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A review on advanced technique for assessment the quality of milk and milk products

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Abstract
This paper presents the advanced instrumental analytical techniques used in milk and milk products analysis. Milk is a complex matrix consisting of different components and because of this, it often becomes impossible to accurately analyze one component in the presence of others using the classical method of analysis. The major advantages of advanced instruments analysis are more sensitive and rapid. Milk and milk products are analysed for a variety of reasons, e.g. compliance with legal and labelling requirements, assessment of product quality, composition, determination of nutritive value, detection of adulteration, and in research & development. The present paper briefly discussed various application of GCMS, HPLC, AAS, FTIR, DLS, NALDI-TOF and MALDI-TOF in dairy sector.

Keywords: GCMS, HPLC, AAS, FTIR, NALDI-TOF, MALDI-TOF

Introduction
India has become the leading milk producer in the world and near about 176.4 million tonnes according to the latest estimate (NDDB 2018; Ranvir *et al.,* 2015) [46]. Milk and milk products are nutritious food items containing numerous essential nutrients such as fat, protein, vitamin, minerals (Haug *et al.*, 2007) [19]. Milk and milk products analysis is the discipline dealing with the development, application and study of analytical procedures for characterizing the properties of foods and their constituents. Concerns over food safety and quality are increasing worldwide. They are priority issues for governments, food producers, industry, traders and consumers alike. The burden of foodborne disease is significant in all parts of the world, and for some important foodborne hazards the reported incidence of disease seems to have increased over the last decades (FAO/WHO, 2002) [3]. Government bodies regulate the permitted levels of contaminant compounds; much of this advancement has been driven by increased sensitivity and specificity of determination e.g. using analytical instruments (Desai *et al.*, 2007) [11]. Food Analysis serves as a unique and invaluable tool for all food scientists, technologists and regulatory authorities for quality assurance and control of food products, to study the different aspects of food products. Due to complex nature of food matrix, it often becomes impossible to accurately analyze one component in the presence of others using the classical method of analysis. More often than not, interferences are encountered during the measurement of minor components in the presence of the components present in bulk quantities (Nielsen, 2010). All this may lead to inaccurate and unreliable results and sometimes erroneous and false results because of lack of specificity and sensitivity of classical method. Therefore, in order to achieve the reliability of results, today the instrumental analytical techniques have become mandatory in development, quality control and safety, exports of food products and meeting the regulatory norms of food products

Gas Chromatography–Mass Spectrometry (GC-MS)
GCMS is a hyphenated analytical technique that combines the separation properties of gas-liquid chromatography with the detection feature of mass spectrometry to identify different substances within a test sample. GC is used to separate the volatile and thermally stable substitutes in a sample whereas GC-MS fragments the analytes to be identified on the basis of its mass. The further addition of mass spectrometer in it leads to GC-MS/MS. The identification of analyte is based on retention time which represents the mass of a given particle (m) to the number (z) of electrostatic charges (e) that the particle carries. The term m/z is measured in Da/e. GCMS commonly uses electron impact (EI) and chemical ionization (CI) techniques (Chauhan *et al.*, 2014) [7]. The main features of enhanced molecular ion, improved confidence in sample identification, significantly increased range of thermally labile and low volatility samples amenable for analysis, much faster analysis, improved sensitivity

particularly for compounds that are hard to analyse and the many other features and options provide compelling reasons to use the GC-MS in broad range of areas of analysis (Amirav *et al*., 2013; Ho & Reddy 2010) [2].

