ARTICLE

EVALUATION OF THE AMALTHEYS® ANALYZER

The Amaltheys® analyzer is a conventional fluorescence-based patented sensor manufactured and commercialised by Spectralys. Soluble proteins (proteins in solution in the liquid phase, not integrated in the micellar system) can be quantified in milk and liquid dairy products using the principle of tryptophan fluorescence (excitation 280 nm and emission 337 nm) and products of the Maillard reaction. The calculation used is as follows: FAST index = [fluorescence of the Maillard products (excitation 340 nm and emission 430 nm)] / [tryptophane fluorescence (excitation 280 nm and emission 337 nm)] x 100.

This instrument contains internal software, which ensures the signal processing, calibrations and adjustments. To apply the method, the samples need to be prepared (selective precipitation with a buffer supplied and filtration), and the filtrate measured.

The reagents and consumables are supplied by the manufacturer in the form of kits. Freeze-dried standards and controls are also supplied by the manufacturer, to be reconstituted extemporaneously.

The tests:

The evaluation tests were performed in Actilait-Cecalait's physico-chemistry laboratory (instrumental and reference analyses) from April to July 2012. Following the preliminary tests regarding stability, linearity and detection/quantification limits for soluble proteins (PS) and the "Fast" index (IF), the repeatability and accuracy in milk and concentrated whey were evaluated.

For PS, a freeze-dried control sample was used to calibrate the analyzer on the basis of a reference value (non casein nitrogen: ANC – non protein nitrogen: ANP). Following the calibration, the analyzer was verified using a freeze-dried milk sample on the basis of an ANC-ANP value. The analyzer was also checked before each series of measurements.

A- PRELIMINARY TESTS

A.1- Evaluation of the short-term stability

16 series of 3 milk samples (raw, pasteurised and UHT) were analysed in consecutive duplicate every 15 minutes over about 4 hours. PS and IF were noted.

The relative standard deviations of reproducibility obtained for PS varied between 2.9 and 5.0 % according to the type of milk.
The relative standard deviations of reproducibility for IF were equivalent for all the types of heat treated milk (between 4.1 and 4.3 %).

A.2- Evaluation of the linearity for soluble proteins

A set of 10 milk samples ranging evenly from about 0 to 6 g/l was obtained by mixing raw and UHT milk. The volume/volume dilutions were obtained according to the adjusted weight of the densities. Each sample in the range was analysed in consecutive duplicate.

On the basis of the results observed, the response of the analyzer is linear for PS values between 0 and 6 g/l.

A.3- Evaluation of the detection and quantification limits

Regular dilutions of UHT milk and water, below PS values of 50 mg/l, were obtained according to the adjusted weight of the densities. The samples were analysed in quadruplicate. The detection and quantification limits were determined according to standard NF V 03-110: 1998.

On the basis of the results observed, the limits were calculated as follows: Uld (detection) = 4 mg/l and Ulq (quantification) = 11 mg/l.

The detection and quantification limits of the Amaltheys analyzer are relatively low for "soluble protein" with regard to the other existing methods for proteins.

B- MILK

B.1- Samples

The tests were performed on raw, pasteurised and UHT milk samples. Raw milk consisted of tanker and farm milk. The pasteurised and UHT milk was bought in supermarkets and hypermarkets. Bronopol was added to the samples to give a final concentration of 0.02 %.

Three series of 15 samples were made up. Each series was prepared with a type of milk (raw, pasteurised or UHT) and a corresponding bulk milk (raw-pasteurised, pasteurised-UHT and UHT-pasteurised) to produce a range of concentrations.

B.2- Procedure

The repeatability of the analyzer was evaluated using all the milk samples for both parameters (PS and IF). The accuracy was evaluated using all the milk samples for the PS parameter. The quantitative analysis of each sample was carried out in consecutive duplicate and a control milk sample was analysed at the beginning and at the end to verify the stability of the instrument.

The following reference methods, ISO 17997 / IDF 29 for non-casein nitrogen (ANC), NF EN ISO 8968-5 / IDF 20-5 for non-protein nitrogen (ANP) and method according to Rowland[(1)] (non-casein nitrogen after ANC_d denaturing) were used to evaluate the accuracy.

