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Bernard Corrigan Technologist Moorepark

o Technologist with Teagasc since 2001.

o   Previously worked in PAL and PHL Dublin.

o Three years pharma. experience with GSK and Genzyme in UK

o Areas of interest & expertise: Standard dairy powder & liquid testing, Chromatography including HPLC, Particle size analysis & Rheology. Mineral analysis including ICP-MS.

o Worked in the area of casein analysis since joining Phil Kelly's & Brendan O' Kennedy's group in 2001.

Goals of this presentation

o To Address some of the current problems in analysis of casein and whey fractions in dairy products.

o To present a rapid rpHPLC method which can identify and quantitate the major casein and whey proteins found in milk and dairy products

o To look ahead at some of the challenges presented and possible ways to overcome these challenges.

o How will we get to an "all in one" universal method.

Importance of Caseins

o Casein important is an foodstuff which provides an important source of protein in the diet as well as inorganic elements such Ca and P.

o Found as a colloidal dispersion in milk with four main subunits. Kcn, As2cn, AS1cn and beta casein.

o Precipitation of caseins as well as re-solubilisation of caseins can be a major problem in analysis.

o Ratio of casein to whey is important in particular with regard to the heat stability of dairy protein mixtures.

o Ratio of casein to whey as well as degree of denaturation of whey is important in dairy products as this can effect the physico-chemical characteristics such as water holding capacity, viscosity, gelation and heat stability.

o Important factor in being able to monitor the success of process operations such as membrane filtration and ultrafiltration

o Amount of casein important as an emulsifier of fat in emulsions systems such as cream liqueurs.

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Some Methods for the identification and quantification of caseins in bovine milk

There are a number of different methods for quantification and identification of caseins in milk these include:

- Gel based methods Urea and SDS gel electrophoreses and Capillary electrophoreses.

- Immunological based methods.

- Chromatographic methods including cation and anion exchange, Size exclusion, hydrophobic interaction chromatography and reverse phase HPLC (rpHPLC).

- Other methods including Lab. on a chip methods.

- Traditionally most work on dairy products has been done on rpHPLC and Gels and this will be the focus of this talk.

Some problems with traditional methods

12% tris gel

A 25 kDa
B 24 kDa
K 19 kDa

Urea gel

G cn
Bcn
Acn
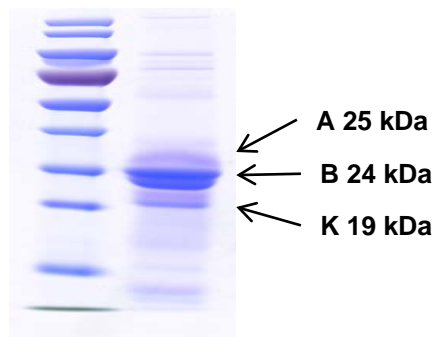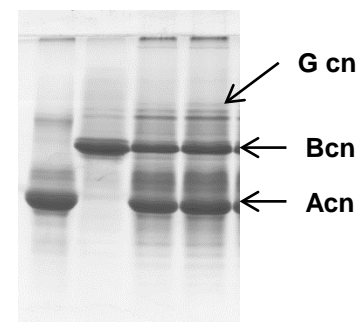
- ❑ Problems encountered with all methods must be used in tandem.
- ❑ Urea gels traditionally used for the separation of caseins. SDS gels for whey's
- ❑ UREA gels will not give a distinct or separate band for Kappa Casein. Good separation of Alpha and Beta Caseins.
- ❑ Modern mini gels give good resolution of caseins, but some bands still unresolved. Poor reproducibility of staining across gels and between gels.
- ❑ Time element involved including preparation of gels and reagents as well as staining and image processing and digital quantification.
- ❑ Immuno-assays expensive owing to preparation of specific antibodies, also problems of antigen recognition with heat degraded samples