Application of GCMS

Pesticide residue Determination

Detection of pesticide by GC/MS/MS provides good precisions and has very low limits of detection (ng/kg) for the analysis of samples. Several Pesticides such as Acephate, Aldrin, Amitraz, Azinphos-ethyl, Azinphos-methyl, Benzene Hexachloride, Carbaryl, Carbofuran, Chlorfenvinphos, Chlorpyrifos, Endosulfan, Hexachlorobenzene, DDD (Dichloro Diphenyl Dichloroethane), DDT (Dichloro Diphenyl Trichloroethane) can be detected from milk and milk products by using GC-MS. Generally, HCH (Hexachlorocyclohexane), DDE, Aldrin, Dieldrin, BHC (Benzene Hexachloride), Endosulfan, Methyl Parathion, Malathion, Dimethoate, DDT have been frequently detected in trace amount in milk samples from several locations in different countries. The extraction of pesticide is regularly carried out by using QuEChERS or solid-phase extraction and final chromatographic estimation by GC/MS/MS (Hernández *et al*., 2013, Ciscato *et al.*, 2015; Nasir *et al.*, 2016) [22, 8].

Fatty Acids (FA) Analysis

The determination of FAs in foods is most often carried out by gas chromatography (GC) and usually involves lipid extraction from foods, a derivatization procedure; fatty acid methyl ester (FAME) extraction, and GC-MS determination. Fatty acid methyl esters (FAMEs) in commercial milk samples are analysed by gas chromatography coupled with flame ionization detection as well as GC-MS using mass analyser. Predominant SFA analysed are: palmitic acid (16:0), stearic acid (18:0), and myristic acid (14:0). The conjugated linoleic acid (CLA) isomer 18:2 cis-9, trans-11 can also be identified and quantified using GC-MS (French *et al.*, 2000; Simionato *et al.*, 2010) [14, 50].

Volatile Compound Analysis

Dairy products like: butter, ghee, cheese upon their manufacturing and storage release several volatile and flavour compounds. However, other techniques such as: Solid-Phase Extraction (SPE) and Solid Phase Microextraction (SPME) are used for extraction and concentration of released volatile compounds in conjugation to GC-MS analysis. Applications include: identification of developed of flavour compounds upon ripening in cheese, analysis of volatile organic compounds of spoiled milk contaminated with psychrotrophic bacteria, etc. Using GC-MS compounds like:3-Methylbutan-1-ol, 2 methylpropan-1-ol, 3-hydroxybutan-2-one, 2, 3-butan dione, butanoic and hexanoic acids have been recognized as chemical markers of spoilage indexes of milk spoilage (Desimo 2014). Recent advances include identification of developed flavour compounds upon heating in ghee using SPME-GC-MS technique, is widely in practice. Furthermore, same method is in vogue for analysis of the volatile compounds of butter, yoghurt and cheese (Morales *et al.*, 2003) [33].

Detection of Adulterants

Milk and infant formula adulterated with melamine is detected using a silica-based strong cation exchanger (SCX) SPE phase and analysed by GC-MS/MS. The adulteration of butter or milk fat using lard, cheaper oil can also be identified by fatty acid profiling using GC-MS. Also, the authentication of milk fat methods includes analysis of fatty acids, sterols and tocopherols (Naviglio *et al.*, 2017) [35].

Other applications

GC-MS has become a highly recommended tool for monitoring and tracking organic pollutants in the environment. The determination of chloro-phenols in water and soil, polycyclic aromatic hydrocarbons (PAH), unleaded gasoline, dioxins, dibenzofurans, organo-chlorine pesticides, herbicides, phenols, halogenated pesticides, sulphur in air is very convenient to be screened by this technique (Chauhan *et al.*, 2014) [7].

High Performance Liquid Chromatography

High-performance liquid chromatography (or High pressure liquid chromatography, HPLC) is a specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds (Martin *et al.*, 2005) [31]. A large number of compounds can be separated rapidly and at high sensitivity by HPLC. The performance characteristics and applicability of the technique are continually improving, principally due to the large and ever increasing choice of column packing, and the growing number of solvent combinations. Separation times are decreasing, and there is a reduction in the volume of sample needed. The increasing sensitivity of the technique enables even very small amounts of substances to be detected. The combination of HPLC and mass spectrometry (MS) has increased the range of applications. The apparatus used for such analyses has become progressively smaller and smaller. This miniaturisation has been driven principally by concerns regarding the cost and ecological impact of the large volumes of solvents that used to be needed in older systems. For some compounds that are important for their use in traceability and food authentication, HPLC is the optimal technique (Malviya *et al.*, 2010) [29].

