RESEARCH REPORT 1990-2

INTERNATIONAL ASSOCIATION OF FISH MEAL MANUFACTURERS

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Project A2.89

THE REPEATABILITY AND REPRODUCIBILITY OF ANALYTICAL METHODS FOR QUALITY CONTROL OF SPECIAL PRODUCT FISH MEALS

REPORT A - PROTOCOL AND ANALYTICAL METHODS

REPORT B - STATISTICAL ANALYSIS OF RESULTS

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Cambridge, UK

August 1990

Project A2.89

To test the repeatability and reproducibility of analytical methods for quality control of special product fish meals

Background and objectives

Different member countries of the IAFMM use a variety of chemical analytical methods as a means of quality control of premium quality fish meals for specialized animal feeds, particularly for farmed fish and early weaned pigs.

A separate IAFMM project will be testing the suitability of these methods for determining biological quality differences (projects A1.88 and A1.89). At the same time it would be prudent to assess the repeatability and reproducibility of these methods within or between laboratories respectively. A method conducted in one laboratory might reliably indicate the quality of the fish meal, but unless the method can be repeated in another laboratory producing the same results, this method could not be used as a means of reassurance between buyer and seller.

The following methods should be tested by all laboratories;

1. Pepsin digestibility (Torry)
2. Titration to pH10
3. pH stat (multi-enzyme digestibility)

In addition participating laboratories are encouraged to test the following methods in order to obtain a preliminary assessment of their suitability.

4. -SH groups
5. Water binding capacity

Samples of meal

Twelve samples of fish meal (100g/sample) will be sent to each participating laboratory. The samples will be taken from commercial meals prepared in four or five different countries. Each country will have submitted samples of meal prepared from fresh fish using "regular" or "gentle" processing conditions. Two hidden duplicates will be included.

Torry Research Station (TRS) will be responsible for grinding the commercial meals to pass through a 20 mesh sieve, sampling the ground meal into representative sub-samples of 100g, packaging the sub-samples into airtight polypropylene containers, and coding the containers. A set of each sub-sample will be sent to each participating laboratory by TRS.

The samples and reagents will be distributed during August 1989.

Reagents

TRS will be responsible for sending to each participating laboratory the following reagents:

Pepsin (1:10,000 - Merck Catalogue No. 7190).

Trypsin T-0134 (16950 BAEE units/mg; 92% - enzymes from Sigma).

Chymotrypsin C-4129 (41 units/mg - enzymes from Sigma).

Peptidase P-7500 (50 units/mg - enzymes from Sigma).

Sodium caseinate (DIFCO, USA).

Analysis

The samples should be stored in a refrigerator until required. Before opening, the container should be thoroughly shaken and equilibrated to room temperature. The samples should be ground more finely in a pestle and mortar to pass through a 80 mesh sieve and sub-sampled into four equal fractions by coning and quartering. Two of the quarters should be combined to form one sample and the remaining two quarters further divided into four quarters by coning and quartering. Thus a total of five representative samples will be obtained one of about 50g and the remaining four of about 12g each. The larger sub-sample should be used for titration to pH10.

Sub-samples not required for immediate analysis should be stored in moisture tight containers at room temperature for no longer than ten days.

Analyses will be conducted using the methods set out in Appendices 1-5.

All analyses will be performed in triplicate and no results will be rejected.

Results and statistical treatment

All results will be recorded on the enclosed Results Sheet and returned to headquarters for statistical analysis. The results will be analysed by an appropriate statistical method in order to establish the reproducibility and repeatability of each method.

Analysts are encouraged to make comments on the analytical techniques particularly with reference to:

a) Ease or difficulty of understanding the written method.

b) Ease or difficulty of performing the analysis.

c) Any other relevant comments.

Participating Laboratories

Contact Person	Organization	Full Postal Address	Methods to be tested
Mr. Eugenio Ossa S.	**CESMEC LTDA**	Av. Marathon 2595, Casilla 14036, Correo 21, Santiago, **Chile.**	1-5
Chief Chemist	**Instituto de Formento Pesquero**	Pedro de Valdivia 2633 Santiago, Casilla 1287, **Chile.**	1-5
Mr. Pablo Herrera L.	**Fundación Chile**	Marine Resources, Av. Parque Antonio Rabat Sur 6165 Casilla 773 Santiago, **Chile.**	1-5
Mr. Max Rutman	**Inual**	General Ekdahl 159, Santiago, **Chile.**	1-5

Participating Laboratories (Continued)

Contact Person	Organization	Full Postal Address	Methods to be tested
Mr. Torben P. Kristensen	**FF**	Technological Laboratory, Ministry of Fisheries, North Sea Centre, DK-9850 Hirtshals, **Denmark.**	1-5
Mr. Niels Christian Jensen	**Andelssildeolie-fabriken AMBA**	Postboks 1049, Fiskerihavnsgade, Esbjerg, 6701, **Denmark.**	1-5
Mrs. Kirsten Kjaer,	**Thyboron Andels Fiskeindustri**	Postboks 40, Sydhalen, 7680, Thyboron, **Denmark.**	2+3
Mr. Peter Sandbøl	**Vestjyk Fiskemelsfabrik AmbA**	Badebrogade 4, Postboks 98, Esbjerg, DK 6701, **Denmark.**	2+3+5
Mr. Peter Sandfeld	**Fiskernes Fiskeindustri AmbA**	Postboks 164, Havnevagtvej 12, Skagen DK 9990, **Denmark.**	2
Mr. Finn Hauge Madsen	**Hanstholm Fiskemelsfabrik A/S**	Molevej 54, Hanstholm, DK 7730, **Denmark.**	2+3
Mr. Snorri Thorisson	**Icelandic Fisheries Lab.**	Skulagata 4, 121 Reykjavik, **Iceland.**	1-5
Mr. Berni Sheridan	**IAWS**	Fishmeal Factory, Killybegs, Co. Donegal, **Ireland.**	1-5
Dr. G.P. Savage Senior Lecturer in Biochemistry	**Lincoln College**	Canterbury, **New Zealand.**	1-5
Mr. Nils Urdahl	**SSF**	5033 Fyllingsdalen, Bergen, **Norway.**	1-5
Dr. James Burt	**Torry Research Station**	P.O. Box 31, 135 Abbey Road, Aberdeen AB9 8DG, **U.K.**	1-5
Dr. Eric Miller	**University of Cambridge**	Department of Applied Biology, Pembroke Street, Cambridge CB2 3DX. U.K.	3+4
Mr. Earl Louviere	**Zapata Haynie Corporation**	1605 North Morrison Hammond, La. 70401. **U.S.A.**	1-5
Mr. E. Von Jan	**Central Laboratory**	Klussmannstr. 3, Bremerhaven 1, P.O. 101248, **West Germany.**	1-5

Participating Laboratories (Continued)

Contact Person	Organization	Full Postal Address	Methods to be tested
Corpesca S.A.	**Corpesca S.A.**	Corpesca S.A. Huerfanos 863 9th Floor Santiago **Chile**	1
Dr. J.P.H. Wessels	**Fishing Industry Research Institute**	Lower Hope Street Rosebank 7700 Cape Province **South Africa**	1-3, 5

PEPSIN DIGESTIBILITY TEST

The pepsin digestibility test is carried out according to Torry Research Station's recommendations with slight modifications. Solvent extraction of the meal is not employed.

