophoresis^{56,58}. It was stored in TE buffer and was used as the unknown sample.

Standard native DNA : Non-labeled plasmid DNA (pUC18) was prepared similarly to the case of native *DNA and was used as the carrier. The concentration was determined by densitometry and spectrophotometry. Absorption coefficient is 20 (cm²/g) at 260 nm.

Reagent for the reaction with DNA : HindIII (Takara Shuzo Co. Ltd,) solution (10 unit/μl) was diluted with the designated dilution buffer (DB) to the concentration of 1 unit/μl and it was stored at 20°C.

DNA solvent : TE Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) was used as DNA solvent.

Dilution Buffer (DB) : HindIII was diluted with DB (10 mM potassium phosphate, 200 mM potassium chloride, 0.5 mM EDTA, 1 mM dithiothreitol, 0.01 % bovine serum albumine , 50% glycerol, pH 7.5).

Reaction Buffer (RB) : HindIII digestion was carried out in the presence of 10 mM Tris-HCl pH7.5, 7 mM MgCl₂, 60 mM NaCl. HindIII was diluted with the same RB just before reaction.

Electrophoresis buffer : TAE buffer (0.04 M Tris-acetate, 0.01 M EDTA, pH 8.0) was used for electrophoresis buffer.

Gel ; 1% agarose gel was used for DNA separation.

ESB : Enzyme reaction was stopped by adding EBS (0.1 M EDTA,

25% sucrose, 0.2% bromophenol blue).

5.3.2 Measurement of activity

CCC-, OC- and L-form DNA were separated by mini gel electrophoresis^{56,58}). The pieces of the gel containing these bands were cut and placed in the vials for liquid scintillation counting. 30 μ l of 6 M HCl was added in the vial and the gel piece was dissolved using a electronic range. After that, 6 ml of EX-H scintillator cocktail(Dojin Chemical Corp., Japan) was added and the radioactivity was measured with liquid scintillation counter.

5.3.3 Procedure

The native ^{*}DNA was determined by the procedure stated in the principle. Each value was $x = 0.25 \mu\text{g}$ (this value was determined by densitometry in comparison with the commercial standard plasmid DNA (pUC18)), $y = i \times 0.125 \mu\text{g}$ ($i = 0, 1, 2, 3, 4$), $V_1 = V_2 = V_3 = 5 \mu\text{l}$, $k = 2$, $M = 0.25$ unit.

5.4 Results and Discussion

In order to investigate the optimum conditions for the determination of native DNA using enzyme, author examined the

factors affecting on the enzyme reaction.

5.4.1 Effect of the reaction time on the yield of L-form DNA

As shown in the previous paper²¹⁾, Subst-IDA may be possible for the determination of DNA in the case that constant amount of L-form DNA is produced irrespective of the content of the substrate, DNA. For this purpose, enzyme reaction must be of the zeroth order with respect to the concentration of the substrate. According to Michaelis-Menten equation, the reaction rate is of the first order as to the substrate concentration in the low concentration region, but it gradually approaches to the zeroth order with increase in the concentration. Therefore, the conditions required for Subst-IDA may be fulfilled at the high concentration range.

The relationship between the yield of the L-form and time was examined for the purpose to find out the conditions of the zeroth order reaction. The experimental conditions are shown in Fig. 22 and Fig. 23. When [HindIII] is 0.2 U/ μ l, the reaction was of the zeroth order at [DNA]= 0.06 μ g/ μ l but deviates from it at [DNA]= 0.02 μ g/ μ l.

Next, a similar experiment was performed at the lower concentration of HindIII, 0.03 U/ μ l, and it was proved that zeroth order reaction proceeded at [DNA]=0.02 ~ 0.06 μ g/ μ l.

This condition ensures me to achieve the determination by Subst-IDA. Further, it becomes the optimum condition for SSE-IDA and will give a sufficient result. In this experiment, however, author adopted a rather difficult condition for Subst-IDA, namely the reaction order of between zeroth and first, because our purpose is to show the usefulness of SSE-IDA.

