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The Determination of Pyrimethamine by Nuclear Magnetic Resonance Spectroscopy

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A procedure is described for the quantitative analysis of pyrimethamine by NMR spectrometry using caffeine as an internal standard.

The drug pyrimethamine (2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine) is widely used as a suppressant of malaria. It can be assayed in pharmaceutical preparations either gravimetrically[1] by precipitation as the phosphotungstate, spectrophotometrically[1] from its ultra-violet absorption at 272·5 nm in 0·1 N HCl, or by titration in a non-aqueous medium.[2] A gas-chromatographic method has been developed for its determination in tissues.[3] The British Pharmacopoeia 1973[4] describes the method of non-aqueous titration with perchloric acid, using quinaldine red as indicator. The pharmacopoeial procedure for the assay of Pyrimethamine Tablets involves the non-aqueous titration of the residue obtained after evaporating an acetone extract of the powdered tablets. An NMR procedure is described here which is specific both for the assay and the identification of pyrimethamine as the powder or in tablets.

Apparatus

Perkin-Elmer R-12 (60 MHz) NMR spectrometer or equivalent instrument.

Reagents

1. *Pyrimethamine B.P.*
2. *Pure anhydrous caffeine*, M.Pt. 237°C.: Caffeine may be purified if necessary by crystallisation from ethanol to give the anhydrous form, or from hot water to give the monohydrate from which the anhydrous form is obtained by drying at 80°C.[5]
3. *Trifluoroacetic acid.*
4. *Tetramethylsilane.*

Method

Weigh accurately into a small sample tube or centrifuge tube a portion of powder or of powdered tablets equivalent to about 200 mg of pyrimethamine.

Add about 78 mg, accurately weighed, of pure anhydrous caffeine, followed by about 2 ml of trifluoroacetic acid. Warm the tube gently on a water bath for about 30 seconds, and centrifuge if necessary to separate the solution from insoluble tablet excipients. If a clear supernatant solution cannot be obtained in this way, extract an accurately weighed quantity of the powdered tablets equivalent to about 200 mg of pyrimethamine with successive quantities of acetone, according to the method described in the B.P. 1973 under Pyrimethamine Tablets. Use the residue obtained on evaporation of the combined acetone extracts to prepare a solution in trifluoroacetic acid containing anhydrous caffeine as standard, as described above.

Transfer about 0·5 ml of the clear solution obtained to an NMR tube, add about 1 per cent. of tetramethylsilane, and record the NMR spectrum, adjusting the instrument gain, Rf field and sweep rate to obtain a convenient integral presentation, at the same time ensuring that the conditions chosen do not produce saturation effects which might affect relative peak areas. Paulsen and Cooke[6] have described the selection of a non-saturating mode of operation from measurements of the ratio of the areas of sample and internal standard peaks at progressively increasing Rf levels. The optimum level is the one just below that at which a change in the value of the ratio is observed. Run the integrals of the peaks of interest at least twelve times, and determine the mean value of the integral height. Measure peak positions with reference to the tetramethylsilane signal. Calculate the content of pyrimethamine, $C_{12}H_{13}ClN_4$, as follows:

Content of pyrimethamine per g of sample =

$$\frac{I_{Sample}}{I_{Caf}} \times \frac{\text{g of anhydrous caffeine}}{\text{g of sample}} \times \frac{E_{Pyr}}{E_{Caf}}$$

I_{Sample} = integral value of sample peak (equivalent to 3 methyl protons of the ethyl group) centred near τ8·74 (triplet).

I_{Caf} = total integral value of two caffeine peaks (equivalent to 6 methyl protons) near τ6·21 and 6·42 (singlets).

E_{Pyr} = weight of pyrimethamine (M.W. 248·7) equivalent to 1 proton = 248·7/3 = 82·9 for the sample integral.

E_{Caf} = weight of caffeine (M.W. 194·2) equivalent to 1 proton = 194·2/6 = 32·37 for the caffeine integral.

Results and Discussion

The NMR spectrum (Fig. 1) of pyrimethamine in trifluoroacetic acid showed two doublets centred at τ2·38 and τ2·72 which are due to the four aromatic protons of the benzene ring, together with a quartet centred at τ7·40 and a triplet centred at τ8·74 representing respectively the CH_2 and CH_3 moieties of the ethyl group attached to the pyrimidine ring. Caffeine gave rise to a singlet at τ1·12 produced by its single iminazole ring proton, and another three sharp singlets at τ values of about 5·67, 6·21 and 6·42, due to the three CH_3

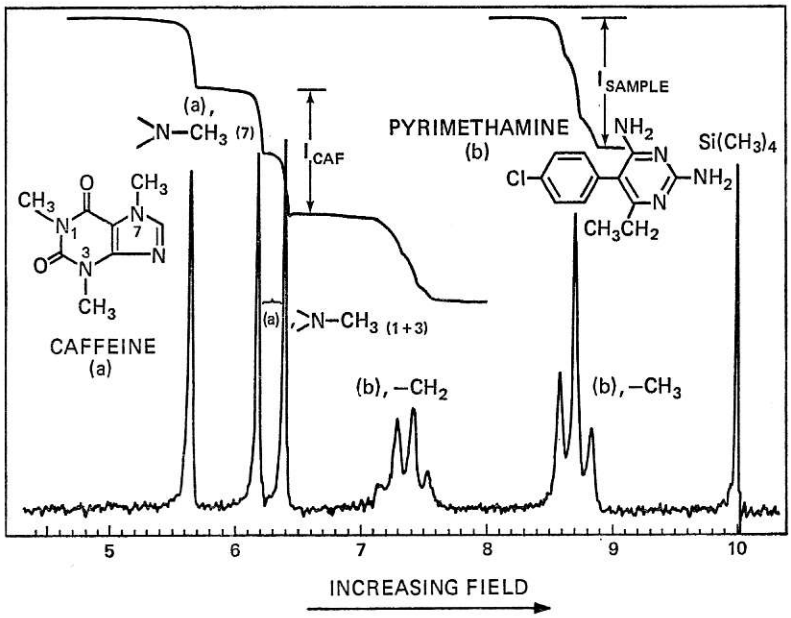

FIG. 1. 60-MHz NMR spectrum of a mixture of pyrimethamine (0·2041 g) and caffeine (0·0770 g). Peak positions are measured on the abscissa with reference to the $Si(CH_3)_4$ peak set at a tau (τ) value of 10,