1. Use an agitator with horizontal movements.
2. Grind the meal through mesh 80. This seems essential for obtaining reproducible results.

Pepsin test

Apparatus and supplies:

1) Incubator, 45° C, with agitator
2) Buchner funnel 150 mm
3) Filter paper. Whatman No. 541; 18.5 cms.
4) Sample glass with screw cap. 200 cc
5) Shaker/incubator: Bench top G24 environmental incubator shaker. New Brunswick Scientific Co. Inc, U.S.A. (the shaker platform is rotated horizontally in a 19mm diameter orbit. Rate of agitation is regulated to 150 rpm).

Reagents:

1) Pepsin 1:10.000 (stored cool and dry) Merck Catalog No. 7190
2) 0.075 N HC1 (6.3 cc cone HC1 liter)
3) 0.0002 % freshly prepared pepsin/HC1 solutions (pepsin 1:10.000 dissolved in 0.075 N HC1)
4) Thompson and Capper "Kjeltabs M"; 5g potassium sulfate and 0.25g mercuric oxide.

Procedure:

1) Grind and weigh accurately 1.000 gms of herring meal into a 200 cc sample glass with screw cap. Add 150 cc 0.0002% pepsin-HC1 solution (reagent 3) and heat to 42° - 45° C.
2) The sample glass is closed and connected to an agitator which is placed in an incubator at 45° C.
3) The sample is incubated 16 hours under continuous agitation.
4) Filter through a Buchner funnel fitted with a 589² S&S filter paper. Wash properly with hot distilled water with a temperature of approximately 95°C.
5) The filter with residue is transferred to a 500 cc Kjedahl flask and digested according to the ordinary Kjeldahl method (IAFMM Technical Bulletin No. 8) using catalyst (reagent 4).
   Calculate as % residual nitrogen = A.
6) Prepare and conduct an acid insoluble determination simultaneously and identical in all respects but without pepsin (150 cc reagent 2).
7) Calculate as % residual nitrogen = B.

$$\text{Pepsin digestible} = \frac{\%\text{ residual nitrogen B} - \%\text{ residual nitrogen A}}{\%\text{ residual nitrogen B}}$$

Reproducibility in our laboratory is quite satisfactory. We do not accept greater differences between parallel determinations than 0.5 per cent in the digestibility coefficient.

Tjaereviken, January 2nd 1969.

TITRATION VALUE

1. Scope and field of application

This paper specifies a method for determination of the titration value.

The method is used for fish meal.
Dry matter determination is part of the analysis.

2. Reference

Sandfeld, P., Association of Fish Meal and Fish Oil Manufacturers in Denmark.

3. Principle

The titration value is expressed in consumed ml 0.1 N NaOH per 10g fish meal dry matter. Consumption is read at pH 10.

4. Reagents

Sodium hydroxide (NaOH) 0.100 N.

5. Apparatus

Analytical balance, pH-Meter, autoburette, titrator, magnetic stirrer, beakers 250 ml, stopwatch, and equipment for dry matter determination (IAFMM Technical Bulletin No. 9).

6. Procedure

1. Weigh 10.0g fish meal in a 250 ml beaker
2. Add 100ml H_2O.
3. Start the automatic titration (end point pH 10.0, titrant 0.100 N NaOH, speed 30 ml/min, constant stirring). pH 10.0 should be obtained in less than 8 min. Read the consumption of 0.100 N NaOH after exactly 10 min.
4. The titration value (ml 0.1 N NaOH/10g fish meal dry matter) is equal to

$$TV = \frac{ml \times 100 \times 10}{SW \times FMDM}$$

Where

TV	titration value ml 0.1 N NaOH/10 g fish meal dry matter
ml	ml 0.100 N NaOH consumed
SW	sample weight g
FMDM	fish meal dry matter %

July 1988

MULTIENZYME - pH-STAT PROCEDURE

1. Scope and field of application

This paper species a method for prediction of protein digestibility by an in vitro enzymatic pH-stat procedure.

The method is for fish meal.

2. Reference

Pedersen, B. & B.O. Eggum: Prediction of protein digestibility by an in vitro pH-stat procedure. *Zeitschrift für Tierphysiologie, Tierernährung und Futtermittelkunde.* **49** (1983), 265-277.

3. Principle

The fish meal is digested by a three-enzyme solution, and the pH is kept constant 8.00 during the 10 min incubation. The consumption of alkali to maintain pH at 8.00 is used to estimate the in vitro digestibility.

4. Reagents

Sodium hydroxide (NaOH) 0.10 N.

Sodium caseinate (DIFCO, U.S.A.)

Enzyme solution - Trypsin T-0134 (16950 BAEE units/mg; 92%),
Chymotrypsin C-4129 (41 units/mg),
Peptidase P-7500 (50 units/g)
The enzymes are from Sigma.

Dissolve

$$\frac{0.92 \times 16950 \times 14.56}{\text{declared units/mg}}$$ mg trypsin

$$\frac{41 \times 45.36}{\text{declared units/mg}}$$ mg chymotrypsin

$$\frac{50 \times 10.40}{\text{declared units/g}}$$ mg peptidase

in 10.0 ml H_20 (add 5 ml H_20, shake gently, and add 5 ml H_2O).

Adjust pH to 8.00 at 37°C over a 2 min (exactly) period.
Immediately store the enzyme solution in an ice bath.
Prepare a new solution every day. Control the solution on sodium caseinate - see note 1.

5. Apparatus

Analytical balance, mortar and pestle or other grinding procedure, sieve (mesh size 80), pH-Meter, autotitrator, magnetic stirrer, water bath, beakers 25 ml, pippettes 1 and 10 ml, stopwatch.

6. Procedure

1. Grind the fish meal to pass through an 80 mesh sieve

2. Weigh the sample in a 25 ml beaker.

   The amount should correspond to 10 mg N.

   $$\text{(Amount} = \frac{6.25}{\%\ \text{Protein}}\ \text{g)}.$$

3. Add 10.0 ml H_2O.

4. Store the sample 1 hr (max. 24 hrs) in a refrigerator.

5. Heat the sample to 37.0°C on a water bath, adjust pH to 8.00 (titrant 0.10 N NaOH, speed 0.5 ml/min, constant stirring - avoid vigorous stirring). pH should be constant over a period of 7 min.

