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Current status in the development of a microwell method for the Analysis of Alkaline Phosphatase Activity

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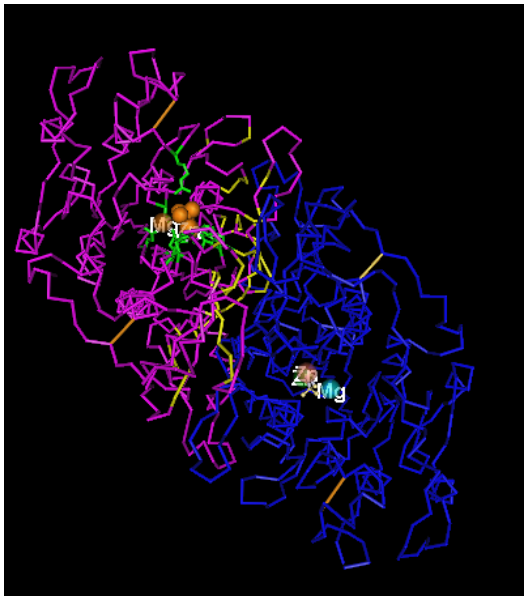
Agroscope, Institute for Food Sciences, Switzerland

Workshop EURL, Maison-Alfort, Paris 2015

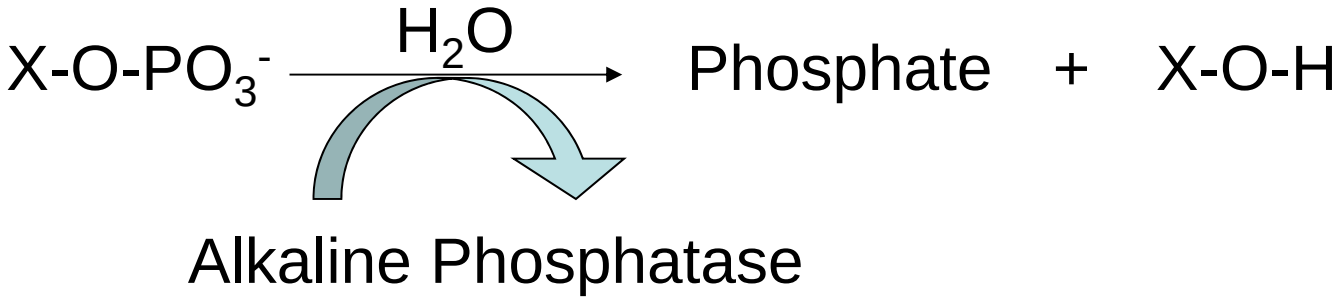
Alkaline Phosphatase (ALP) activity



- Homodimer
- One Mg^{2+} and two Zn^{2+} are bound to the active site and needed for ALP activity
- Inorganic phosphate is held firmly in the active site by the two Zn^{2+} and Arg166
- Monomer of 524 aa length

Conditions for optimal activity

- pH optimum: 10.5
- Optimal temperature: 37°C
- Heat sensitive (denatured after 15 s at 71.7°C)
- ALP catalyzes the hydrolysis reaction via phosphoseryl intermediate to produce inorganic phosphate and the corresponding alcohol



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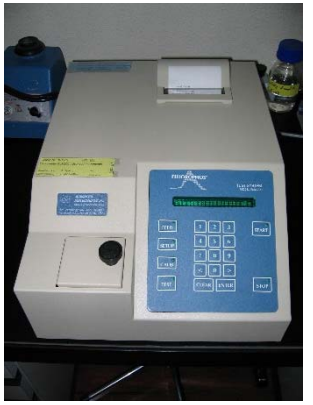
Analytical methods for ALP activity



- Reference method: ISO11816-1 IDF155-1/ ISO11816-2 IDF155-2
- Instrument and Chemicals provided by Advanced Instruments

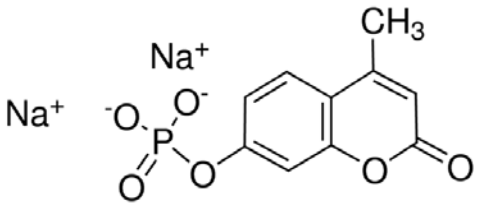
Excitation: 439 nm
Emission 560 nm

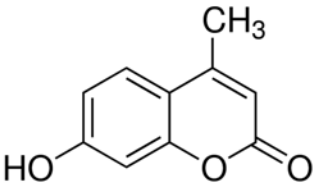
$$\text{Fluorophos}^{\circledR}\text{-O-PO}_3^- \xrightarrow[\text{ALP}]{\text{pH 10}} H_3PO_4 + \text{Fluoroyellow}$$

