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ANALYTICAL TECHNIQUES FOR CHEMICAL CONTAMINANTS IN MILK AND MILK PRODUCTS

This bulletin includes technical and latest development on products, systems, techniques etc. reported in journals, companies' leaflets and books and based on studies and experience. The technical information on different issues is on different areas of plant operation. It is hoped that the information contained herein will be useful to readers.

The theme of information in this issue is **Analytical Techniques for Chemical Contaminants in Milk and Milk Products**. It may be understood that the information given here is by no means complete.

In this issue:

1. INTRODUCTION

Food safety is becoming ever important in ensuring public health owing to increased customer awareness and changing customer demands and patterns. To ascertain that the food is safe for consumption, various legal requirements have been made at the national and international levels. The Government of India enacted the 'Prevention of Food Adulteration (PFA) Act' in 1954 to protect consumers from food borne health hazards. Maximum levels for toxic metals, aflatoxins and pesticides are prescribed in this Act. At the international level, the food trade is governed by the standards set by the Codex Alimentarius Commission. The Codex standards include maximum levels for aflatoxin M_1, lead, pesticides residues and veterinary drugs residues in milk and milk products. The Technews issues 25 (March-April 2000), 33 (July-August 2001) and 44 (May-July 2003) provide information on Codex requirements with respect to these contaminants in milk and milk products.

The food producers are required to monitor the levels of contaminants in their food products and take necessary actions so that these levels do not exceed the established legal requirements. The national authorities also carry out surveillance to this effect.

To be effective, the monitoring / surveillance activities need to be supported by availability of validated, easy to use and economical methods of analysis. Literature presents different methods that may be used for the analysis of these contaminants for routine and confirmatory purposes. This issue of Technews provides information on analytical techniques available and internationally adopted methods for analysis of contaminants for which maximum levels have been prescribed by Codex in milk and milk products.

2. ANALYTICAL TECHNIQUES FOR AFLATOXIN M_1

The following paragraphs provide brief details on the analytical techniques for aflatoxin M_1 [1, 2, 3, 4].

A. Internationally recognized methods

The methods adopted by the IDF / ISO / AOAC for analysis of aflatoxin M_1 in milk are listed in the Annex. These methods follow the general pattern outlined in Figure 1.

Sampling → Extraction → Extract clean-up → → Concentration → Separation of extract components → Detection and determination → → Confirmation of identity

Figure 1: Basic steps in analysis of aflatoxin[1] and physicochemical methods for veterinary drugs

a. Sample preparation. Aflatoxin M_1 is uniformly distributed in milk and, therefore, representative samples are easy to obtain.

Extraction of aflatoxin, to efficiently separate the toxin from milk matrix, is accomplished by using an organic solvent like chloroform.

Extract clean-up to remove co-extracted substances is done by silica gel column chromatography or liquid-liquid partitioning or both. The extraction and its clean-up could also be accomplished in a single step utilizing C18 cartridge combined with silica gel column or immunoaffinity column.

Finally, the cleaned-up extract is available after eluting the columns with appropriate eluting solvents (e.g. acetone, dichloromethane, acetonitrile). The solvent from the purified

extract is partially or fully removed by evaporation under vacuum / nitrogen to concentrate the toxin in the extract. This concentrated / dried extract is then used for determination of aflatoxin.

b. Determination. Aflatoxin M_1 is determined by either Thin Layer Chromatography (TLC) or Liquid Chromatography (LC).

In **TLC,** a known volume of sample extract is spotted on to a silica gel plate. In addition, a series of different concentrations of standard solutions of aflatoxin M_1 are spotted. The plate is then developed in a solvent system (e.g. chloroform:acetone:isopropanol :: 87:10:3). After the plate is developed, quantification can be done visually or by using densitometer by comparing the intensity of the spots from the sample extract to those from the standard solutions.

