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Combined Residual Bromine in Bromine Treated Swimming Pools

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The formation of combined residual chlorine due in part to urine contamination is already well established in chlorine treated pools, but although bromine is now widely used as a disinfectant for swimming pools no reports have been made of the presence of combined residual bromine in such pools.

The results obtained by applying the diethyl-p-phenylenediamine (D.P.D.) test to bromine treated pools are reported. In pools treated with liquid bromine combined residual bromine is always found, but it is less than its chlorine counterpart and does not build up in the same way, it therefore has little value in judging pool purity. In pools treated with 1-bromo-3-chloro-5,5-dimethylhydantoin (dihalo), on the other hand, much higher apparent combined residual bromine may be obtained but this can be due in part to unreacted dihalo.

The reactions between ammonia and urine with bromine in aqueous solution are discussed and the reactions given in the D.P.D. test by freshly prepared solutions of dihalo are described.

In a previous publication the Author[1] discussed the formation of combined residual chlorine in swimming pools, pointing out that it included a substantial proportion of chlorinated urine residues. Consequently, the value provided a useful guide as to the purity of the pool, a high value indicating the need for dilution with fresh water. It is the Author's experience that pool waters showing high combined residual chlorine (in excess of 3 mg per litre) often give high figures for total solids, albuminoid nitrogen, and permanganate values that in themselves would indicate a need for dilution.

The Department of the Environment (D.o.E) in its publication "The Treatment and Quality of Swimming Pool Water"[2] recommends that if the combined residual chlorine exceeds 1·0 mg per litre, and monochloramine is absent, the pool should be diluted with fresh make up water. No reference, however, is made to the possibility of combined residual bromine in bromine treated pools although limits for free bromine are given. Furthermore the Author knows of no publication dealing with this subject. Possibly the universal experience that bromine treated pools do not give rise to tastes and odours, in the same way as their chlorine counterparts, has led to the belief that combined residuals are unimportant.

At the Author's laboratory, an examination of bromine treated pools has always included a complete D.P.D. test.

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Total Combined Residual Bromine

The method used to determine the total combined residual bromine by D.P.D. was that described in the D.o.E publication "The Purification of the Water of Swimming Pools"[3], all reagents being prepared as described therein. A brief summary follows:

To 100 ml of pool water add 1 g of potassium iodide, stand for 2 min. and add to a mixture of 5 ml buffer solution and 5 ml of reagent, titrate immediately with standard ferrous ammonium sulphate solution. Deduct the titration for free bromine, obtained in a similar manner but omitting the addition of potassium iodide.

This procedure follows that used for some years for total combined residual chlorine and has proved valuable in establishing the purity of chlorine treated pools.

Liquid Bromine as Bromine Source

Some of the results obtained are given later in the paper (Table II) and these show varying amounts of apparent monobromamine and significant combined residual bromine. The presence of apparent monobromamine is interesting since the D.o.E in its monograph on Chemical Disinfecting Agents in Water and Effluents and Chlorine Demand[4] states that Stage 1 of the D.P.D. test measures both free bromine and bromamines (these constituents are equally effective germicides). Therefore, if only ammonia and bromine are concerned no titration should have been obtained in Stage 2 of the D.P.D. titration.

On the subject of the possible formation of bromamines, there appear to be uncertainties, with some publications stating that such compounds if formed are rapidly destroyed by excess bromine. This statement is probably correct since some textbooks on analytical chemistry describe a method for the determination of ammonium compounds based on the measurement of the volume of nitrogen produced on treatment with bromine in solution. The concentrations of the chemicals in this test are of course much greater than those likely to be present in pool water.

In order to obtain a clearer understanding of the reactions taking place in pool waters treated with bromine, a study was made of the effect of adding bromine water to distilled water and tap water containing ammonia and to tap water containing small amounts of urine.

The first tests were carried out with distilled water containing 4 mg of ammonia nitrogen per litre, added in the form of ammonium chloride, to which freshly prepared bromine water was added. The pH adjusted to 7·5 and the solution allowed to stand for 1 h at 27°C. The D.P.D. test showed free bromine but no reaction at Stage 2 of the test and no evidence of any combined residual bromine. A substantial loss of free bromine was found that was a little in excess of that required to convert the ammonia into free nitrogen.

Tests were then made using tap water containing 1 and 2 mg of ammonia nitrogen per litre; once again no reaction was obtained in Stage 2 of the test but some combined residual bromine was indicated due no doubt to a reaction between bromine and traces of organic impurities present in the tap water.

The effect of small amounts of urine in tap water on bromine treatment was

then studied. Tap water containing 0·1 per cent. of urine was treated with bromine water containing 140 mg of bromine per litre. This sample was adjusted to a pH of 7·5 and allowed to stand at 27°C for 1, 2 and 3 days. The following results were obtained.

TABLE I

TREATMENT OF TAP WATER CONTAINING 0·1 PER CENT. OF URINE WITH BROMINE WATER: D.P.D TEST RESULTS

Time *Days*	Free bromine as Br. *mg/l*	Apparent mono-bromamine as Br. *mg/l*	Total combined residual bromine as Br. *mg/l*
1	6·03	0·54	1·62
2	1·35	0·45	1·80
3	–	0·32	1·12

These results show evidence of apparent monobromamine and of combined residual bromine; the amounts of the latter, however, are not as large as the corresponding amounts of combined residual chlorine that would have been produced if chlorine had been used instead of bromine. Additional tests, in which the bromine was added in increments, showed that initially the whole of the bromine is taken up destroying the urea with no formation of apparent monobromamine nor combined residual bromine. Only subsequently does the bromine combine with trace urine constituents, resulting in the apparent monobromamine and combined residual substances.