- Alternative method applied in serum and cell culture samples
- Open method

Excitation: 365 nm
Emission 450 nm

$$\text{4-methyl-umbelliferyl-PO}_3^- \xrightarrow[\text{AP}]{H_2O} H_3PO_4 + \text{4-methyl-umbelliferone}$$





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Alternative ALP method for Dairy Products

Journal of Food Protection, Vol. 76, No. 5, 2013, Pages 892–898
doi:10.4315/0362-028X.JFP-12-302

Research Note

Fluorometric Detection of Active Alkaline Phosphatase and Gamma-Glutamyl Transferase in Fluid Dairy Products from Multiple Species†

GEORGE C. ZIOBRO[1]* AND KEVIN M. McELROY[1,2,3]

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MS 12-302: Received 11 July 2012/Accepted 3 January 2013

Alternative ALP method for Dairy Products

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Draft Method at ISO/IDF →

Draft Method to the Standing Committee on Analytical Methods for Processing Aids and Indicators

Milk and Dairy Products – Determination of Alkaline Phosphatase Activity Using a Microwell Fluorometric Method

1 Scope

This method has been proven to be appropriate for determining the amount of alkaline phosphatase activity for a variety of different dairy products including milk, cheese, and cream from bovine, ovine, caprine, etc., as an indicator for pasteurization.

2 Definitions

For the purposes of this part of ISO, the following definitions apply:

2.1 **ALP**: Alkaline phosphatase. Measurement of the presence of the active form of this enzyme is determined by this method.

2.2 **Unit (U)**: Amount of alkaline phosphatase enzyme that catalyses the transformation of 1 µmole of substrate per minute.

2.3 **4MU**: 4-methylumbelliferone, the fluorescent product resulting from the action of ALP on the substrate 4MUP.

2.4 **4MUP**: 4-methylumbelliferone phosphate, the non-fluorescent substrate for the action of ALP.

Alternative ALP method: Equipment and assay protocol

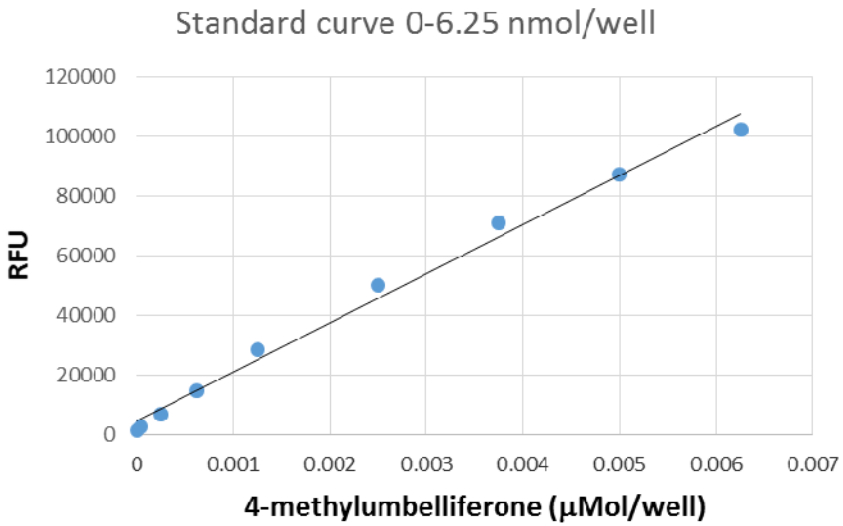
- Instrument:
  - Fluorescence microplate reader (e.g. Molecular Device, Biotek, Labtech, Millipore…)
  - Black brka2ell plates