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HPLC

o **DEAE, Anion exchange and size exclusion used for the separation of caseins since the 1950s.**

o **HPLC gives information on the makeup of the protein fractions as opposed to the total protein values given by traditional methods such a kjeldahl.**

o **Visser wrote a seminal paper on in 1992 based on the use of rpHPLC.**

o **A Number of further notable studies followed including Bordin, Veloso, Bobe and Bonfatti.**

o **Some of these studies replaced the urea with Guanidine and the mercaptoethanol with DTT another reducing agent.**

o **Typically these methods are run at higher than ambient temperature**

Method selection

- Simple and robust.
- Good separation of caseins with preferably 1.0m baseline separation and no co-elution of proteins.
- Reagents easy to prepare and safe to use.
- Good reproducibility.
- Good precision.
- Good Linearity.
- Good Accuracy.
- Sensitive.
- Good value for money.
- High throughput ability.

HPLC system and column characteristics

- Agilent 1200 series HPLC with MWD plus column heater and 99 well auto sampler

- Agilent Poroshell c18 column 2.1* 75 mm ID cheap and robust column

- Short diffusion path. Higher mass transfer when compared to fully porous column.

- Pore size greater than 300 Ang. Capable of running at elevated temperature.

- Chemstation software easy to use especially for calibration purposes.

Sample Chromatogram of Bovine Milk

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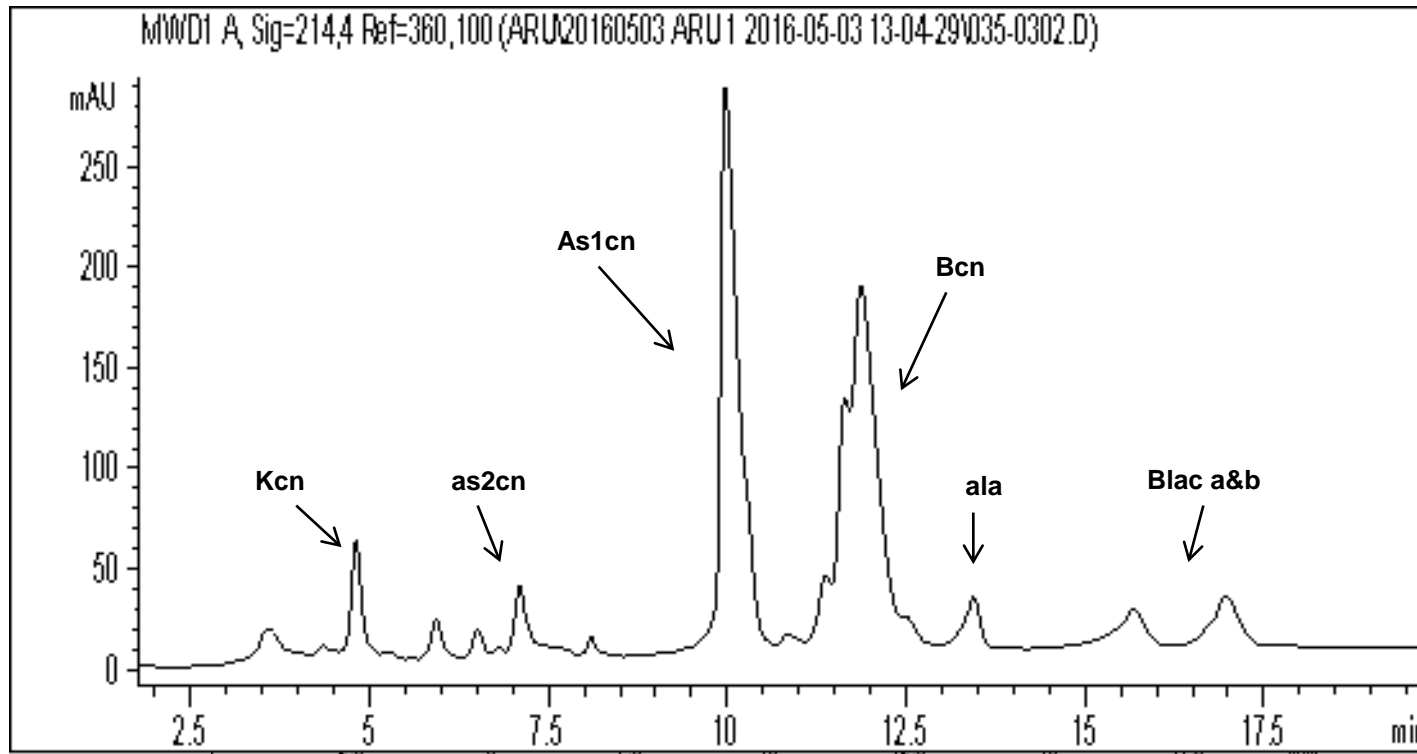
Linearity