Pools Treated with Liquid Bromine

Table II shows a cross-section of results that have been obtained in these pools. Results are given for free bromine, apparent monobromamine and total combined residual bromine. In some cases the figures for inorganic bromide are also given.

TABLE II

TREATMENT OF SWIMMING POOLS WITH LIQUID BROMINE. TYPICAL RESULTS (D.P.D TEST)

Free bromine as Br. *mg/l*	Apparent mono-bromamine as Br. *mg/l*	Total combined residual bromine as Br. *mg/l*	Inorganic bromide as Br. *mg/l*
1·13	–	1·35	
1·08	0·36	1·04	
0·77	0·36	0·72	
0·14	–	0·50	
1·04	0·77	2·10	
0·27	0·23	1·08	
0·63	–	1·85	712
1·04		1·40	68
0·90		0·70	1322
1·12		1·12	686
0·95		0·41	579
0·23		0·68	509
0·05		0·18	1016
0·68		0·72	1280

The foregoing results show that some pools examined contained low levels of apparent monobromamine but since they are not true bromamines no useful purpose is served in carrying out this stage of the test. The first stage of the D.P.D test will indicate both free bromine and the true bromamines; any apparent bromamines indicated in the second stage will be due to organically combined bromine residuals, that will be included in "Total combined residual bromide". All waters contained combined residual bromine and on the basis of chlorine treated pools a maximum limit of 2·25 mg of bromine per litre would be regarded as satisfactory; none of the pools examined exceeded this limit. The inference from these results is that combined residual bromine does not have the same value in judging pool purity as combined residual chlorine. In some of the results the figures for inorganic bromide are given, and it will be seen that there is no relation between combined residual bromine and the content of inorganic bromide; indeed some pools containing high inorganic bromide show only trivial quantities of combined residual bromine.

Total inorganic bromide is probably one of the best guides of the purity of pools treated with liquid bromine and dilution with fresh water is usually advised if this figure exceeds 500 mg per litre. Inorganic bromide does not build up to the same extent in pools treated with dihalo but chlorides increase significantly.

Dihalo as Bromine Source

Dihalo, the trade name for 1-bromo-3-chloro-5,5-dimethyl hydantoin, is being used increasingly in the disinfection of swimming pools. Although free bromine is the active agent the material is handled easily and in the concentrated form it has none of the hazards that are associated with liquid bromine.

A selection of the results obtained in water from dihalo treated pools is shown in Table III.

TABLE III

TREATMENT OF SWIMMING POOLS WITH DIHALO. TYPICAL RESULTS (D.P.D TEST)

Sample	Free halogen as Br. *mg/l*	Apparent monohalamine as Br. *mg/l*	Total combined residual halogen as Br. *mg/l*
1	10·6	1·8	2·9
2	1·8	0·5	1·4
3	0·2	0·1	2·1
4	3·4	0·5	0·7
5	11·7	0·2	5·0
6	9·2	2·4	4·5

In general the pools concerned were small ones and some of the results obtained were unexpected (i.e. those obtained from pools treated with liquid bromine). For example pools 1 and 6 showed the presence of significant amounts of apparent monohalamines that were usually much lower in pools treated with liquid bromine. This would not be expected in the presence of a

substantial amount of free bromine, and moreover in pools 1, 5 and 6 the total combined residual halogen was high. In order to find an explanation of these results a study was made of the behaviour of solutions of dihalo in tap water in the D.P.D. test. Thirty milligrams of dihalo were dissolved in London tap water, the pH adjusted to 7·6 and the solution tested by D.P.D. after different intervals at 27°C. Tap water treated with bromine at the rate of 10 mg/litre was also examined at the same time for comparison.

Table IV shows the results obtained.

TABLE IV

TREATMENT OF TAP WATER WITH DIHALO AND WITH BROMINE: COMPARISON OF D.P.D. TEST RESULTS

	Dihalo		Bromine
Time	4 h *mg/l*	24 h *mg/l*	4 h *mg/l*
Free halogen as Br.	14·0	11·3	8·8
Apparent monohalamine as Br.	4·1	2·0	–
Total combined residual halogen as Br.	17·3	5·9	0·4

By the addition of glycine in the first stage of the D.P.D. test it was found that the free halogen consisted entirely of bromine. The end point in the monohalamine stage of the test was poor in the 4 h test and continued to return; the first colour change was taken.

In the D.P.D. test, dihalo solution resulted in significant amounts of apparent monohalamine and large amounts of total combined residual halogen.

Liquid bromine treated tap water showed very different results. These tests were repeated employing different amounts of dihalo and also in distilled water. Similar results were obtained including the loss of total halogen after 24 h.

The literature issued by the supplies of dihalo in the UK implies that dihalo when dissolved in water yields both free bromine and free chlorine. This was not borne out by our tests since only free bromine was detected. In a paper on "the chemistry of ozone related to the treatment of swimming pool and spa waters" R. G. Rice[5] states that dihalo reacts with water to yield free bromine and chlordimethyl hydantoin and that the latter compound can subsequently react with bromide to give free bromine. This was checked by adding potassium bromide and ammonium sulphate to solutions of dihalo in tap water; the ammonium ion will react with free bromine to produce hydrobromic acid and nitrogen. For this purpose one litre portions of tap water each containing 20 mg of dihalo and adjusted to pH 7·6 were prepared and separate portions treated with potassium bromide (40 mg) and ammonium sulphate (5 mg).

Table V shows the results obtained after standing for 4 h at 27°C.