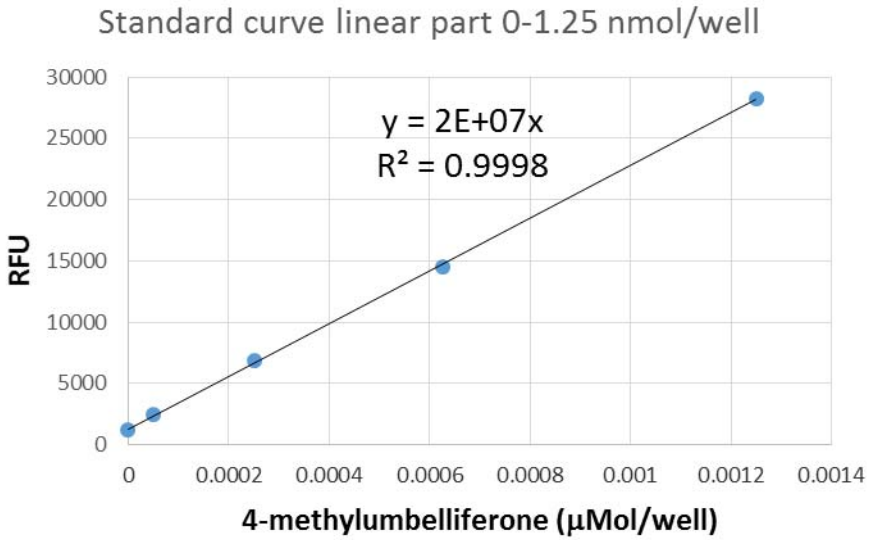
- Kinetic Assay in microplate:
  - Primary sample dilution 1:10 in diethanolamin buffer
  - Generation of blank for each sample by heating one portion at 95°C for 5 min
  - Pipetting 50 µl per well of sample and blank in duplicates
  - Addition of substrate 4-MU-Phosphate
  - Kinetic fluorescence measurement (365/460 nm) at 37°C for 15 min
  - Calculation of slope from linear part of the curve
  - Calculation of activity by using the standard curve measured with the fluorescent product 4-MU

brka2 hata die Farbe eine Bedeutung?

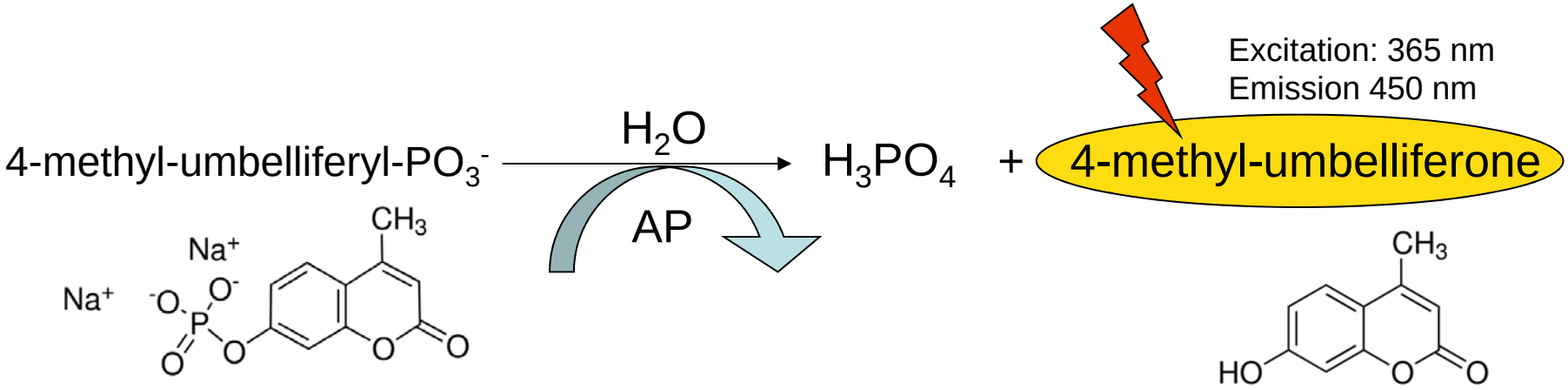
Breme Katharina Agroscope; 01.10.2015

Alternative ALP method: Standard curve





- Linearity of fluorescent 4-MU Standards: 1.25 nmols/well (25 µmol/L)