6. Start the automatic titration (titrant 0.10 N NaOH, speed 0.125 ml/min), and add quick 1.0 ml enzyme solution (time = zero). Stir the sample during the incubation at 37.0°C.

7. Record the amount of alkali added after exactly 10 min and stop the titration.

8. The multienzyme digestibility (%) ME is equal to

   $$ME = 76.2 + \left(\frac{50.5 \times 6.25}{SW \times PR}\right) \times \left(\frac{0.485 \times A_{10}}{C_{10}}\right)$$

   where

   ME multienzyme digestibility %.

   SW sample weight g.

   PR protein content %.

   A_{10} added amount of 0.10 N NaOH ml.

   C_{10} added amount of 0.10 N NaOH ml to the control (Note 1).

7. Notes

1. Use sodium caseinate to control the activity of the enzymes. Weigh 0.0742 g sodium caseinate and analyse it. C_{10} should be between 0.460 and 0.510 ml. Average 0.485 ml.
2. It is necessary to follow the procedure exactly to obtain reproducible results.

July 1988.

Appendix 4

DETERMINATION OF SULPHYDRYL GROUPS AND DISULPHIDE BONDS IN FISH MEALS

1. Scope and field of application

This paper specifies a method of determining the content of disulphide (S-S) and sulphydryl (-SH) bonds in fish meal protein.

2. Reference

Opstvedt, J., Miller, R., Hardy, R.W. and Spinelli, J. (1984) "Heat - Induced Changes in Sulphydryl Groups and Disulphide Bonds in Fish Protein and Their Effect on Protein and Amino Acid Digestibility in Rainbow Trout (Salmo gairdneri)" J. Agric. Fd Chem., July- August, 929-935.

3. Principle

Heating of fish protein results in oxidation of -SH bonds to S-S bonds. Disulphide bonds reduced with sodium borohydride are determined as -SH groups along with the native -SH groups. Native -SH groups are determined alone by not employing sodium borohydride reduction. SH groups react with DTNB to produce an intense yellow colour with maximum absorption at 412 nm. A difference between these results gives the number of S-S bonds.

4. Apparatus

Analytical balance, centrifuge to 3000g, spectrophotometer capable of measuring absorbency at 412 nm.

5. Determination of total -SH groups + SS bonds

5.1 Reagents:

0.6M $NaBH_4$ (sodium borohydride) in 8 M urea:
(2.22 g $NaBH_4$ + 48 g urea dissolved to 100 ml dist. water).

Octanol.

2.0 N HC1

2.0 N NaOH

0.2 M Tris buffer, pH 8.2, 0.02 M EDTA Na_2:
(Dissolve 24.2 g Tris buffer + 7.44 g EDTA Na_2 in 1000 ml dist. water, adjust pH to 8.2 with 1 N HC1 and adjust volume to 1000 ml with dist. water).

0.005 M DTNB (5,5' - dithiobis-2-nitrobenzoic acid):
(dissolve 100 mg DTNB in 50 ml methyl alcohol).

Methyl alcohol.

Watman filter paper no. 4.

5.2 Analysis

An accurately weighed sample (W1) corresponding to approx. 35 mg protein is added to 4.0 ml 0.6 M $NaBH_4$ in 8 M urea solution. In order to avoid foaming, 2 dr. octanol are added prior to the addition of the sample. The solution is allowed to stand at room temperature for two hours (dried samples for 3 hours) (until the solution is clear). Remaining $NaBH_4$ is destroyed by the addition, drop by drop, of 1.1 ml 2 N HCl (until pH = 6.5).

The pH of the solution is adjusted to 8.2 through the addition of 0.9 ml 2 N NaOH. 1.5 ml 0.2M Tris buffer, pH 8.2, 0.02 M EDTA Na_2 solution, 0.2 ml 0.005M DTNB and 7.8 ml methyl alcohol is added to a 0.5 ml aliquot. The solution is mixed and then allowed to stand for 15 min. in covered test tubes at room temperature before being centrifuged at 3000 x G for 15 minutes and then filtered through Watman filter paper no. 4. The absorption in the filtrate is determined at 412 nm.

6. Determination of total -SH groups

6.1 Reagents:

0.2 M Tris buffer pH 8.2, 0.02 M EDTA Na_2, 2% SDS (dodecyl sodium sulphate) solution.

0.016 M DTNB (dissolve 317 mg DTNB in 50 ml methyl alcohol).

Methyl alcohol.

Watman filter paper no. 4.

Nitrogen must be blown on all the reagents prior to use.

6.2 Analysis

Suspend an accurately weighed sample (W2)corresponding to approx. 30 mg protein in 8.0 ml 0.2 M Tris buffer, pH 8.2, 0.02 EDTA Na_2, 2% SDS solution.

Blow the solution with nitrogen and then allow to stand at room temperature for 2 hours. Then add 0.5 ml 0.016 M DTNB and 31.5 ml methyl alcohol. After allowing to stand in covered test tubes for 15 min., centrifuge at 3000 x G for 15 min., whereupon the solution is filtered through Watman filter paper no. 4, and the absorption is determined at 412 nm.

7. Calculation of Results

$$\% \text{ of intact -SH groups} = \frac{\text{Absorbance @ 412} \times W1 \times 100}{\text{Absorbance @ 412} \times W2}$$

Water - Binding Capacity

Principle: When fish meal and water is blended, the meal will absorb water and increase in weight and volume. The fraction between absorbed water and meal, corrected for watersoluble dry material and carried out under specified condition is defined as swelling factor.

Apparatus

1) 25 ml graduated cylinder with stopper
2) Balance precision 0.001 gram

Procedure:

2 gram of fish meal is transferred into a 25 ml graduated cylinder. Add 20 ml water and shake well and make sure no caking occur. Shake additionally 3 times with one hour's interval and let the mixture settle over night.

Read the total volume V4 and the volume of the sediment V3. Watersoluble dry material (c) is estimated by drying an aliquot of the supernatant in a drying cabinet for 5 hours at 103 degrees C. Calculate as gram dry material per ml supernatant.

Calculation: See also Fig.