where $\tau = 10 - \delta$

and $\delta = \dfrac{\text{distance of peak from } Si(CH_3)_4 \text{ signal (Hz)} \times 10^6}{\text{operating frequency (Hz)}}$

groups of the molecule. There was thus no overlapping of peaks in the combined spectrum of the two compounds. Trifluoroacetic acid was chosen as solvent, since pyrimethamine is insoluble in water, and its solubility in chloroform (1 in 125 parts)[1] is insufficient to be of use. A 220 MHz spectrum obtained for an accurately prepared mixture of pure caffeine with the pyrimethamine sample showed very minor impurity peaks between the two caffeine peaks at τ5·67 and 6·21. In order to eliminate these, all assay results were calculated from the combined integral value of the two peaks near τ6·21 and 6·42. Prepared mixtures of pyrimethamine and caffeine were analysed by the procedure described (using the 60 MHz spectrometer), as well as a commercial pyrimethamine tablet preparation, which required a preliminary extraction with acetone. The results are shown in Table I.

By this method, the mean result of 18 assays of the pyrimethamine sample was 99·7 per cent., with an average deviation from this mean of $\pm$1·5 per cent. The alteration of the ratio of caffeine to pyrimethamine in some of the assays did not appear to have any significant effect on the results obtained. The standard deviation for the assay was 1·96 per cent. The manual measurement of peak areas in this assay is precluded by the difficulty of measuring

TABLE I

THE DETERMINATION OF PYRIMETHAMINE IN PREPARED MIXTURES AND IN A COMMERCIAL TABLET PREPARATION

Sample	Caffeine added *mg/ml*	Pyrimethamine added *mg/ml*	Pyrimethamine found *mg*	*per cent*
Prepared mixture				
1	38·5	102·1	102·8	100·7
2	38·3	102·7	104·1	101·4
3	41·3	101·3	102·0	100·7
4	38·2	101·7	102·5	100·8
5	40·3	100·2	99·0	98·8
6	40·7	102·5	103·6	101·1
7	42·8	99·7	98·9	99·2
8	39·9	100·7	100·8	100·1
9	39·2	100·1	99·8	99·7
10	40·0	102·5	101·1	98·6
11	37·3	100·6	95·8	95·2
12	44·4	96·9	93·1	96·1
13	26·9	97·8	96·8	99·0
14	27·5	105·2	107·8	102·5
15	28·0	92·2	93·0	100·8
16	25·8	101·6	103·5	101·9
17	108·9	106·6	107·2	100·6
18*	27·9	99·3	96·6	97·3
25 mg Tablet preparation	26·7	503·2†	99·6	101·3

* Result obtained from a 220 MHz spectrum.
† mg of powdered tablets (average weight per tablet 128 mg).

the triplet at τ8·74. It was therefore not possible to determine whether the variation in results is due to differences in signal response or in integrator response. The precision obtained is obviously somewhat less than would be obtainable by the titration method of the B.P. 1973. The method has, however, the great advantage of specificity, relying as it does upon a characteristic resonance pattern being obtained for the pyrimethamine, and a comparison of signal areas which are directly proportional to the number of protons producing them. Tablet excipients did not interfere when the procedure was applied to the assay of a tablet preparation.

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NOTE ON NMR TECHNIQUE BY THE AUTHORS

NMR is concerned with the resonance of nuclei of hydrogen atoms of organic compounds in a strong magnetic field in response to an applied radio frequency. For different strengths of field and for nuclei in different environments, different values of radio frequency are required. In practice, the radio frequency is kept constant and the magnetic field varied to bring different nuclei into resonance successively as the field is increased. In order that results from different instruments may be comparable the following procedure is used:

The positions of the various peaks are measured with reference to the peak due to tetramethylsilane which is taken as an arbitrary zero. The distance from the tetramethylsilane peak (which is called Δ value) in cycles per second (Hz) on the spectrum is known as the chemical shift. This is expressed as follows to give a dimensionless chemical shift value δ which is independent of the instrument used.

$$\delta = \frac{\Delta}{\text{Operating frequency of instrument in Hz}} \times 10^6 \text{ p.p.m.}$$

The τ value $= 10 - \delta$ and is used to give an expression for the chemical shift which increases with increasing magnetic field.

The Public Analyst and The Baking Industry

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This article is adapted from a lecture delivered on 13th November, 1973, to the British Chapter of the American Society of Bakery Engineers, to whom the author is indebted for permission to publish. Legal aspects of the functions and duties of Public Analysts are discussed with some regard to difficulties arising from the Bread and Flour Regulations 1963, and to principles applied to labelling problems.

In the days before the 1939–45 war, there were, with few exceptions, no quantitative requirements of composition for specific foods. Butter and margarine had to contain not more than 16 per cent. of water; there were compositional standards for condensed milk; and there had been presumptive minimum standards for the fat and non-fatty solids of liquid milk ever since 1901. In other respects the Public Analyst, when trying to decide whether a sample was genuine or not, had to make up his own mind in the light of his knowledge and experience. He worked very largely under the principal provisions of the Food and Drugs Act itself, and particularly under Section 2 of the Act. This reads as follows:

"If a person sells to the prejudice of the purchaser any food or drug which is not of the nature, or not of the substance, or not of the quality, of the food or drug demanded by the purchaser, he shall, subject to the provisions of the next following section be guilty of an offence."

This is the foundation of a Public Analyst's work when deciding whether or not a given sample of food is adulterated. There has always been some controversy regarding the exact meaning of the words "nature", "substance" and "quality", but with one or other as a basis, it is usually possible to characterise the offence committed. Although the use of the word "demanded" makes it appear that purchasers of foods are aggressive in their habits, it is normally held that the word simply means "expected".

It must be emphasised, because there seems to be so frequently a complete misunderstanding of the position by manufacturers and retailers, that the duty is laid firmly on the Public Analyst to decide, as regards each and every sample submitted to him, whether it is of the nature, substance and quality that the ordinary purchaser—not an expert, but an ordinary reasonable housewife—would expect to receive if she asked for the food by the name under which it is submitted to the Public Analyst. He cannot evade the issue by saying to himself "there is no quantitative standard of composition for this product and therefore anything goes". It is obvious, for example, that marzipan containing only 1 per cent. of ground almonds, or even none at all, is not of the quality expected; it is equally obvious that marzipan containing 50 per cent.

of ground almonds mixed with sugar is of very good quality (though a hundred years ago it would have been of normal average quality). Somewhere in between these two extremes, the Public Analyst has to draw a line which separates marzipan sheep from marzipan goats, so to speak. This type of decision is forced upon him every time he is faced with a sample for which there is no quantitative standard laid down by the law.