5.4.2. The effect of native DNA concentration on the quantity of L-form DNA

Author examined how the concentration of native DNA affects on the quantity of L-form DNA under the condition near zeroth order. The result is shown in Fig. 24. It is evident that the yield of the L-form DNA is not constant but increases gradually with increase in the native DNA concentration. As stated before, it seems that SSE-IDA gives an accurate value in contrast to Subst-IDA even in this concentration range.

5.4.3. Stability of the enzyme

It is well known that the enzyme loses activity by heating or adding acid. Besides, the activity is sometimes lost by dilution. The reason is not explicit but one possible reason is attributed to the adsorption of the enzyme on the wall of the vessel. In this experiment, author did not

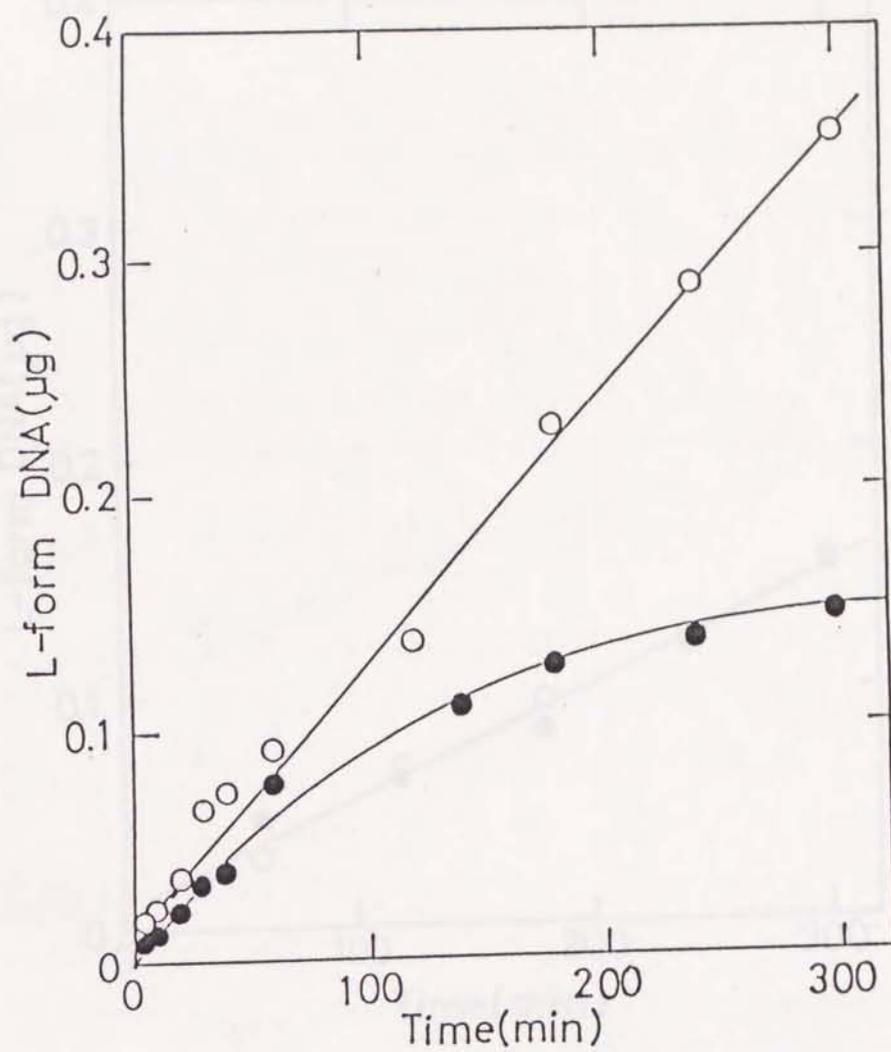


Fig. 22 Relationship between reaction time and the amount of L-form DNA produced.

● pUC18 native [* DNA]=0.02 $\mu\text{g}/\mu\text{l}$,

○ pUC18 native [* DNA]=0.06 $\mu\text{g}/\mu\text{l}$,

Experimental conditions ; [HindIII]=0.2 U/ μl , total vol. 10 μl , reaction : 37^o C, 1h.