Types of HPLC

There are two major types of HPLC on the basis of mobile phase and stationary phase which are generally used in dairy and food products analysis (Malviya *et al.*, 2010) [29].

Normal phase chromatography: Also known Normal phase HPLC (NP-HPLC), this method separates analytes based on polarity. NP-HPLC uses a polar stationary phase and a non-polar mobile phase. The polar analyte interacted with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase increases the elution time.

Reversed phase chromatography: Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the

Application of HPLC

Analysis of carbohydrates: HPLC analysis using an amino-based stationary phase is the most popular technique for the

routine analysis of simple sugars. This analysis uses isocratic elution (e.g., acetonitrile: water, 75:25) and a refractive index detector (RID). In N-phase HPLC, the elution order of carbohydrates, monosaccharides and polyhydric alcohols then di saccharides followed by oligosaccharides (Ball, 1990) [4]. This technique has been successfully used to analyse the carbohydrate content of honey beverages, breakfast cereals, ice creams, cakes, malts, infant foods, fruits, vegetables, meat and so on. Reverse phase chromatography has been used to analyse the sucrose, raffinose and stachylose content of soybeans and soy bean products (Kennedy *et al.*, 1985) [26]. In addition, juices, syrups and beverages have been analysed for invert sugar, sucrose, maltose and malto triose (Walker *et al.*, 2015) [59].

Analysis of proteins: HPLC technique shows a growing importance for the analysis of specific protein components in foods and food products. The mixture of proteins to be separated is introduced into mobile phase and separated on the basis of the difference between protein components for the stationary phase in the column. Three basic separating principles can be discerned: ion-exchange chromatography (IEHPLC), size exclusion chromatography (SE-HPLC) and reverse phase chromatography (RP-HPLC). In ion exchange - HPLC proteins are separated on the basis of their net charge (Huang *et al.*, 2018) [24]. Modern IE supports are mainly based on silica or hydrophilic organic polymers. The latter being more stable at high pH values, which allows the use of more stringent separation procedures to extend the life time of column (Sykora *et al.*, 2019) [52]. In size exclusion HPLC, proteins are separated on the basis of their molecular size. Protein detection is usually achieved in the far (220nm) or near (280nm) UV region. SE-HPLC has also been proposed as a fast and non-destructive method for the separation of unreduced flour proteins into glutenin, gliadin and albumin globulin components (Hong *et al.*, 2012) [23]. In RP chromatography (RPC) separation is based on difference in surface hydrophobicity between the protein molecules. Hydrophobic amino acid residues of proteins are bounded through hydrophobic interaction to stationary matrix composed of a non-polar surface (usually C2-C18 alkyl chains) and silica based (Hedhammar *et al.*, 2006) [20].

Fat Characterization: The importance of fat characterization is evident in many aspects of the food industry including ingredient technology, product development, quality assurance product shelf life and regulatory aspects (Nielsen, 2010). Now-a-days with the reverse phase C18 column allowing the separation of individual components from the simplest natural mixtures of triacylglycerols, a detection system permitting the quantitation of peaks directly on chromatographs (e.g. Differential spectrometer, a mobile phase leading to separation of triacylglycerols, and a suitable identification method (Palmar *et al*., 1989).

Analysis of Vitamins

The three most used types of methods currently used for the analysis of vitamins – bioassays and microbiological and physico-chemical assays (Bates, 2006) [5]. Bioassays are extremely time consuming. Their uses are generally limited to those instances in which no suitable alternate methods are available or in cases in which bioavailability of the analyte is desired. Therefore, due to relative simplicity, accuracy and precision, the chromatographic methods using HPLC are preferred (Nielsen 2003) [37]. Vitamin assays are used to ensure product quality and to verify nutritional label claims. In addition, assays are important in developing manufacturing and storage processes because many vitamins are light- and/or air sensitive. C18 HPLC column features a retentive, high-purity packing that is ideal for separating a range of fat-soluble vitamins (Xue *et al.*, 2008) [63].