The free halogen in both solutions consisted of bromine. The presence of potassium bromide has reduced very considerably both the apparent monohalamine and the combined residual halogen thus confirming the above theory. The presence of ammonium ions produced sufficient bromide to bring about a

TABLE V

ADDITION OF AMMONIUM SULPHATE AND POTASSIUM BROMIDE TO TAP WATER CONTAINING DIHALO: D.P.D. TEST RESULTS AFTER 4 H

	Control (No addition) *mg/l*	Ammonium sulphate 5 mg *mg/l*	Potassium bromide 40 mg *mg/l*
Free halogen as Br.	10·0	–	17·2
Apparent monohalamine as Br.	1·8	1·6	0·6
Total combined residual halogen as Br.	9·8	5·4	1·8

nearly 50 per cent. reduction of the combined residual bromide but was not sufficient to reduce it to zero.

The foregoing results show that in pools containing recently dissolved dihalo high combined residual bromine can be due to unreacted chlordimethyl hydantoin.

The reaction of dihalo with pool water contaminated with urine was studied in a manner similar to that of bromine. Sixty milligrams of dihalo were dissolved in 1 litre of tap water containing 0·2 per cent. of urine and the pH adjusted to 7·6. After 24 h at 27°C the solution gave the test results shown in Table VI.

TABLE VI

TREATMENT OF TAP WATER CONTAINING 0·2 PER CENT. OF URINE WITH DIHALO: D.P.D. TEST RESULTS

	Control *mg/l*	After adding 20 mg potassium bromide and standing 1 h *mg/l*
Free halogen as Br.	7·4	7·8
Apparent monohalamine as Br.	1·9	1·3
Total combined residual halogen as Br.	3·0	2·6

The results show that after 24 h a significant amount of combined residual halogen remains, and moreoever the addition of potassium bromide did not result in a major reduction of the combined residual bromine. Whilst impurities in pool waters, particularly urine, can clearly give rise to apparent monobromamine and combined residual bromine care must be exercised in judging the results since high values can be due to unreacted parts of the original dihalo molecule.

Acknowledgement

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Community Law and Methods of Analysis

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The uses to which methods of analysis are put are considered against the backdrop of Community legislation and food law enforcement. It is argued that there is a need for developments in food law enforcement policy and for an Association of Official Community Analysts which would assume responsibility for the development and dissemination of Official methods of analysis.

The origins of this paper came from the suggestion that consideration should be given to the effects of Directives and Regulations on methods of analysis. In fact, developments in the last 30 years have resulted from interaction between a considerable number of influences—*Codex Alimentarius*, development of the collaborative study process, advances in analytical chemistry, particularly instrumentation and much more—and it would not be correct to see the generation of Directives and Regulations as a major impetus to the development of new methodology or a major influence on the way we look at methods of analysis. Most of the methods incorporated in the Directives are not new. The requirement under Directive 85/591[1] to ensure that those included are collaboratively tested is very much to be welcomed.

Community Directives and Regulations relating to foodstuffs often include standards, compositional requirements or maxima or minima for additives or contaminants. Two situations arise—either the parameter is self-defining or it can be defined only by a method of analysis. An example of the former would be the amount of lead contaminating a food. The amount is an absolute number expressed as a concentration and all methods of analysis are attempts to discover that one true value. As an example of the second type of parameter, the amount of fat in a food can be defined only by the method used to determine it. In a complex food matrix, a method to determine fat may include in that value lecithins, fat-soluble vitamins and other components in addition to triglycerides. The result of a fat analysis will thus vary with the method used. The method chosen must be appropriate to the food, recognising the fact that the method is matrix dependent.

Methods of analysis vary in their characteristics and can be subdivided and classified in a number of ways, as in Table I.

Official Community methods exist in many cases for the resolution of legal dispute in support of Directives that are themselves developed to promote the internal market. This may involve a blithe disregard for the technical distinctions mentioned above.

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TABLE I

CLASSIFICATION OF METHODS OF ANALYSIS

Classificatory criterion	Varieties of method
Status	Official, reference, non-official
Precision	Qualitative, semi-quantitative, quantitative
Use	Screening, limit, routine, forensic
Methodology	Titrimetry, colorimetry, chromatography, etc.
Analytes	Single analyte, multiresidue, method-generated parameter, e.g. "fat"

Before Directives

Developments in which Directives and Regulations have played a part have to be considered against the background of the situation existing before Community legislation got underway. In those days, the analyst chose from

(a) A method incorporated in national legislation
(b) A Codex method
(c) An Association of Official Analytical Chemists (AOAC) method
(d) A national standards organisation or International Standards Organisation (ISO) method
(e) An industry method such as those of the International Dairy Federation (IDF), the International Organisation of Cereal Chemists (OICC), the Office International de la Vigne et du Vin (OIV), the Federation Internationale de Producteurs de jus de fruits (IFJU), the International Commission for Uniform Methods of Sugar Analysis (ICUMSA), etc.
(f) Other organisations such as the International Union of Pure & Applied Chemistry (IUPAC), the Society of Public Analysts(SPA)
(g) A method taken from the scientific literature, one developed in the analyst's own laboratory, or one obtained by private communication.

Of the above list, the minority that had been collaboratively tested included those of the AOAC. They were sometimes regarded as needlessly long-winded and complicated and it would be interesting to know to what extent those of that opinion were taking unjustifiable short cuts.

Community Methods

Directives adopted under the Food Harmonisation Programme include the phrase "methods of analysis and sampling shall be drawn up to verify the provisions of the Directive". Adoption follows the Standing Committee for Foodstuffs procedure. Criticisms of the Community about its approach to methods of analysis are well rehearsed and are beginning to be addressed, but there is still much to do.