- ❖ Multipoint Calibration curves prepared for each of the casein and whey standards
- ❖ Standards prepared in triplicate using certified Sigma standards.
- ❖ ESTD method used.
- ❖ RSD% for all standards found to be acceptable
- ❖ R^2 values found to greater than 0.99 for all standards
- ❖ No separate standard present for As1 and As2 cn at present so estimate based on relative areas must be used.
- ❖ Care must be taken with commercial standards to get true protein values as values based on PAGE .

Accuracy

- 10 Bulk milk samples repeated on four separate days average result compared with Sprint analyser from CEM. Sprint values checked on standard milks against Kjeldahl TP and DairySpec FT.

- % Recovery from milk studies found to be greater than 90% in all cases for true protein.

- Results for Bovine milk also in agreement with values found in literature.

- Farrell, H. M. Jr., R. Jimenez-Flores, G. Bleck, E. Brown, J. Butler, 2004. Nomenclature of the proteins of cows' milk—Sixth revision. J. Dairy Sci. 87:1641–1674.

	% Total Prot. HPLC	% Total Prot. Sprint	%Recovery
day1	3.49	3.69	94.5
day2	3.47	3.74	92.68
day3	3.63	3.75	96.75
day4	3.46	3.72	92.88

Repeatability – precision

- Initial precision study taken over four days from the same bulk tank.

- Forty separate samples prepared.

- Relative standard deviation calculated for both the retention time and areas under the curve for each of the casein and whey fractions.

- Sample lots were not identical however and thus were more challenging than a single population.

- Room for larger and or further studies to improve precision values. as well as use of frozen lyophilised samples to ensure reduction in sample errors.

	Kcn		As2cn		As1cn		Bcn	
	rt	area	rt	area	rt	area	rt	area
RSD %	2.37	11.19	1.77	7.85	1.44	4.13	2.02	6.39
	ala		Blac A		Blac B			
	rt	area	rt	area	rt	area		
RSD %	1.64	11.92	1.67	13.14	1.69	8.4		

Reproducibility

- Bovine milk sample taken from bulk tank on one day and from this ten separate samples were prepared
- Method found to give reproducible result with a relative standard deviation below 5% in most cases and below 10% for all fractions.

	kcn		as2cn		as1cn		bcn	
	rt	area	rt	area	rt	area	rt	area
RSD%	0.711	3.8231	0.649	6.572	0.335	0.538	1.088	0.733
	ala		blaca		blcb			
	rt	area	rt	area	rt	area		
RSD%	0.963	5.0145	0.718	2.326	0.804	2.042		

LOD/LOQ

	LOD = 3.3α /S (µg)	LOQ = 10α /S (µg)
kcn	0.071	0.214
as2cn	0.024	0.074
as1cn	0.076	0.23
bcn	0.098	0.298
ala	0.055	0.168
blac a	0.123	0.372
blac b	0.037	0.113

- Estimates of the LOD and LOQ can be made from either the calibration curves or from the repeat blank samples.

- Care should be taken that all samples have at least a 1:4 signal to noise ratio

- Care must be taken that all samples lie above the Limits of quantification

- All samples should lie within the range of analysis of the graph and also be in the linear region.

Standard milk

- One of the challenges faced is that there is no standard milk powder for caseins at present.

- There is a ERM certified standard available for minerals but not for proteins.

  Poroshell method tied in well with Bordins findings for Beta casein, as2 casein, Kappa casein and alpha lactalbumin. However lower findings for as1 cn than Bordin. Beta lactalbumin levels higher than Bordin but at widely accepted levels of 3.2 g/L of beta for a standard milk these values look more correct.

