Vitamin B_{12} in Muscle Foods. Comparison of a Microbiological Assay and a Fully Automated Chemiluminescence System for the Determination of Vitamin B_{12} in Fresh and Processed Meat.

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Abstract— **Aim of this study was to assess the performance of a fully automated chemiluminescence analyser in the determination of Vitamin B_{12} in fresh and processed meat. To this end Vitamin B_{12} values obtained by the chemiluminescence method (CHM) were compared with the reference microbiological assay (MBA)(Official method AOAC 952.20). The efficacy of two different extractive protocols from meat matrices for Vitamin B_{12} were also evaluated.**

A full factorial design was applied to develop an appropriate analytical protocol (extraction and analytical methods) for the assay of the vitamin in fresh and processed meat. The experimental design was based on two methods of extraction of the vitamin (enzymatic hydrolysis vs thermal hydrolysis) and two analytical methods (CHM vs MBA).

Fresh meat muscles (N= 20) as well as dry cured and cooked meat products (N= 21) were from local market.

Significant correlation between the methods was observed (r= 0,99) for all the meat matrices.

Validation data of CHM showed that the method is more selective and precise than the MBA.

Thermal hydrolysis released a larger amount of vitamin in fresh and dry cured meat samples while enzymatic hydrolysis proved more effective in cooked meat samples where non-meat ingredients are usually present.

It can be concluded that the automated chemiluminescence method enables Vitamin B_{12} to be determined in fresh and in processed meat products in a more precise, rapid way than with conventional microbiological assay.

Keywords— **Vitamin B_{12}, meat and meat products, chemiluminescence method.**

I. INTRODUCTION

Vitamin B_{12} is mainly present in animal food material, including milk, eggs and above all, meat and meat products. Assay of naturally occurring B_{12} in food matrices is difficult owing to the low concentration normally present (less then 1 μg/100g) and the cobalamin forms lability [1].

Methods frequently used for Vitamin B_{12} determination in food include a microbiological assay that uses *Lactobacillus leichmannii* or *delbrueckii lactis* ATCC 7830 as a test organism, radioisotopic assay, spectrophotometric, electrophoretic, various chromatographic methods, chemiluminescence assay and MALDI and TOFMS techniques [1-3]. All the above mentioned methods have their own analytical advantages but at the same time they have certain drawbacks, including high costs, time-consuming test or less specificity and often they require handling of cyanide salts to make the cobalamin forms stable.

Aim of this study was to assess the performance of a fully automated chemiluminescence analyser in the determination of Vitamin B_{12} in fresh and processed meat. To this end Vitamin B_{12} values obtained by the chemiluminescence method (CHM) were compared with the reference microbiological assay (MBA) (Official method AOAC 952.20). The efficacy of two different extractive protocols from meat matrices for Vitamin B_{12} were also evaluated.

II. MATERIALS and METHODS

A full factorial design was applied to develop an appropriate analytical protocol (extraction and analytical methods) for the assay of B_{12} Vitamin in fresh and processed meat. The experimental design adopted was based on two methods of extraction of the vitamin: Enzymatic hydrolysis (ENZ) vs Thermal hydrolysis (TRM) and two analytical methods: Chemiluminescence (CHM) vs Microbiological Assay (MBA), as shown in Table 1.

Table 1: The experimental ranges and levels of independent variables.

Levels	*Analitical method*	*Extraction protocol*
−1	Chemiluminescence assay	EnzymaticHydrolysis
+1	Microbiological assay	Thermal Hydrolysis

Different meat matrices (N=3) were tested to evaluate the analytical performances of the methods. A fresh pork fillet cut, dry cured ham and cooked ham were purchased from the local market and analysed. N=10 replicates for each extraction protocol and for each analytical methods were analysed for every meat matrix according to the full factorial design requirements. (N=40 data for each meat matrix).
In order to evaluate the content of Vitamin B_{12} in meat products and validate the analytical procedure, several fresh pork meat cuts (N= 20) as well as dry cured (N=11) and cooked meat products (N= 10) were submitted to both ENZ and TRM hydrolysis. Vitamin B_{12} were quantified by CHM method. Regression analyses were performed to study the effect of different extraction protocols on each meat matrix.

Microbiological assay: Microbiological test kits for the determination of Vitamin B_{12} in meat samples were from Immunodiagnostik (Bensheim, Germany): the ID-Vit® Vitamin B_{12} is a microtiter plate test kit based on a microbiological assay (*Lactobacillus delbrueckii lactis* ATCC 7830) which measures the total Vitamin B_{12} content in samples according to the AOAC Method 960.46. [4]

Chemiluminescence assay: A fully automated chemiluminescence analyser ACCESS 1 (Beckman Coulter, Milan) was used for the quantitative determination of Vitamin B_{12} level in samples: the Access Vitamin B_{12} assay is a competitive binding immunoenzymatic assay. Samples are automatically added to a reaction vessel along with alkaline potassium cyanide and dithiothreitol, converting all forms of Vitamin B_{12} to the cyanocobalamin form. After neutralization, intrinsic factor conjugate (IF) and particles coated with monoclonal anti-intrinsic factor are added to the sample in order to strictly bind Vitamin B_{12} to the intrinsic factor conjugate, and wash away all the other compound to avoid interferences. Then, the chemiluminescent substrate is added to the vessel and the light generated by the reaction with B_{12}-binding protein (IF) is measured with a luminometer. The light production is inversely proportional to the concentration of Vitamin B_{12} in the sample. The amount of analyte in the sample is determined from a multi-point calibration curve. [5]

Thermal hydrolysis: Vitamin B_{12} active compounds are extracted in phosphate buffer 0.1 M containing 1% citric acid and 1% sodium metabisulfite 1% in order to protect the cobalamins throughout the extraction. (AOAC Official method 952.20, [4]). 10 g of meat sample are homogenised with 40 ml of phosphate buffer, homogenised wit an Ultraturrax for 1 minute. Autoclave at 121°C for 10 minutes to complete the extraction of Vitamin B_{12}. After the thermal hydrolysis pH sample solution have to be adjust to to 4.5 for the following MBA analysis, for CHM determination to 7.0. Adjust the final volume to 50 ml.