1. Atomic Absorption Spectroscopy (AAS)

Atomic spectroscopy is used for the qualitative and quantitative determination of 70 to 80 elements. The methods can be based on absorption, emission, or fluorescence. Detection limits for many of these lie in the sub-parts-per-million range. AAS is a widely used and accepted technique. Capable of determining trace (µg/ml) and ultra-trace (sub-µg/ml) levels with great accuracy and acceptable precision. AAS was discovered independently by Walsh Alkemade and Melatz in the early to mid-1950s. Atomic absorption spectroscopy can be simply defined as the absorption of radiant energy by atoms. This absorption and its quantitative correlation with the concentration of metal ions originally present in a sample solution serves the basis of AAS. Atomic absorption spectroscopy may also be defined as a method for determining the concentration of an element in a sample by measuring the intensity of external radiation absorbed by atoms of the sample at a wavelength characteristic for that element (Farrukh, 2012; Leo *et al.*, 2016) [12].

Atomic absorption spectroscopy quantifies the absorption of electromagnetic radiation by well-separated atoms or ions in the gaseous state.

Atomic emission spectroscopy (AES) measures emission of radiation from atoms excited by heat or other means.

Application of Atomic Absorption Spectroscopy:

Agricultural and food stuffs (White *et al.*, 2010):

a. Analysis of soil, soil extracts, fertilizers and plants for Na, K, Ca, Mg and trace elements like Cu, Mn, Zn, Fe, Mo and B.
b. Fruits, vegetables, fish and meat products require either wet or dry ashing.

Dairy Industry (Wong *et al.*, 1978) [60]

a. AAS is used to determine various minerals in milk and milk products.

Fourier Transform Infra-Red (FTIR)

Analysis of liquid milk samples has traditionally been performed by FT-IR based methods. This process is based on a transmissive infrared measurement that requires uniform particle sizes to avoid light scattering (King *et al.*, 2004) [27]. For this reason, FT-IR systems utilize an initial sample homogenization to obtain uniform fat globule sizes, while also eliminating entrapment of protein (Di *et al.*, 2016). The past few years have seen rapid growth in the use of infrared spectroscopy for at-line, on-line, and even in-line analysis. This progress has been made possible by developments in the design of both FTIR instruments and equipment to interface these instruments to chemical processes. It has been driven by the need for real-time monitoring of the chemistry underlying various processes and by infrared's ability to provide a wealth of information about chemical structure. IR spectroscopy is a technique for the determination of the structure and identification of different compounds (Garhwal *et al.*, 2011). The greatest advantage of this technique is that it can be used

for almost any sample. Because of their simplicity and non-destructive nature, IR analytical techniques have been used in a wide range of applications. Commercially, even though IR spectrometers have been around since 1940s, it was not until more recently after the introduction of FTIR spectrometers and the advances in computer technology, that spectrum quality and data time turnover has improved greatly. These aspects of IR spectroscopy have made it an important technique for studying and identifying biological molecules rapidly, with minimum sample preparation, giving IR spectroscopy advantages over other analytical techniques. This technique has been used to distinguish successfully a number of biological systems including proteins, fats and carbohydrates, food and pharmaceutical products as well as cells and tissues from plants and animals. However, recent developments in FTIR spectroscopy have made FTIR technique a powerful tool for authentication of food products. Moreover, there is a continual need for development of rapid instrumental based methods that meet or exceed the detection levels as well as certainty of currently available analytical methods for authentication of milk and milk products. The knowledge of IR spectroscopy is important to understand FTIR (Ranvir *et al., 2018*) [45].