In case of **LC**, a liquid chromatography system is required. It consists of a chromatographic column (e.g. Octadecylsilane columns), pump, injector, fluorescence detector and a recorder. The equipment is stabilized by maintaining the required flow rate of the mobile phase (solvent system, e.g. acetonitrile – water). Optimal conditions are checked with aflatoxin M_1 standard solutions and spiked milk before analyzing test materials. A standard curve is prepared from either peak heights or peak areas to ensure linear relationship. The sample extract (as such or derivatized with trifluoroacetic acid) is then injected into the stabilized equipment under the same conditions as for the standard solutions. Injection of standard solution is continued intermittently to ensure accurate quantification.Calculations are done as described in the method being used.

B. Analytical kits

Immunochemical procedures to determine aflatoxin M_1, especially Enzyme-Linked-Inmmuno-Sorbent-Assay (ELISA) are available. ELISA based kits have been developed to enable rapid screening

of milk for routine monitoring. These tests are based on antigen-antibody reaction.

3. ANALYTICAL TECHNIQUES FOR LEAD

The following paragraphs provide brief details on the analytical techniques for lead[5,6,7,8,9,10].

A. Internationally recognized methods

The methods adopted by the IDF / ISO / AOAC / Codex for analysis of lead in milk and milk products are listed in the Annex. The method of choice for routine analysis is Atomic Absorption Spectrophotometry (AAS).

Basic steps involve ashing or digestion of the sample to free it of the organic matter followed by instrumental analysis using Atomic Absorption Spectrophotometry (AAS) or Anodic Stripping Voltametry (ASV).

a. Sample preparation. The element (lead) is required to be freed from the organic matter that forms the matrix of the sample. Organic matter would otherwise result in interferences during subsequent steps. This is done either by 'ashing' or 'digestion'.

In **Ashing, t**he sample is gradually heated and held for sufficient time at a temperature of about 500ºC to get an organic matter free ash. The ash is then dissolved in an acid (usually hydrochloric or nitric acid) followed by the estimation by AAS or ASV.

In **Digestion, t**he sample is mixed with acid(s) and then heated at about 150°C for sufficient period to digest the organic matter. Depending upon the requirements, other reagents may be added to the samples with the acids to selectively precipitate the lead that is then recovered and analyzed. A method for digestion using microwave heating also exists. The acid solution containing lead or the recovered lead in the precipitated form, as the case may be, is used for analysis by AAS or ASV.

b. Determination. Lead estimation is done by **Atomic absorption spectrophotometry (AAS)** or **Anodic stripping voltametry (ASV).**

In **AAS**, the energy absorbed during conversion of an atom from its 'ground state' to an 'excited sate' is measured. The basic instrument for AAS requires a light source, an atom source, a monochromater to isolate the specific wavelength of light, a detector, some electronics to treat the signal, and a data display. The light source is usually a hollow cathode lamp.

For determination of lead, generally graphite furnace (flameless) AAS is used. However, there are methods that use flame AAS for estimation of lead. The wavelength, gas mixture / temperature programme, and other instrumental parameters that are most appropriate are found in the manuals supplied with the instruments.

The **Anodic stripping voltametry** is a method for the electrochemical detection of trace metals. In this, the metal is concentrated by electrodeposition on the electrode surface. The concentration is done by cathodic deposition at controlled time and potential. The metal is then stripped from the electrode by scanning the potential anodically (at a given potential according to the metal being analyzed). Traditionally one uses mercury on the electrode, so that a mercury-metal amalgam is formed, leading to a significant increase in the sensitivity of the method.

A polarograph or voltametric analyzer with all necessary anodic stripping accessories is required for ASV based analysis. In case of voltametric analyzer, it should be equipped with cells, electrodes, recorders, Hg capillaries, micrometer or similar device for adjusting drop size, stirring motors etc.

B. Recent techniques

A recent addition to the list of methods for lead estimation is the **Inductively Coupled Plasma Mass Spectrometry (ICP-MS).** In

ICP-MS, the sample is prepared in a similar way as that described above.

Inductively Coupled Plasma (ICP) is an argon plasma (or gas consisting of ions, electrons and neutral particles) maintained by the interaction of a radio frequency field and ionized argon gas. The sample is injected into this plasma, which is at temperature to the tune of 10,000 K, resulting in heating of samples at 5000 – 8000 K. At these temperatures, the elements in the sample matrix are atomized and ionized. These resulting ions are then passed through a series of apertures (cones) into a high vacuum mass spectrometer (MS). The function of MS is similar to that of monochromater in AAS – it separates the ions from the ICP according to their mass-to-charge ratio. The intensity of a specific peak in the mass spectrum is proportional to the amount of the elemental isotope from the original sample[10] .