Often commodity Directives have been adopted and the Directive relating to sampling and analysis negotiated later, leaving the parent Directive subject to national laws and practices.

The important point has been made that many of the parameters that must be determined under the requirements of Directives are method dependent. The standard is not defined until the method is defined. This issue has been fudged in

the past by the inclusion of methods which had not been collaboratively tested and hence were not adequately defined. The issue remains fudged to the extent that sampling is not statistically based and analyses are still not always carried out by accredited laboratories with adequate quality assurance programmes.

Method Criteria

The Community Methods of Sampling and Analysis for Foodstuffs Enabling Directive[1] has a technical annex detailing the criteria with which methods should comply if they are to be incorporated in a Directive. The applicability, practicability, specificity, sensitivity, accuracy, precision and limit of detection must be considered as well as any other relevant criteria. The repeatability and reproducibility of the method must be determined and the figures included in the method.

Commission Decision 87/410[2] of 14th July 1987 on the methods of analysis for hormone residues in meat is a good example of the detail necessary to specify the methods adequately. This details method criteria, taken from ISO 3534-1977[3].

Collaborative Studies

It is now accepted within the Community that methods for official use must be collaboratively tested, using an internationally recognised protocol such as ISO 5725: 1986[4]. The U.K. Laboratory of the Government Chemist has recently published very useful Guidelines for the Development of Standard Methods by Collaborative Study[5] which can be used by the organiser of a trial, and these include suggested forms for notifying participants at the various stages of the trial. The IUPAC recommendations have also been published recently[6].

There is a need for existing Community methods to be examined to the new criteria, and for further collaborative studies to be carried out where the existing data are not of adequate quality.

The necessity for collaborative studies is exemplified by the story of Regulation 4154/87. This prescribes the methods to be used in enforcing Regulation 3033/80 on classifying for tariff purposes certain starch and sugar products. The analysis involves treatment with caustic soda prior to enzymic hydrolysis of the polysaccharides to glucose, which is estimated. The method gives erroneous results because the caustic soda treatment renders some of the carbohydrates resistant to the enzymes, due to reactions called aldol condensation reactions. These were reported at least as early as 1872[7], and by 1968, the time of the last comprehensive review, they had been the subject of over 2300 scientific papers.

Food Law Enforcement

In the broadest terms, the purposes of food analysis in the official sphere, and in most other instances are related to economic value and health. Customs laboratories use analysis to classify foods according to tariffs, in essence to protect the revenue of governments and ultimately of the Community. Consumer protection laboratories analyse food so that commodity standards can be enforced to protect the pocket or health of the consumer and the pocket

of the honest trader, using the courts when necessary. The health of the population at large may be protected via analysis carried out in these laboratories, and in other laboratories not involved with the legal process.

These two functions of protecting government revenue and protecting the consumer have to be carried out cost effectively. It is no use spending more on analysis than the revenue lost by mis-classification. There is little point in spending a great deal of money on ensuring compliance of a minor article of the diet with a standard of dubious significance for health, for example the limit of 100 mg kg^{-1} for zinc in food colours, when the evidence tends to suggest a deficiency of zinc in the diet rather than excess.

This major question of measuring outcome of activities is not addressed in several areas. For example in a recent paper on the U.K. National Health Service[8], it is pointed out that hospital information systems are so designed that there is no real distinction made between patients who leave hospital alive and those who die there. Both outcomes are to all intents and purposes identical as far as one can deduce by subsequent inspection of hospital statistics. The situation is worse than in the time of Florence Nightingale who at least classified those she discharged as "relieved", "unrelieved" or dead[9].

In the same vein, there is no measurement of the benefits to society of a food law enforcement policy. Indeed, there is no policy. The need to harmonise enforcement has been appreciated in the Community context of removing non-tariff barriers to trade. This is enforced through national organisations, and provides only one part of the operational basis of those organisations. Variations in the intensity of enforcement will not lead to barriers any more than national legal requirements lead to barriers. However, bias in enforcement will lead to non-tariff barriers and must be avoided. Thus, although the Community generally adopts the minimalist approach of enforcing the Treaty and may find difficulty engendering powers to do anything else, we are entering a phase when an agreed approach to food law enforcement policy may be required, as an essential third party control over the first-party (i.e. self-certification) and second party (i.e. accreditation) that is likely to be introduced.

This current debate about how food quality and integrity are to be controlled may seem irrelevant to the question of methods of analysis, but it is important to examine the framework in which analysts use methods because there exists a dichotomy between real life and the idealistic and scientifically correct road now being taken by the Community. Statistically valid sampling and quality assured analysis by collaboratively tested methods are expensive activities. How does one relate them to cost-effective analysis? Attempts to grapple with this have been made for many years by "informal sampling", that is, taking samples by simple purchase, not using the legally defined procedure of sampling, or analysis; by screening tests; by use of methods which have not been collaboratively tested; and by omitting or skimping quality assurance.

Thus the balance between good science, forensic credibility and cost effectiveness has not yet been found.

Choice of Method

A Regulation or Directive may give rise to standards based on analytical

values, resulting in the specification of methods of analysis in the Directive. These methods can be of secondary interest to the enforcement analyst. The situation is exemplified by the Honey Directive (74/409)[10]. The analyst wishes to satisfy himself, firstly, that the sample calling itself honey is not adulterated, and secondly that it complies with the standards. The analytical determinations recommended to detect adulteration[11] and those implied by the Directive are listed in Table II.