W1 = weight of sample (2 gram)
W2 = weight of supernatant = volume V2 (spec. weight = 1.0)
W3 = weight of sediment calculated (W4-W2) = W4 - V2.
W4 = total weight (sample + added water) (2 + 20 = 22 gram)

V2 = volume of supernatant = V4 - V3
V3 = volume of sediment
V4 = total volume
C = gram watersoluble dry material in one ml supernatant.

$$S = \frac{W3-(W1 - V2 \times C)}{W1 - V2 \times C}$$

Example:

Weight of sample = 2 gram (W1)
Volume of supernatant = V4 -V3 = 13.5 ml
W3 = W4 -W2 = 22 - 13.5 = 8.5 gram
Watersoluble material C = 0.0238 gram/ml

$$\text{Swelling factor } S = \frac{8.5 - (2.00 - 13.5 \times 0.0238)}{2.00 - 13.5 \times 0.0238} = 4.075$$

Summary of Analysis of Fish Meal Data

Although these data appeared an ideal vehicle for a variance component analysis, whereby the various sources of variation are separated, quantified, and interpreted, it was found that numerous unsatisfactory features of the data argued against that formal approach. The presence of atypical laboratories, and the detection of unexpected systematic variation, necessitated a very careful scrutiny and a non-standard evaluation of the data. The findings may now be summarised as follows.

(1) The supposed 'between estimation' error within the laboratories, as computed from the triplicate measurements, showed immense variability, as may be appreciated from the column headed S.D. in Tables 1-4. In view of this inconsistency, it does not seem at all appropriate to pool this source of variability to supply a single 'between estimation' error, which would otherwise be a very useful 'global' statistic to quote.

(2) The variation between 'hidden duplicates' was often significantly greater than the 'estimation error' as obtained from the triplicate observations. This feature may be noted from the proliferation of asterisks in the column headed Du in Tables 1-4, or by comparing the relevant standard deviations, in columns S.D., and BD. This result implies that the 'between duplicate' error is a far more reliable estimate of the variation within a specific laboratory, than the triplicate variation. However, the vast differences in this measure of variability between laboratories (see column BD in Tables 1-4), suggests that it would be imprudent and misleading to pool this variation to produce a composite figure.

(3) The analyses of variance (See Appendix 1) demonstrated quite conclusively that there were systematic differences between the laboratories, and furthermore, that these differences were not consistent across samples. The very large variation 'Between Laboratories' in the tables displayed in Appendix 1 provides ample evidence for the the first proposition, and the significant Lab x Samples interaction provides evidence of the absence of consistency. Although it is not an easy matter to demonstrate visually, some of the important deviations may be in fact be observed in the mean values contained in Tables 1-4.

(4) The statistical technique of Principal Component analysis provides a means of quantifying the overall differences in the performances of the laboratories; what might be termed the 'Distance'. This method attempts, in the present context, to compress the information contained in the 12 samples, and provide a concise summary of the overall performance. Using this method, a number of truly 'outlying' laboratories were detected for three of the methods as follows;

PEPSIN	3 outliers out of 12 laboratories
ph Stat	4 outliers out of 12 laboratories
Titration	2 outliers out of 17 laboratories
Water Binding	No outliers detected

Whereas for the first three methods, the outliers could be detected quite easily, the variation in the Water Binding method did not permit such a selection.

(5) It would appear therefore, from these findings, that the erratic and inconsistent results obtained from the various laboratories preclude the use of 'within laboratory' variation as reliable estimate of measurement error. The error to be expected on a single estimate should therefore

reflect the 'between laboratory' variation, and a summary of the relevant statistics are presented below, with more detailed figures appearing in Table 5 of the main report. The standard deviations quoted are single composite figures reflecting 'between laboratory' variation on the mean of triplicate measurements.

Method	Overall Mean	Range	St.Deviation	Coeff.Variation
Pepsin	93.7	88.1-96.8	1.88	2.0
pH Stat	89.3	87.5-91.3*	1.19	1.3
Titration	64.2	35.2-96.3	5.58	8.7
Water Binding	2.81	2.5- 3.3*	0.322	11.4

* The narrow range of values obtained among the 12 test samples, when considered in conjunction with the standard deviations quoted, suggests that the these methods are unlikely to be able to detect even moderate differences in the quality of the meals.

Report on the Analysis of the Fish Meal Data

The data as presented consisted of four types of chemical analyses (PEPSIN, TITRATION, PHSTAT, & WATER) carried out on 12 samples submitted to 20 laboratories. The data structure was not completely balanced in that few laboratories provided results for all four types of analysis. There were in fact only 10 distinct types of material, since the 12 samples contained two sets of 'hidden' duplicates. The statistical analysis was thus intended to estimate the various sources of variation in the data set.

In order to explore the relevance of the triplicate measurements, individual analyses of variance were carried out on each Chemical Analysis/ Laboratory, the results being summarised in Tables 1-4. These tables give the mean values for each sample, an estimate of the within laboratory variation, as calculated from the triplicates, and some information about the reproducability of the 'hidden duplicate' samples. The column headed Du indicates whether the variation between the concealed duplicates was significantly in excess of what was expected from the random error as estimated between triplicates (Column S.D., which gives the appropriate standard deviations). The 'Between Duplicate' standard deviations are also tabulated (BD). Two asterisks in column Du indicates that variation between BOTH sets of duplicates (2 v 8, and 3 v 12) was significantly greater than the 'within laboratory' variation, a single asterisk, or no asterisk at all, then having the logical interpretation.

TABLE 1 (PEPSIN)

	Sample Number														
Lab	1	2	3	4	5	6	7	8	9	10	11	12	S.D.	Du	BD
1	84.9	73.6	88.3	90.0	62.4	89.8	75.7	67.6	97.2	97.3	93.8	94.0	1.34	**	7.22
2	98.6	95.1	97.7	98.5	96.0	100	92.1	95.4	101	102	100	98.6	0.68		0.79
3	96.2	94.8	94.3	96.2	94.3	96.8	88.0	94.8	97.4	97.7	97.4	91.9	0.40	*	2.08
5	95.6	91.4	93.9	94.9	93.1	95.8	90.0	92.3	97.3	97.5	95.5	92.9	0.53	**	1.18
7	93.7	90.2	92.5	95.3	86.6	96.2	88.7	87.6	96.9	94.1	87.0	92.9	2.66		2.25
12	95.1	91.9	94.0	95.2	93.4	95.3	88.8	94.4	97.2	97.0	97.4	93.0	0.16	**	2.26
13	91.7	90.3	91.4	93.0	92.0	93.9	87.8	91.5	95.6	96.3	95.3	91.5	0.18	*	1.01
15	94.1	90.4	91.9	96.7	93.6	96.4	84.4	89.5	97.4	98.2	96.8	92.8	1.09		1.10
16	95.4	95.7	93.9	95.6	94.6	97.3	89.8	95.0	97.9	98.4	97.9	94.5	0.53		0.76
18	92.6	87.1	89.6	92.7	92.5	91.1	77.3	87.5	90.2	95.3	93.5	89.8	0.75		0.40
19	93.5	87.9	92.6	95.1	92.5	96.1	87.3	90.8	96.5	97.5	97.0	94.4	0.26	**	2.93
20	93.8	91.8	92.1	92.4	92.8	94.3	88.5	91.3	94.6	94.6	95.4	91.2	0.78		0.96