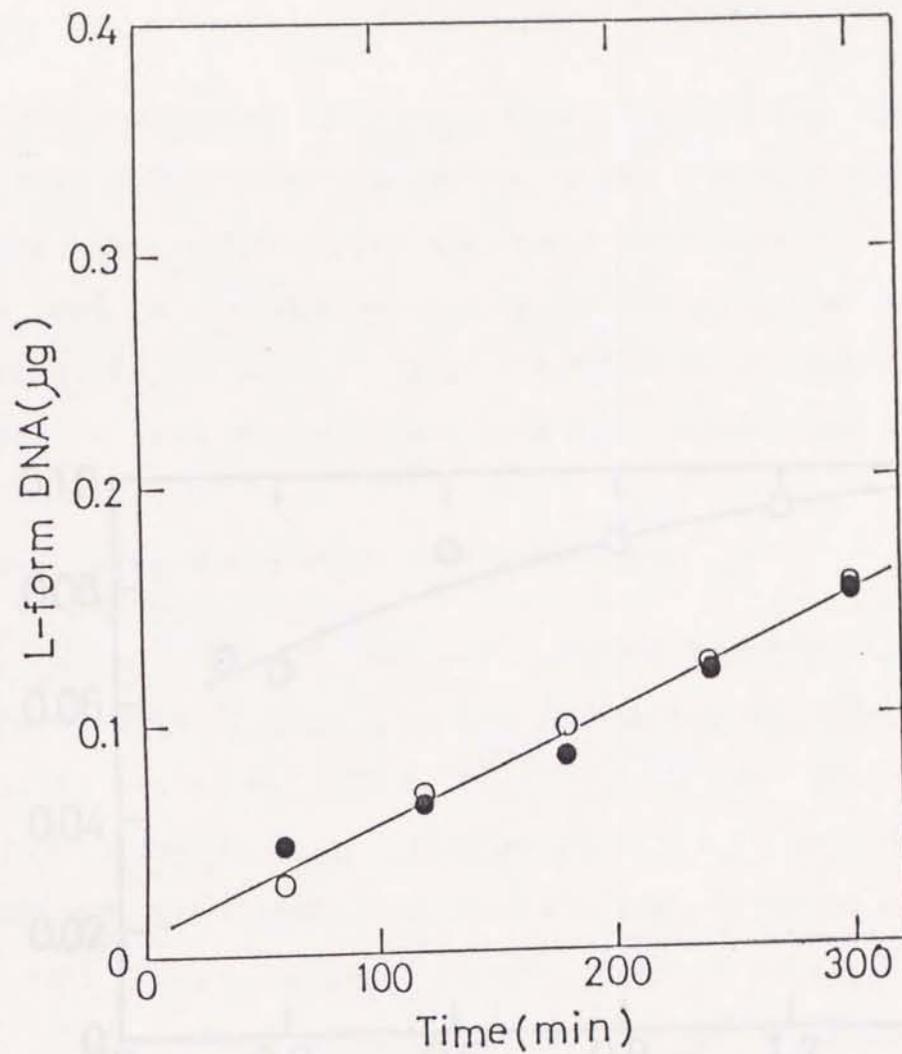


Fig. 23 Relationship between reaction time and the amount of L-form DNA produced.

● pUC18 native [^{*}DNA]=0.02 µg/µl

○ pUC18 native [^{*}DNA]=0.06 µg/µl

Experimental conditions ; [HindIII]=0.03 U/µl,
total vol. 10 µl, reaction : 37^o C, 1h.