This necessity of decision making can sometimes put the Public Analyst in considerable difficulty, particularly when the sample is what is usually referred to as a "complaint" sample, that is to say, one that has been submitted to the Public Analyst because an ordinary purchaser has complained to the Local Authority that the food purchased is not what it ought to be. Many such complaints can be dealt with readily because the offence is clear-cut —a finger bandage in a bun or a mouldy meat pie—but others are not so simple. Some time ago, I had occasion to report against a sample of blackcurrant pie; the purchaser had alleged that the fruit content was too low and I found myself in agreement with him. The manufacturer took strong objection to the idea that I should criticise his product on this ground at all, because there was no regulation governing the fruit content of a pie. In a letter he wrote to the Local Authority he said "one might just as well ask the Public Analyst to decide how many currants there should be in a currant bun". He presumably thought this was a crushing rejoinder and that his argument constituted a *reductio ad absurdum*, but in fact it was the manufacturer who was being absurd. If I had a complaint from an aggrieved housewife that the article she bought as a currant bun did not contain sufficient currants to justify the term, I should then be forced to consider and answer the very question that the manufacturer had so scornfully posed.

FOOD STANDARDS REGULATIONS

Fortunately, since the 1940's, the Public Analyst has been relieved to a large extent of the necessity for making standards for himself by the enactment under the Food and Drugs Act of regulations governing the composition of foods. For example, it is no longer necessary for a Public Analyst to have to decide how much meat there should be in a meat pie; the law now requires that at least 25 per cent. of meat shall be present in an article sold under that name. There are many regulations of this kind, and this has the great advantage that the situation cannot arise, as it did occasionally at one time, that the Public Analyst in one area would adopt a different standard from the Public Analyst somewhere else, so that what it was legal to sell in Yorkshire could be sold only illegally in Essex. Again, the manufacturer now knows exactly where he is when deciding on formulations for his products. Regulations do lead to difficulties and I shall come back to this point shortly, but on the whole, consumers, manufacturers and enforcing authorities are all much better off with regulations than without them.

DESCRIPTIONS THAT DECEIVE

Section 6 of the Act is almost as important to the Public Analyst as is Section 2. It makes it an offence to label or advertise a food sold or exposed for sale in a manner which "falsely describes the food or drug, or is calculated to mislead as to its nature, substance or quality". Once again, the legal draughtsman does not always use words with their ordinary meaning and in this context the word "calculated" merely means "likely".

There was always the difficulty, when Section 6 of the Food and Drugs Act was the only guide to correct labelling, that the Public Analyst had to decide for himself the point at which a label becomes misleading. Section 6 was undoubtedly inserted in the Act primarily for the purpose of dealing with statements that were misleading in themselves. For example, the phrase "Rich in Vitamins", if applied to a product containing only negligible amounts thereof, is obviously misleading in any size of type. When one has to consider whether a label is misleading not because of what it says but because of the size of type in which it says it, one requires more detailed guidance, and an attempt to provide it was made by the Labelling of Food Order, 1953. Experience showed that this was not sufficiently detailed, and it has now been superseded by the Labelling of Food Regulations, 1970, which came into force on 1st January, 1973.

LEGAL DIFFICULTIES

The Public Analyst is thus enabled to deal successfully with the unscrupulous or ignorant or careless manufacturer who commits an offence that would be regarded as such by every reasonable person. The great majority of food manufacturers have every wish to comply with the law and do their best to do so. Unfortunately they then frequently have great difficulty in deciding what the law is in relation to the product they are considering, because of obscurities in the wording, not so much of the Act as of the Regulations. Both as a Public Analyst and also as a consultant, I have often had great difficulty in advising a manufacturer how he should comply with the law, and an appeal to the Ministry by either the enforcing authority or the manufacturer frequently produces a non-commital answer. One must admit that it is extremely difficult to word a legal document that will contain no ambiguities and that will provide for every kind of food product covered by the Regulation in question, even including new products that were not on the market when the Regulation was published. The fact remains that in my opinion many Regulations could have been drafted very much better. The Bread and Flour Regulations offend in this respect in more than one way. The worst offence, because it is so fundamental, is the definition of the word "bread".

WHAT IS BREAD?

The definition in the Regulations begins as follows: " 'Bread' means bread in any form intended for sale for human consumption". This leaves us precisely where we were before we started, except perhaps that it excludes bread intended

for sale for non-human consumption, which does not seem in this context worth saying. It then goes on "and includes the following, and any part of any of the following, that is to say, rolls, baps, fancy loaves and speciality bread but does not include potato bread". This does answer a few questions about "near-breads", provided, that is, that everyone is quite sure what is meant by baps, fancy loaves and speciality breads, because these are not defined further. The definition says nothing about any other articles that might be considered as in the fringe area between bread and non-bread, which is just the area in which a good definition is essential.

For example, a product that appeared on the market a few years ago had the shape of a loaf of bread, a similar appearance, texture and taste, and was intended to be used instead of bread. But it had a very different composition from ordinary bread. It was eventually agreed between the manufacturers and the Local Authorities that provided the word "bread" was not applied to the product on labels and advertisements it would be accepted that it was outside the scope of the Regulations; but the point was never tested in the Courts.

Again, I had submitted to me as Public Analyst some time ago an article described as a "bread mix". The ingredients included baking powder but no yeast. The label bore the words "Simply add water and bake. Makes 2 delicious bread loaves". The question immediately arose, can bread be made by a process that does not involve panary fermentation? I decided that the best approach to the problem would be by a practical test. The contents of the packet were processed according to the instructions and the final baked product was then examined by myself and other members of my staff. The product looked and tasted more or less like bread and was quite pleasant to eat. We all agreed that if the loaf had been handed to an ordinary housewife and she had been asked what it was, she would have replied at once that it was bread and would wonder why there was any doubt about it. I therefore accepted the product as correctly described; but many orthodox bakers would stoutly deny that any article produced in this way could possibly be described as bread without qualification, and I am still wondering why the Ministry did not foresee this problem when they originally drafted their so-called "definition" of the word "bread".