TABLE 2 (TITRATION)

	Sample Number														
Lab	1	2	3	4	5	6	7	8	9	10	11	12	S.D.	Du	BD
1	36.3	58.0	68.3	99.9	41.5	85.7	62.8	58.8	97.1	110	67.7	71.7	0.92	*	2.98
2	38.8	59.0	72.6	109	47.4	85.9	63.3	61.7	96.0	109	65.8	68.8	0.47	**	4.06
3	49.7	69.0	68.1	131	52.3	87.7	68.7	62.2	100	113	74.6	73.9	6.88		7.72
4	32.9	50.2	60.1	97.0	39.5	96.1	60.8	59.1	91.7	105	60.2	63.0	1.14	**	8.09
6	32.1	49.9	54.1	90.2	36.7	73.5	54.0	51.0	54.4	57.8	81.3	89.1	0.28	**	30.3
7	39.8	53.6	58.1	92.1	40.5	76.1	59.1	56.0	87.1	98.3	58.2	60.1	0.19	**	2.68
8	33.3	52.9	58.3	92.1	36.9	75.2	52.7	52.8	85.2	93.9	55.9	57.1	2.17		1.01
9	33.6	52.8	59.5	94.4	38.9	78.6	59.8	52.6	87.6	98.4	58.5	61.5	0.11	**	1.74
10	36.6	56.2	58.6	96.4	49.3	79.2	63.9	55.7	89.6	102	62.4	64.4	0.32	*	5.04
11	31.6	49.4	57.3	91.4	34.1	72.7	54.4	49.3	81.7	92.0	54.2	55.1	0.29	*	1.93
12	33.3	52.3	58.1	92.0	38.6	70.8	52.5	52.5	82.2	90.5	54.8	56.0	0.61	*	1.86
13	38.0	56.6	60.9	91.4	38.8	71.4	54.5	50.8	85.8	92.1	56.2	54.3	1.26	**	7.60

15	35.3	53.8	57.1	88.8	38.1	71.3	55.1	49.3	[illegible]	51.2	53.7	56.4	0.51	*	3.88
16	37.3	54.5	62.8	98.3	42.0	79.5	58.9	56.5	87.8	101	60.1	62.7	0.73	*	1.76
18	34.2	50.7	54.0	85.6	36.0	63.6	54.5	53.2	74.2	83.0	51.0	53.5	0.83	*	2.26
19	36.7	55.5	63.7	98.5	39.4	75.3	53.7	54.6	86.5	96.9	57.9	62.2	0.34	**	1.53
20	31.0	48.4	50.7	80.2	34.7	65.0	55.5	48.5	77.1	82.2	49.2	52.9	6.16		1.85

TABLE 3 (PHSTAT.)

	Sample Number														
Lab	1	2	3	4	5	6	7	8	9	10	11	12	S.D.	Du	BD
1	86.8	88.9	89.6	90.1	90.9	90.9	87.9	87.2	90.6	92.0	91.2	92.2	0.66	**	2.75
2	86.4	87.3	88.8	87.6	87.7	91.3	90.6	87.3	90.8	91.8	90.7	90.1	0.88		1.13
6	88.0	87.9	88.8	88.5	87.1	89.8	87.7	87.6	90.6	90.8	88.8	88.6	0.26		0.23
7	87.5	88.3	89.4	89.3	87.0	91.2	89.7	89.8	91.1	92.5	91.3	90.0	0.15	**	1.42
8	85.6	88.5	89.2	87.9	87.0	89.1	88.0	87.6	90.1	91.3	89.9	89.8	0.37	*	0.74
9	87.7	88.0	89.5	91.0	88.5	89.7	88.2	88.2	90.5	91.1	89.6	90.0	0.18	*	0.47
11	91.7	89.0	91.2	88.3	89.1	90.4	89.5	90.3	89.6	91.2	91.8	92.4	0.16	**	1.49
15	85.4	87.6	89.2	87.5	86.5	91.2	80.5	87.9	90.6	91.6	91.8	88.2	0.83		0.92
16	84.8	83.4	85.6	84.7	84.5	86.6	85.3	82.9	86.6	89.2	88.0	84.8	0.92		0.84
18	83.5	86.3	86.2	88.4	86.1	86.7	84.8	85.3	85.7	84.8	86.0	86.7	1.04		0.97
19	86.3	87.7	88.1	87.1	86.7	90.8	86.4	85.6	89.8	90.0	90.6	88.9	0.50	*	1.96
20	84.6	85.0	85.6	86.2	83.6	87.6	86.5	83.8	87.9	88.2	87.7	86.3	0.92		1.14

TABLE 4 (WATER)

	Sample Number														
Lab	1	2	3	4	5	6	7	8	9	10	11	12	S.D.	Du	BD
1	3.93	3.00	2.67	3.00	3.17	3.43	2.40	2.90	2.43	2.57	3.03	2.50	0.78	*	1.68
2	3.31	2.67	2.81	3.02	2.70	2.76	2.19	2.48	2.29	2.73	3.00	3.31	1.51	*	4.61
3	2.88	2.41	2.28	2.85	3.37	3.77	2.43	3.11	2.45	2.87	3.15	2.54	0.59	**	6.49
4	3.29	2.79	2.58	2.83	2.71	3.18	2.70	2.80	2.42	2.67	2.73	2.43	0.73	*	1.27
6	2.83	2.30	2.40	2.63	2.40	2.77	2.47	2.33	2.37	2.77	2.40	2.43	0.55		0.41
7	3.10	2.50	2.61	2.80	2.84	3.59	2.80	2.57	2.68	3.22	3.08	2.78	2.12		1.57
9	3.92	2.98	3.24	3.35	3.23	2.97	2.46	3.10	2.76	2.93	3.19	2.94	0.39	**	2.80
13	2.98	2.52	2.47	2.46	2.76	2.80	2.48	2.02	2.13	2.18	2.52	2.41	0.85	*	4.39
15	3.40	2.57	2.40	2.87	3.03	3.23	2.40	2.40	2.33	2.40	2.87	2.33	0.93	*	1.55
16	3.48	2.59	2.47	2.82	2.71	3.04	2.39	2.50	2.25	2.51	2.87	2.51	0.92		0.87
18	22.0	9.07	4.64	1.98	5.48	17.4	13.1	8.82	2.79	2.85	3.89	3.52	5.88	*	10.0
19	2.93	2.90	2.60	2.70	2.50	2.73	2.57	2.57	2.77	2.77	3.20	2.80	0.65	**	3.37
20	3.82	3.34	3.37	3.41	3.25	3.84	3.09	3.21	3.11	3.35	3.39	3.10	2.00		2.63

N.B. For the sake of convenience, the Standard Deviations for WATER have been multiplied by 10.