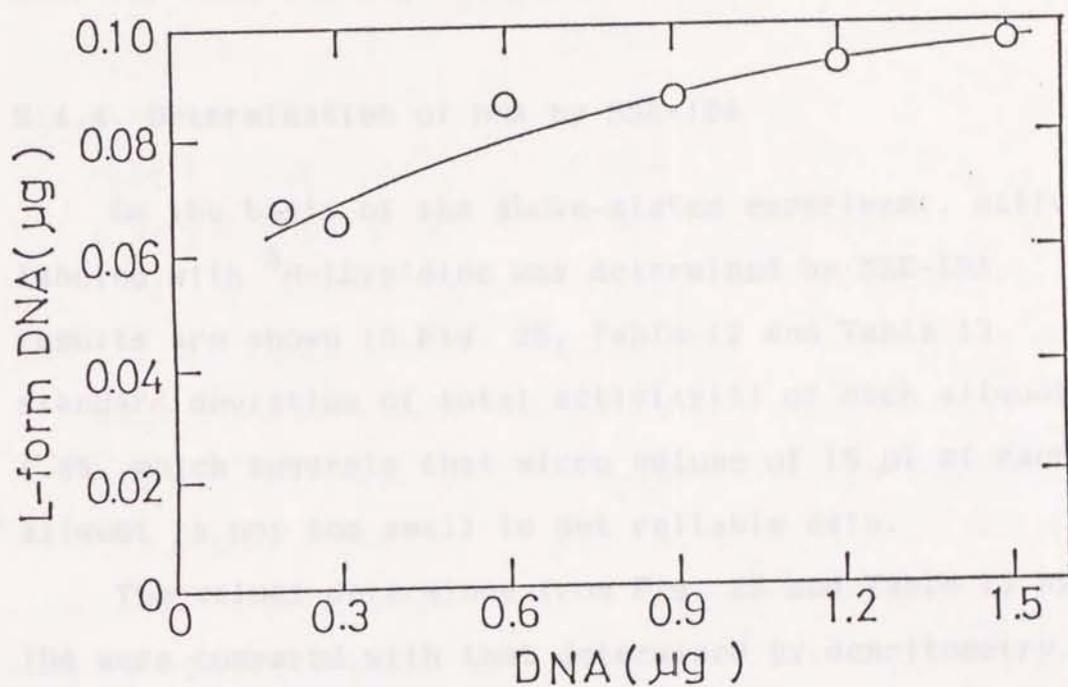


Fig. 24 Effect of native [^{*}DNA] on the amount of L-form DNA produced.

Experimental conditions ; HindIII= 0.45 U,
total vol. 15 µl, reaction : 37^o C, 2h.

use the so-called dilution buffer, because the solution was so viscous that it was impossible to mix DNA with enzyme in a short time. Therefore, the reaction buffer of low viscosity was used and the dilution with the solution was performed just before the reaction. Thus diluted HindIII perfectly lost the activity when the concentration was lowered below 10^{-3} U/ μ l.

5.4.4 Determination of DNA by SSE-IDA

On the basis of the above-stated experiment, native DNA labeled with 3 H-thymidine was determined by SSE-IDA. The results are shown in Fig. 25, Table 12 and Table 13. The standard deviation of total activity(A) of each aliquot is 1.4%, which suggests that micro volume of 15 μ l of each aliquot is not too small to get reliable data.

The values determined from Fig. 25 and Table 13 by SSE-IDA were compared with that determined by densitometry. They coincided with each other within about 10 %.

It is well known that radioactive measurement is usually more accurate and sensitive than other methods.

Autoradiography and radioimmunoassay are commonly used in the biochemical field. Here, a new method, SSE-IDA is proposed, and it is possible to determine DNA by this method.

A problem left here is the inactivation of enzyme in diluted solution. This is important because it relates to the limit of detection.

Table 12 Activity of L-form DNA isolated from each aliquot
(Sample No 1, Experimental conditions as for Fig.25)

Series	Amount of native DNA		Activity	
	(*DNA + DNA)		L-form DNA	(CCC+OC+L)-form DNA
	x	+ ly (μg)	a _{x+ly} (dpm)	A (dpm)
1st	0.25	+ 0 = 0.25	24,765	93,908
	0.25	+ 0.125 = 0.375	-----	-----
	0.25	+ 0.250 = 0.500	15,519	93,230
	0.25	+ 0.375 = 0.625	10,980	-----
	0.25	+ 0.500 = 0.750	9,164	92,822
2nd	kx (μg)		a _{kx} (dpm)	kA (dpm)
	2 x 0.25	= 0.500	28,864	2 x 95,600 = 19,1200
	2 x 0.25	= 0.500	27,907	2 x 95664 = 191,328

Average A = 94,479 + 1,316 (S.D.=1.4%)

The values on the dotted lines were missed by the failure of the experiment. But this fact did not seriously affect on the reliability of the result, as taken from Fig. 25.