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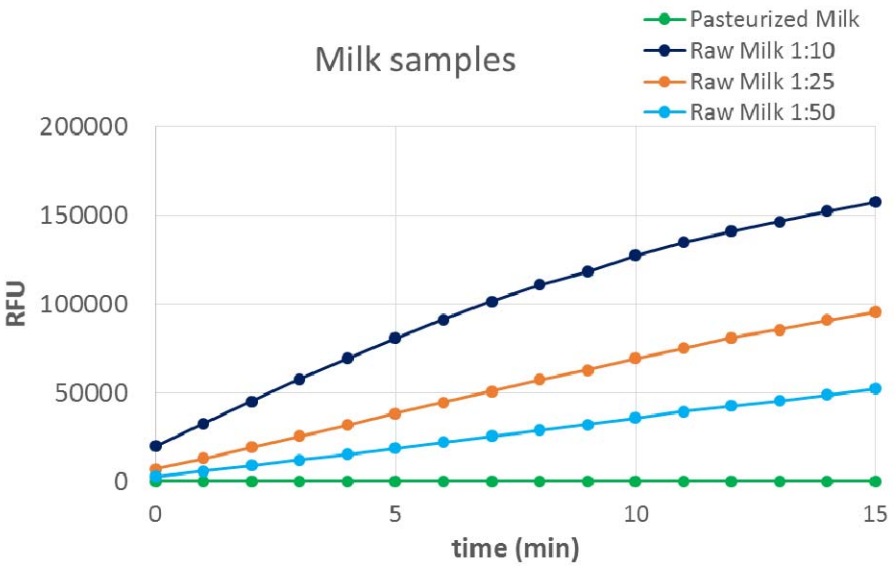
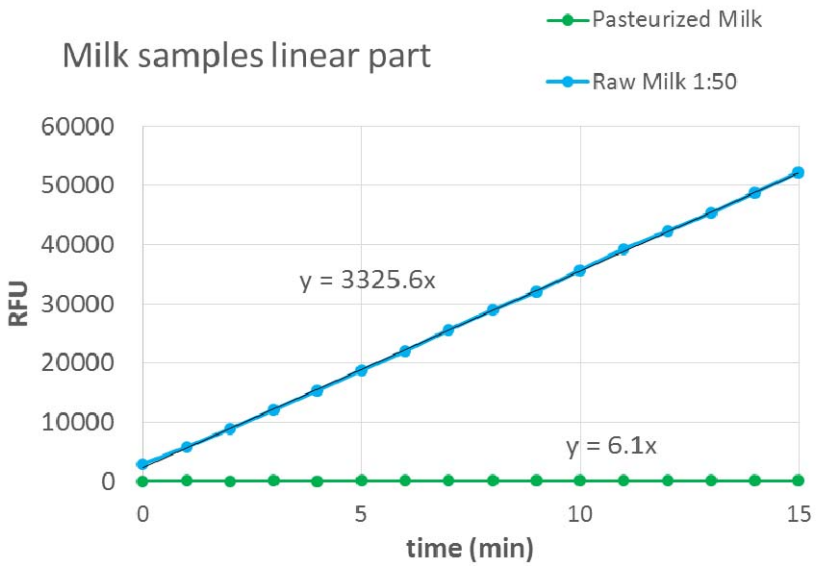
Alternative ALP method: Raw and pasteurized milk





- Definition of ALP activity: 1 U = 1µmol of 4-MU formation / Min
- Activity Calculation: (Slope Sample-Slope Blank)/Slope Standard Curve)* k * m * 20'000= U/L

	Raw Milk 1:50	Pasteurized Milk
Slope Sample - Blank	3'326	6
Slope Standard Curve	21'487'649	21'487'649
Dilution factor (k) 5 ul Sample in 45 ul Buffer	10	10
Secondary Dil.-factor (m) with heated milk	50	1
*20000 (to obtain Liter/kg)	20'000	20'000
Activity U/L	1'548	0
Activity mU/L	**1'547'661**	**56**

