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Standardised methods for the determination of alkaline phosphatase activity in dairy products: Comparison for effective use

Scientific excellence
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Why determine the alkaline phosphatase activity?

The determination of the alkaline phosphatase (ALP) activity is used to verify the process of conventional pasteurisation of (bovine) dairy products. Pasteurisation inactivates/denatures the ALP that is naturally present in raw milk. The ALP test was initially established based on the finding that ALP in milk had similar inactivation kinetics to the inactivation of the pathogenic bacteria *Coxiella burnetii* and *M. tuberculosis*. Therefore, when ALP is inactivated to a legally established level, it serves as a marker indicating that the milk has been adequately treated. Table 1 shows some examples of the regulatory ALP levels.

Pasteurisation is a thermal process widely used in the dairy industry. An overview of key technical, microbiological and nutritional aspects of pasteurization can be found in the Bulletin of IDF N° 496/2019 (International Dairy Federation, 2019).

ALP activity measurement is traditionally applied to bovine milk immediately after conventional pasteurisation treatment (72 °C for 15 seconds). More recently ALP testing has been considered for application to dairy products such as cheese, as a means to presumptively check the pasteurisation status of dairy products (Egger et al., 2016). For this application, microbial ALP may also be present. This may arise from post-processing microbiological contamination, from starter culture activity etc., so there is a need for methods to determine ALP activity that are rapid, easy-to-use, can distinguish microbial from bovine milk ALP, and are effective for a diversity of dairy products.

Table 1. Examples of regulatory ALP activity levels in pasteurised milk, and reference methods

Country	ALP activity limit	Reference method	Reference
International	10 µg p-nitro-phenol equivalent/ mL	Phenol method	FAO & WHO (2004)
Europe	350 milliunits of enzyme activity per liter (mU/L[1]) in cow's milk	ISO 11816-1 | IDF 155-1 (IDF & ISO, 2013) or equivalent	Commission Implementing Regulation (EU) No 2019/6272[2]
	No legal limit has been set for cheeses		
United States	Less than 350 milliunits/L for fluid products and other milk products by approved electronic phosphatase procedures.	M-a-98 Official Grade "A" Pasteurized Milk Ordinance (PMO) Regulatory Laboratory Tests for Grade "A" Milk and Milk Products and Grade "A" Dairy Farm and Milk Plant Water	U.S. Grade A Pasteurized Milk Ordinance 2019Rev[3] FDA GAMS M-a-98[4]
	• 20 µg phenol/1 g for brick, semisoft, and semisoft part-skim cheeses; • 16 µg phenol/1 g for Limburger cheese; • 12µg phenol/1 g for all other cheese and related cheese products.	Phenol equivalent value[4]	U.S. Food & Drug Administration, 21 Code of Federal Regulations part 133 – Cheeses and related cheese products[5]
New Zealand	350 mU/L in cow's milk, cream, flavoured milk & cheese	ISO 22160 |IDF 209 (IDF & ISO, 2007)	NZ Ministry of Primary Industries - Guidance document: Alkaline phosphatase testing. 7 April 2022

1. One unit of ALP activity is the amount of ALP enzyme that catalyses the transformation of 1 micromole of substrate per minute. To note that there is no harmonised conversion factor between the methods of phenol and enzyme activity, although there is certainly a strong correlation between the two.
2. Commission Implementing Regulation (EU) 2019/627 of 15 March 2019 laying down uniform practical arrangements for the performance of official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of the European Parliament and of the Council and amending Commission Regulation (EC) 2074/2005 as regards official controls. OJ L 131, 17.5.2019.
3. US Pasteurized Milk Ordinance 2019 REV.
4. M-a-98 https://gams.fda.gov/active/M-a-98_Revision1_FINAL.pdf
5. Protocol: https://www.fda.gov/food/laboratory-methods-food/bam-chapter-27-screening-method-phosphatase-cheese

Limitations of testing ALP activity

Although the ALP test is considered as being the most appropriate method of verification of conventional pasteurisation for bovine milk, there are several factors affecting its useability in practice.

- Milk from different species contains different levels of ALP with different activities and different susceptibilities to heat treatment. This should be taken into account when establishing the criteria for ALP analysis since the test has been mainly validated and tested in products from bovine milk. Typically, raw cow's milk shows an ALP activity about five times higher than goat's milk, and about three times lower than sheep's milk (Klotz et al., 2008). This also varies depending on breed within species and individual factors (Raynal-Ljutovac et al., 2007). As pasteurization results in a tenfold reduction of the initial level, the post-pasteurization residual level will vary with the initial level in the raw milk. Consequently, different interpretation according to the milk origin is necessary. Cut-off values have not legally been established for products of sheep and goat origin. The report from the European Food Safety Authority (EFSA) suggested an ALP activity below a limit of 300 mU/L and 500 mU/L for goat's and sheep's pasteurized milk, respectively (EFSA et al., 2021). However, they recommended collecting further data. For equine milk, the current test sensitivity does not allow the use of ALP testing as the endogenous ALP activity is very low. Camel milk also contains low levels and a heat-stable ALP, hence, ALP testing as a means to check proper pasteurisation is not appropriate for these species either (Malissiova et al., 2022).