4. ANALYTICAL TECHNIQUES FOR PESTICIDE RESIDUES

The following paragraphs provide brief details on the analytical techniques for pesticide residues [1,11,12]

A. Internationally recognized methods

The methods adopted by the IDF / ISO / AOAC / Codex for analysis of pesticides in milk are listed in the Annex. In addition, the following Codex standards provide useful information/guidance for analysis of pesticides:

- Recommended Methods of Sampling for the Determination of Pesticide Residues for Compliance with MRLs (CAC/GL 033-1999)
- Guidelines on Good Laboratory Practice in Pesticides Residue Analysis (XOT08-1993)
- Recommended Methods of Analysis for Pesticide Residues (XOT 09-1995)

A flow diagram showing the basic steps involved in pesticide analysis is given below[1] :

Sampling → Separation into aqueous or fatty compartments according to the residues looked for → Group specific raw extract → Extraction → Extract clean-up → Concentration → Separation of extract components → Detection and determination → Confirmation of identity, usually by Mass Spectroscopy (MS)

a. Sample preparation. Pesticides are uniformly distributed in milk and, therefore, representative samples are easy to obtain.

The analytical step for the first crude extract depends on the chemical nature of the pesticide, e.g. polar versus non-polar, and determines whether the aqueous – proteinaceous or lipid phase is submitted to the due step(s).

The clean-up procedures comprise either liquid-liquid partitioning steps, the different chromatographic techniques employing solid phases including the reversed – phase techniques, and/or combination of both procedures with more or less sophisticated auxiliary procedures.

The final extract is a concentrated aliquot of the original sample and should be as free as possible from co-extracted impurities. This is then subjected to determination.

b. Determination. The final determination is dependent on high-tech equipment and employs gas chromatography (GC), high-performance liquid chromatography (HPLC), spectrometry, thin layer chromatography (TLC), or the electrochemical procedures. GC and HPLC can be coupled with mass spectrometry (MS) for

confirmatory techniques. The detectors used are specific to certain moieties of the molecules and thus help to make the specific analysis. The mass flow in the detection system in modern equipment is in the order of picomoles / second or several femtograms per time unit.

The Table 1 provides different categories of pesticides with examples and analytical technique applicable.

Table 1: Different categories of pesticides and applicable analytical techniques [complied from 11]

Pesticide group (Examples)	Analytical technique
Benzimidazoles (Carbendazim, Thiabendazole, Albendazole, Fenbendazole)	HPLC with UV and fluorescence detection. GC not suitable but still necessary for confirmation of the HPLC analysis
Carbamates (Aldicarb, Carbaryl, Methomyl, Methiocarb, Carbofuran)	HPLC with post column modification / derivatization to form fluorescent compounds. GC not commonly used.
Halogenated Hydrocarbon [Organochlorine] pesticides (Lindane, Chlordane, Aldrin, Heptachlor, DDT, Dieldrin)	GC with Electron Capture Detector or Electrolyte Conductivity Detector
Organophosphates (Chlorpyrifos, Fenthion, Parathion, Malathion)	GC with Electron Capture Detector.
Pyrethrins (Cypermethrin, Permethrin, Allethrin, Fenvalerate)	GC with Electron Capture Detector. HPLC also used.
Alkyl Phenolic Acid Herbicides (2,4-D [2,4-Dichlorophenoxyacetic acid])	HPLC with UV detector. GC possible after esterification.

The pesticides have been classified according to similarities in their chemical structures. Generally, an analytical method developed for a particular group of pesticides will work for most of the pesticides in that group.

B. Immunochemical techniques

The ELISA techniques have become more and more important in pesticide residue analysis. Their simplicity, low cost, rapidity and use of environmentally harmless chemicals in minute amounts make them a valuable tool in serial analysis, at least as screening tests.