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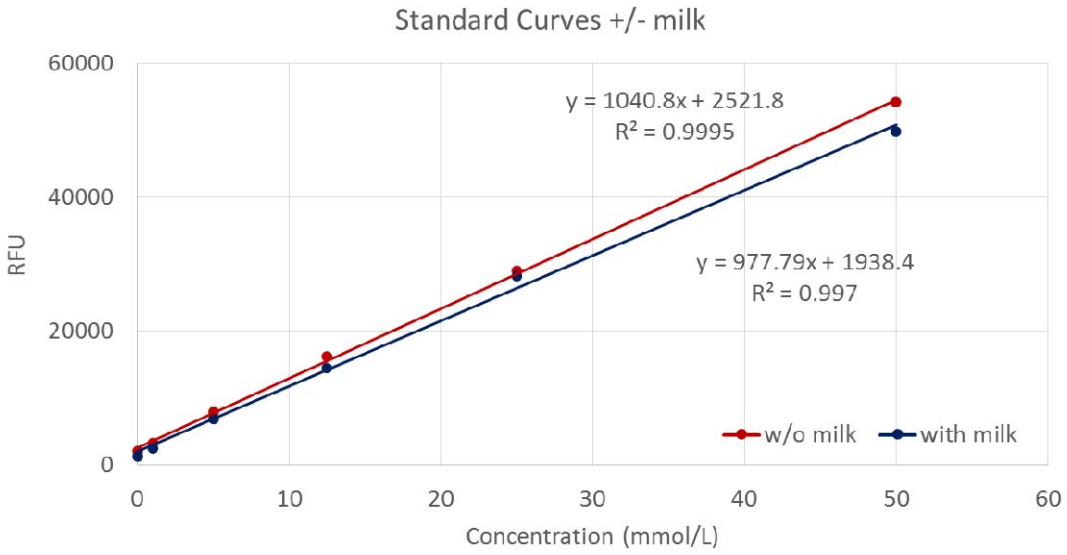
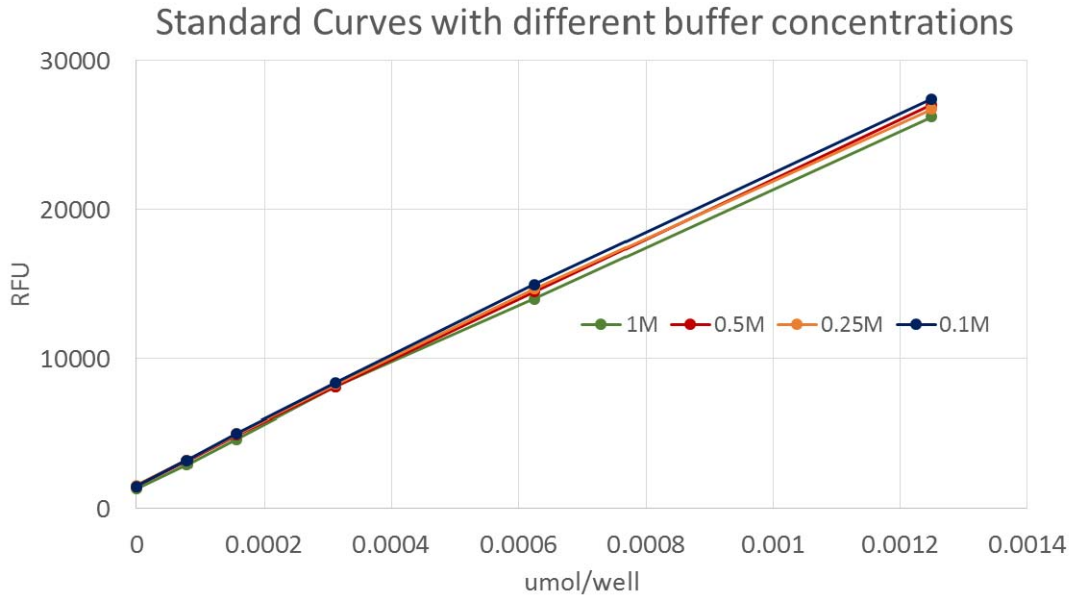
Alternative ALP method: Optimizations



- Presence of milk in standard curve reduces fluorescence
  → Same volume of milk in standard samples