- Bordin mentions this low beta lac value in his paper. This lower whey value could also serve to elevate the casein levels a percentage in the method overall.

- Chromatography superior to an number of previous studies aiding quantitation.

Identification and quantification of major bovine milk proteins by liquid chromatography,Bordin G., Raposo F.C., B. de la Calle, Rodriguez A.R. Journal of Chromatography A, 928(2001) 63-76.

Tie in with other methods and publications

- Method has been used in a number of peer reviewed papers and journals
- Found to give good recovery of protein when compared with LECO, SPRINT and Kjeldahl True protein (TP) ie .>90%
- Used recently for a study on the separation of beta casein for next generation IMF. Results cross checked by Maldi-TOF and found to concur.

Stability of b-lactoglobulin ⁄micellar casein mixtures on heating in simulated milk ultrafiltrate at pH 6.0, Mounsey J.S and O'Kennedey B.T., Vol 62, No 4 November 2009 International Journal of Dairy Technology

The effect of native and modified konjac on the physical attributes of pasteurized and UHT-treated skim milk,John T. Tobin a,b, Sinead M. Fitzsimons a, Alan L. Kelly b, Mark A. Fenelon a, International Dairy Journal 21 (2011) 790-797

The effect of dietary crude protein and phosphorus on grass-fed dairy cow production, nutrient status, and milk heat stability

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Challenges ahead

- "Universal method" Still not with us.

- Like other rpHPLC Methods, this one only looks at major caseins and whey fractions

- How will we measure the minor whey proteins such as BSA, LF/LPO and IGG

- How will we measure the missing caseins y casein 1-3

- Pseudo proteins such as proteo peptones

- rpHPLC Methods separate only three beta casein iso-proteins. Beta casein has at least 10 iso -proteins present with different degrees of phosphorylation and and glycosylation.

Processing challenges

o Highly heated/Processed samples

o HPLC fine on native samples

o IMF MPC samples.

o WPC esp. the presence of CMP's

Conclusions and future work

- rpHPLC well accepted robust and simple method for the quantification of major caseins and whey's in bovine milk.

- Values tie in well with total protein values given by other methods such as LECO and Kjeldahl.

- Provides key data about the different levels of the casein and whey's in milks.

- Very quick and cheap method with good reproducibility when compared to traditional gel based methods

- Apart from initial capital outlay on equipment cost of consumables quite low in comparison with other methods

- Does not use dangerous and/ or toxic mobile phases.

- How do we go towards a future universal method?

- Investigate the use of LCMS to convolute the mixed signals from beta casein/ y caseins.

- Use of mixed mode columns for the separation of caseins on more than one level

Selected publications

Phenotyping of bovine milk proteins by reversed-phase high performance liquid chromatography, Visser. S., Slangen . C. and Rollema, Harry. S

Journal of Chromatography, 548 (1991) 361-370.

Sample Preparation Affects Separation of whey protein by Reversed-Phase High-Performance Liquid Chromatography, Bobe. Gerd, Beitz. Donald C., Freeman. Albert E. and Lindberg Gary L.

j. Agric. And Food Chem. 1998,46, 1321-1325.

Identification and quantification of major bovine milk proteins by liquid chromatography,Bordin G., Raposo F.C., B. de la Calle, Rodriguez A.R.

Journal of Chromatography A, 928(2001) 63-76.

Validation of a new reversed-phase high-performance liquid chromatography method for the seperation and quantification of bovine mik protein genetic variants, Bonfatti. Valentina, Grigeletto. A, Cecchinato, A., Gallo. L., Carnier. P,

Farrell, H. M. Jr., R. Jimenez-Flores, G. Bleck, E. Brown, J. Butler,

L. Creamer, C. Hicks, C. Hollar, K. Ng-Kwai-Hang, and H. Swaisgood. 2004. Nomenclature of the proteins of cows' milk—Sixth revision. J. Dairy Sci. 87:1641–1674.

Journal of Chromatography A, 1195 (2008)101-106.

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Thank's for your attention!