It is curious that although bread is defined so vaguely, *white bread* is defined as "dough, made from flour, yeast and water, which has been fermented and subsequently baked". The word "fermented" is not defined further, but aeration by means of chemical substances is certainly not fermentation. Consequently, although the do-it-yourself product described above can properly, in my opinion, be described as bread, it cannot legally be described as "white bread", even though this is an accurate description of its colour!

WHEATMEAL OR WHOLEMEAL

Brown bread or wheatmeal bread has a definition (Reg. 6) that begins with the same words as the definition of white bread, but continues "and shall contain wheatmeal and not less than 0·6 per cent. of fibre (calculated

by weight on the dry matter of the bread)". The word "fibre" is defined (Reg. 2) as "the organic matter contained in the dry de-fatted residue obtained by digesting a sample of flour or of bread successively with boiling acid and boiling alkali". Even those whose knowledge of food chemistry is only basic will appreciate that this definition is completely ridiculous, because there is no reference whatever to the strength of the acid, the strength of the alkali, the time of boiling, and so on. The words quoted are apparently intended to imply the use of the analytical method, employed by chemists all over the world, which is specified in great detail in the Fertilisers and Feeding Stuffs Regulations; and it would have been very much better if the Ministry had either said so or omitted a definition of the word "fibre" completely from the Bread and Flour Regulations, because then all chemists would have accepted the word as having a defined meaning elsewhere. As it is, it would be quite easy for several chemists working on the same sample of brown bread to obtain results for the fibre content anywhere between nil and 1 per cent. or more by applying different analytical processes, all of which would nevertheless fulfil the requirements of the definition of fibre quoted above.

"Wheatmeal flour" is also undefined except in terms of its "fibre" content, the requirement being exactly the same as that for wheatmeal bread. Even if we accept that everyone will interpret this term in what we may call the orthodox sense, the fact remains that there must be a great number of ways of re-combining the various fractions from the milling of wheat so as to produce a mixture complying with the specification. There is no such doubt about the definition of "wholemeal", which states, simply and clearly, that it "shall contain the whole of the product derived from the milling of cleaned wheat".

This leads me, however, to a matter that is currently causing me some concern. One Local Authority recently decided to survey the wholemeal bread sold in its area, so that I received half a dozen samples from as many different shops. The fibre content of the samples (calculated on the dry matter) was found to vary from 1·17 to 1·87 per cent. Now the fibre content of wholemeal flour is given by Kent-Jones and Amos in "Modern Cereal Chemistry" as 1·6 to 2·1 per cent. at 13 per cent. moisture, which is equivalent to 1·84 to 2·41 per cent. of the dry matter. It is closely correct to say that 1 lb. of the dry matter of bread contains 1 lb. of the dry matter of the flour from which it was made; so there is a clear inference that at least some of my samples were not made from wholemeal flour.

It may be that some of the purchasers of these misdescribed loaves are neither prejudiced nor defrauded. I was told by a retail baker that the person who asks for a wholemeal loaf often merely wants a brown loaf and accepts a wheatmeal loaf without question. He added that people who know the difference between a brown or wheatmeal loaf and a wholemeal loaf, and want to buy the latter, almost always go to a Health Food Stores and get one with a well-known proprietary name. The fact remains that to sell one thing in response to a request for another is a *prima facie* fraud, and those who do it are at risk of prosecution.

TRADITION AND PROGRESS

The primary duty, imposed by Parliament on Public Analysts, of protecting the ordinary consumer against being led into purchasing food that is "not of the quality demanded" has frequently led to controversy with manufacturers who do not agree with our opinion of what the housewife expects to get when she asks for an article of food under a certain name or sees that name on a packet. In forming our opinion we always try to adhere to certain principles that have been fought for by Public Analysts ever since they came into existence, and which can be illustrated by examples.

First, then, if a compound food derives its essential character from an ingredient that is expensive relatively to the others, we strive to maintain a satisfactory content of that ingredient. In many cases, our task is made easier by the existence of a Statutory Instrument that prescribes what the minimum requirement shall be. The fruit content of jam is controlled in this way, the actual figure varying with the variety, and meat pies must contain at least 25 per cent. of meat. But when there is no official standard, we have to make one for ourselves, and the difficulty of doing this is made worse by the continual degradation brought about by competition in this highly competitive field.

As an illustration, consider the story of marzipan. Sixty or seventy years ago, all the cookery books agreed that this was a mixture of ground almonds and sugar in about equal parts, and the housewife who wanted marzipan as a cake coating or filling bought these two ingredients and made it herself, perhaps adding white of egg as a binder. But then two things happened. After the first World War, almonds became increasingly more expensive; during the second World War, they disappeared altogether, so that "marzipan" became a mixture of sugar and soya, with almond flavouring! When almonds came back after the war, they soon became five times more costly than sugar. Further, in line with the general move towards pre-packing everything, pre-packed marzipan had appeared on the market before 1939, and early specimens thereof were of reasonably good quality, the percentage of almond substance being about 40 per cent., which is in agreement with a recipe in "Mrs. Beeton" in an edition of the 1930s. But these were soon undercut in price by products containing less almond, and by the late 1940s, some of these so-called "marzipans" contained less than 20 per cent. of almond substance while others, containing none at all, were made with peach kernels and even peanuts. The Association of Public Analysts realised that if this process of degradation were permitted to continue, marzipan of traditional type would never return, and we opened negotiations with the leading manufacturers of pre-packed marzipan, who were themselves concerned at the position. We were unable by this time to persuade them to accept a minimum almond content of 40 per cent., as we would have liked. This attempt to put the clock back to before 1939 was regarded by them as quite unrealistic with almonds at their post-war price, and we had to agree that events had overtaken us. We eventually compromised by accepting a minimum of 25 per cent. almond substance and no other nut. Then, armed

with a Code of Practice embodying these requirements, it became possible to prosecute some of the worst offenders, knowing that the reputable traders would be with us and not against us, and so the Code of Practice became accepted. But if an agreement had been negotiated before 1939, the quality of pre-packed products sold as marzipan could have been pegged at a higher level and the housewife would have been assured of a product nearer to what her grandmother made.

This brings me to my second principle, which is almost a corollary of the first. If a traditional product or recipe has become expensive because of the high cost of certain ingredients, there can be no objection to the marketing of a less expensive substitute containing a smaller proportion of the costly ingredients, or even made from other cheaper substances in place of the traditional ingredients; but the substitute must be so labelled and described as to make its nature quite clear to the prospective purchaser and avoid any suggestion of "passing off". A mixture of ground peach kernels and sugar, for example, is sold as "Persipan" on the Continent and may also be available in Britain. This is, of course, quite legal and unobjectionable. But if the product were sold as marzipan, this would be as much a fraud as selling margarine under the name of butter.