- Buffer concentration has no impact on standard slope

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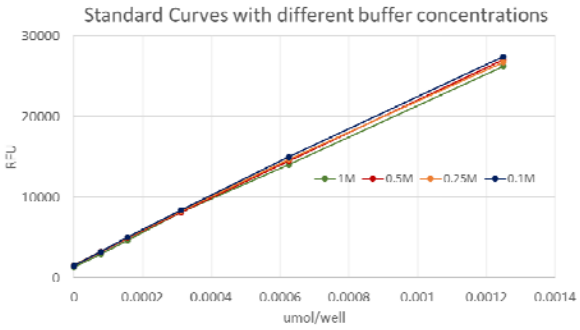
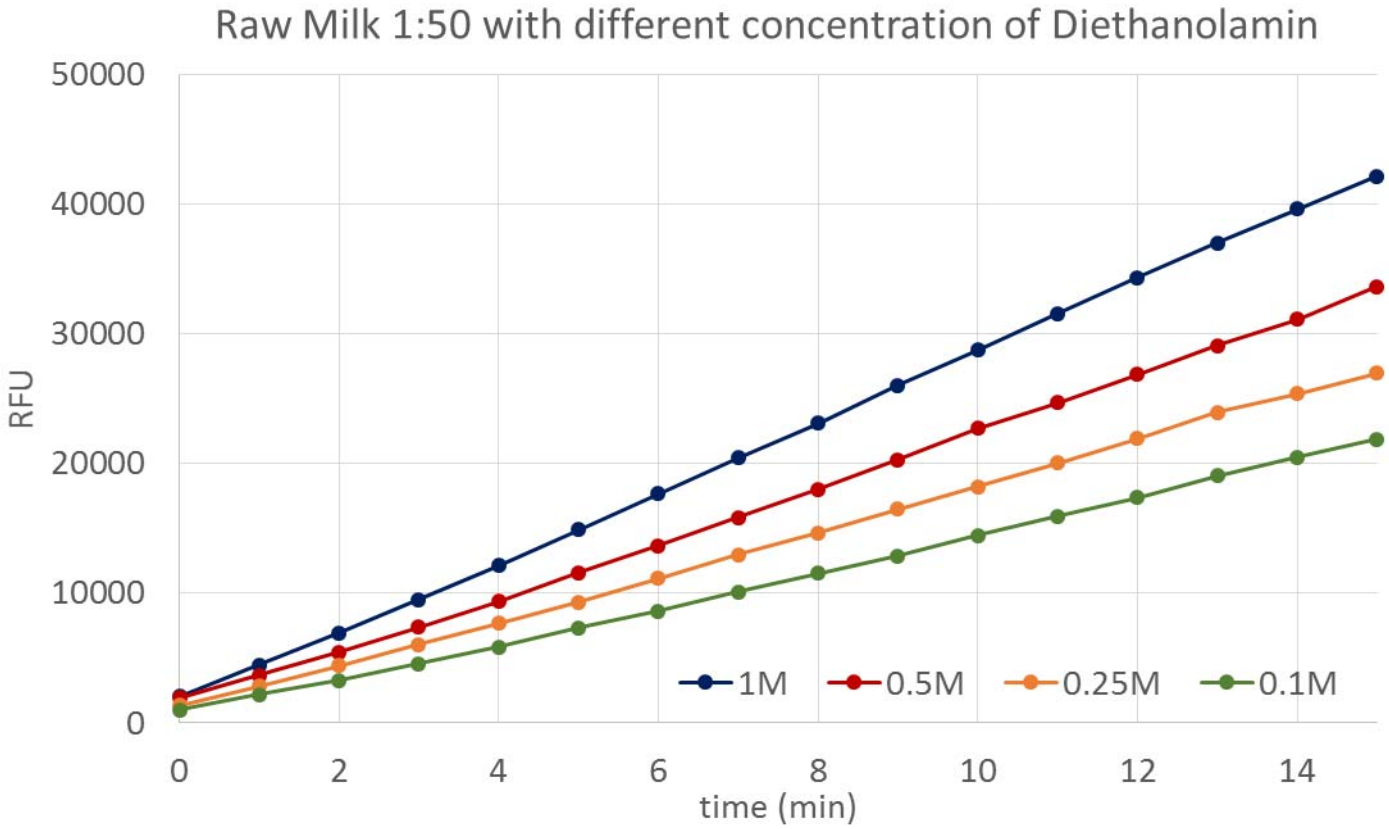
Alternative ALP method: Optimizations



- Buffer concentration has no impact on standard slope
- But: has a large impact on ALP activity!



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Alternative ALP method: further considerations

- ALP activity is dependent on Mg^{2+} → Addition of MgCl to the extraction and reaction buffer
- ALP stability is optimal at a pH between 7.5 and 9.5 (depending on the buffer) → pH and buffer in the optimal pH range (e.g. diethanolamine Buffer pH 9.8)
- Dilution of samples → in case the enzyme reaction curve is not linear (regression < 0.98), the sample needs to be diluted
- Dilution of samples in heated milk
- Standard curve done with the same volume of milk in the buffer brka5

brka5 added to the buffer?

Breme Katharina Agroscope; 01.10.2015

Alternative ALP method: Current status within ISO/IDF

- AW 2015: Work item under consideration
- Aim: Definitive method protocol until Analytical Week 2016, Project leaders: Hanène Ghezzal (EURL), Charlotte Egger (Agroscope)
- Proposal as New Work Item at AW 2016
- Generation of a Draft ISO Standard
- Ringtrial within interested laboratories for precision data collection
- Final draft and publication of the new ISO Standard

Thank you for your attention

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Additional Slides….

