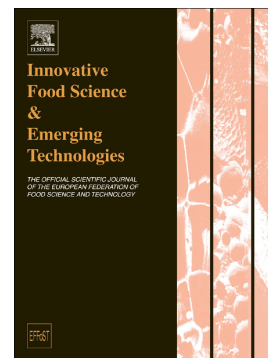# Accepted Manuscript

Fluorescence-based analyser as a rapid tool for determining soluble protein content in dairy ingredients and infant milk formula

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Fluorescence-based analyser as a rapid tool for determining soluble protein content in dairy ingredients and infant milk formula.

Lisa E. Henihan[*,†], Colm P. O'Donnell[†], Carlos Esquerre[†], Eoin G. Murphy[*,1] and Donal J. O'Callaghan[*]

[*]Food Chemistry and Technology Department, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[†]School of Biosystems and Food Engineering, University College Dublin, Belfield, Dublin 4, Ireland

[1]Corresponding Author:

      Email: Eoin.Murphy@teagasc.ie

      Tel: +353 76 1112525

Abbreviations

Abbreviation	description
agg	Agglomerated powder
CIF	Commercial infant milk formula
k	number of measurements
MIF	Model infant milk formula
min	minutes
n	number of samples
NCN	Non-casein nitrogen
NPN	Non-protein nitrogen
RP-HPLC	Reverse phase high performance liquid chromatography
RSD	Relative standard deviation, SD/mean x 100.
SD	Standard deviation
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEest	Standard error of the estimate, measure of the variability of predictions in a regression
SMP	Skim milk powder
SP	Soluble whey protein content g/100g
$SP_{Kjeldahl}$	Soluble whey protein content by Kjeldahl
std	non agglomerated powder
TN	Total nitrogen
TS	% total solids
WPC	Whey protein concentrate
WPN	Undenatured whey protein nitrogen, mg per g of non-fat milk powder
WPNI	Whey protein nitrogen index
$WPNI_F$	Whey protein nitrogen index by fluorescence based analyser
$WPNI_{GEA}$	Whey protein nitrogen index by GEA Niro method

Abstract

Milk protein, in particular native whey protein, is of interest to dairy manufacturers as a measure of functional and nutritional quality. However, quantification of soluble whey protein (SP) is time consuming; giving rise to the need to develop rapid, accurate, and portable at-line process analytical technology. In this study, the performance of a fluorescence-based analyser($_F$) (Amaltheys II, Spectralys Innovations, France) was evaluated for quantification of SP_F and whey protein nitrogen index $(WPNI)_F$ in skim milk, whey protein concentrate and infant formula powders. Rehydration of powders prior to analysis was a key factor for ensuring repeatability and reproducibility. A comparison of the analyser with reference methods for SP_F and $WPNI_F$ resulted in coefficient of determination $(R^2) >$ 0.993 for both $SP_{Kjeldahl}$ method and $WPNI_{GEA}$. The results show the fluorescence-based analyser to be rapid, compact, and accurate device, suited for providing reliable support to dairy ingredient and infant formula manufacturers.

Industrial Relevance

The fluorescence based analysis investigated in this article is suitable for application in the dairy industry where it can be used as a rapid, at-line PAT tool for both liquid and powder samples. The technology has the potential to replace well-established methods for measurement of soluble protein. The main benefit to industry is the ability to respond more rapidly to variations in soluble protein without compromising on the accuracy associated with more time consuming methods.

1   Introduction

The effect of processing on proteins, in particular whey proteins, is a key concern for the dairy industry. The quality of dairy ingredients and infant milk formula is highly dependent on whey protein functionality which, if not controlled, can lead to rapid aggregation, fouling and in some cases gelling of protein in heat exchangers during processing (Buggy, McManus, Brodkorb, Mc Carthy, & Fenelon, 2016; Murphy, Fenelon, Roos, & Hogan, 2014). As a result many methods have been reported for quantification of soluble/native whey protein content e.g. precipitation of casein and denatured whey followed by quantification using Kjeldahl, Dumas, chromatographic or spectroscopic methods (IDF, 2001; Jovanovic, Barac, Macej, Vucic, & Lacnjevac, 2007; McSweeney & Fox, 2013; Mimouni, Deeth, Whittaker, Gidley, & Bhandari, 2010; Murphy, Tobin, Roos, & Fenelon, 2013)