THE "LAW" OF FIRST IMPRESSION

Finally, there is the principle, which is embodied nowadays in the Labelling of Food Regulations, 1970, not only that the description under which a food is sold must inform the prospective purchaser of the true nature of the food, but also that the label as a whole, including any illustrations, must convey to the purchaser a reasonably accurate and honest idea of what the food is. Moreover, this idea must be conveyed by what the purchaser sees on the front label, at a reasonable distance, within the first few seconds, or in short, under normal conditions of purchase. This is what in the United States is called "the law of first impression"; it is not a law in the strict sense at all, but numerous cases taken by the Food and Drugs Administration of that country have established it as a guide-line for judges who have to decide whether a product is mislabelled.

This attitude towards labelling has developed over the last thirty years or so because of the enormous increase in the production and sale of the pre-packed foods and the accompanying—indeed, the consequent—enormous increase in self-service food retailers. The modern housewife has to decide for herself what she will buy entirely on the evidence presented by the labels on the goods before her, without consulting the vendor as her grandmother would have done. Moreover, the latter would never have dreamed of buying a ready-prepared Irish Stew or Christmas Pudding, or even a Cake Mix; she would have bought the raw ingredients and made the compound food herself, and if the ingredients were wrongly proportioned it was her own fault, whereas her grand-daughter does not even know what the ingredients are, unless she is the exceptional sort that reads all the small type. The vague and non-

quantitative wording of parts of the Labelling of Food Regulations, requiring, for example, only that certain important words shall be "conspicuous" without defining that word, causes discussions and indeed disputes between manufacturers and Local Authorities following criticisms of labels by Public Analysts; but if all manufacturers would accept and conform with the *spirit* of the Regulations by looking at their labels in the light of the "Law of first impression", a great deal of time and trouble would be saved by all concerned and the consumer we all want to serve would be much benefited.

CONCLUSION

In principle, the Public Analyst's duty is easy to define—it is to examine and report on all samples submitted to him under the Food and Drugs Act, whether it be by the authorised officer of a Local Authority or by the private citizen, and thereby to protect purchasers and consumers from attacks upon either their health or their pockets. In practice, in spite of the wide powers provided by the Act and a host of Regulations made thereunder, the task leads him into consideration of many complicated and subtle problems and forces him to make many difficult decisions, which are made no easier by imperfections in the wording of the laws he has to try to interpret. I am not in a position to form an impartial judgment, but I think that a review of the work of Public Analysts over the last hundred years, and in particular—coming down to modern times—since the passing of the Food and Drugs Act, 1955, will show that on the whole they have made a good job of it.

Selenium: A Review

by L. E. COLES

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Selenium was identified 150 years ago by Berzelius in the residue from sulphuric acid production—the name coming from the Greek word "selene" meaning the moon because of its resemblance to tellurium, an element which had been discovered earlier and was named from the Latin word "tellus", meaning the earth.

It is a rather rare element, there being no large deposits anywhere and it is mainly obtained commercially as a by-product of the electrolytic refining of copper. Compounds of selenium are used in photo-electric cells, *e.g.* in photographic exposure meters, photometers, counting devices and light controlled switches; as orange and maroon pigments, when calcined with cadmium sulphide, for plastics and ceramics; as decolorisers of glass and for the manufacture of ruby glass; to increase the resistance of rubber to heat, oxidation and abrasion; and as lubricants to increase the machinability of stainless steel.

As a therapeutic agent selenium sulphide is used as a shampoo for certain scalp conditions and isotopic labelled selenium compounds have been used to evaluate pancreatic morphology[1].

In most farming areas, the virgin soils contain so little available selenium that cultivated crops cannot absorb more than traces. However, the quantities absorbed vary from 0·1 to 30 p.p.m. depending on the solubility in water of available selenium compounds[1]. Chronic poisoning from selenium in animals occurs in the form of "blind staggers" which is characterised by loss of hair, hooves, nails and teeth and a particular kind of paralysis. Grain containing 10 p.p.m. of selenium causes the typical "alkali disease" in pigs which produces emaciation and lack of vitality[2].

A survey has been carried out in over a hundred families in a seleniferous area. The manifestations of the disease were bad teeth, yellowish discolouration of the skin, skin eruptions, chronic arthritis in some, diseased nails and subcutaneous oedema[1]. There is some evidence from human epidemiological studies that selenium may be a factor contributing toward increased susceptibility to dental caries[1].

In industries where selenium compounds are used the atmospheric concentration should be below 1·0 mg/m^3 of air. The threshold limit given by the American Conference of Government Industrial Hygienists for hydrogen selenide is 0·2 mg/m^3 of air[1].

Under ordinary conditions the threshold of toxicity to animals and probably also for man is placed at 3 to 4 p.p.m. in the diet and more recently it has been stated that a concentration of 5 p.p.m. in common foods or one tenth of this concentration in milk or water is potentially dangerous[1]. The presence of

methyl selenide in the breath, making it smell of garlic, is one of the early symptoms of selenium poisoning and this may occur with a daily ingestion of only a few milligrammes. It is probable that continued ingestion of 5–10 mg/day would lead to nervous symptoms and gastro-intestinal disorders. It is claimed that the more refined and processed foods usually contain less selenium, that protein diminishes the toxicity of selenium and that it is not so poisonous in high protein foods as in high carbohydrate foods[3,4]. It is also claimed that some of the ingested selenium is stored as a protein compound in the tissues.

TABLE I

SELENIUM CONTENT OF FOODS[5,6]

Most fruits and vegetables except garlic, mushrooms and radish which contain 0·25, 0·13 and 0·04 p.p.m. respectively	less than 0·01 p.p.m.
Grain products vary widely, e.g.	
Corn flakes	0·025 p.p.m.
Barley cereal	0·67 p.p.m.
Whole wheat flour, whole wheat bread and brown sugar contain 2–4 times more selenium than white flour, white bread and white sugar.	
Milk products	
Table cream	lowest 0·005 p.p.m.
Skim milk	highest 0·05 p.p.m.
Dried skim milk powder	0·10–0·25 p.p.m.
Meat products	
Chicken muscle	0·1 p.p.m.
Pork kidney	0·2–0·5 p.p.m.
Sea foods	0·4–0·7 p.p.m.