TABLE II

METHODS OF ANALYSIS FOR HONEY

To detect adulteration	Honey Directive
1. Carbon 12/13 isotope ratio	1. Diastase activity
2. Thin-layer chromatography for the saccharides of high fructose corn syrup	2. Apparent reducing sugar content
3. Hydroxymethyl furfuraldehyde	3. Moisture content
4. Total mono-, di- and higher saccharides	4. Apparent sucrose content
5. Glucose, fructose and sucrose	5. Water-insoluble solids content
6. Nitrogenous constituents	6. Ash content
7. Polarisation (for honeydew)	7. Acidity
	8. Hydroxymethyl furfuraldehyde
	9. Pollen analysis

A number of conclusions can be drawn from examples of this sort. There is a need for authenticated samples of natural products. Community methods can be mandatory (provided there is compliance with Directive 85/891), but must not be the only permitted methods. The analyst must be able to choose other methods appropriate to the problem. These must be chosen and used in a way that is adequate for forensic credibility, whether by accreditation or other means. There is a need to review the relationship of the Community approach to that of the courts. The fossilisation of methods in Directives can cause difficulty. Progress could be made by specifying a method in a Directive with the statement added that any other method can be used provided it is as good as or better than the prescribed method when judged by the criteria of Directive 85/591.

The Need for Community Initiative

"Entia non sunt multiplicandur praeter necessitatem". This, the razor wielded by William of Occam, is well applied to the setting up of new organisations. However, there is a need for an Association of Official Analysts of the Community. Highly technical arguments between analysts before the courts are not conducive to good enforcement, favouring the most eloquent witness as much as the best science. This is rightly being replaced by official Community methods. Directive 85/591 introduces the requirement for the methods to be collaboratively tested. There is a need to go further. In the United States, the methods in the manual of the Association of Official Analytical Chemists are part of legislation by incorporation. This enables the development of methods to take place at the natural pace demanded by the needs of industry, the government administration and the courts taken together. It keeps the

technology with the technicians and generates the maximum amount of development activity, allowing funding to come from non-governmental as well as governmental sources, but provides a formal procedure for the review of collaboratively tested methodology.

This approach would require a new initiative, altering article 4 of Directive 85/591. Under the new Directive, a new Association with a small secretariat would be created. All methods (and only those methods) adopted by the Association would be official within the EC. Membership of the Association would be restricted to nationals of Community countries and companies with registered offices in those countries. The Association would also usefully have a role in accreditation of laboratories. The need for "non-official" methods will remain but the quality of results from those methods would have to be assured, and accreditation is an appropriate means of achieving this.

Conclusion

There has been considerable progress in the Community in the field of analytical chemistry as it relates to Directives and Regulations. This is particularly true in relation to collaborative studies. There is still a great deal to do. There is a need to develop food law enforcement policy and within that policy the achievement of a synthesis of good science, forensic credibility and cost effectiveness in the laboratories that provide the evidence on which enforcement is based.

The purpose of Community methods of analysis is largely to settle legal disputes and for such activities as customs control. Food analyses generally are used for quality control, screening, checking against limits and other purposes. The quality of the results should be assured by the accreditation of the laboratories in which the work is done and by the validation of the methodology. Technological change is more rapid than the administrative and legal procedures for incorporating it. The Community would derive the most benefit from new technology in the fields of food law enforcement and of removal of barriers to trade by the formation of an Association of Official Community analysts, acting as an umbrella for the generation, validation and collaborative testing of official methods of analysis.

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The Loss of Erythorbic Acid in Minced Meat on Storage at Refrigerated and Freezing Temperatures

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The effect of different storage temperatures on the level of erythorbate added to mince is described. It was found that minces stored at 2–5 °C for a week lost more than 90 per cent. of the original 1000 or 500 mg of erythorbic acid per kg. Frozen minces retained less than half the original amount of 2000, 1000 or 500 mg of erythorbic acid per kg after two and a half months and less than one-third after six months. Meat which had contained 2000 mg of erythorbate per kg was analysed for oxalic acid after two months when the erythorbate levels had dropped to less than 100 mg/kg. No evidence of the presence of oxalate in the meat could be found.

Ascorbic acid and/or erythorbic acid (*iso*ascorbic acid) and their sodium salts are not permitted in minced meat, chopped meat, sausages and sausage meat under Australian Model Food Legislation[1]. Exception is made for corned, pickled, cured or salted meat and cooked manufactured meats. Thus, by definition antioxidants are not permitted in minced, chopped or sausage meats.

It has been known for at least a decade that erythorbic acid and/or ascorbic acid have been added to minced meat by the retail trade with the intention of prolonging the shelf life of the product. The preferred form of antioxidant is sodium erythorbate rather than ascorbic acid, which is almost twice as expensive.

Food legislation sometimes requires that samples taken by food inspectors for analysis be divided into three (3) portions. If a difference in analysis occurs between the Government - tested and vendor - tested portions, the magistrate may order a joint analysis of the third part kept in the interim by the Food Inspector. Months, even years may elapse before the third portion may be analysed. Thus information on the breakdown of erythorbic acid with time would assist courts in understanding how samples stored at refrigerated or freezing temperatures and analysed at different intervals will vary in erythorbic acid (or ascorbic acid) content. This problem is the subject of this investigation which also tried to establish whether erythorbate lost had been converted to oxalic acid.

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Materials and Methods

Batches of minced beef were prepared to contain 2000, 1000, 500 and 100 mg of erythorbic acid per kg of meat. Sub-samples of 5 g from each batch were weighed and placed into polythene "stomacher" (paddle homogenizer) bags which were stored at 2–5°C and −20°C, respectively.

