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INDUSTRIAL FEED MIXTURES WITH AN ADDITION OF METHIONINE HYDROXY ANALOG

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ABSTRACT

HMB (2-hydroxy-4-(methythio)butanoic acid), which is the precursor of methionine in the organism, is widely used in the production of industrial feeds as an addition supplementing the deficiency of methionine proper. HMB does not have any amino group, which is the reason why it cannot be marked within the analysis of the aminoacidic composition using the reaction with ninhydrine reagent. In the method of marking HMB using HPLC technique the conditions of chromatographic division were modified. The applied system of chromatographic columns and movable and eluating phases enabled effective separation of HMB from the elements of differentiated matrixes of industrial feed mixtures. The analytical proceedings are characterized by good validation parameters, i.e. high selectivity, high repeatability and a high degree of recovery of contents occurring in the production practice of industrial feeds ranging from 0.05 to 0.4%.

Key words: industrial feed mixtures, methionine hydroxyl, determination method.

INTRODUCTION

Contemporary production of industrial feeds is connected with the use of aminoacid additives, which enable to supplement the shortage of exogenous aminoacids, for example methionine, to the level corresponding to the animals' demand. In industrial practice, two types of additives supplementing the level of methionine are used: synthetic DL-methionine (Met) and methionine hydroxy (HMB), which in the animals' organism is the precursor of methionine proper. A limited access to natural sources of methionine (for example, a ban on the use of animal utilization meals), increased technological possibilities of precise dosage of liquid components as well as the economic factor cause that the use of methionine hydroxy in the production of industrial feeds has a wider and wider range. For that reason, both the producers and receivers of feeds express their interest in controlling the HMB level in industrial feed mixtures.

The natural property of HMB is the tendency to create polymerized forms – dimmers and trimmers, which, like monomer, are biologically useful. A satisfying control of the HMB content in industrial feeds requires marking

polymeric forms, aside monomer. Ontiveros et al. [3] analyzed the content of HMB in feeds with the use of HPLC technique, through the marking of monomer content; however, after earlier alkaline depolimerization of polymeric forms. HPLC technique applied to determine HMB content in feeds is also recommended by the producer of the commercial preparation Rhodimet^{TM} AT 88 [1].

The use of so-called variant recipes in the production of industrial feeds causes that in practice the analyst encounters a differentiated and unknown composition of so-called matrix, i.e. the elements of a feed mixture that in many cases made it hard to isolate and at times even make it impossible to mark HMB in the environment of a feed mixture.

The purpose of the paper was to develop and evaluate a modified method of determining HMB (with the use of HPLC) in industrial feed mixtures with differentiated content of raw materials.

MATERIAL AND METHODS

The material for studies consisted of industrial feed mixtures with an addition of HMB (tab. 1). Those were full-ratio feed mixtures intended for the poultry with a required high level of methionine, differentiated composition of raw material and with a dominating proportion of Polish feed material (cereals, oil meal, fish meal). The aim was to check the effect of differentiated matrixes on the results of markings. Totally, 15 industrial feed mixtures with an addition of HMB were used in the analytical tests.

Table 1. The composition and content of HMB In the analyzed industrial feed mixtures