Enzymatic hydrolysis: 10 g of meat sample are suspended in 30 ml of phosphate buffer 0.1 M pH 4,5 and disrupted by an homogenizer. Add 1 ml of 5% α-amilasi solution and incubate at 38-45°C for 4 hours, then 5 minutes at 90°C to stop the enzymatic reaction and adjust the volume to 50 ml. PH has to be adjust as before reported.

III. RESULTS

Table 1 shows results of full factorial design related to each meat matrix sample. P-values of GLM analysis show the significance of the two principal factors on Vitamin B_{12} determination. Although mean values from microbiological assay are similar to those determined by the chemiluminescence method, they are always significantly lower with all the meat matrices analysed, except for cooked ham, where mean values are not significantly different.

Moreover the thermal extraction protocol - in presence of a protective compound - results in statistically higher values than with the enzymatic procedure.

Vitamin B_{12} contents in cooked meat samples extracted by the enzymatic protocol were always statistically higher than those obtained after the thermal hydrolysis.

Between-run precision of microbiological and chemiluminescence method were also different; in fact the coefficients of variation ranged from 10.5 to 27.6% with the microbiological method and from 3.9 to 9% using the chemiluminescence method. (Data not shown)

Table 2: Data (mean ± std. dev.) of full factorial design related to principal factors (Analytical method and Extraction protocol). Each data set is referred to a single meat matrix. Data are expressed in μg/100g.

			ANALYSIS METHOD	
			CHM	MBA
FILLET	***EXTRACYION METHOD***	*ENZYMATIC .*	0.34 ± 0.01	0.28 ± 0.05
		THERMAL.	0.50 ± 0,04	0.45 ± 0.07
		P analysis	**0.0001**	
		P extraction	**0.0001**	
DRY CURED HAM	***EXTRACYION METHOD***	*ENZYMATIC*	0.68 ± 0.06	0.58 ± 0.09
		THERMAL.	0.75 ± 0.07	0.65 ± 0.07
		P analysis	**0.0001**	
		P extraction	**0.003**	
COOKED HAM	***EXTRACYION METHOD***	*ENZYMATIC .*	0.13 ± 0.01	0.12 ± 0.03
		THERMAL.	0.09 ± 0.01	0.09 ± 0.01
		P analysis	**0.206**	
		P extraction	**0.001**	

Several meat samples (N=41) including raw meat (liver, fillet, loin and shoulder), dry cured meat (salami and dry cured ham) and cooked meat samples (cooked ham and mortadella) were analyzed by Chemiluminescence after both enzymatic and thermal extraction, in order to confirm data obtained from previous set of samples and to verify the best extraction protocol for each meat matrix.

Comparison of Thermal and Enzymatic extraction protocol in different meat matrices is reported in figure 1, where each regression line is referred to a single meat matrix (raw meat samples, dry cured meat products and cooked products).

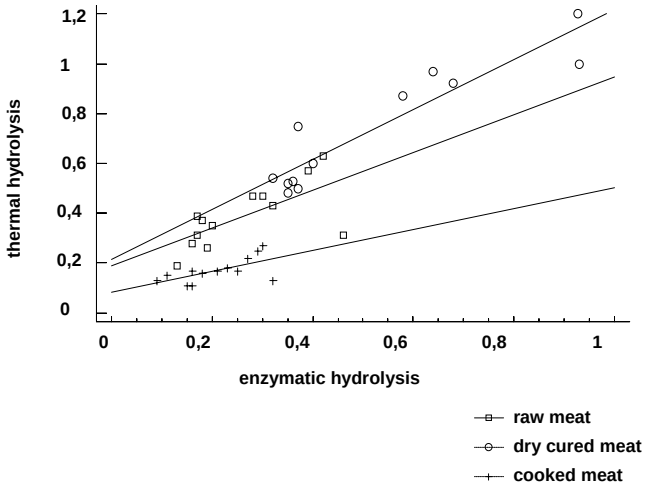

Fig.1 Comparison of B_{12} contents obtained after Thermal and enzymatic hydrolysis in different meat samples (raw meat, dry cured meat and cooked meat samples).

For raw meat (including liver samples) and dry cured meat, the observed correlation coefficient between the extraction method is 0.986, indicating that the two methods are well correlated. For cooked meat products correlation coefficient is 0.628.

IV. DISCUSSION

For all the meat matrices the chemiluminescence method was more precise and selective than the microbiological one, also thanks to the most specific B_{12}-binding protein (IF). Chemiluminescence method is fully automatic and allows not to handle toxic compounds.

For raw meat (including liver samples) and dry cured meat, thermal hydrolysis is able to release larger quantities of Vitamin B_{12} than the enzymatic

extraction while for most of cooked meat products enzymatic hydrolysis yields larger amounts of Vitamin B_{12}.

V. CONCLUSIONS

The automated chemiluminescence method enables Vitamin B_{12} to be determined in fresh and in processed meat products in a more precise, rapid way than with conventional microbiological assay.

Thermal hydrolysis releases a larger amount of vitamin in fresh and dry cured meat samples while enzymatic hydrolysis proves cooked meat samples where non-meat ingredients are usually present.

VI. ACKNOLEDGMENTS

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VII. REFERENCES

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