The Kjeldahl method converts all nitrogenous substances to ammonia by digestion with sulfuric acid followed by distillation; nitrogen content is then determined by titration. The Kjeldahl method for quantifying total nitrogen (TN), non-casein nitrogen (NCN), non-protein nitrogen (NPN) and soluble protein ($SP_{Kjeldahl} = NCN - NPN$) include several time-consuming steps, including various purification/separation operations to isolate the milk fraction in question (IDF, 2001; Mimouni et al., 2010). In contrast, the Dumas method determines the protein content based on the conversion of nitrogenous substances to N_2 gas by combustion and catalytic reduction (Mimouni et al., 2010). Whey Protein Nitrogen Index ($WPNI_{GEA}$) is widely used as an indication of soluble whey protein and to classify dairy products based on severity of received heat treatments (GEA-Niro, 2012). $WPNI_{GEA}$ involves precipitation of caseins and denatured whey proteins followed by acidification of the remaining whey protein to induce turbidity which is then measured and correlated to a standard curve (Sikand, Tong, & Walker, 2008). Reverse phase high performance liquid chromatography (RP-HPLC) methods and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) are not considered as

accurate as the Kjeldahl and Dumas methods, but can be considered slightly more time-efficient as sample preparation and analysis is quicker (Jovanovic et al., 2007; McSweeney & Fox, 2013; Murphy et al., 2013) . However, a major disadvantage associated with all methods mentioned above is a significant amount of time is still required to prepare and analyse samples.

Fluorescence spectroscopy is a rapid and sensitive technique, increasingly employed for food research applications, which exploits the environmentally dependent properties of fluorescent compounds. The natural fluorescence of the amino acid tryptophan, inherent in most milk proteins, may be used to characterise changes at molecular level resulting from environmental stresses such as heat treatment (de Sereys et al., 2014; Henihan, O'Donnell, Esquerre, Murphy, & O'Callaghan, 2018; Lacotte et al., 2015; Lakowicz, 2007; Ramachander et al., 2008). The use of fluorescence-based analysers can provide key indicators relating to milk, dairy powders and dairy derivatives, namely soluble protein by fluorescence (SP_F) and WPNI by fluorescence ($WPNI_F$), as described by Lacotte et al (2015). The aim of this study was to independently investigate the performance of a novel fluorescence-based analyser for routine, rapid analysis of soluble protein and heat classification during dairy ingredient and infant milk formula manufacture.

2   Materials & methods

2.1   Materials

Skim milk powder (SMP) (% w/w protein) was manufactured from fresh raw milk obtained from a dairy farm at Teagasc (Moorepark, Cork, Ireland). Fresh milk was skimmed at 50 °C, evaporated to 30% total solids (TS) under low heating conditions (maximum temperature of 60 °C) and dried on single stage spray dryer (Anhydro F1 Lab; Copenhagen, Denmark), at inlet/outlet temperatures of 180°C/95 °C, respectively. Acid whey protein concentrate

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powder (WPC) (35% w/w protein) was supplied by Arrabawn Co-op Society Ltd. (Nenagh, Ireland). Vitamins and lactose were supplied by Glanbia (Kilkenny, Ireland). Sunflower oil was obtained from a local retail outlet.

2.2 Formulation, heat treatment and drying of powders used for evaluation

The products used for the evaluation trials were SMP, WPC powder, model infant milk formula (MIF) powder and commercial infant formula (CIF) powder. SMP, WPC and MIF powders were produced in the bio-functional food engineering pilot plant at Teagasc Food Research Centre, Moorepark, Cork, Ireland. Table 1 shows the conditions under which the various samples used in the study were produced. SMP and WPC powders were rehydrated to 10% TS. 1st stage model MIF were formulated at 30% TS with protein/fat/lactose ratios of 1.3/3.6/7.3. The whey to casein ratio in MIF batches was 60:40. Batches were subjected to various heat-treatments using a MicroThermics Lab heat exchanger (MicroThermics, North Carolina, U.S.A.) with a holding time of 15 s. MIF batches were homogenized at 65 °C using an in-line, two stage, valve-type homogenizer (Model NS2006H, Niro Soavi, Parma, Italy) at 15/5 MPa. Prior to spray drying, batches were concentrated at 65 °C using a single-effect falling film evaporator (Anhydro F1 Lab; Copenhagen, Denmark). Non agglomerated SMP and WPC powders were produced using a pilot-scale single stage spray dryer (Anhydro F1 Lab; Copenhagen, Denmark) equipped with a rotary atomisation system. Agglomerated MIF powders were manufactured using an Anhydro 3-stage dryer with fines return (SPX Flow Technology, Soeberg, Denmark) equipped with a two-fluid nozzle atomiser. The SMP and WPC powder samples prior to rehydration and heat treatment were used as control samples. 3 different CIF brands were obtained from local retail outlets. Each MIF powder and CIF brand were stored at both 15±2 °C and 37±2 °C for up to 1 year.