No.	Kind of feed mix	Basic elements	Declared HMB content in g/kg	Determined HMB content, in g/kg
1.	For broiler chickens, Starter	Maize, wheat bran, soybean ground grain, fish meal, plant oil, chalk, calcium phosphate, mineral-vitamin pre-mix	2.54	2.48
2.	For broiler chickens, Starter	Maize, wheat, wheat bran, soybean ground grain, fish meal, yeast, chalk, plant oil, calcium phosphate, mineral-vitamin pre-mix	2.55	2.47
3.	For broiler chickens, Finisher	Maize, wheat, barley, soybean ground grain, sunflower ground grain, barley seedlings, plant oil, chalk, calcium phosphate, mineral-vitamin pre-mix	2.11	1.98
4.	For broiler chickens, Starter	Maize, wheat, wheat bran, soybean ground grain, fish meal, plant oil, chalk, calcium phosphate, mineral-vitamin pre-mix	2.55	2.42
5.	For broiler chickens, Starter	Maize, wheat, soybean ground grain, fish meal, plant oil, chalk, calcium phosphate, mineral-vitamin pre-mix	2.59 a	2.22 b
6.	For layers	Maize, barley, wheat bran, soybean ground grain, sunflower ground grain, chalk, calcium phosphate, mineral-vitamin pre-mix	1.33	1.28
7.	For layers	Maize, barley, wheat bran, soybean ground grain, sunflower ground grain, chalk, calcium phosphate, mineral-vitamin pre-mix	1.51	1.50
8.	For layers	Maize, barley, wheat, soybean ground grain, sunflower ground grain, chalk, calcium phosphate, mineral-vitamin pre-mix	1.42	1.38
9.	For layers	Maize, barley, wheat bran, soybean ground grain, sunflower ground grain, chalk, calcium phosphate, mineral-vitamin pre-mix	1.34	1.29
10.	For breed chickens	Maize, wheat, wheat bran, soybean ground grain, plant oil, chalk, calcium phosphate, mineral-vitamin pre-mix	1.91	2.01
11.	For breed chickens	Maize, wheat, wheat bran, barley, soybean ground grain, sunflower ground grain, barley seedlings, plant oil, chalk, calcium phosphate, mineral-vitamin pre-mix	1.41	1.37
12.	For breed chickens	Maize, wheat, wheat bran, soybean ground grain, plant oil, chalk, calcium phosphate, mineral-vitamin pre-mix	2.00	2.03
13.	For breed chickens	Maize, wheat, wheat bran, soybean ground grain, plant oil, chalk, calcium phosphate, mineral-vitamin pre-mix	1.97	2.03
14.	For household layers	Maize, wheat, wheat bran, soybean ground grain, chalk, calcium phosphate, mineral-vitamin pre-mix	1.43	1.39
15.	For household layers	Maize, wheat, wheat bran, soybean ground grain	1.43	1.40

Values marked with different letter are statistically significantly different at $P < 0.05$

Apparatus and reagents. The basic laboratory equipment (laboratory mill, shaker, test-tube centrifuge, analytical balance) as well as a set HPLC Dionex with a feeder of samples and with Array's diode applied as a detector were used for the analyses.

The reagents used to make the motion phase were of chromatographic purity – for HPLC, while the others, used to prepare the sample, i.e. orthophosphorus acid, potassium hydroxide were pure for the analysis.

Sample preparation. 10 g of finely ground feed was weighed into a conical flask with the volume of 200 ml (the size of the particles was 0.5 mm at the maximum) with the accuracy of 0.01 g. Exactly 100 ml of 10% extract solution (10% acetonitrile in water) was added and shaken off onto a shaker for 30 minutes. The exact was taken to a glass centrifugal tube and separated by centrifuge at 4000 g_n for 10 minutes. Exactly 5 ml of clear supernatant was taken to a propylene centrifugal tube containing 0.2 ml of 50% solution of potassium hydroxide. The tube was shaken for 30 seconds. Next, 0.2 ml of 85% orthophosphorus acid was added to the tube. The whole was shaken again for seconds, and then separated by centrifuge at 4000 g_n for 5 minutes. The clear supernatant was filtered through a nylon syringe filter with the pores of 0.2 μm to the flask of the sample feeder and portioned on the column.

HPLC analysis. Two chromatographic columns with the diameter of 4.6 mm and the total length of 500 mm, the grain size of 5 l'm, the reversed phase C18 were connected in series (LiChrospher, Merck). The elution was performed using a mixture of acetonitrile and the hydrous solution of orthophosphoric acid with the concentration of 0.01 mol/l, mixed in the proportion of 1:9 (v/v) – movable phase "A", while the column was cleaned for 15 minutes with a mixture of water and acetonitrile in the proportion 1:1 v/v as the movable phase "B". Besides, before each analysis the system of columns was conditioned washing with the movable phase "A" for 10 minutes.

Conditions: temperature of the column 25°C, the flow of the eluent 0.8 ml/min, time of the analysis 30 min, the length of the detector's wave 214 mm, the volume of the dosage 20 l'm, the measurement of the peak area HMB.

Calibration. An 88% preparation was used as the model substance, where the content of HMB was precisely determined using the method of biamperometric titration with sodium hypobromiate [2].