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Recent Publication of cheese data using the Reference Method ISO 11816-2/IDF 155-2

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journal homepage: www.elsevier.com/locate/lwt

Short communication

Alkaline phosphatase activity in cheese as a tracer for cheese milk pasteurization

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[b] National Reference Laboratory for Milk and Milk Products (EURL), Avenue du Général de Gaulle 23, 94706 Maisons Alfort Cedex, France
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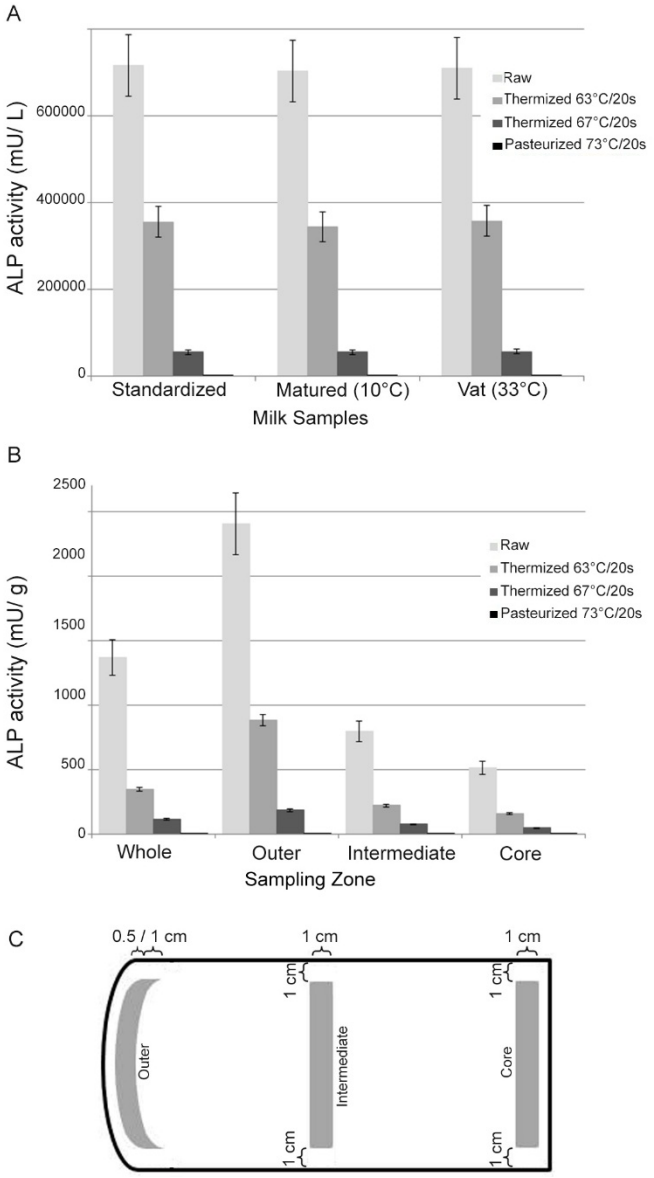
Recent Publication of cheese data using the Reference Method ISO 11816-2/IDF 155-2



- Cheese production under controlled conditions with different thermization temperatures:
  - no heat treatment
  - 63°C/ 20 s
  - 67°C, 20 s
  - 73°C, 20 s (pasteurization)

- Cheese sampling from the outer portion is necessary for cheeses with big wheels to allow a clear indication of proper pasteurization

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Target Limit of 10 mU/g Cheese

Fig. 2

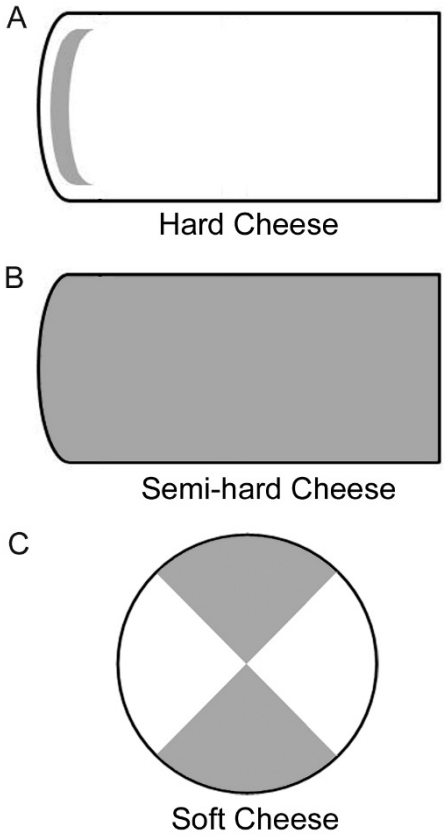
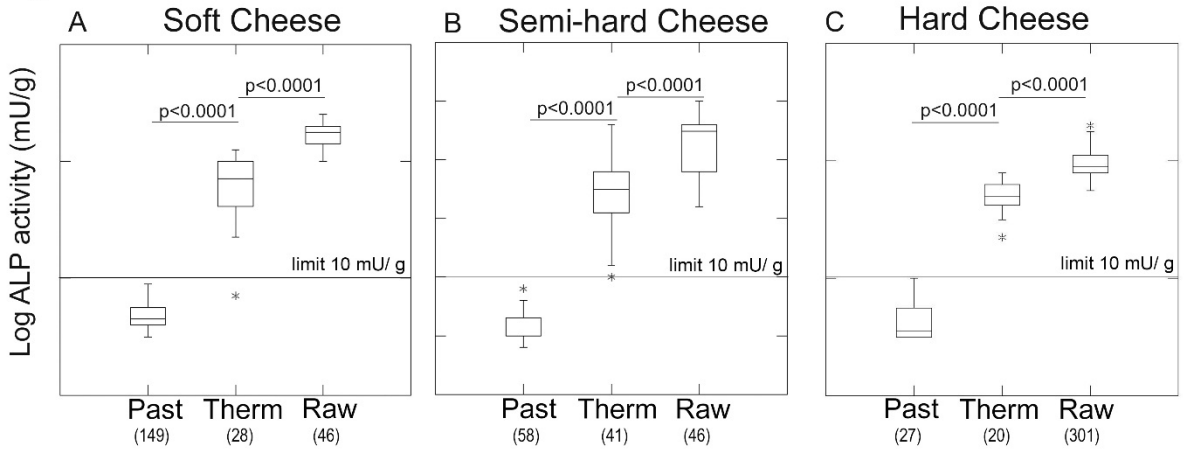