. Erythorbic acid was determined by the method of Archer, Higgins and Perryman[2] using high performance liquid chromatography. Determination of erythorbic acid continued for 37 days for samples stored at 2–5°C then discontinued as the residue of erythorbic acid in the 2000 mg/kg sample had virtually disappeared. For samples stored at −20°C determination was discontinued after 180 days, by which time the trend was apparent.

Two methods were used to detect oxalate. The meat was extracted with 0·1 HCl with a paddle homogenizer and after filtration, taken to dryness by rotary evaporation. The residue was methylated with BF_3/methanol (10 mins)[3], and the resulting methyl esters were examined by gas chromatography. When this method was applied to (i) 5 g of meat with added oxalate (100 mg/kg) and (ii) 5 g of meat with added oxalate (100 mg/kg) and added succinate (100 mg/kg) as internal standard, peaks were obtained corresponding to the added dicarboxylic acids.

The second method (Hodgkinson and Williams)[4] relies on precipitation of calcium oxalate, reduction to glycolic acid and colorimetric estimation, a procedure used widely in many food products. Results are given as the average of two separate extractions and determinations.

Results and Discussion

Table I gives the amounts of erythorbic acid found in minced meat after storage.

No oxalate was found either with method 1, on meat which originally contained erythorbate with added succinate as internal standard, or with method 2.

TABLE I

ERYTHORBIC ACID IN MINCED MEAT HELD AT 2–5°C and −20°C

	Cold room 2–5°C Erythorbic acid *mg/kg*				Freezer −20°C Erythorbic acid *mg/kg*			
Day no.	2000	1000	500	100	2000	1000	500	100
1	2058	1075	517	88				
2	1843	590	367	54				
8	429	83	21	0	1133	710	397	38
21	212	43	27	0	982	616	283	0
37	38	0	0	—	878			
76	—	—	—	—	819	369	151	0
180	—	—	—	—	553	302	61	—

Day 1 = immediately sample was prepared.
Recovery of added erythorbic acid was 99·15% ± 0·86.

Conclusion

The effects of storage at different temperatures on the level of erythorbate added to minced meat was studied. Minces stored at 2–5°C for a week had lost more than 90 per cent. of the original 500 or 1000 mg of erythorbic acid per kg. Frozen minces retained less than half the original amount of erythorbic acid after two and a half months and less than one-third after six months.

Meat which had contained 2000 mg of erythorbate per kg was analysed for oxalic acid after two months when the erythorbate levels had dropped to less than 100 mg/kg. No evidence of the presence of oxalate in the meat could be found.

Acknowledgements

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Quantitative Determination of Cows' Milk as an Adulterant of Goats' Milk by ELISA

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A quantitative immunoassay (ELISA) has been developed which can be used to determine whether goats' milk has been adulterated with cows' milk. The method has been verified using samples of goats' milk which contained known quantities of cows' milk within the range 0–75 per cent.

The ELISA method described is suitable for use in Public Analyst Laboratories and could be a valuable tool in the detection of adulteration.

In recent years the popularity and availability of milk from species other than cow has increased. Goats' milk, ewes' milk and their products such as yoghurt, cheese and ice-cream have become more commonplace. Some consumers choose these products for reasons of preference, whereas others may do so because they are allergic to cows' milk. Non-bovine milk and its products generally command a higher price and there exists the possibility of substitution or adulteration of these foods with cows' milk which is cheaper and more freely available.

In the past, various chemical and immunological methods have been published which attempt to detect the presence of cows' milk in ewes' or goats' milk (Bret[1], Durand *et al.*[2], and Furtado[3]). More recently a method has been reported for the detection of cows' milk in cheeses ostensibly made from sheep's and/or goats' milk which relies on isoelectric focusing of the soluble protein fractions (Ruiz and Santillana[4]). These methods have their merits in the qualitative aspects of adulteration, but the need has arisen for reliable quantitative determinations and suitable methodology that can find application in the enforcement of legislation.

Soluble proteins present in cows' milk should be distinguishable from those in milk from other species by use of an antibody specifically directed towards the former. Such an immunological approach, because of the inherent specificity and sensitivity associated with immune reactions was thought capable of yielding a viable method.

This paper describes the development of an ELISA technique that is capable of the quantitative detection of cows' milk as an adulterant of goats' milk.

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Materials and Methods

Antibodies

The antibodies used in the development work were produced by Beeches Biotech and supplied as stock capture antibody and conjugate (horseradish peroxidase labelled antibody). They were prepared by methods briefly described below:—

The antigen used for production of antisera in rabbits was prepared from decaseinated cows' milk. Immunoglobulin (IgG) was isolated from antisera by affinity chromatography using Protein A-Sepharose CL-4B (Sigma). The antibodies directed specifically towards soluble cows' milk proteins were isolated from IgG preparations by immunoaffinity chromatography. The affinity medium was prepared by linking antigen to Sepharose 4B. Antibodies with cross-reactivity to goats' milk were also removed by immunoaffinity chromatography. The resulting preparation was used as capture antibody.

Antibody-enzyme conjugate was prepared by labelling IgG with horseradish peroxidase by the method of Nakane and Kawaoi[5].