The reference values were calculated as follow: PS = (ANC – ANP) x 6.38 and proteose-peptone fraction (PP) = (ANC – ANC_d) x 6.38.

B.3- Results

The results obtained are presented in the tables and figures below:

		n	min	max	M	Sx	Sr	Sr (%)	r
RAW MILK	**PS (g/l)**	15	4.51	5.37	5.00	0.26	0.10	2.07	0.29
	IF	15	0.00	8.33	2.70	2.43	0.17	6.24	0.47
PASTEURISED MILK	**PS (g/l)**	15	1.46	5.25	3.95	1.27	0.11	2.81	0.31
	IF	15	3.36	27.84	9.68	8.09	0.34	3.56	0.96
UHT MILK	**PS (g/l)**	15	0.78	2.25	1.28	0.40	0.09	6.76	0.24
	IF	15	19.41	75.49	41.31	17.23	2.26	5.46	6.25
OVERALL	**PS (g/l)**	45	0.78	5.37	3.41	1.76	0.10	2.95	0.28
	IF	45	0.00	75.49	17.90	20.14	1.32	7.38	3.66

Table 1: AMALTHEYS repeatability criteria for PS and IF in milk samples
n: number of results; min and max: minimum and maximum values; M and Sx: mean and standard deviation of the results; Sr and Sr%: absolute and relative standard deviation of repeatability; r: maximum deviation of repeatability in 95 % of cases.

		n	min	max	Y	Sy	d	Sd	Sy,x	Sy,x%	b	a
RAW MILK	**PS (g/l)**	15	4.87	6.12	5.67	0.38	-0.67	0.15	0.099	1.99	1.427	-1.46
PASTEURISED MILK	**PS (g/l)**	15	1.77	5.40	4.12	1.21	-0.17	0.12	0.104	2.65	0.948	0.37
UHT MILK	**PS (g/l)**	15	0.99	2.08	1.54	0.37	-0.26	0.15	0.140	10.96	0.858	0.44
OVERALL	**PS (g/l)**	45	0.99	6.12	3.78	1.88	-0.37	0.26	0.241	7.08	1.059	0.17

Table 2: AMALTHEYS accuracy criteria for PS in milk samples
n, min, max: number of results, minimum and maximum values; Y,X: mean results using the reference and instrumental methods; Sy: standard deviation of the results from the reference method; d, Sd: mean and standard deviation of deviations; Sy,x and Sy,x%: absolute and relative residual standard deviation; b, a: slope and intercept of the linear regression

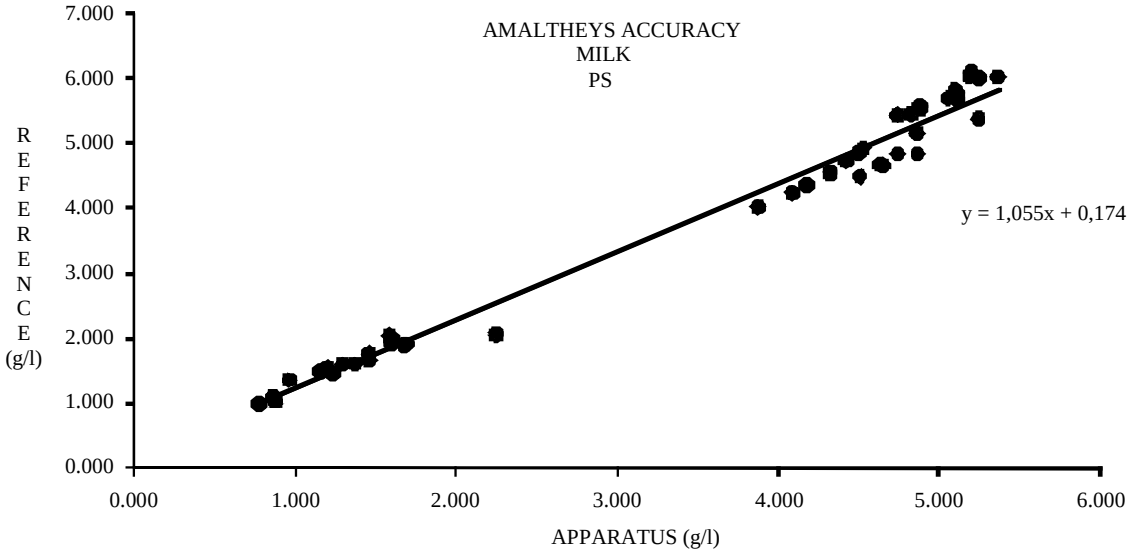

Figure 1: Relationship between the AMALTHEYS and reference results for PS in milk samples