5. ANALYTICAL TECHNIQUES FOR VETERINARY DRUGS

It seems, from the available reference material[13,14,15,16] that the methods for analysis of veterinary drugs are not so standardized (or rather are in the process of being standardized) as compared to those for other contaminants.

A. Internationally recognized methods

The methods adopted by the IDF / ISO / AOAC for analysis of veterinary drugs in milk are listed in the Annex. In addition, the Codex 'Guidelines for the Establishment of a Regulatory Programme for Control of Veterinary Drug Residues in Foods (CAC/GL 16-1993)' provides useful guidance on selection of methods of analysis for veterinary drugs. Part II of this document provides general considerations with respect to analytical methods for residue control and Part III deals with the attributes of such analytical methods.

These techniques of analysis can be broadly categorized into two types based on the principle of analysis involved: microbiological and physico-chemical.

a. Microbiological tests. These are commonly applied for screening analysis of antimicrobials in milk due to their simplicity of use and ability to detect all the members of several antibiotic families simultaneously, at low individual cost [17]. Microbiological tests are two types: microbiological inhibitor assays and microbiological receptor assays.

The test principle in **microbiological inhibitor assays** is detection of growth inhibition, noticed visually by interpreting the colour change of a pH –indicator in the test medium. The following bacteria are widely used in microbiological tests: *Bacillus stearothermophilus* var. *calidolactis, Streptococcus salivarius* ssp. *thermophilus, Bacillus subtilis, Bacillus cereus and Micrococcus luteus.* The detectable concentrations of various antimicrobials vary between different tests. In these tests, while a positive reaction should generate suspicion on the safety and suitability of milk, a negative reaction cannot be relied upon to exclude the presence of antibiotics, especially those which are not covered by such tests (e.g. chloramphenicol and dapsone). Use of other methods is therefore necessary, at least periodically[(1)].

Major drawbacks of microbiological tests lie in the non-specific nature of the bacterial response, which may be inhibited by native antimicrobial substances present in milk (especially mastitic milk) and lipids[(1,17)].

In **microbiological receptor assays**, the principle involved is specific affinity binding of certain antibiotic on certain specific sites on the bacterial cell walls. Radio-labelled antibiotic and a bacteria are added to the test milk sample. Antibiotic in the test sample competes with the radio-labelled antibiotic for binding sites on the bacterial cell wall. Amount of labelled antibiotic bound to the cell wall is measured and compared with the control to determine the antibiotic.

b. Physico-chemical methods. The physico-chemical methods are necessary for identification and/or quantification of residues. These are almost invariably based on chromatographic purification of residues followed by spectroscopic quantification.

They are applicable to all drug categories, require relatively short development times, and in particular permit unambiguous confirmation of drug identity by techniques such as MS[(17)]. The IDF bulletin 358/2000 reviews various attributes, including the

limits of detection, of available physicochemical methods. It also lists at least one method for quantification of residues at a concentration below corresponding MRL.

A schematic representation of basic steps in physico-chemical analysis is provided in Figure 1.

Most physico-chemical methods involve extraction of homogenized samples into an appropriate solvent, removal of interferences (clean-up) by liquid-liquid partition, and/or low-resolution chromatography followed by reverse phase HPLC quantification of the drug.

Commercially supplied prepacked solid phase extraction (SPE) cartridges have largely replaced laboratory-prepared clean-up columns and instrumentation has improved substantially over the past few decades, particularly with regards to data storage. One innovation has been the development of on-line dialysis-concentration column HPLC methods. This method has considerable potential for rapid monitoring of residues in milk[17].

The drawbacks of physico-chemical methods include application of protracted sample preparation schemes in order to attain adequate specificity, which severely limits the sample throughput. Furthermore, chemical determination of residues at trace levels requires considerable skill in manipulating sample extracts to achieve an acceptable recovery of analyte reproducibly[17].

B. Immunochemical analysis

These methods fall into two groups: immunoassay and immuno-affinity-columns (IAC) for sample clean-up[17].

Immunoassay is rapid, selective and sensitive and is most efficiently applied to long sample runs.