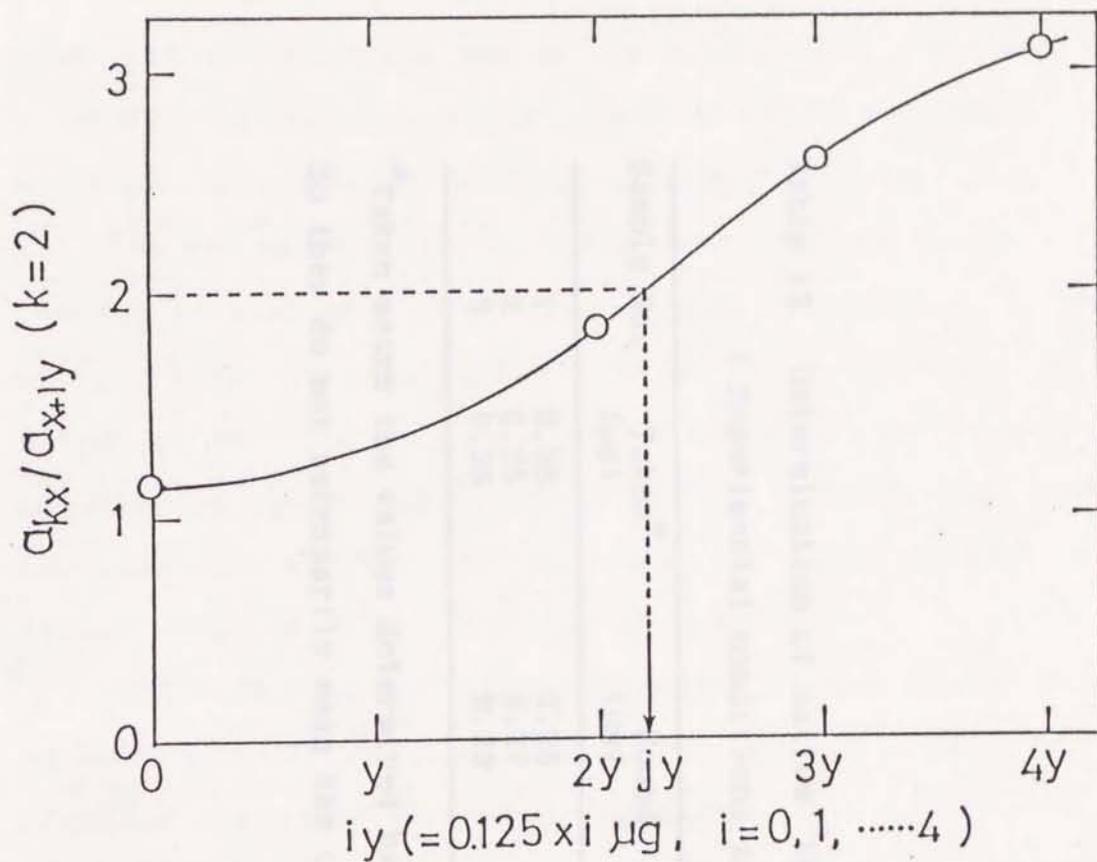


Fig. 25 An example of the determination of native ^{*}DNA by SSE-IDA.

$$x = jy/(k-1) = 0.28 \mu\text{g}$$

Experimental condition ; HindIII = 0.25 U,

Total vol. 15 μl , reaction : 37^o C, 1h.

[HindIII] = 0.017 U/ μl ,

native [DNA] = 0.017-0.05 $\mu\text{g}/\mu\text{l}$

Table 13 Determination of native *DNA by SSE-IDA
 (Experimental conditions as for Fig. 25)

Sample No.	Taken* (μg)	Found (μg)	Error (%)
1	0.25	0.28	+12.0
2	0.25	0.27	+ 8.0
3	0.25	0.23	- 8.0

*Taken means the values determined by densitometry.
 So they do not necessarily mean the correct values.