2.3 Fluorescence-based analyser

The fluorescence-based analyser (Amaltheys II, Spectralys Innovations, Romainville France) is designed for rapid at-line measurement of pH 4.6-soluble whey proteins. The model evaluated in the present study quantified SP in SMP, WPC and infant formula. The instrument also generated results for whey protein nitrogen index (WPNI), which is a standard analysis in SMP manufacture. Two analysis programs were available depending on the level of SP_F; SMP and WPC were analysed using Program 1 (0.5-24 g SP/100g) and Program 2 (24-94 g SP/100g) respectively. SP_F content of MIF and CIF were measured in the range 0.5-24 g/100g. SMP was analysed for $WPNI_F$, an output from the analyser which is analogous to the $WPNI_{GEA}$ method as described by GEA-Niro (GEA-Niro, 2012). After powder rehydration, the total time associated with measurements was less than 5 minutes, including the precipitation step to remove pH 4.6-insoluble proteins and the fluorescence-based analysis step.

An external laboratory equipped with the same instrument (Spectralys Innovations, Romainville, France) was used to carry out an initial inter-lab reproducibility assessment. For initial assessment of repeatability and inter-lab reproducibility, samples were prepared by rehydrating a 0.5 ml scoop of powder in 10 ml of distilled water and vortexed until the powder was visually dissolved. Subsequent experiments used varying dissolution periods to investigate the effect of varying the rehydration time. In all cases, a target relative standard deviation (RSD) of ≤ 5 % for analysis evaluation was required, as confirmation of good repetition. Each sample was analysed in triplicate, unless otherwise stated.

2.4   Reference methods

Non-casein nitrogen (NCN) and non-protein nitrogen (NPN) were determined by Kjeldahl (IDF, 2001), and a nitrogen-to-milk protein conversion factor of 6.38 was used for determination of soluble whey protein, g/100g powder;

$$SP_{Kjeldahl} = (NCN - NPN) \times 6.38 \qquad (1)$$

$WPNI_{GEA}$ of SMP was determined using the GEA Niro method, and expressed as mg undenatured whey protein nitrogen per g of non-fat milk powder (mg WPN/ g powder) (GEA-Niro, 2012). Each sample was analysed in duplicate.

2.5   Statistical analysis

Standard deviation (SD) was used as a primary measure of variability between samples. Repeatability of instrument or of a method step, such as rehydration time, was determined by carrying out a series of repeat measurements of the same test material under identical conditions, by the same instrument and the same operator. Repeatability is expressed using relative standard deviation (RSD) which is computed as

$$\frac{SD \times 100}{\bar{x}} \qquad (2)$$

where $\bar{x}$ is the mean. Instrument reproducibility was determined by carrying out repeat measurements of the same test material by different instruments of the same type in two laboratories; reproducibility is expressed by employing RSD under those conditions (Bartlett & Frost, 2008). Least squares regression was used to fit analyser readings to respective reference measurements, and to determine slope and intercept constants of potential

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calibration curves. Coefficient of determination (R^2) and standard error of estimate (SEest) were computed as statistical measures of fit to the regression line. All data handling and linear regression analyses were performed using Microsoft Excel.

