"All arguments speak for a bio-efficacy of 65%"

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Executive summary

- The perception of the nutritional value of methionine hydroxy analogue products is of high economic importance not only for the feed industry, but also for the producers of methionine hydroxy analogue.
- Evonik and other organizations such as the European Food Safety Authority, Centraal Veevoederbureau (NL) or the National Research Council (USA) recommend a bioefficacy of around 65% for methionine hydroxy analogues relative to DL-methionine.
- While slope-ratio or multi-exponential regression are established and validated methods to determine the bioefficacy of nutrient sources relative to a reference, producers of methionine hydroxy analogue try to introduce alternative statistical approaches for evaluating their product. With those they aim to show nutritional equivalency of their product to DL-Methionine and at the same time discredit the nutritional superiority of DL-Methionine.
- The lower bioefficacy of methionine hydroxy analogue can be well explained by various physiological research findings. Producers of methionine hydroxy analogue recently published reviews which left out publications which were not in favor of their product and in which emphasis was put on discrediting established methods in physiological research which showed shortcomings of methionine hydroxy analogue.
- The producers of methionine hydroxy analogue made several attempts to assign additional value to their products to realize cost savings in feed formulation. Among those are a high energy value, acidifying properties for replacing organic acid additives, or high anti-oxidative capacity. All these aspects are scientifically doubtful.
- All the above listed activities will not change the fact that DL-Methionine is the superior Methionine source and applying a relative bioefficacy of 65% for methionine hydroxy analogue is recommended. This recommendation is based on validated and scientifically established methodologies and has repeatedly been proven under various scientific and practical conditions.

Introduction

In their review published already in 1995, Lewis and Baker reported on relative bioefficacy of amino acids a bioavailabilty of 66% for liquid hydroxy analogue of methionine– free acid (**MHA-FA**) relative to DL-methionine (**DL-Met**) on weight basis in practical -type diets already [1]. This means 100 parts MHA-FA can be replaced by 66 parts DL-Met in feed without impacting performance. Latest since then a debate on relative bioefficacy of methionine sources is ongoing although already in the early 1980ties bioefficacies between 63% and 70% for liquid MHA-FA relative to DL-Met were reported [2–4].

In the year 2003 main producers of DL-Met and MHA-FA were invited by the Centraal Veevoederbureau (**CVB**), Netherlands, a) to agree on the methodological approach to determine the biological efficacy of MHA-FA in pigs and poultry and b) to contribute publications and data to this study. Accordingly, Aventis Animal Nutrition (today

Adisseo), Novus International Inc, Degussa AG (today Evonik Industries GmbH) and CVB agreed on a protocol to determine the bioefficacy of MHA-FA relative to DL-Met by simultaneous linear or exponential regression. The outcome of this project suggested that MHA-FA is on average 68% as efficient than DL-Met in broilers, the species with by far highest number of suitable data sets [5]. Moreover, the analysis revealed that this difference is not random but statistically significant although some variation has been found. Indeed, for laying hens, turkeys, and swine results were less clear because of very limited number of studies. It was recommended to generate further data. More recently, the European Food Safety Authority (**EFSA**) concluded that "there is convincing evidence" that MHA-FA (and its calcium salt) has a significant lower bioefficacy than DL-Met in all non-ruminant species [6]. Accordingly, MHA-FA was reported to be 67% as efficient as DL-Met. Also, other organizations such as the National Research Council (US), concluded that "it is reasonable to assume" that the bioefficacy of MHA-FA in fish is about 67% to 71% that of DL-Met [7].

Based on own comprehensive meta-analyses, Evonik recommends a bioefficacy of 65% for all MHA products (MHA-FA; MHA-Ca) for all farmed monogastric terrestrial and aqua species under any production condition [8–13]. In addition, there is a lot of research providing evidence on physiological mechanisms behind the lower bioefficacy of MHA-FA [6]. Moreover, the 65%-recommendation can be challenged under any production and nutritional condition without compromising performance [14–20,11,21–26,13]. Finally, applying our recommendation will save money for the feed mill and avoid animal performance losses. Consequently, it will have impact on the price MHA-FA producers would achieve which is most likely the major reason for them doing the utmost to discredit our recommendation as well as established methodologies behind. MHA-FA producers are particularly (mis)using the scientific platform to confuse and misinform nutritionists.