Immunoassays may be preferred in circumstances where analyte concentrations below 1 μg/kg must be measured and a mass

spectrometer is not available. It is often difficult to achieve selectivity at these low levels with other physico-chemical techniques. However, in common with all analytical methods, immunoassays require considerable skill from the analyst to achieve consistent results[17].

In **Immuno-affinity-columns (IAC)** clean-up for veterinary drugs, the immobilized antibody isolates drugs cleanly from the crude sample extracts prior to chromatographic or immunoassay end determination. Thus the selectivity of antibody binding is coupled with chromatographic separation of eluted residue enabling individual quantification of cross-reacting metabolites and MS confirmation of analyte identity. Several different antibodies can be bound in one column to provide multi-analyte capability.

However, IAC requires availability of antibody in substantial amounts; antigen-antibody complex formation is slow; the gel supports currently in use are soft and subject to lengthy load-elute-wash cycle times; and relatively large volume of eluent containing desorbed analyte are obtained necessitating some form of concentration before chromatography[17].

ANNEX

Internationally recognized methods[a] for analysis of contaminants in milk and milk products [18,19].

IDF/ISO/AOAC/ CODEX	Description / Principle
I. Aflatoxin M_1	
AOAC 980.21	TLC
IDF 171: 1995 ISO 14 501: 1998	Immunoaffinity column & HPLC
AOAC 986.16	LC
AOAC 2000.08	Immunoaffinity column & LC; applicable for determination at > 0.02 ppb (ppb=μg/kg)

II. Lead	
AOAC 972.25	Digestion, Atomic absorption spectrophotometry
AOAC 982.23	Ashing, Anodic stripping voltametry; estimated quantification limit based on 10 g sample is 0.01 ppm (ppm=mg/kg)
AOAC 986.15	Digestion, Anodic stripping voltametry
AOAC 999.10	Microwave digestion, Atomic absorption spectrophotometry; approximate limit of detection is 0.1 ppm
AOAC 999.11	Ashing, Atomic absorption spectrophotometry; formula provided for calculation of detection limit
NMKL[b] 139 (1991)	Atomic absorption spectrophotometry
III. Pesticide residues	
AOAC 970.52	GC; Covers Aldrin, DDT, Dieldrin, Heptachlor and Lindane among Codex pesticides MRLs
AOAC 960.41	Colorimetric; applicable for DDT
Codex XOT 09 – 1993	Methods referenced[c] against each pesticide irrespective of food commodities; users required to study the references in this standard and identify applicable methods for commodity of interest
IV. Veterinary drugs residues	
AOAC 988.08	Microbial receptor assay; validated detection levels are higher than Codex MRLs
AOAC 979.14	Zones of inhibition of bacterial growth; qualitative; applicable to Penicillin; validated detection level is higher than Codex MRL
AOAC 982.15	Competitive affinity adhesion of β-lactam antibiotics in sample and radio-labelled β-lactam antibiotics to enzymes on cell wall of bacteria
AOAC 982.17	Zones of inhibition of bacterial growth; qualitative; applicable to Penicillin
AOAC 982.16	Zones of inhibition of bacterial growth; qualitative; applicable to β-lactam antibiotics

AOAC 962.14 F	Zones of inhibition of bacterial growth; qualitative; applicable to β-lactam antibiotics; conclusive quantitative method for penicillin
AOAC 962.14 A-E	Zones of inhibition of bacterial growth; qualitative; applicable to β-lactam antibiotics
AOAC 995.04	LC, applicable to tetracycline, chlortetracycline and oxytetracycline; validated detection level are lower than Codex MRLs

[a] Methods for aflatoxin M_1 and veterinary drugs residues are for milk; those for lead and pesticides residues are for milk and milk products.

[b] Nordic Committee on Food Analysis

[c] Official Manuals of several countries are included in addition to individual scientific papers. E.g Pesticides Analysis Manual of the Food and Drug Administration, USA.

Methods adopted by the Codex are shown in bold.

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Dr. N.N. Varshney
National Dairy Development Board
PB No.40
Anand 388001
Gujarat

Fax No. (02692) 260157
Email : nnv@nddb.coop