CONCLUSION

Since isoconcentration principle was introduced into Subst-IDA to develop a new method of SSE-IDA, the range of determination was made wider compared with Subst-IDA. In addition, accuracy and sensitivity were also improved.

The SSE method has the same advantages as Subst. The largest advantageous point is the high selectivity. That is to say, when the interfering ions exist in the solution, the effect of the ions is suppressed by using substoichiometric quantity of the reagent, and the selectivity of the reagent increase with decrease in the quantity of the reagent used. It was shown in Fig. 1 in section 1.1.3.

SSE method cancel the many errors caused in the procedure because only the ratio of the activities are used. So, the accuracy and sensitivity are increased.

The following description is also one of the advantageous points. Determination in the low concentration range is generally difficult. It is attributable to the fact that the reaction rate becomes slower in the lower concentration range, so it takes much time to reach the equilibrium and the determination is carried out under the non-equilibrium state. Other possible reason is that the partial adsorption of the ions on the sample container's wall becomes larger in the range. However, so far as the concentrations in two

aliquots are the same, every conditions including the amount of the adsorbed ions are maintained the same. Therefore, all the effects of these troubles are canceled and SSE-IDA gives satisfactory results.

On the other hand, even when the reagent having not so large equilibrium constant is used, in other words when the quantitative separation is impossible, accurate determination is still possible. This is evident from the experimental results in chapter 2~5. It will be hopeful to apply SSE method to the determination of the substance in a complicated system such as biological molecules and to the measurement of stability constants of complexes and solubility of the precipitates, etc.

Finally, the principle of SSE method is widely applicable not only to IDA and RA, but also to the analysis using radioactivity including NAA and IEA.

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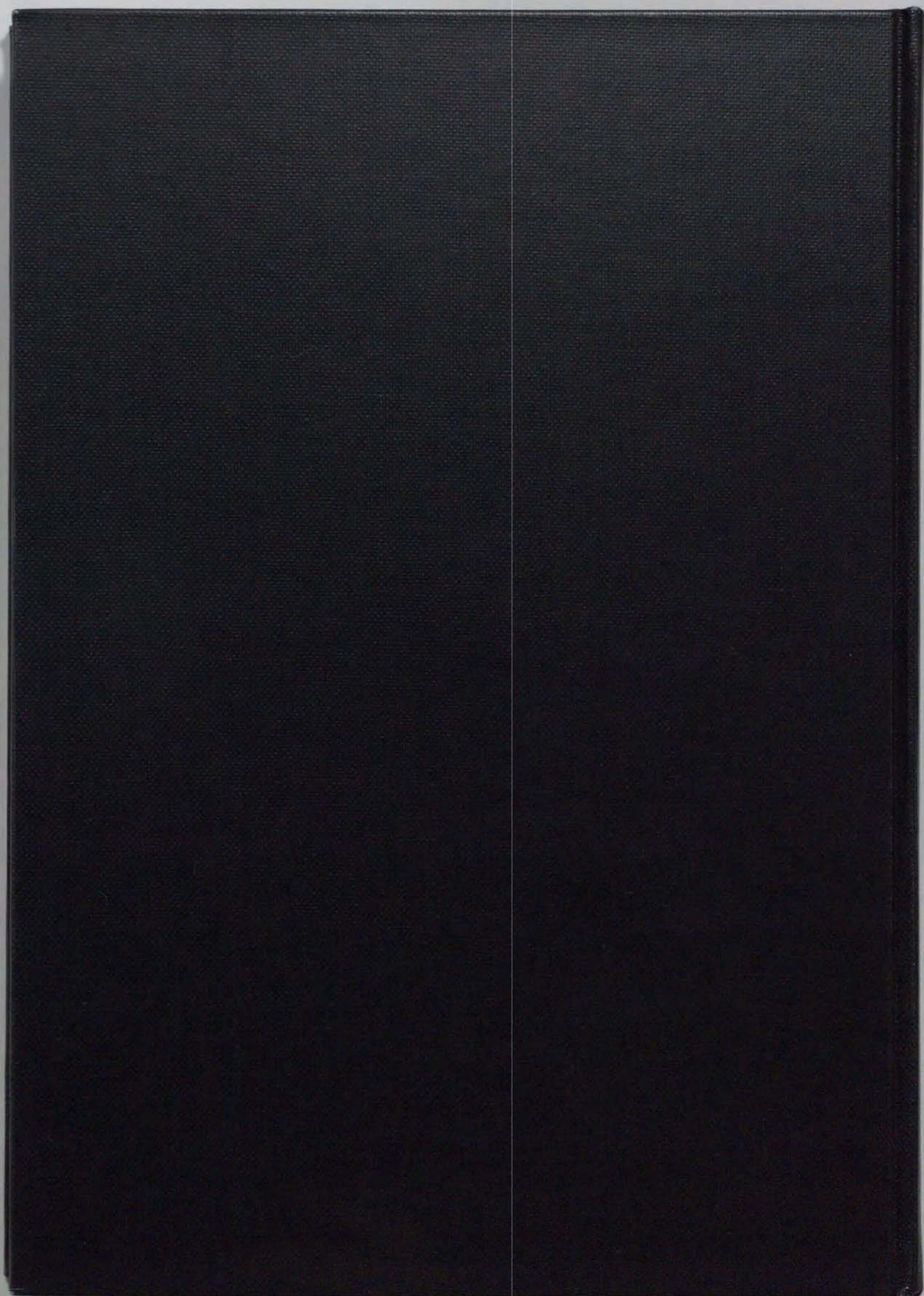
ACKNOWLEDGMENT