- Fat content of the milk, which is influenced by season and lactation stage, influences the ALP activity. ALP is readily absorbed on fat globules, thus increasing fat levels results in increased ALP activity (Painter & Bradley, 1997). The typical concentrations of endogenous ALP in bovine milk are 400 µg/mL for skimmed milk, 800 µg/mL for whole milk, and 3500 µg/mL for 40% cream (FAO & WHO, 2004). Therefore, the influence of compositional factors might be of consideration in the regulatory limits in the future.

- Microorganisms intentionally added to dairy products may produce microbial ALP that may interfere with tests for residual ALP. Therefore, in order to obtain valid results, the test should be performed immediately after heat treatment. In case of positive result after ALP test, the American Public Health Association recommends the repetition of the pasteurisation process in order to distinguish between microbial or bovine ALP. If the activity is not considerably reduced after the re-pasteurisation, the original ALP activity is due to the presence of bacterial ALP. However, some bacteria can produce both heat-labile and heat-stable ALP (Murthy & Kaylor, 2020). Therefore, differentiating bovine and microbial ALP can be problematic in some cases.

- ALP can be reactivated in many milk products (cream, cheese, etc.) over time (FAO & WHO, 2004). Reactivation has been reported in UHT milk and high-fat products. Therefore, in order to obtain valid results, the test should be performed immediately after heat treatment.

There are some alternative measurements to verify pasteurization. To learn more, the readers are referred to the comprehensive review by EFSA (EFSA et al., 2021). It is important to note that the use of alternative analytical methods is acceptable when they are validated against the reference method in accordance with internationally accepted protocols and rules of good laboratory practice.

Comparison of current standardised methods

IDF jointly with the ISO develops standards for the analysis and sampling of milk and milk products, including the determination of ALP activity. The following table shows the existing IDF/ISO standardised methods. It compares the methods to facilitate the decision-making of their selection and use. All these methods are based on chemical reactions with specific substrates but may have different principles of determination. Regulatory recognition, as well as equipment availability and technical support of the method may weigh into a decision on what method is suitable. It is important to note that there are other non-standardised methods in the literature (Shaban et al., 2022).

Table 2. Comparison of standardized methods for the determination of ALP activity.

Reference	Method principle	Sample scope	Detection limit	Unit definition	RSD_R (%)	Limitations	Other features (including substrate and product, wavelength)
ISO/TS 4985 | IDF/RM 255 (IDF & ISO, 2023)	Fluorimetry	Dairy products (fluid and solid)	0.006% pasteurised cow's milk	U = µmol/min	4.1	• Not tested in products from different species.	• Assay in a 96-microwell plate. • Substrate: 4-methyl-umbelliferone-phosphate, Product: 4-methyl-umbelliferone. • Ex 365 nm/Em 450 nm.
ISO 11816-2 | IDF 155-2 (IDF & ISO, 2016)	Fluorimetry	Cheese (soft, semi-hard and hard cheeses), provided that any mold is only on the surface and not in the inner part.	0.006% pasteurised cow's milk 5 mU/Kg	U = µmol/min	4.3	• Does not specify if the method is applicable to cheese from other species different from cow. • Proprietary method. • Specific sample treatment for hard/ soft cheeses.	• Fluorogenic Substrate: monophosphoric ester Fluorophos®, Product: Fluoroyellow®. • Ex 440 nm/Em 520 - 560 nm.
ISO 11816-1 | IDF 155-1 (IDF & ISO, 2013)	Fluorimetry	Liquid dairy products from cow, sheep, and goat. Raw and heat-treated whole milk, semi-skimmed milk and flavoured milks. Milk powder after reconstitution.	0.006% pasteurised cow's milk 15 mU/L	U = µmol/min	4.4	• Proprietary method.	• Fluorogenic Substrate: monophosphoric ester Fluorophos®, Product: Fluoroyellow®. • Ex 440 nm/Em 520 - 560 nm.
ISO 3356 | IDF 63 (IDF & ISO, 2009)	Colorimetry	Milk, milk powder, buttermilk and buttermilk powder, whey and whey powder.	0.1 - 0.2% pasteurised cow's milk	1 µg phenol/mL	NR	• Does not specify if the method is applicable to samples different from cow's milk. • Low sensitive method. • Possible interferences in coloured dairy products.	• A result of ≥ 2.5 µg of phenol indicates a milk that has not been properly pasteurized. • Substrate: phenol, Product: dibromoindophenol. • Measurement at 610nm
ISO 22160 | IDF 209 (IDF & ISO, 2007)	Chemiluminescence	Milk and milk-based drinks from cow, goat, buffalo and sheep. This includes whole, skimmed and flavoured milk. Also, 20% fat cream.	0.005 - 0.2% pasteurised cow's milk 15 mU/L	U = µmol/min	7.5	• Low-sensitive method. • Proprietary method.	• No requirement of sample preparation • Chemiluminescent Substrate: 3-(2'-spiroadamantanane)-4-methoxy-4(3"-phosphate phenyl-1,2 dioxetane disodium salt (Charm reagent AP®), Product: adamantly1,2-dioxetan • Measurement at 540 nm

ALP: alkaline phosphatase; Ex: Excitation; Em: Emission; RSD_R: relative standard deviation of reproducibility; NR: not reported

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