The basic model solution with concentration of about 4.4 mg HMB/ml was prepared in a measuring flask of 100 ml, weighing 0.5 mg of the preparation and complementing the content of the flask to the mark with a 10% solution of acetronitile in water (solution for extraction). Considering the result of titration, a precise content of HMB in the solution was established.

The working model solutions HMB in 10% acetonitrile were prepared introducing, respectively, 1, 2, 5, 8 and 10 ml of the basic model solution into the successive 100 ml measuring flasks. The content of the flasks were filled up to the mark with 10% acetonitrile. The content of HMB in the working model solutions was calculated.

Considering the procedure (size of the weighed amount and the dilution) the enumerated concentrations of HMB model working solutions covered the range of HMB content in the mixtures between 0.05 and 0.4%.

Next, for the purpose of hydrolysis of polymeric forms HMB, strictly 5 ml of each working model solutions (in three repetitions) was sampled into the centrifuge flasks and the following parts of the analysis were performed. The mean peak area HMB of each concentration was calculated. A calibration curve was drawn, marking on y-axis the concentrations of HMB after hydrolysis in mg/ml, while on a-axis the corresponding mean areas of the peaks were marked and the equation of linear regression was calculated.

HMB concentration in the studied solution (mg/ml) was calculated from the mean peak area obtained after a twice repeated dosing of the solution of the examined sample and from the equation of regression of the calibration curve. The content of HMB in the sample of the feed mixture was calculated according to the formula:

$$W = \frac{C \cdot V}{M} \ (g/kg)$$

where:
C – HMB concentration in the solution of a given sample, in mg/ml
V – volume of the sample extract, in ml
M – mass of the analytical sample, in g.

Evaluation of the method. The fixed procedure of the analysis was subjected to evaluation determining the precision of the method in repeatable conditions, as variability coefficient with the value of the means of 15 parallel

determinations of HMB content in a typical feed mixture and establishing the degree of recovery of HMB from the feed mixture with its content ranging from 0.05 to 0.4%, i.e. within the limits of the method's application.

The significance of differences of mean results of the determinations of HMB content in feed mixtures with the declared value was checked with t-Student test at $p > 0.05$.

RESULTS AND CONCLUSIONS

Calibration results are presented in figure 1. The desired linear dependence of the calibration curve was obtained, which went through the beginning of the coordinate system, with a very high correlation coefficient – 0.999.