All these values suggest that a diet well balanced in other nutrients is probably also nutritionally adequate with regard to selenium.

In livestock, selenium deficiency results in white muscle disease in calves and liver malfunctioning and muscle degeneration in pigs[1]. In 1957, the role of selenium in nutrition became important when it was demonstrated that a diet, to which selenium had been added at a level of 0·5 p.p.m., prevented dietary liver necrosis in rats[7]. There is also a relationship between selenium and vitamin E in reducing the incidence of retained placentae in dairy cows receiving a diet deficient in selenium[8]. A number of papers have been published on the protective action of selenium against the toxic effects of cadmium and mercury,[9,10] and it appears that there may be some nutritional adaptation of the organism to selenium[11]. Preliminary work in Jamaica also suggests that protein malnutrition disease may also be associated with selenium deficiency[1].

Apparently selenium deficiency is more widespread than has been suspected in the past and Scott, in an editorial article in the journal *Nutrition* which is called "The Selenium Dilemma"[12], attempts to balance the problem of dealing with selenium deficiency and cancer with the evidence that in certain circumstances the element and its compounds are carcinogenic.

METHODS FOR THE DETERMINATION OF SELENIUM IN BIOLOGICAL SUBSTRATES

The first reliable analysis of biological material appeared in 1964[13], when an oxygen flask technique was applied and 5-litre flasks were used with one gramme samples.

The general method of A.O.A.C.[14] may be sufficiently accurate for high concentrations of selenium, for example, from 1–5 p.p.m. using a five-gramme sample, but for microgramme amounts it is not sufficiently sensitive, *e.g.* 1 ml 0·001 N $Na_2S_2O_3$ = 19·8 μg of Se. Further practical experience shows that mercuric oxide is not a good fixative if one is trying to recover very small amounts.

A colorimetric method giving a blue colour with codeine in concentrated sulphuric acid does not seem to have gained popularity[15] but possibly could be used as a screening test after wet oxidation.

The method chosen as a standard method in the analysis of raw, potable and waste waters[16] consists of isolation of the selenium from interfering substances by ion-exchange and subsequent determination as the colloidal element produced by reduction with either ascorbic acid or hydrazine sulphate. This method has been chosen because of its simplicity and the avoidance of distillation and use of known carcinogens (*e.g.* di-aminobenzidine). Selenium is conveniently absorbed on to an anionic resin and separated from many other compounds, for example mercury, by passing through a cationic resin. The reactions with mercaptobenzoic acid[17] or mercaptobenzothiazole[18] give fine absorptiometric linear graphs but anyone who has tried it will find that it does not work in the presence of many other ions such as are found after wet oxidation. A limit of detection of selenium in the range of 50–100 picogrammes has been claimed for waste waters using atomic absorption carbon rod technique[19], but this has been done by using large samples and preconcentration techniques. Matrix interferences have not yet been overcome. Similarly gas/liquid chromatography using an electron-capture detector has been used to detect 0·002 μg in a 1-ml organic extract of sea water[20]. A recently published spectrophotometric method involves oxidation of hydroxylamine hydrochloride to nitrous acid by selenious acid followed by a diazotisation and coupling reaction. However, although there do not appear to be any common interferences, the range is only from 0·01 mg to 0·20 mg of selenium which is not sensitive enough[21]. A sensitivity down to 0·1 μg has been claimed in water analysis by taking advantage of the specific reaction of the catalytic effect of selenium on the sulphide reduction of methylene blue, which can be measured photoelectrically and the reduction time documented on a recorder[22].

The main problem is to destroy the organic matter without losing selenium. It can be done in the absence of fatty material, for example in plant material, although it is very necessary to maintain oxidising conditions at all stages of wet oxidation and this requirement explains the efficiency of oxidising mixtures containing perchloric acid. However, if the biological substrate is particularly difficult to destroy, *e.g.* fatty material, and the digestion procedure has to be modified by raising the temperature to complete the wet oxidation, there is the

likelihood that selenium will be lost. Controlled experiments have shown that some selenates are volatile above 200°C and, therefore, during the nitric acid/peroxide/perchloric acid digestion the temperature has to be carefully watched[23]. There is no completely satisfactory standard that can be used in checking recoveries of selenium from biological materials unless good oxidising conditions are maintained throughout because the organic forms of selenium can be converted to volatile selenides during decomposition of the sample[24,25].

Workers who have carried out surveys on selenium in biological materials have usually concluded that the best approach has been the fluorescent method which is based on the piazselenol formed between selenium and diaminonaphthalene. The limit of sensitivity is extremely low, in the region of 0·05 microgramme and for this reason there are difficulties[23,26]. The fluorescence is not absolutely specific for selenium; substances likely to give false positives are direct light, oxygen and certain solvents and it is, therefore, not easy to get a satisfactory blank value. Mercury interferes with the development of fluorescence.

The immediate problem that agricultural analysts have to face is to be able to rely on a method for the determination of selenium at a level of 0·5 p.p.m. in a feeding stuff and, if possible, to develop a screening or limit test for routine work. The draft directives of the E.E.C. in relation to colours, preservatives and antioxidants include in their general purity criteria that they should not contain, amongst other things, selenium in detectable quantities. A calibration curve can easily be obtained between the range 0·1–0·5 of a microgramme of selenium using the fluorimetric procedure.

The advice of the author on the analysis of the ordinary compound feeding stuff is to extract the oil in the usual way by soxhlet extraction. Wet oxidise the defatted material with nitric acid, hydrogen peroxide and perchloric acid and determine the selenium by fluorimetry after the formation and extraction of the 4,5-benzopiazselenol.

Summary

(*a*) Selenium is an essential element for nutritional reasons.

(*b*) The nutritional requirement lies in the range of 0·1 to 0·3 p.p.m. of selenium.

(*c*) Selenium can be an extremely toxic element in moderately excessive quantities.

(*d*) Levels in the region of 2–10 p.p.m. produce chronic toxic symptoms.

(*e*) Studies suggest that in some way or other it has an effect in the action of various carcinogens and may itself be carcinogenic.

(*f*) Wet oxidation of defatted material followed by fluorimetric determination using diaminonaphthalene is the method of choice for selenium.

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The Assessment of Rancidity of Oils on a Common Chloroform Extract with Special Reference to TBA Values

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A modified procedure for determining TBA values which can be applied to a common chloroform extract of fats and oils is described. Results are compared with those obtained for free fatty acids and peroxide values. Although the results obtained suggest that the increase in TBA values is relatively small in the incipient stages, it should be useful as confirmatory evidence of the degree of deterioration of vegetable oils and butter.