The proliferation of asterisks in Tables 1-4, and the fact that BD is often greatly in excess of S.D., casts very serious doubts on the integrity of the triplicate measurements as reliable estimates of within laboratory variation. It would be prudent therefore to disregard this stratum of variation, and concentrate henceforth on the mean values of the triplicates.

A visual inspection of Tables 1-4 suggested that some laboratories deviated consistently from the others in the estimates presented. In order to confirm this visual impression, and make an objective identification of any outlying laboratories, a principal component analysis was carried out on the Laboratory/Sample mean values. The complete representation of the variation in these tables would require 12 dimensions, and principal component analysis attempts to compress the variation into a few (perhaps 2) dimensions in order to identify outliers. It thus summarises the 'between laboratory' variation into as few dimensions as

possible. The accompanying diagrams contain plots of the first two principal components for the four chemical analyses. The laboratories represented by points which were distant from the main set were then removed, for all subsequent investigations. This device was moderately successful for PEPSIN, TITRATION, & PHSTAT where 92%, 90%, and 81% respectively of the total variation was contained in the first two principal components. The laboratories excluded as outliers were 1, 2, & 18 for PEPSIN; 3 & 6 for TITRATION; 15, 16, 18 & 20 for PHSTAT. (See diagrams.)

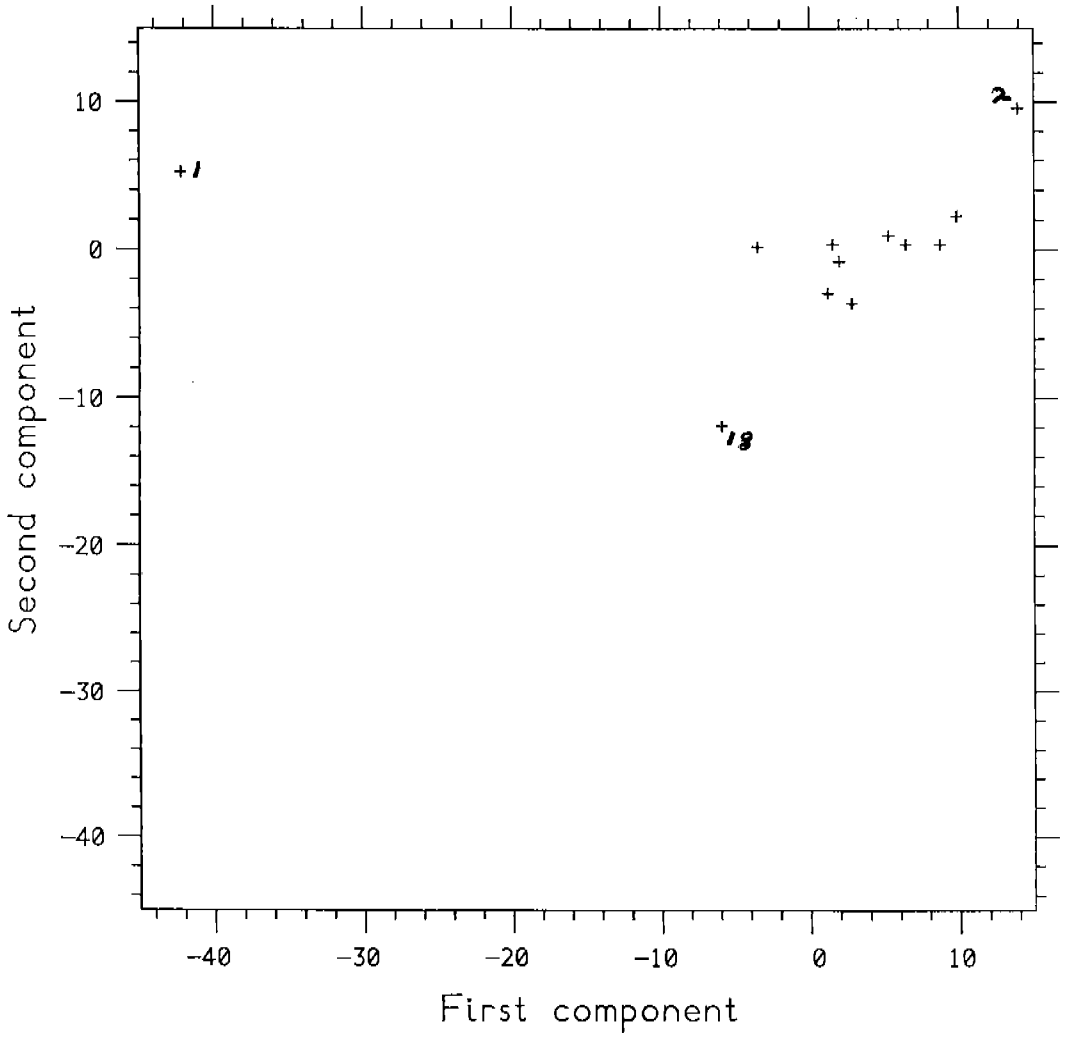
In view of the unsatisfactory features discovered in the analyses described above, perhaps the most meaningful measure of variability to be presented would be that between the means of triplicates for individual samples, only over those laboratories which were not classified as outliers. These figures are presented in Table 5, which gives the 'Between Laboratory' standard deviation (S.D.) for each of the four analyses and 12 samples. The interpretation of these statistics is, that if a randomly selected laboratory were asked to analyse a sample in triplicate, and to present the resulting mean value as an estimate, then these figures give an indication of the errors to be expected on such estimates. The overall mean values are also quoted in order to gauge the percentage error.