Figure 1. Calibration curve of methionine hydroxy analog

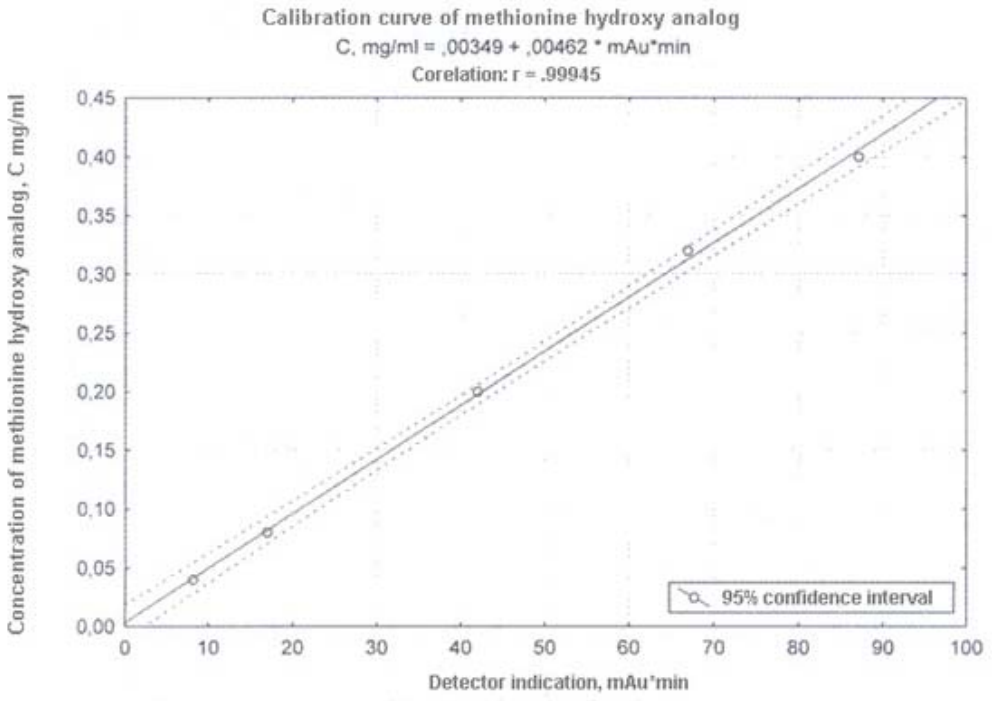


Figure 2. Chromatogram of methionine hydroxy analog extracted from the feed mixture

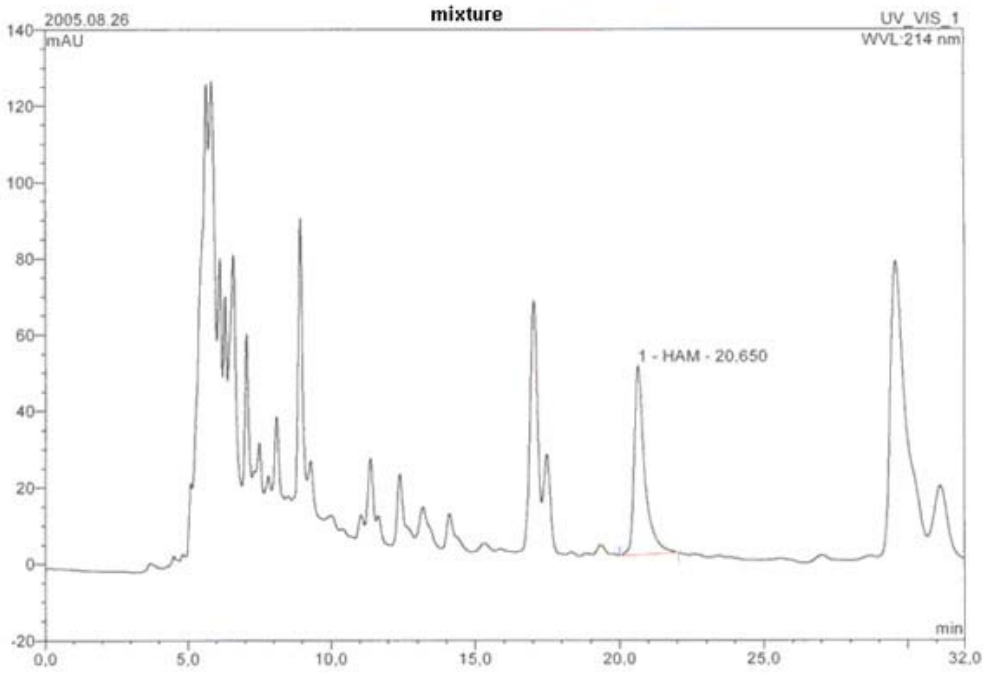
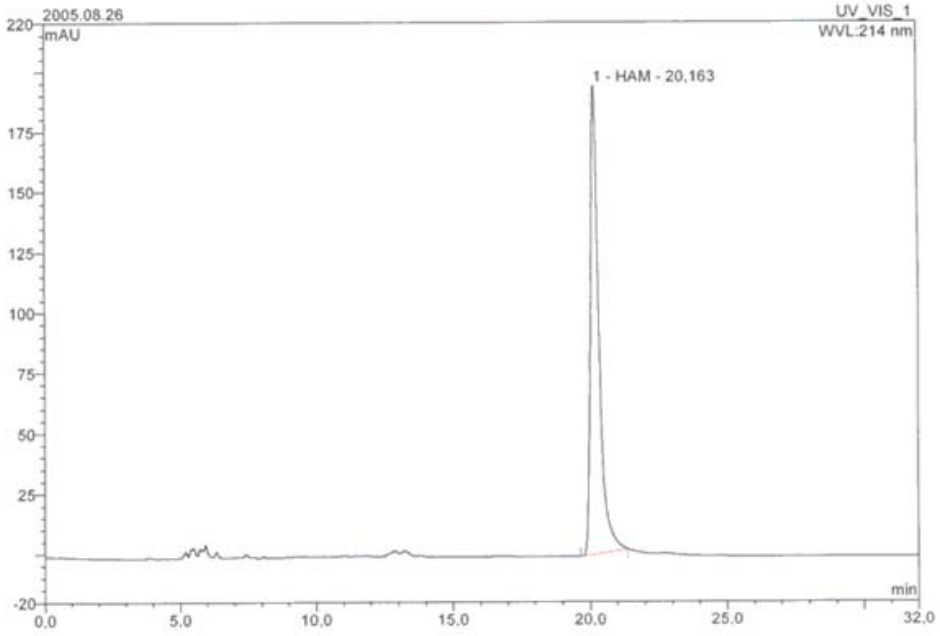