Methodology for bioefficacy determination

The methodology to determine the relative bioefficacy has been described by Littell et al. [27]. Basically, two (multiple) dose response data sets are analysed by either linear (slope-ratio) or exponential regression simultaneously. Steepnesses from regression equations are related to each other taking – in this case – DL-Met as reference. The resulting coefficient suggests how much DL-Met is needed to replace MHA-FA for same animal performance independent of general supplementation level and performance level. This approach assumes that the maximal achievable performance (asymptote) is similar for both products.

Several attempts have been made to provide evidence that this method would not be applicable for MHA-FA. Vazquez-Anon et al. (Novus International) reported four simultaneous dose response studies and concluded that nature of dose-response curve would differ between MHA-FA and DL-Met fed broilers [28]. Interestingly, when data were analysed separately for each trial, linear, exponential and quadratic regressions fit best to DL-Met and MHA-FA responses but were different between trials and products whereas a meta-analysis of combined data of these four trials suggested a linear response to MHA-FA with not achieving an asymptote and a quadratic response for DL-Met [28]. In the same year these author's identified quadratic responses for both products [29] whereas a further broiler trial suggested linear responses to both products [30]. Thus, the team around Vazquez-Anon and Knight from Novus International demonstrated biologically fallacious and inconsistent conclusions but stated no difference between product efficacies. At least the linear responses were used for comparing slopes [30] basically accepting slope-ratio approach. However, there were no significant responses of broilers to either product which would be prerequisite for reliable slope ratio. After an unconventional processing of data, a meta-analysis sponsored by Adisseo accepted and applied the slope-ratio approach, too, and concluded that efficiency of MHA-FA would not differ from that of DL-Met [31]. Concerns were addressed particularly with respect to data treatment and selection of publications considered [32], authors only partly could justify their conclusions [33] apart from the fact that it is impossible for the reader to reproduce this analysis. Kratzer (who co-authored [29]) and Littell (who originally described the slope-ratio and multi-exponential approach for methionine sources [27]) referred to the above mentioned study by CVB [5] (which was accepted by all companies involved) and concluded that MHA-FA and DL-Met would not result in same asymptote and, therefore, the conclusion of lower bioefficacy of MHA-FA by Jansman et al. [5] would not be valid [34]. An immediate response to this article announced doubts

[35] and a deep-dive meta-analysis then provided clear evidence that asymptotes for both products are similar while the dose needed to get to this maximum differs between products [36]. Since then, the methodological approach has not been questioned any more in the scientific forum although the International Methionine Analogue Association (IMAA) which was founded in 2012 used above publications to “promote the reputation” of MHA-FA [37].

However, the ultimate evidence for validity of multiple-exponential regression is provided by using diluted DL-Met as an internal standard. With a known concentration of only 65% DL-Met in a premix (DL-Met65), the simultaneous dose-response trial should reveal a bioefficacy of about 65% relative to pure DL-Met. Broiler trials using DL-Met65 suggested an average bioefficacy of 63% which, in addition, was identical to the bioefficacy determined for MHA-FA [38,18,20]. Interestingly, these studies were published before 2006 and were, therefore, known already before the above publications suggesting different regression models, pre-treating data for slope ratio, etc. became available. Further DL-Met65 trials are meanwhile available also validating both method and bioefficacy of products [11,16], and thus disproving the concept of same bioefficacy, different asymptotes or difference nature of responses. An amusing side information is that Rhône-Poulenc which is the original predecessor company of Adisseo once developed the method of including diluted DL-Met as internal standard into the experimental design resulting in similar conclusions [39].