The author would like to express her greatest acknowledgement to professor Nagao Ikeda, Department of Chemistry, Tsukuba University, for his valuable suggestions and discussions during the preparation of this dissertation.

The author wishes to express her sincere gratitude to professor Kunihiro Hasegawa, Radiochemistry Research Laboratory, Faculty of Science, Shizuoka University, for his continuous encouragement and helpful suggestions and discussion through the course of this study.

The author is deeply indebted to an emeritus professor Tomihisa Kambara, Shizuoka University, for his warm encouragement and interest.

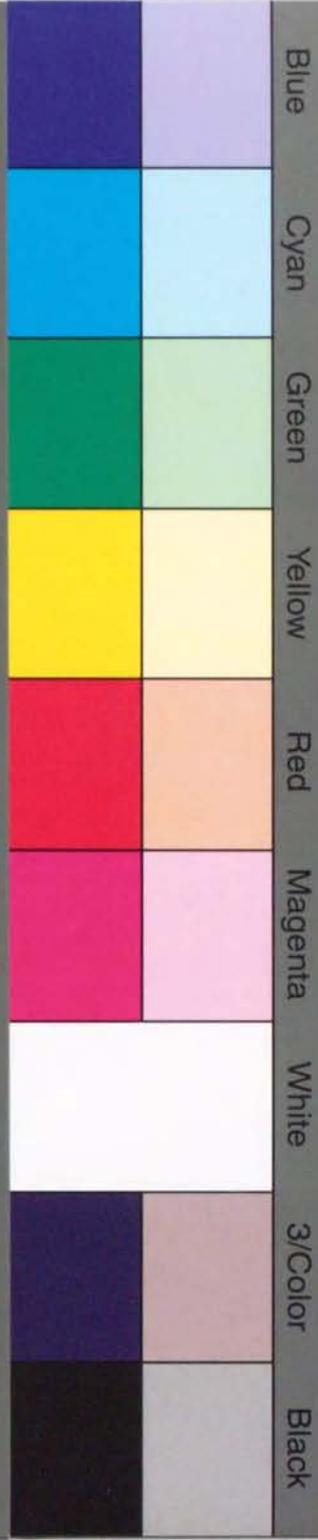




Inches 1 2 3 4 5 6 7 8
cm 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

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