Fig. 3



- Over 700 cheese samples analyzed in duplicates
- All complied to the target value of 10 mU/g for proper milk pasteurization

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Average ALP values of Cheeses from France, Italy, and Switzerland

	Cheese Type	Country	Pasteurized			Thermized			Raw		
			Average (mU/g)	SD (mU/g)	N	Average (mU/g)	SD (mU/g)	N	Average (mU/g)	SD (mU/g)	N
Soft Cheeses	**Brie**	F, CH	**3**	1	37	**60**	40	3	**1913**	565	12
	Camembert	F, CH	**3**	1	61	**648**	314	12	**3687**	928	20
	Acid curd cheese	F	**2**	1	3						
	Coulommiers	F	**2**	1	24	**920**	1109	2	**2999**	1449	5
	Stracchino	I	**2**	1	4						
	Limburger	CH	**1**	2	4						
	Vacherin Mont d'Or	CH				**456**	500	9			
	Tomme	F, CH	**2**	3	5	**1589**	26	2	**4306**	1869	8
Semi-Hard Cheeses	**Appenzeller**	CH							**1284**	895	10
	Cream Cheese	F, CH	**1**	1	8	**93**		1	**3260**		1
	Crotto	I	**2**	1	3						
	Flösser Cheese	CH				**325**	132	5			
	Mountain Cheese	CH				**499**	437	9			
	Raclette	F, CH	**2**	2	16				**2643**	1679	6
	Raschera	I				**562**	110	3	**3522**	1073	6
	St. Paulin	F, CH	**2**	2	7	**77**		1	**9691**		1
	Taleggio, Quartirolo	I	**5**	2	2				**5060**		1
	Tête de Moine	CH							**3636**	631	15
	Tilsiter	CH	**0**	0	6	**1639**	1508	6	**2274**		1
	Vacherin Fribourgeois	CH				**102**	103	4	**3733**	518	4
	Valtellina Casera	I	**1**	1	2						
	Winzer Cheese	CH				**326**	251	5			
Hard Cheeses	**Bernese Hobelcheese**	CH							**3342**	350	2
	Comté	F							**1846**	597	4
	Emmental	F	**2**	2	12						
	Emmental Switzerland	CH							**1117**	681	13
	Generic Grana Type	I	**2**	1	5	**318**	173	18	**1435**	745	8
	Grana Padano	I							**954**	288	134
	Gruyère	CH							**1163**	785	12
	Parmiggiano Reggiano	I							**781**	299	114
	Sbrinz	CH							**1236**	711	6
	Switzerland Swiss	CH	**0**	0	6						

	Cheese Type Single Analysis (N=1)		Pasteurized mU/g	Thermized mU/g	Raw mU/g
Soft Cheeses	Chaource	F	5	864	
	Mascarpone	I	1		
	Paradiso	I	2		
Semi Hard Cheeses	Caciotta Valle Alpina	I			1977
	Fontina	I			1447
	Montasio	I			300
	Spicy Max	CH		36	

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