Principle

The enzyme linked immunosorbent assay (ELISA) is performed in Dynatech M129A 96 well plates or 16 well strips using the Double Antibody Sandwich Technique shown diagrammatically in Fig. 1. Essentially, cows' milk specific antibody (prepared as described above) is adsorbed on to the solid phase and acts as a capture antibody. The test milk sample (goats' milk) is incubated with the solid phase and any cows' milk protein is captured. Conjugate is added to the wells where it binds to any captured cows' milk protein. Finally the enzyme

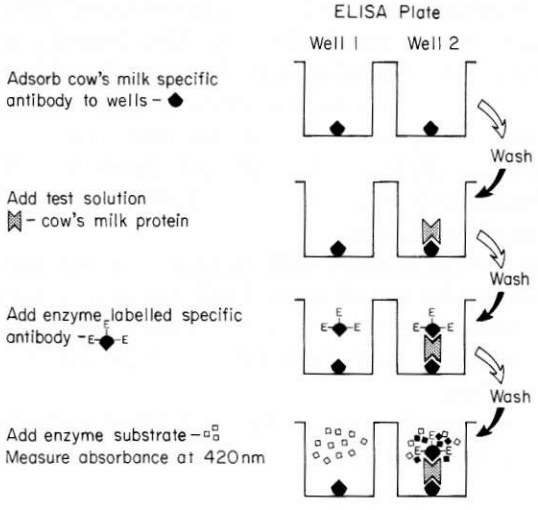

Fig. 1. Schematic representation of the Double Antibody Sandwich ELISA technique showing the sequence of events occurring in two wells of the plate. In well No. 1, no cows' milk is present whereas in well No. 2 the presence of cows' milk protein results in the production of the chromophore. The wash step in between each stage of the procedure represents six separate washings of each well as described in the text (Section ii) of the ELISA procedure.

substrate is added which reacts with bound conjugate to produce a chromophore, the intensity of which is proportional to the amount of cows' milk present in the sample. Between each stage of the ELISA the wells are washed to remove unreacted reagents.

Reagents

1. Coating buffer (carbonate-bicarbonate buffer, pH 9·6). Dissolve sodium carbonate (1·59 g) and sodium bicarbonate (2·93 g) in 1 litre of distilled water. This solution can be stored for 2 weeks at 4°C.

2. Phosphate buffered saline (PBS) pH 7·4. Prepare by dissolving sodium chloride (8·0 g); di-sodium hydrogen orthophosphate dodecahydrate (2·89 g); *potassium phosphate* (0·2 g); and potassium chloride (0·2 g) in 1 litre of distilled water.

3. PBS-tween (PBST). PBS containing 0·1% Tween 20.

4. Substrate buffer (phosphate-citrate pH 4·1). Dissolve citric acid (5·25 g) in 250 ml water (solution A) and di-sodium hydrogen orthophosphate anhydrous (7·10 g) in 250 ml water (solution B). For 100 ml substrate buffer add 62 ml of solution A to 38 ml of solution B.

5. ABTS substrate solution. Dissolve 50 mg ABTS [2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid)] in 100 ml substrate buffer. Add 5 µl hydrogen peroxide (20 vols) to the assay solution just before use.

6. Stopping solution. Prepare by dissolving sodium fluoride (1 g) in 100 ml of distilled water.

ELISA procedure

(i) Coat wells with capture antibody

Dilute capture antibody solution 1–200 with coating buffer (100 µl in 20 ml). Using a multichannel pipette, transfer 200 µl to each well of the ELISA plate, leaving the last well of each row empty as a control. Cover the wells with clingfilm and incubate at 25°C for 4 h and then place in a refrigerator overnight or until required (maximum 3 days).

(ii) Washing procedure

At each stage of the ELISA the wells are washed using a standard procedure. This can be done either manually or automatically. In either case the wells are three-quarters filled with PBST and emptied immediately. This is repeated six times in total, but allowing a soak time of 2 min. after washes four and five. Wells should be left containing PBST until required.

(iii) Incubation of samples with capture antibody

Dilute goats' milk samples 1 : 1 with water and centrifuge at 3000xg for 10 min. Remove the upper fat layer and dilute the sample 1–25 with PBST (dilution effectively 1–50). Make three serial dilutions 1 : 1 of each milk sample in disposable tubes with PBST as diluent. Dilute a sample of authentic cows' milk of known protein concentration (total nitrogen × 6·38) 1–50 with PBST, and make six serial dilutions of this as standards for the assay.

Treat samples and standards in identical ways and place in the washed,

capture antibody coated wells for exactly 5 min. at ambient temperature (16–20°C). (The most convenient way of achieving this is to load the samples into another 96 well microtitration plate prior to transfer to the assay plate. Pipette about 0·3 ml of each solution into the microtitration wells and at the start of the incubation period transfer 200 µl of each into the assay wells using a multichannel pipette.) Place 200 µl PBST in sample blank wells and antibody blank wells. A minimum requirement is that samples should be determined in duplicate.

Remove well contents immediately after the incubation period and wash the wells.

(iv) Incubation with antibody-enzyme conjugate

Dilute the antibody-enzyme conjugate solution 1–200 with PBST (100 µl in 20 ml). Dispense 200 µl into each well, cover with clingfilm and incubate at 16–20°C for 4 h. Remove well contents and wash wells as before.

(v) Substrate addition

Dispense 200 µl of substrate solution in each well and allow to incubate for 40 min. at 16–20°C, swirling the plate a few times every 5 min. to prevent product inhibition occurring at the surface of the solid phase. Add stopping solution (50 µl) to each well and swirl vigorously for a few seconds.

Allow the substrate colour to stabilize for 5 mins. and measure absorbances at 410 nm using an ELISA plate reader. Check that sample blank wells have a lower value than the sample test wells.

(vi) Treatment of data

Subtract the average of the sample blank readings from all of the test readings and construct a calibration curve by plotting log [protein concentration (mg/l)] of the authentic cows' milk samples against their absorbance readings. Determine cows' milk protein concentration in unknown samples by interpolation on the standard curve.