For PS, it can be noted a regression slope of 1.055, significantly different from 1.00 at the 5 % threshold, and an intercept of +0.174 g/l (not significant at the 5 % threshold). The residual standard deviation of regression is 0.214 g/l. The causes of the deviations between the two methods were examined (-0.17 to –0.67 g/l). It was found that one of the major "soluble protein" fractions, the proteose peptones (from the proteolysis of β-casein by plasmin, present in quantities of about 1 g/l in raw milk), do not contain a tryptophan residue and consequently are not measured by the Amaltheys instrument.
An additional examination of the data was then performed to compare the results obtained with the Amaltheys instrument calibrated with the ANC-ANP-PP fraction (with a coefficient, calculated on the basis of the control sample composition, being applied directly to the results obtained for the ANC-ANP calibration) and the soluble protein content obtained using the Kjeldahl method minus the proteose peptone concentration (i.e. ANC-ANP-PP).

The results are summarised in the table and figures below:

	n	min	max	Y	Sy	d	Sd	Sy,x	Sy,x%	b	a
PS-PP (g/l)	45	0.99	6.12	3.78	1.88	0.36	0.23	0.142	5.61	1.139	-0.71

Table 3: AMALTHEYS accuracy criteria for PS-PP and (PS-PP + α-LACTA) in milk samples
n, min, max: number of results, minimum and maximum value; Y,X: mean results using the reference and instrumental methods; Sy: standard deviation of the results from the reference method; d, Sd: mean and standard deviation of deviations; Sy,x and Sy,x%: absolute and relative residual standard deviation; b, a: slope and intercept of the linear regression

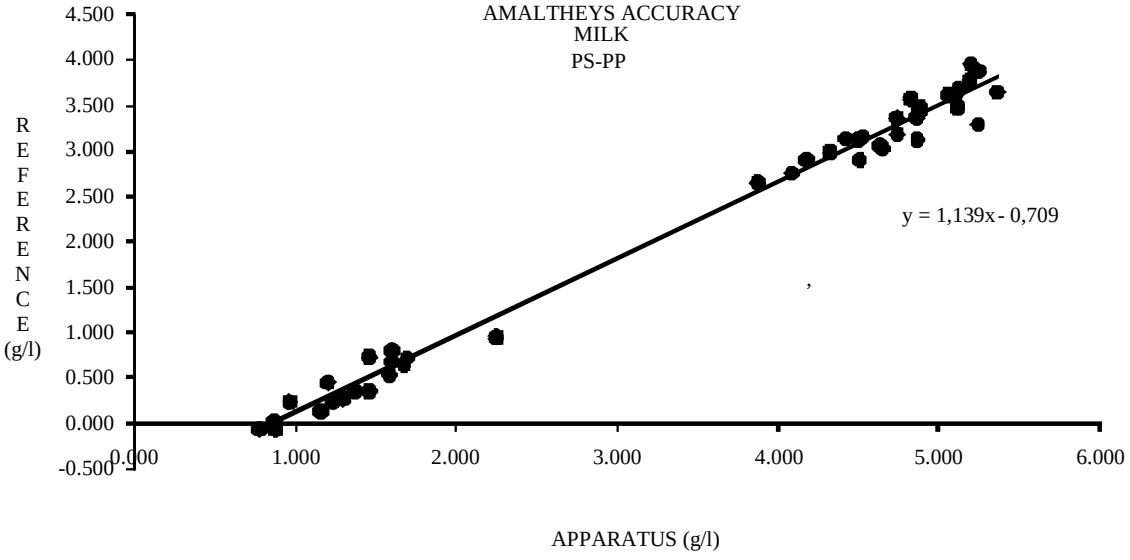

Figure 2: Relationship between the AMALTHEYS and reference methods for PS-PP in milk samples

For PS–PP the regression slope and the intercept are 1.139 and –0.709 respectively, significantly different from 1.00 and 0.00 at the 5 % threshold. The residual standard deviation of this regression is 0.142 g/l.