Figure 3. Chromatogram of methionine hydroxy analog standard



In order to divide HMB, Ontiveros et al. [3] used a column with a reversed phase NH_2, while Wauters et al. [4] used a single column with a reversed phase C18, at the same time proving its better effectiveness as compared to column LiChrosorb C18. It follows from the v = chromatograms presented in figures 2 and 3 that the use of two 25-cm columns connected in series and with a reversed phase C18 and the properly chosen system and sequence of movable phases makes it possible to effectively divide HMB monometer from other numerous elements of the feed mix matrix with a very rich composition, for example with sunflower ground grain. In the applied chromatographic conditions, manometer HMB eluated in the 21st minute with the flow of movable phase A and it was completely divided from the other elements of the matrix of the feed mix extract. Introducing movable phase B with a 50% content of acetonitrile removed from the chromatographic column those elements of the feed mix matrix that did not eluate at movable phase A, which lengthened the viability of separating efficiency of the columns. On the other hand, the consecutive conditioning of the columns with movable phase A not only made it possible to achieve a good repeatable division and repeatable retention time of HMB but it was also a factor affecting the high precision of the method. Repeatability expressed with variability coefficient was high, V < 3% (tab. 2).

Table 2. Characteristics of the analytical method

Kind of feed mix	Basic elements	Number of markings	Declared content of HMB, g/kg	Result dispersion g/kg	Mean value M	Standard deviation SD	Coefficient of variability V%
For broiler chickens Grower	Maize, wheat, wheat bran, soybean ground grain, plant oil, mineral-vitamin pre-mix	9	2.00	1.91-2.09	2.03	0.0564	±2..9

The degree f recovery of the method was determined analyzing the content of HMB in the feed mix enriched with HMB to the level within the range of contents determined by the limits of the method's use (tab. 2). The mean HMB recovery from the feed mix was related to its content. When HMB was added to the feed mix at the level of about 0.05%, the man recovery was approx. 93%, while when its was added in the amount of 0.1% and moree, the mean recovery of HMB turned out to be almost 100% and it was within the range between 99 and 101%.

The method was additionally checked by the analysis of HMB content in 15 full-portion feed mix for poultry, with the recipe content of this element declared by the producer. The determinations were made in three repetitions. The obtained results (tab. 1) prove full agreement of the mean content of HMB in the mixtures with the declaration (no significant differences, t-Student test), which not only confirms good validation parameters of the method but also points to high precision of technological dosing and mixing devices in feed production.

Table 3. Evaluation of the method's recovery

Kind of feed mix	Basic elements	Added HMB g/kg	Recovered HMB g/kg	Mean recovery %	Standard deviation of recovery, SD	Coefficient of variability of recovery, V
For layers	Maize, barley, wheat bran, soybean ground grain, sunflower ground grain, pre-mix, chalk, calcium phosphate	0.470	0.436	92.8	0.151	±2.32
For broiler chickens	Maize, wheat, wheat bran, soybean ground grain, fish meal, plant oil, pre-mix, chalk, calcium phosphate	0.951	0.957	100.6	1.626	±1.62
For broiler chickens	Wheat, wheat bran, soybean ground grain, plant oil, pre-mix, chalk, calcium phosphate	3.550	3.530	99.7	1.775	±1.78

Basing on the analyses and calculations, the usefulness of the developed analytical method (tab. 3) for the determination of the content of methionine hydroxy in industrial feed mixtures can be stated.

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