3   Results and discussion

3.1   Instrument repeatability and inter-lab reproducibility

Five repeat samples of SMP95, WPC$_{control}$ and SMP95 were analysed to assess sample repeatability on each analyser (Table 1). The inter-lab reproducibility was also assessed by comparing the overall relative standard deviation (RSD) across both instruments, at their respective laboratories, to that of the individual instruments. Repeatability and reproducibility of measurements are shown accordingly in Table 2. A target RSD of ≤ 5 % for sample repeatability was achieved for both instruments individually; however, the overall RSD for all measurements made on both instruments was ≥ 6.8 % for both SP$_F$ ranges and WPNI$_F$. Table 2 shows that, on average, readings from Instrument 2 were consistently higher than Instrument 1 across all types of product; this is perhaps indicative of sensitivity of results to sample preparation and rehydration. The effect of varying rehydration time is discussed in section 3.2.

3.2   Influence of rehydration method

To test the effect of rehydration conditions, SP$_F$ analysis of non-agglomerated powders (SMP and WPC) was studied under two conditions, (i) where samples were analysed immediately after mixing, upon visual observation of rehydration and (ii) after 30 minutes of rehydration. WPC$_{control}$ and SMP115 samples were used to represent high and low levels of SP$_F$ content, respectively. Results listed in Table 3 show that with an increase in rehydration time there

was a decrease in RSD %. This was most likely due to non-agglomerated powders, such as SMP115 and WPC$_{control}$, requiring longer rehydration time, to ensure that the proteins in the sample are fully rehydrated.

The effect of rehydration time on agglomerated infant formula was investigated at two rehydration times, (i) hand shaken for 1 min and (ii) hand shaken for 1 min followed by 10 minutes of standing and then hand shaken for 30 sec, (total hydration time = 11.5 min). MIF72 and MIF115 were chosen as they were at the high and low end of the SP$_F$ range, respectively. Results obtained indicted that rehydration of powders prior to analysis is critical for accurate results (Table 4). Agglomerated powders showed better repeatability, with a % RSD range of 1.7 % - 2.6 % after 11.5 minutes in comparison to the non-agglomerated powders with % RSD range of 5.0 % - 6.6 % after 30 minutes. This would suggest that more attention to rehydration of non-agglomerated powders is required to ensure robust repeatability of results.

3.3   Performance against reference methods

Fifteen batches of non-agglomerated dairy powders (11 SMP and 4 WPC) with various heat treatment profiles were rehydrated (30 mins hold time) and analysed for SP content, by the fluorescence-based analyser (SP$_F$) and by the Kjeldahl (NCN-NPN) method (SP$_{Kjeldahl}$). SP$_F$ and SP$_{Kjeldahl}$ results were highly correlated, with an R^2 value of 0.9931 (Figure 1). The SEest obtained was 0.78 g/100g powder, indicting good precision and reliability of the regression line.

Twelve samples of CIF stored at 2 temperatures (15 and 37 ºC) were rehydrated (10 mins hold time) and analysed for SP content, by the fluorescence-based analyser and by Kjeldahl (NCN-NPN) method (Table 5). The overall accuracy of the fluorescence-based analyser versus Kjeldahl was < 0.2 g/100g powder for CIF brands 2 and 3; however, for brand 1 the

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overall accuracy was between 0.86 and 1.18 g/100 g powder. This is perhaps due to the higher content of tryptophan-rich alpha-lactalbumin in the formula which may have skewed the instrument response (Kelly, Woonton, & Smithers, 2009). Therefore, users must be careful when drawing comparisons between products which exhibit significant differences in tryptophan rich or deficient proteins.

Seven batches of non-agglomerated SMP powder which were heat treated at various temperatures were rehydrated (30 min hold time) and $WPNI_{GEA}$ was analysed by the GEA Niro method and $WPNI_F$ was measured by the fluorescence-based analyser. $WPNI_F$ and $WPNI_{GEA}$ results were highly correlated, with an R^2 value of 0.9932 (Figure 2). Very good precision and reliability of the regression line was confirmed with a SEest of 0.18 g/100g powder.

4   Conclusions and recommendations

The fluorescence-based analyser was evaluated for rapid measurement of SP_F and $WPNI_F$, using the highly sensitive fluorophore, tryptophan, to measure structural changes to the protein. The instrument performed rapid measurements of SP_F and $WPNI_F$, which were highly correlated ($R^2 > 99\%$) with the more time consuming reference methods. However, rehydration time was found to be an important parameter affecting repeatability of results. This was especially true for non-agglomerated powders which due to relatively slower rehydration kinetics exhibited more variability than agglomerated powders. It is recommended that defined sample preparation and rehydration protocols be specified to ensure instrument repeatability and inter-lab reproducibility. It should also be noted that while the current study focused on measurement of SP in dairy powders, the fluorescence based analyser also has potential application for at-line monitoring of SP in liquid dairy and

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IF streams prior to spray drying. Overall, it may be concluded that the fluorescence based analyser (Amaltheys II) when employed according to the recommendations of this study is a robust, rapid at-line PAT tool for measurement of soluble whey protein and heat classification.