B.4- Conclusion

Concerning soluble proteins, the standard deviations of repeatability obtained are similar for the 3 types of milk at a level of about 0.10 g/l. The values obtained are in accordance with the recommendations of the ISO 17997/IDF 29: 2004 standard for the determination of non casein nitrogen in milk (Sr = 0.092 g/l).
Concerning the Fast index, the standard deviations of repeatability obtained vary according to the levels observed in the samples. The overall relative standard deviation (for all the samples) is 7.4 %.

For PS, the residual standard deviation of linear regression (calculated for all the types of milk) is 0.241 g/l, hence an estimation accuracy for this method and this parameter of ± 0.482 g/l. For PS-PP, the residual standard deviation of regression is 0.142 g/l (estimation accuracy of ± 0.28 g/l) but there is a significant slope adjustment defect (about 14 %) and a mean bias of 0.36 g/l for the products and range studied.

C- CONCENTRATED WHEY

C.1- Samples

The tests were performed on concentrated whey samples. Three whey samples (fabrication of pressed cooked cheese, pressed cheese and soft cheese) were collected and then concentrated by ultrafiltration on a 10KD membrane. Bronopol was added to the samples to give a final concentration of 0.02 %.

A set of 12 samples was constituted by mixing the different concentrated whey samples to produce a range of concentrations.

C.2- Procedure

The repeatability and the accuracy of the instrument were evaluated for PS using all the concentrated whey samples. The quantitative analyses of each sample were carried out in consecutive duplicate and a control milk sample was analysed at the beginning and at the end to verify the stability of the instrument.

To evaluate the accuracy, the same reference methods as for milk were used with suitable test samples (cf. B.2).

C.3- Results

The results obtained are summarised in the tables and figures below:

	n	min	max	M	Sx	Sr	Sr (%)	r
PS (g/l)	12	32.11	50.67	41.52	6.50	0.99	2.38	2.74

Table 4: AMALTHEYS repeatability criteria for PS in concentrated whey samples
n: number of results; min and max: minimum and maximum values; M and Sx: mean and standard deviation of the results; Sr and Sr%: absolute and relative standard deviation of repeatability; r: maximum deviation of repeatability in 95 % of cases.

	n	min	max	Y	Sy	d	Sd	Sy,x	Sy,x %	b	a
PS (g/l)	12	31.12	51.42	41.27	7.02	0.25	2.88	3.015	7.26	0.986	0.35

Table 5: AMALTHEYS accuracy criteria for PS in concentrated whey samples
n, min, max: number of results, minimum and maximum value; Y,X: mean results using the reference and instrumental methods; Sy: standard deviation of the results from the reference method; d, Sd: mean and standard deviation of deviations; Sy,x and Sy,x%: absolute and relative residual standard deviation; b, a: slope and intercept of the linear regression