TABLE 5

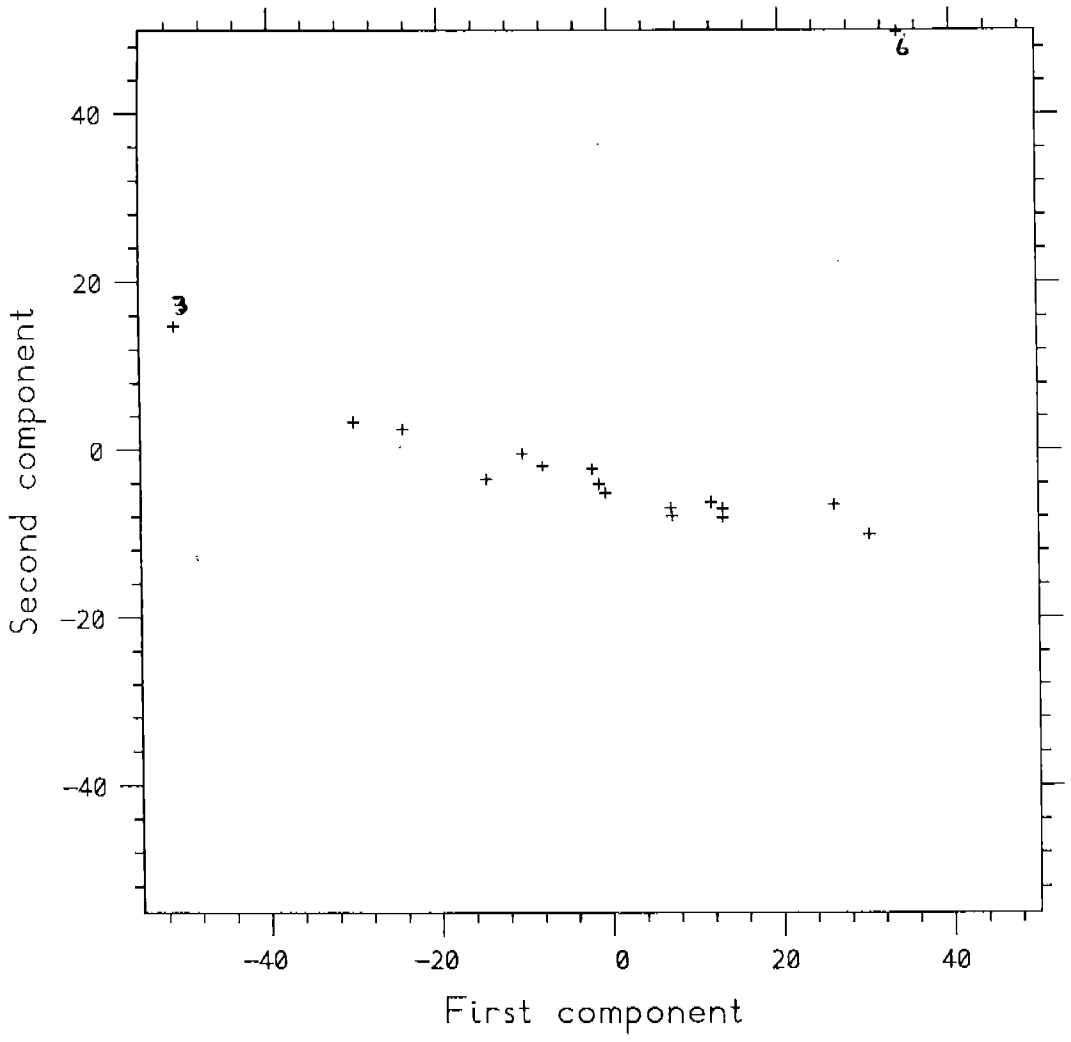
		Sample Number											
		1	2	3	4	5	6	7	8	9	10	11	12
PEPSIN	S.D.	1.39	2.39	1.07	1.40	2.38	1.11	1.65	2.51	1.04	1.52	3.32	1.17
	Mean	94.3	91.6	93.0	94.9	92.6	95.8	88.1	91.9	96.7	96.8	95.5	92.8
TITR.	S.D.	2.66	3.12	5.35	6.74	4.16	8.36	4.00	3.89	6.28	8.26	5.07	5.63
	Mean	35.2	53.6	60.0	93.8	39.7	76.4	57.4	54.1	86.0	96.3	57.7	60.0
PHSTAT	S.D.	1.89	0.59	0.90	1.32	1.42	0.77	1.34	1.50	0.52	0.76	1.00	1.37
	Mean	87.5	88.2	89.3	88.7	88.0	90.4	88.5	88.0	90.4	91.3	90.5	90.3
WATER	S.D.	0.40	0.30	0.33	0.27	0.31	0.40	0.24	0.36	0.28	0.33	0.29	0.31
	Mean	3.32	2.71	2.66	2.90	2.89	3.18	2.53	2.67	2.50	2.75	2.95	2.67

If the 12 standard deviations for each analysis are now pooled to give a single composite figure, we obtain a pooled figure of 1.88 (2.0%) for PEPSIN, 5.58 (8.7%) for TITRATION, 1.19 (1.3%) for PHSTAT, and 0.322 (11.4%) for WATER.

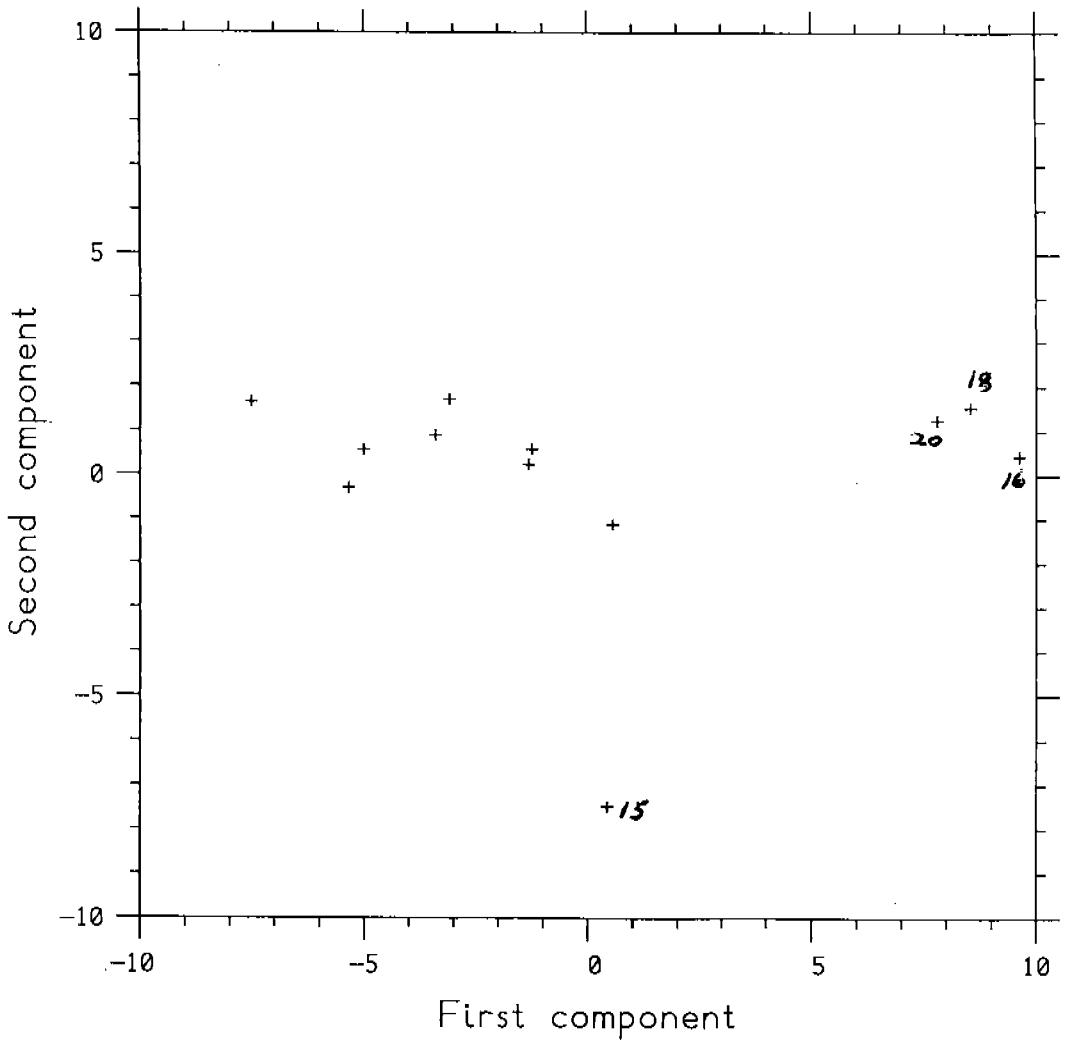
Plot of first two principal components, PEPSIN (92%)