Acknowledgement

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Figure legends

Figure 1. Linear regression (---) between fluorescence-based analyser (SP_F) method and Kjeldahl (NCN-NPN) ($SP_{Kjeldahl}$) method for non-agglomerated dairy powders (n=15) (y = 1.063x-0.7629, R^2 = 0.9931, SEest = 0.78 g/100g powder).

Figure 2. Linear regression (---) between $WPNI_F$ and $WPNI_{GEA}$ for non-agglomerated SMP (n=7) (y = 1.1411x - 0.3499, R² = 0.9932, SEest =0.18 g/100g).

Table 1. Process conditions for SMP, WPC and MIF

Product	Reconstituted TS %	Heat treatment	Homogenisation pressure (1st/2nd stage)	Evaporated dry matter	Drying conditions (Inlet/Outlet)
	% w/w	°C	bar	% w/w	°C
SMP$_{control}$^{std}		n/a	n/a	n/a	n/a
SMP72^{std}	10%	72	n/a	30%	180/95
SMP95^{std}	10%	95	n/a	30%	180/95
SMP115^{std}	10%	115	n/a	30%	180/95
WPC$_{control}$^{std}		n/a	n/a	n/a	n/a
WPC72^{std}	10%	72	n/a	30%	180/95
WPC95^{std}	10%	95	n/a	30%	180/95
WPC115^{std}	10%	115	n/a	30%	180/95
MIF$_{control}$^{agg}		none	150/50	50%	175/90
MIF72^{agg}	30%	72	150/50	50%	175/90
MIF95^{agg}	30%	95	150/50	50%	175/90
MIF115^{agg}	30%	115	150/50	50%	175/90
CIF Brand 1^{agg}	Purchased from local retailer; α-lactalbumin enriched				
CIF Brand 2^{agg}	Purchased from local retailer				
CIF Brand 3^{agg}	Purchased from local retailer				

Abbreviation: std – non agglomerated powder; agg – agglomerated powder; SMP – skim milk powder; WPC – whey protein concentrate; MIF – model infant milk formula; CIF – commercial infant milk formula.

Table 2. Repeatability and reproducibility of fluorescence-based analyser

Program	$SP_F{}^1$	$SP_F{}^2$	$WPNI_F$
Product	SMP95	WPC$_{control}$	SMP95
Instrument 1^3 ± RSD (%, n=5)	4.0 ± 4.8	27.6 ± 2.5	3.5 ± 4.0
Instrument 2^4 ± RSD (%, n=5)	4.6 ± 5.0	30.4 ± 2.2	3.9 ± 2.9
Overall RSD (%, n=10)	11.1	6.8	6.8

Abbreviation: SMP – skim milk powder; WPC – whey protein concentrate; SP_F – Soluble protein by Fluorescence-based analyser; $WPNI_F$ – whey protein nitrogen index by Fluorescence-based analyser.

[1]Program range 0.5-24g/100g
[2]Program range 24-94g/100g
[3]Internal laboratory based analyser (Ireland)
[4]External laboratory based analyser (France)

Table 3: Effect of rehydration time on soluble protein content (g/100g of powder) of non-agglomerated powders as measured by the fluorescence-based analyser

Product	Time after mixing, t (min)	k=1	k=2	k=3	k=4	average	SD	RSD (%)
SMP115	0	2.90	2.97	2.34	2.29	2.63	0.36	13.7
SMP115	30	2.61	2.40	2.40	2.62	2.51	0.12	5.0
WPC_{control}	0	42.11	31.28	28.57	27.48	32.36	6.69	20.7
WPC_{control}	30	27.5	27.74	31.28	27.36	28.47	1.88	6.6

4 repeat measurements (n=4) were made, as indicated by k = 1, 2, 3 or 4. Abbreviation: SMP – skim milk powder; WPC – whey protein concentrate.