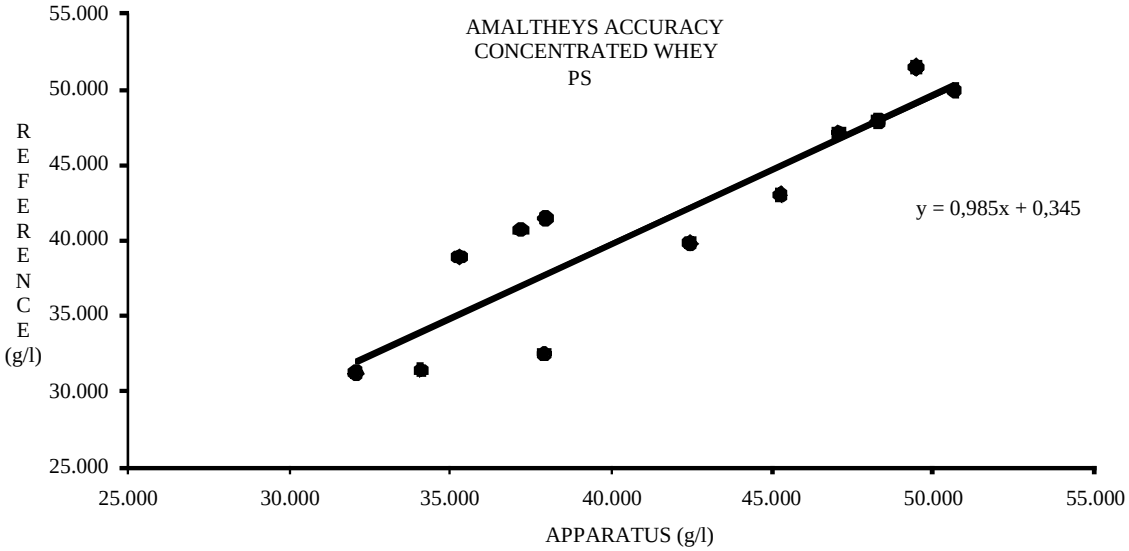

Figure 3: Relationship between AMALTHEYS and the reference results for PS in concentrated whey samples

For PS, a linear regression slope of 0.985 and an intercept of +0.345 were observed, not significantly different from 1.00 and 0.00 respectively (at the 5 % threshold). The residual standard deviation is equal to 3.015 g/l.

An additional examination of the data was performed using the same approach as for milk. The results are presented below:

	n	min	max	Y	Sy	d	Sd	Sy,x	Sy,x %	b	a
PS-PP (g/l)	12	25.15	39.08	32.21	5.08	-1.45	0.75	0.759	2.47	1.043	0.12

Table 6: AMALTHEYS accuracy criteria for PS-PP in concentrated whey samples
n, min, max: number of results, minimum and maximum value; Y,X: mean results using the reference and instrumental methods; Sy: standard deviation of the results from the reference method; d, Sd: mean and standard deviation of deviations; Sy,x and Sy,x%: absolute and relative residual standard deviation; b, a: slope and intercept of the linear regression.

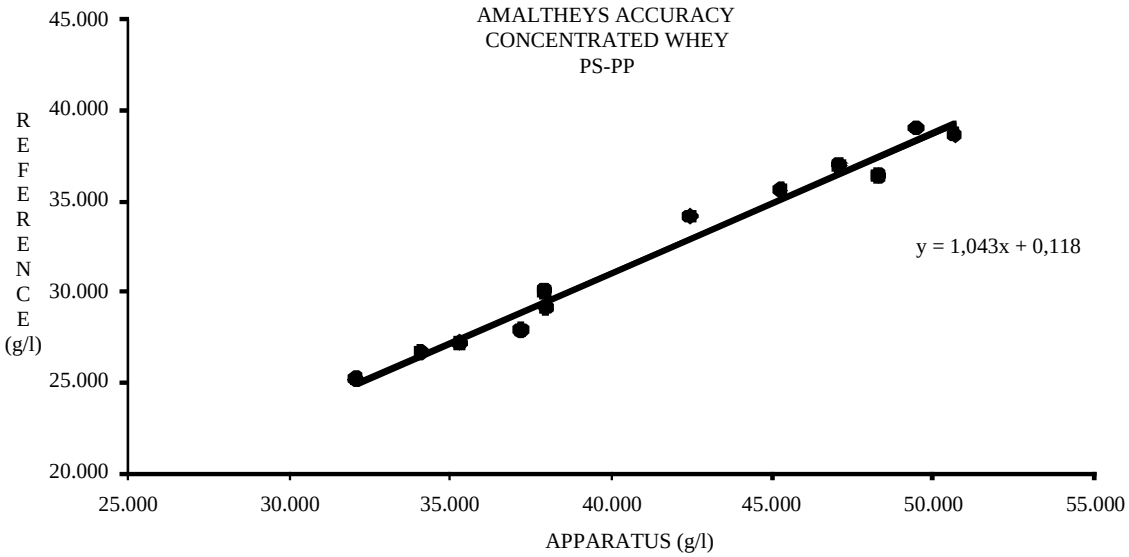

Figure 4: Relationship between AMALTHEYS and reference results for PS-PP in concentrated whey samples

For PS-PP, a regression slope and an intercept 1.043 and –0.118 respectively were observed. The residual standard deviation is 0.759 g/l.