In a previous paper the author[1] described a general scheme for the assessment of the rancidity of fats and oils. This involved for convenience the determination of several rancidity values on a common chloroform extract or solution and included the estimation of the thiobarbituric acid value (TBA) by the method of Koning and Silk[2]. Further work has indicated that it is more convenient to apply a procedure based on the reagents recommended by Sidwell *et al.*[3], particularly in view of its superior reproducibility. It must be borne in mind however that, unlike the procedure of Tarladgis *et al.*[4], the method does not estimate changes in the non-extractable lipids.

Experimental Procedures

PREPARATION OF CHLOROFORM EXTRACT[1]

Either dissolve the oil or fat in chloroform or prepare a filtered macerate. Obtain the filtrate concentration by evaporating 10 ml and weighing.

THIOBARBITURIC ACID NUMBER (TBA)

Apparatus

1. Separating funnels, capacity 50–100 ml.
2. Unicam SP600 spectrophotometer or equivalent instrument.

Reagents

1. *Glacial acetic acid.*
2. *TBA solution.*
   (*a*) *Solution A:* Heat 0·07 g of 2-thiobarbituric acid in 40 ml of water on a boiling water bath until dissolved. Then wash into a 100 ml volumetric flask, cool and make up to the mark.
   (*b*) *Working reagent B:* Mix an equal volume of A (freshly prepared) with an equal volume of glacial acetic acid.

Method

Pipette 10 ml of chloroform solution (preferably containing about 3 g of oil or fat) into a small separating funnel and add by pipette 10 ml of TBA working reagent B. Stopper and shake hard for 5 min. Allow the layers to separate and reject the lower chloroformic layer. Pour the aqueous layer into a boiling tube and immerse in boiling water for 30 min. (C). At the same time heat 10 ml of reagent B in the boiling water (D). Cool C and D, use D for standardising the spectrophotometer and measure the optical density of C at 530 nm in a 1-cm cell.

$$\text{TBA value} = \frac{\text{optical density at 530 nm}}{\text{weight of oil or fat taken (per 10 ml of extract)}}$$

If D shows more than a slight pink colour it usually indicates some decomposition of the 2-thiobarbituric acid used in the preparation of A. In this case the test should be repeated using a reagent prepared from fresh powder.

The following procedures were also employed for comparison purposes.

FREE FATTY ACIDS (FFA)

Determine on the chloroform extract as previously described[1].

PEROXIDE VALUE

Apparatus

Reflux apparatus of Sully[5].

Reagents

1. *Glacial acetic acid.*
2. *Potassium iodide.*
3. *0·01 M Sodium thiosulphate.*
4. *Starch solution.*

Method

Add through a Sully condenser down into the flask 30 ml of glacial acetic acid and 5 ml of chloroform and boil to the top of the tube. Whilst refluxing, pour 1 g of potassium iodide dissolved in 1–3 ml of water down the tube. Add 25 ml of chloroform extract and turn off the cooling water to get all the sample into the flask. Turn the condenser water on again. Boil for 3–5 min., cool, dilute with 50 ml of water and titrate with 0·01 M of thiosulphate using starch.

Peroxide value (*milliequivalents per kg*)

$$= \frac{\text{Titration (ml of 0·01 M thiosulphate)} \times 10}{\text{Weight of sample in 25 ml of extract}}$$

TABLE I

VARIOUS RANCIDITY VALUES OBTAINED ON CHLOROFORM EXTRACTS OF VARIOUS OILS AND FATS

Oil	State of freshness	FFA (*as % oleic acid*)	Peroxide value *me/kg*	TBA number (see text)
Butter A	as purchased	0·35	1·4	0·03
Butter A	rancid	0·97	6·3	0·15
Cottonseed oil B	as purchased	0·12	23·3	0·01
Cottonseed oil B	rancid	0·15	39.9	0·15
Arachis oil C	as purchased	0·10	7·7	0·03
Arachis oil C	rancid	0·90	20·3	0·10
Soyabean oil D	as purchased	0·11	14·5	0·03
Soyabean oil D	rancid	0·13	30·8	0·24
Safflower oil E	as purchased	0·12	12·4	0·06
Safflower oil E	rancid	1·16	42·8	0·18
Olive oil F	as purchased	0·31	17·3	0·06
Olive oil F	rancid	1·28	67·1	0·18

Results and Discussion

The results obtained using the various methods for assessing fat spoilage are given in Table I. Butter and various vegetable oils were purchased and examined (*a*) immediately and (*b*) after accelerated storage by heating in beakers at 70°C until they attained a rancid odour. A few of the untreated samples appeared to give higher figures for FFA and the peroxide value than would normally be expected, but the TBA method gave consistently low figures from 0·01 to 0·06. As these corresponded with relatively high FFA and peroxide values in some instances, it is apparent that the TBA method lacks sensitivity in the incipient stages. This may, however, represent an advantage over the distillation procedure of Tarladgis *et al.*[4], which tends to give very inconsistent results with fresh samples[6]. The TBA method using the modified procedure gives figures with rancid oils ranging from 0·10 to 0·18. In general, therefore, although the procedure does not show high sensitivity for distinguishing between fresh and spoiled samples, the results obtained assist by giving supplementary evidence in support of more conventional procedures.

Finally it should be noted that although peroxide values of fats in chloroform extracts do not alter on storage, Kreis values using the method previously described[1] tend to increase[7].

Conclusion

More generally all TBA procedures are empirical, but the figures obtained represent additional evidence of the state of freshness when taken in conjunction with results obtained using other methods.

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Food Microscopy

(An Annotated Bibliography)

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Part IIA. Major Ingredients: Cereals and Flours

A number of ingredients can form the major components in a whole range of manufactured food products. These include cereals and flour, starch and gluten, eggs and egg products, fats and oils, milk and milk powders, sugar and water. Consideration of the microscopy of these ingredients is valuable in that it provides a foundation for the study of more complex products in which these ingredients may be combined in various ways.

Cereals and Flours

The microscopical structure and composition of cereal grains, such as barley, maize (corn), millet, oats, rice, rye and wheat, have been studied by light microscopy, and both histological and histochemical techniques have been employed. Fluorescence microscopy and electron microscopy have also been used. The light microscope is used to identify the pests of cereals and flours, and is also used for the qualitative and quantitative analysis of cereal grains in feeding stuffs. The effects of various reagents (*e.g.* alkali) on cereal grains have been studied microscopically. The wetting of cereal grains and flour has been investigated, especially in relation to wheat conditioning for milling. Much work has been done on the milling of corn and wheat, on the relationship between structure and milling quality, and between milling and starch damage. The fractions produced by air classification have also been characterised.