Table 1: Recent FTIR applications in dairy industry

S.N.	Applications of FTIR	Wavelength used	Instrument used	Chemometric technique used	Significant Results	References
1	Estimation of cholesterol in dairy products using FTIR	2800 and 3200 cm^{-1}	Bio-Rad FTS 6000	Partial least square (PLS) and principal component regression (PCR)	Results indicate that FTIR spectroscopy can determine the cholesterol content in dairy products in approximately 5 min	Paradkarand Irudayaraj, 2002 [43]
2	FTIR to identify adulterated raw milk	5000 – 1000 cm^{-1}	Foss Analytical	Principal Component Analysis	FT-IR can be used for the identification of adulterated milk with 0,05% and 0,075% of sodium bicarbonate and citrate respectively	Cassoli *et al.*, 2011 [6]
3	Presence of beef fat in butter using FTIR	1500-1000 cm^{-1}	Nicolet 6700 FTIR with ATR	Partial least square (PLS) regression	2.42% beef could be detected in butter fat	Nurrulhidayah *et al.*, 2013 [41]
4	Detection of melamine adulteration in dairy milk by FTIR	4000–650 cm^{-1}	Thermo Nicolet Avatar 330 FTIR spectrometer	partial least-squares models	FTIR in combination with partial least-squares models could detect melamine at 2.5 ppm level	Jawaid *et al.*, 2013 [25s]
5	Detection of the microbial spoilage in milk	4000-600 cm^{-1}	ZnSe Gateway ATR Horizontal 6 reflection accessory	Multivariate statistical method, including Principal Component Discriminant function analysis and partial least square regression	Showed reasonable results for bacterial load above 10^5 cfu/ml	Nicolaou and Goodcare 2008 [36]

Dynamic light scattering (DLS) technique

Dynamic light scattering (DLS), also known as photon correlation spectroscopy, is an efficient method used to determine the size, size distribution, and shape of particles in suspension through the Brownian motion and Doppler shift induced by a laser beam. When a suspension of particles in Brownian motion is excited by a monochromatic laser beam the wavelength of the incoming light changes after hitting the moving particles, which creates a Doppler shift, which is a small frequency change in the scattered light compared to the unscattered light (Sakho *et al., 2017;* Hennart *et al.*,2012) [48, 21].

Applications

Detection of aggregation of protein

As milk protein undergoes proteolytic degradation as well as aggregation during various processing and storage conditions in dairy products. In order to investigate its low resolution structure, we required a homogenous preparation devoid of both degradation and aggregation (Hennart *et al.,* 2012, Thomas *et al.,* 2004) [21, 56].

Protein–protein interaction studies

In addition to detection of aggregation behaviour, DLS can also be employed to study protein protein interaction. After studying how individual proteins behave at multiple concentrations in terms of their homogeneity by DLS, we can also investigate their complex using DLS. It is very rapid measurements and requiring low amounts of purified components to study protein–protein interactions (Some *et al., 2012;* Halnon *et al.,*2010) [51, 18].

Homogeneity of milk samples or homogenization efficiency measurement

Dynamic light-scattering considered as an ideal method to study the homogenization efficiency of the milk homogenizer. By measuring the size of fat particle in the homogenized milk samples, a check of homogenizer can be performed (Michalski *et al.,* 2001; Wangdi *et al.,* 2015) [32, 58].

Nanotechnology-assisted laser desorption/ionization time-of flight mass spectrometry (NALDI)

NALDI is among the first commercially available matrix-free

methods for laser desorption ionization (LDI) of small organic compounds (< 3000 Da). Ionization takes place on a surface with densely packed alumina nanostructures (100/μm2) coated with perfluorinated silane. The structures have diameters of approx. 20 nm and lengths of 100–500 nm. The perfluorinated coating adds ultra-hydrophobic properties to the nanostructured surface, which allows dense application of analytes and optimizes ionization, regardless of the type of solvent used. NALDI is somewhat similar to MALDI. For both methods, ionization occurs by illumination of a solid sample with a short laser pulse. In the case of MALDI, the analyte molecules are co-crystalized with a matrix, which absorbs in the wavelength of the laser, and this is spotted on a metal plate (target) (Peterson, 2007; Niu *et al.*, 2008; Gandhi *et al.*, 2018) [44, 39, 45]