Table 4: Effect of rehydration time on analysis of agglomerated powders for SP_F (g/100g g of

powder) (n=4)

Product	Rehydration time	
	1 min	11.5 min
MIF72 ± RSD (%)	5.0 ± 7.2	4.8 ± 2.6
MIF115 ± RSD (%)	1.0 ± 4.9	1.0 ±2.1
CIF ± RSD (%)	2.2 ± 4.5	2.5 ±1.7

Abbreviation: MIF – model infant milk formula; CIF – commercial infant milk formula; SP_F
– soluble protein by Fluorescence-based analyser.

Table 5. Comparison of SP_F with $SP_{Kjeldahl}$ method for commercial infant formula (g/100g of powder)

Product	Storage temperature (°C)	SP_F	$SP_{Kjeldahl}$	$SP_F - SP_{Kjeldahl}$
BRAND 1 G	15	2.58	1.58	1.00
BRAND 1 H	15	2.57	1.61	0.96
BRAND 2 I	15	2.26	2.11	0.15
BRAND 2 J	15	2.34	2.22	0.12
BRAND 3 K	15	2.51	2.41	0.10
BRAND 3 L	15	2.53	2.36	0.17
BRAND 1 G	37	2.55	1.69	0.86
BRAND 1 H	37	2.78	1.60	1.18
BRAND 2 I	37	2.18	2.12	0.06
BRAND 2 J	37	2.24	2.28	- 0.04
BRAND 3 K	37	2.45	2.39	0.06
BRAND 3 L	37	2.41	2.40	0.01

Abbreviation: SP_F – soluble protein by Fluorescence-based analyser; $SP_{Kjeldahl}$ – soluble protein by Kjeldahl method

Highlights

- Rapid and accurate at-line process analytical technology for dairy industry.
- Fluorescence-based analyser for replacement of standard analytical methods.
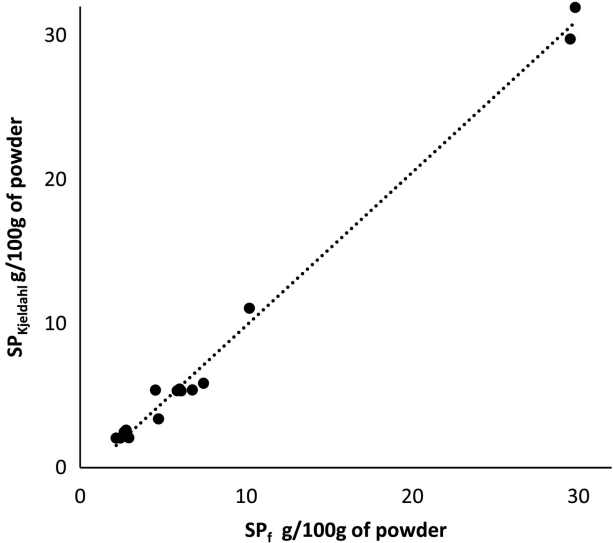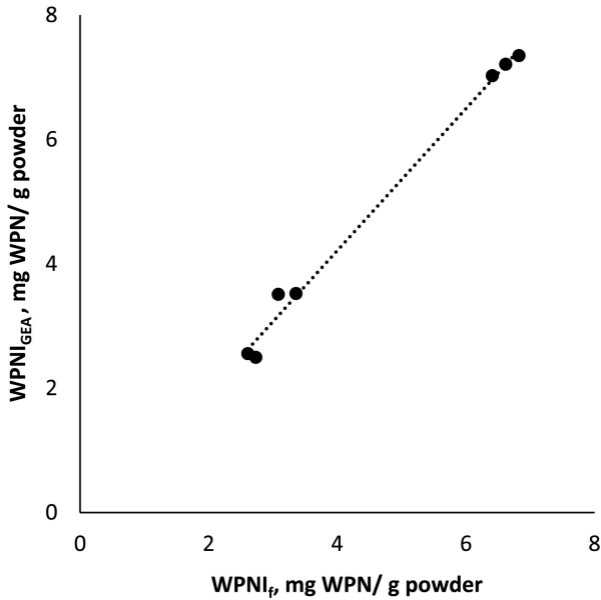- Quantitative measurement of soluble whey protein and whey protein nitrogen index.

Figure 1

Figure 2