The microscopy of cereal flours has been mainly concerned with particle size distribution, and with particle structure. The microscope has been used for the identification of, and detection of adulteration in cereal flours. The determination of bran and germ in flour is also possible. Wheat protein has had detailed structural examination, particularly by electron microscopy.

The microscopical structure of "popped cereals" has been studied, and the cooking and gelatinisation of cereal products, such as rice and wheat flour have been investigated by histological and histochemical techniques.

All these aspects have been covered in considerable detail in the following papers, which constitute a selection of about half the available papers on the microscopy of cereals and flours. Abstracts of papers presented at the Annual Meetings of the American Association of Cereal Chemists (AACC) are published annually in *Cereal Science Today* and are a good reference source, particularly on biochemical and structural aspects.

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ERRATUM

1st Quarter, 1974, page 22.

The author has drawn attention to a statement in the paragraph dealing with the Fertilisers and Feeding Stuffs Regulations, 1973, which is misleading in that it indicates EEC Directives in which methods of analysis may be found. The following, supplied by Mr. Weston of the Government Laboratory, is a correct list of EEC publications relating to fertilisers and feeding stuffs and their methods of analysis.

Fertilisers and Feeding Stuffs

DIRECTIVES ACCEPTED IN TREATY ACCESSION

1. *Council Directive 70/373* dated 20/7/70 establishing Community methods of analysis for sampling and analysis (Minor amendment *72/275* dated 20/7/72).
2. *Council Directive 70/524* dated 23/11/70.
   Additives in feeding stuffs
   (amendments: *1st Commission Directive 73/264*, 27/7/73)
   2nd ,, ,, *73/275*, 27/7/73)
   3rd ,, ,, *74/7*, 13/12/73)
   4th ,, ,, *74/38*, 17/12/73)
   5th ,, ,, *74/180*, 26/2/74)
   6th ,, ,, *74/181*, 26/2/74)
   7th ,, ,, *74/182*, 26/2/74)
   and (*Council Directive 73/103*, 28/4/73)
   (urea replaced by non-protein nitrogenous compounds).
3. *1st Commission Directive 71/250* dated 15/6/71.
   Methods of analysis for feeds: HCN, Ca, CO_3, ash, acid—insoluble ash, chloride chlorine, mustard oil, lactose, potassium, sodium, sugars, theobromine, urea, lupin alkaloids, urease activity of soya.

DIRECTIVES AWAITING IMPLEMENTATION

4. *2nd Commission Directive 71/393* dated 18/11/73.
   Moisture, volatile nitrogen bases, total phosphorus (V) and crude oil.
   (U.K. has entered reservations on the oil method.)
   (Moisture in milk products, animal and vegetable oils and mineral substances excluded by *Commission Directives 73/47* of 5/12/72.)
5. *3rd Commission Directive 72/199* dated 27/4/72.
   Methods for starch (polar), crude protein, 'digestible' protein, free and total gossypol.
6. *4th Commission Directive 73/46* dated 5/12/72.
   Annex I : moisture in oils, Mg (AA), crude fibre.
   Annex II: retinol, thiamine, ascorbic acid.

7. *5th Commission Directive 74/203* dated 25/3/74.
   Annex I: starch (pancreation).
   Annex II: amprolium, ethopabate, dinitolmide, nicarbazin and menadione (K_3).
8. *Council Directive 74/63* dated 17/12/73.
   Maxima for undesirable substances and products in feeds (this should be brought into force 1.1.76).
9. Commission proposal on marketing feeds [*COM(71)93*] currently under discussion March, 1974.
10. Proposed Council Directive on Fertiliser legislation (*COM(71)1500 fin)*.
11. Fertilisers—proposed methods of analysis *111/10388/67.*
12. Sampling of fertilisers: *227/111/71.*

Letter to the Editor

Lead in Drinking Water

Sir,

Owing to the alarmist publicity given in the national and local press on 13th March last concerning the alleged hazard to health caused by the lead content of the Balmoral Castle water supply, it is felt that the position should be clarified and matters put in their proper perspective.

The headlines, "Balmoral Water is Dangerous" (*Western Daily Press*); "Royal Family Warned of Lead in Water Danger" (*Daily Telegraph*); "Balmoral Castle Tapwater is Dangerous" (*The Times*), were the result of a pronouncement by Derek Bryce-Smith, Professor of Organic Chemistry at Reading University. He stated that in June, 1973, he found the water supply to Balmoral Castle, the Queen's Scottish home, to contain 0·1 p.p.m. of lead. This, he said, was twice the maximum limit of 0·05 p.p.m. recommended by the World Health Organisation. Now, this is a case where a university professor, if he intends to enter Public Analyst's preserves, should first be sure, amongst other things, of any existing standard. In this case, the standard is not 0·05 p.p.m. but 0·10 p.p.m., and the figure obtained by Bryce-Smith did not exceed the W.H.O. limit but equalled it. The fact is that in 1971 the W.H.O. maximum was changed from what it had been from 1963 for the twofold reason that the higher figure had been accepted in many countries for years with no evidence of ill effect resulting therefrom and that it had been found difficult in certain circumstances to attain a lower level in countries where lead pipes were still in household use, which even today is true of many houses in the United Kingdom.

In the letters from myself published in *The Western Daily Press* on 20th March and in *The Daily Telegraph* on 16th April, I emphasised the incorrectness of the statement made by Bryce-Smith, who subsequently only indirectly admitted that he had been wrong by being reported in *TitBits*, 9th–15th May, as stating that the Queen's water supply at Balmoral contains exactly one part of lead in ten million. Also, in *Chemistry in Britain*, June, 1974, Vol. 10, No. 6, p. 204, he and his co-author, H. A. Waldron, writing on "Lead in Water", mention Amphlett Williams' references to the decision of the W.H.O. to raise the maximum recommended amount of lead in drinking water from 50 to 100 micrograms per litre.

I deplore the misleading of the public and the quite unnecessary alarm possibly caused to many readers of the daily papers, in particular to those with an insufficient knowledge of the true scientific facts to be able to make their own judgement.

C. H. Manley,

Bristol.