Applications of Naldi-TOF

The most used techniques for analysis of proteins and peptides in different food matrices are electro spray ionization (ESI) and MALDI-TOF MS (Shevchenko *et al.*, 2001; Trauger *et al.*, 2002; Gandhi *et al.*, 2018) [49, 57, 16]. However, there are also reports available that analysis of low molecular weight compounds with MALDI can be complicated because of intense chemical noise from the matrix. These problems can be reduced by using a matrix-free setup like NALDI which is reported to show good performance for molecules up to 3000 Da (Thomas *et al.*, 2001; Daniels *et al.*, 2008; Lewis *et al.*, 2003) [54, 10] conducted a study to identify peptides from colostrum with molecular weight under 3000 Da. NALDI has been used for the analysis of a variety of lipids including PLs, TAGs, DAGs and FFAs derived from both standards and biological extracts (Guenin *et al.*, 2009; Muck *et al.*, 2010; Colantonio *et al.*, 2011) [17, 34, 9].

Matrix-assisted laser desorption ionization is a recent soft ionization technique (MALDI)

MALDI-TOF MS is a soft ionization technique suitable for analysis of peptides, proteins, glycoproteins, oligosaccharides and oligonucleotides etc. Polymer characterization with mass spectroscopy (MS) is not possible as MS requires gas phase ions for a successful analysis, and polymers are composed of large, entangled chains that are not easily converted to gas phase ions. Traditional mass spectroscopic techniques developed for polymer analysis such as pyrolysis gas chromatography use thermal energy to vaporize nonvolatile samples such as polymers. Although thermal energy also decomposed polymers into constituent parts leading to fragmentation, resulting loss of full chemical structural information during vaporization. A new technique has been developed to measure this aspect of polymer sample. This new technique is known as matrix assisted laser desorption ionization MS (MALDI-MS).

Applications of Maldi-TOF MS

Characterization of milk proteins

Polymorphism of goat αs1 casein was confirmed using MALDI-TOF MS (Roncada *et al.*, 2002). Mamone, *et al.*, (2003) [47, 30]. Identified 34 casein components of ovine caseins using combined approach

Post-translational Modifications

Identification of phosphoaminoacyl residue in a peptide sequence, hence detection of phosphoserines and K-casein (Talbo *et al.*, 2001) [53].

Proteolysis

C-terminal truncated forms of β-lb in whey from Romegnola cow's milk (Zappacosta *et al.*, 1998) [64]. Determination of changes in milk protein profiles due to action of microbes during production (Fedele *et al*., 1999) [13]

Complex Dairy Matrices

Determination of different proteolytic enzymes and their specificity in different cheese types (Addeo *et al.*, 1995) [1]. Identification of peptides in Emmental cheese (Gagnaire *et al.*, 2004) [15].

Conclusion

Milk is a complex matrix consisting of different components and because of this, it often becomes impossible to accurately analyze one component in the presence of others using the classical method of analysis. Advanced instruments such as GCMS, HPLC, AAS, FTIR, DLS, NALDI-TOF and MALDI-TOF are widely used for assessing the quality of dairy and food products. GC-MS is an ideal technique for qualitative and quantitative determination of volatile and semi-volatile organic compounds in a wide variety of samples. A detection limit as low as sub-nano gram is possible. HPLC used for rapidly separation of a large number of compounds with high sensitivity. It is widely used for analysis of carbohydrate, protein, vitamin and fat characterization in dairy and food products. AAS is used for the qualitative and quantitative determination of elements in dairy and food products. The methods can be based on absorption, emission, or fluorescence. FTIR spectroscopy technique used for the determination of the structure and identification of different compounds. DLS used for determine the size, size distribution, and shape of particles of casein and fat. NALDI-TOF the most used techniques for analysis of proteins and peptides. MALDI-TOF MS used for characterization of milk proteins.

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