From these results the percentage by weight of cows' milk present in mixtures can be estimated as follows:—

$$\text{Percentage Cows' Milk} = \frac{\text{Cow's Milk Protein Concentration in Samples}}{\text{Average Cows' Milk Protein Concentration}} \times 100$$

For routine purposes assume a value of 3·4 per cent. w/w protein as the average cows' milk protein concentration (Tamime and Robinson[6]).

Results and Discussion

Antibodies directed towards the soluble proteins present in decaseinated cows' milk initially gave unacceptable cross-reactivity with goats' milk, presumably because of the presence of some immunologically similar proteins in the two milks. This problem was not unexpected and was overcome by the removal of goats' milk specific antibodies from the capture antibody preparation by immunoaffinity chromatography.

The ELISA procedure subsequently developed gave quantitative determinations of cows' milk protein in the presence of goats' milk. The immunoassay has

TABLE I

DETERMINATION OF COWS' MILK PROTEIN IN GOATS' MILK (0–20%)

Sample	Cows' milk protein *g/l*		Percentage cows' milk (estimated)
	Actual	Experimental	
1	0	0·47	(1·5)
2	0·66	0·92	(2·9)
3	1·58	1·55	(4·9)
4	3·25	2·78	(8·8)
5	4·20	3·89	(12·3)
6	4·93	4·04	(12·8)
7	5·88	5·94	(18·8)
8	6·41	5·18	(16·4)

N.B. Figures in brackets denote estimated percentage of cows' milk in the mixture, calculated using 31·6 g/l as the cows' milk protein concentration (determined experimentally).

TABLE II

DETERMINATION OF COWS' MILK PROTEIN IN GOATS' MILK (0–75%)

Sample	Cows' milk protein *g/l*		Percentage cows' milk (estimated)
	Actual	Experimental	
1	0	0·60	(1·9)
2	1·17	1·52	(4·8)
3	1·64	1·90	(6·0)
4	3·25	3·48	(11·0)
5	6·29	8·22	(26·0)
6	6·32	7·27	(23·0)
7	15·80	13·59	(43·0)
8	23·70	20·86	(66·0)

N.B. Figures in brackets denote estimated percentage of cows' milk in the mixture, calculated using 31·6 g/l as the cows' milk protein concentration (determined experimentally).

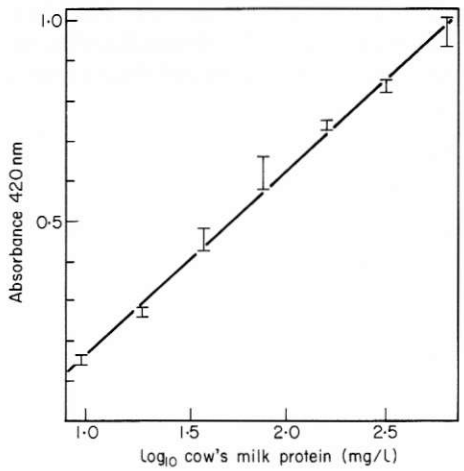

Fig. 2. A typical calibration curve for the immunoassay. $X = 2{\cdot}14y + 0{\cdot}665$; $R = 0{\cdot}990$; $SEE = 0{\cdot}069$.

been assessed using samples of goats' milk containing known quantities of cows' milk (representing the ranges 0–20% and 0–75% cows' milk). The results given in Tables I and II are the results of two blind trials and show the actual cows' milk protein concentration and the experimental levels found.

The results show that there is a low but measurable apparent cows' milk content in pure goats' milk (0·47 and 0·60 g per litre shown in Tables I and II, respectively). This may be due to a very small amount of cross-reactivity remaining in the capture antibody preparation after immunoaffinity chromatography. Alternatively, it could be due to a matrix effect as the results show that there is a tendency to slightly overestimate the cows' milk protein at low levels and underestimate it at high levels of adulteration. However, this effect is thought to be insignificant in practical terms.

A typical calibration curve for the immunoassay is shown in Fig. 2.

The quantitative immunoassay method described can be performed within a working day and is technically simple to perform. As such it would constitute a useful method for use by Public Analysts for the detection of cows' milk adulteration of goats' milk.

Acknowledgements

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Book Review

INDUCTIVELY COUPLED PLASMAS IN ANALYTICAL ATOMIC SPECTROMETRY. Edited by Akbar Montaser and D. W. Golightly. VCH: Weinheim, 1987. 660 pp. Price £79.00.

The book is predominantly concerned with all facets of inductively coupled plasmas (ICP) although the first chapter briefly describes other plasmas such as microwave induced plasmas (MIP) and direct current plasmas (DCP). The remainder of the book is divided into four parts, each part consisting of several chapters contributed by acknowledged experts. Part One deals with ICP-AES (atomic emission spectrometry) and here the chapter by Thompson on "Analytical Performance" deserves special mention.

Part Two deals with complementary ICP techniques such as ICP-AFS (atomic fluorescence spectrometry) and ICP-MS (mass spectrometry).

Part Three deals with sample introduction and plasma generation, and includes chapters on the introduction of solids and gases into plasmas, and on the lesser known techniques of mixed-gas, molecular-gas and helium ICP.

The final section, and perhaps the most relevant to practising chemists, is an overview of analysis by ICP-AES as applied to a wide range of materials, (e.g. biological, geological and waters), complete with a comprehensive list of references. Also included are the prominent spectral lines for most elements that can be determined by ICP-AES.

There is some overlap on certain topics, and for the analyst, while the theoretical discussions are of interest and indeed necessary to his understanding of this rapidly-growing technique, his main preoccupation will be with practicalities. However, Fassel states, in his foreword, the need for a comprehensive reference book on the theory and practice of IPC; there is no doubt that this publication satisfies that need admirably.

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