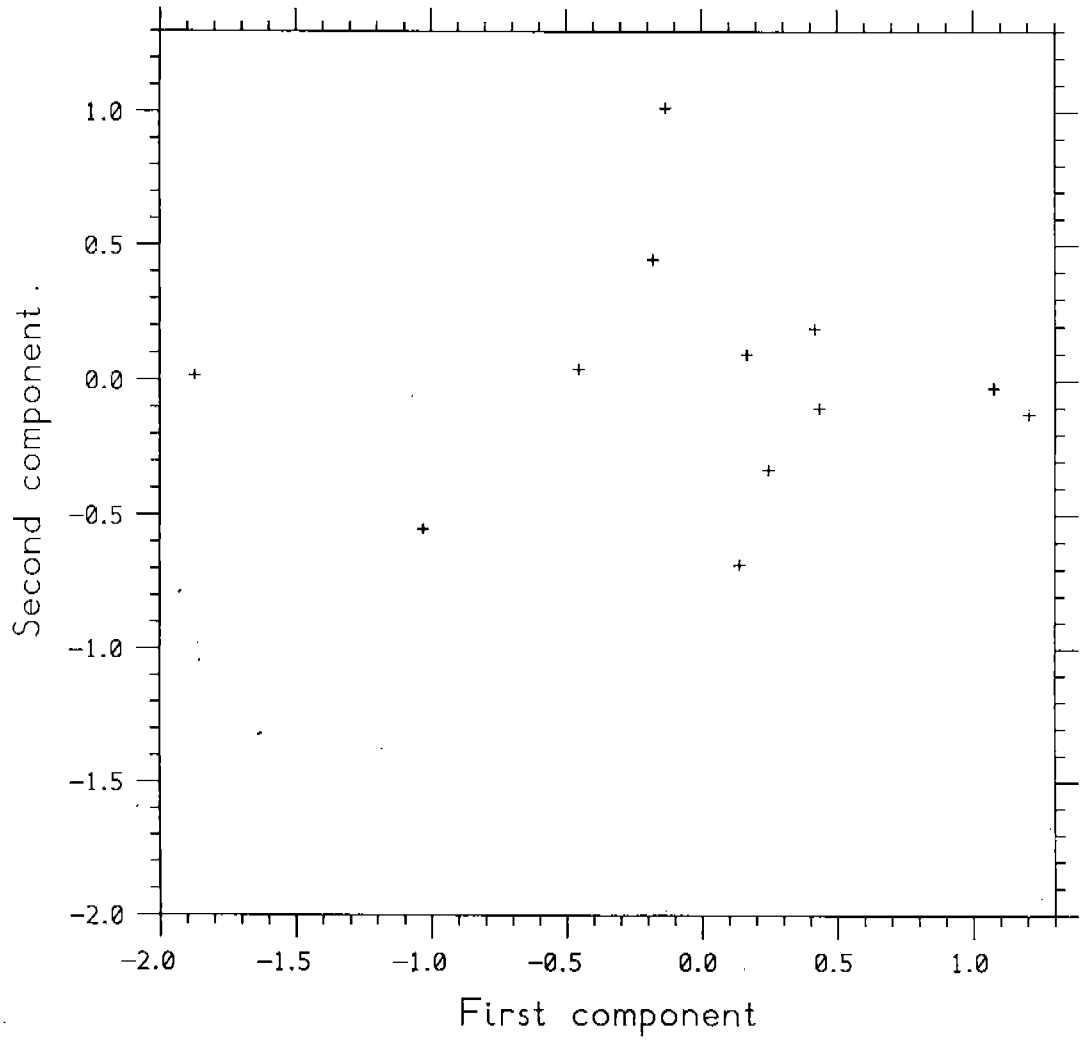
Plot of first two principal components, TITR. (90%)



Plot of first two principal components, PHSTAT (81%)



Plot of first two principal components, WATER (73%)



APPENDIX 1: Analyses of variance for the four analytical methods.

(a) PEPSIN

Source of Variation	S.o.S.	df	M.S.
Between Laboratories	420.36	8	52.55
Between Samples	1881.75	11	171.07
Between Hidden Duplicates	1.65	2	0.82
Remainder	1880.10	9	208.90
Labs x Samples	600.67	88	6.83
Error	233.61	216	1.08
Total	3136.39	323	

(b) PHSTAT

Source of Variation	S.o.S.	df	M.S.
Between Laboratories	132.64	7	18.95
Between Samples	429.38	11	39.03
Between Hidden Duplicates	11.16	2	5.58
Remainder	418.22	9	46.47
Labs x Samples	221.72	77	2.88
Error	40.97	183	0.224
Total	824.71	278	

(c) WATER

Source of Variation	S.o.S.	df	M.S.
Between Laboratories	22.860	11	2.08
Between Samples	24.590	11	2.24
Between Hidden Duplicates	0.047	2	0.023
Remainder	24.543	9	2.727
Labs x Samples	18.214	121	0.151
Error	3.740	288	0.013
Total	69.404	431	

(d) TITRATION (The sums of squares need to be scaled upwards by 100)

Source of Variation	S.o.S.	df	M.S.
Between Laboratories	113.0	14	8.07
Between Samples	1940.0	11	176.4
Between Hidden Duplicates	0.056	2	0.028
Remainder	1939.9	9	215.5
Labs x Samples	44.19	154	0.287
Error	11.72	360	0.033
Total	2108.9	539	