C.3- Conclusion

For PS, the standard deviation of repeatability obtained is 0.99 g/l, which corresponds to a relative value of 2.38 %.

The determination of PS-PP using the right calibration significantly improves the estimation accuracy of the instrument (± 6.030 g/l to ± 1.518 g/l) for concentrated whey.

GENERAL CONCLUSION

Firstly, the good repeatability of the Amaltheys instrument can be noted for the determination of soluble proteins and other fractions measured during the additional examination of the data.

Concerning the accuracy of the instrument for these criteria, it can be concluded that:

A correlation (for milk and whey) between the Amaltheys and reference methods for both PS and PS-PP (with different estimation accuracies) was observed. Indeed, the "proteose-peptone" fraction of milk and liquid dairy products cannot be measured using the Amaltheys method due to the absence of a tryptophan residue on the corresponding peptides. As a result, the estimation accuracy (PE) of this method could be significantly improved by examining the soluble protein fraction minus the "proteose-peptone" fraction (PS-PP = ANC-ANC_d) instead of the soluble protein fraction alone (PS = ANC-ANP), and this for the milk (PE of ±0.48 to ±0.28 g/l) and concentrated whey samples (PE of ±6.03 to ±1.52 g/l).

For milk samples, the slope and the intercept of Amaltheys vs. the reference method are significantly different from 1.00 and 0.00 respectively for both PS (b = 1.059 and a = +.017) and PS-PP (b = 1.1.39 and a = -0.71), indicating a deviation between the two methods (mean deviation of –0.37 and +0.36 g/l respectively. After consideration, it was found that the "proteose peptone" fraction, determined according to Rowland, contains a portion of non-denatured α-lactalbumin (on the basis of the Dannenberg and Kessker[(2)] tables), for which a signal is obtained with the Amaltheys method. This was also confirmed by the qualitative HPLC analysis of the PP filtrate (using Spectralys). Within the context, if the totality of this residual fluorescence was assign to non- denatured α-lactalbumin, the bias of the slope could thus be significantly reduced by integrating this factor. Nevertheless, the intercept would still be significantly different from 0.00, corresponding to the initial PS-PP regression. This statement could be confirmed by specific analyses of α-lactalbumin in the standard and milk samples.

For concentrated whey, the deviations observed were lower (in relative %) than for milk.

To conclude, investigations (and confirmations) must then be carried out to discover a technical and scientific explanation concerning the accuracy deviations observed during this evaluation for the different types of samples tested. Many possibilities could then be studied (precipitation reagent, filtration process, prediction model of the sensor, or milk composition parameter) for a good understanding of the measurement and possible adjustment.

Finally, the repeatability obtained for the Fast index for drinking milk (pasteurised and UHT) was good (Sr % of 3.5 and 5.5 %, respectively). However, a relevant descriptor must be defined for an accuracy study. Indeed, the first tests concerning furosin in UHT milk were not suitable because the milk furosin content decreased during storage, and these tests were carried out on samples for which the duration of storage varied after production.

Bibliography :

[(1)] ROWLAND S.J. The determination of the nitrogen distribution in milk. Journal of Dairy Research, 1938, V. 9, p. 42-46.
[(2)] – DANNENBERG F. et KESSLER H.G. Application of reaction kinetics to the denaturation of whey proteins in heated milk. Milchwissenchaft, 1988, 43, 3-7
[(3)] - CORZO N., LOPEZ-FANDINO R., DELGADO T., RAMOS M., OLANO A. Changes in furosine and proteins of UHT treated milks stored at high ambient temperatures. Lebensmittel-Untersuchung und-Forschung, 1994, 198, 302-306

According to the Amaltheys® analyzer evaluation report (part 1) - X. QUERVEL and Ph. TROSSAT – October 2012