More recently it was argued that a fair comparison should be done at “commercial” supplementation levels at requirement [40–42]. This is a ridiculous suggestions as the maximum performance is achieved even with DL-Met65 at such “commercial” levels [16,18,38,11,20]. Nobody would conclude on same bioefficacy of DL-Met65 compared to pure DL-Met. Also Batonon-Alavo et al. supplemented DL-Met and MHA-FA at equimolar levels to achieve broiler breeder recommendations [43]. They introduced the non-inferiority test as “new statistical approach” to show that MHA-FA is not inferior to DL-Met. However, the application of this test included major errors [44] which the authors of course did not accept [45]. First, the non-inferiority test pre-assumes a lower efficacy of the product under test – in this case MHA-FA – and the question to be solved is just whether this lower efficacy is in an acceptable range. Doing the stats on the data reported correctly reveals that non-inferiority cannot be confirmed for MHA-FA compared to DL-Met [44].
In summary, publications initiated by MHA-FA produces aim to create doubts on correctness of a significantly lower bioefficacy of MHA-FA relative to DL-Met. In order to achieve this aim, they suggested various regression models, they conducted meta-analyses in which data were prepared in a way that a fair comparison was avoided, and they suggest test conditions in which comparisons are not meaningful. At the same time slope ratio and multi-exponential regression is an established method to compare a nutrient source with a reference. This model has been validated not at least by including DL-Met65 while the recommended bioefficacy of 65% for MHA-FA relative to DL-Met can be challenged under any production condition, any climate, with any non-ruminant farm species and with any MHA product without compromising performance but indeed achieving savings and other benefits.

Physiological background

There is quite some research published dealing with the metabolic und physiological fate of DL-Met and MHA-FA. EFSA concluded that major reasons for lower bioefficacy of MHA-FA can be related mainly to larger degradation by intestinal microbes and a particularly lower digestion and utilization of di- and trimers of MHA-FA [6]. Becquet, who is currently president of above mentioned IMAA, and co-workers, who all work for Adisseo and Novus International, published recently two extensive reviews on metabolism of methionine sources [46,47]. The first review focuses on the digestion and absorption of the molecules from digesta and the second review deals with the transformation of D- and L-MHA-FA as well as D-Met into L-Met. While not obvious for a reader with only little insight to these topics, authors were selective regarding chosen references which per definition avoided a comprehensive and scientifically reliable review [48]. It turned out that not only publications which were not in favor of MHA-FA were ignored, but that methodological approaches used in studies revealing disadvantages for

MHA-FA compared to DL-Met were discredited to the utmost. This is also remarkable as all the research on the physiological background of these product would not allow at all to quantify effects and, thus, to revise the determined relative bioefficacy – it just allows to explain the empirical observation. The topics addressed cover those which were listed in the EFSA report [6] and it appears that with scientific publications like this, new "evidence" shall be established which would allow for revising the respective assessment by the authority. Our response letter passed the review process of the journal quickly and accepted that the "chain of argumentation aimed at discrediting studies reporting lower absorption of MHA-FA. The impression of a biased review is strengthened due to the omission of a number of easily accessible publications." [48].

Approaches to increase the nutritional value of MHA-FA

With respect to pricing of DL-Met and MHA-FA, matrix values for least cost feed formulation (LCF) play a central role as those are decisive for the relative attractiveness of an ingredient in the final receipt. For example, if the only nutritional information in the matrix would be the available Met (Met+Cys) content, the value of MHA-FA would be 65% as high as that for DL-Met according to Evonik's recommendation. If the price ratio between products is > 65%, LCF would prefer DL-Met and would suggest a shadow price for MHA-FA which is competitive.

Energy values associated with DL-Met and MHA-FA

However, the matrix can contain further information and the energy content is important in this context. Of course, DL-Met and MHA-FA contain energy which contributes – although little - to the overall energy content of the diet. In poultry and swine nutrition metabolizable and net energy concepts are established. It is assumed that the discussion on relative efficacy also considers dietary energy and respective utilization because growth responses as determined in growth response trials are net effects of all nutrients and energy together. Therefore, a bioefficacy of 65% should be reflected in the energy values entered into the matrices in LCF, too. However, if the energy ratio between the products is higher than 65% the relative attractiveness of MHA-FA compared to DL-Met increases. Adisseo released a publications in which metabolizable energy for MHA-FA is suggested as high as for DL-Met for birds; net energy even 4-5% higher [49,50]. Similar numbers were reported for mammals. Using such values in LCF would overrule the effect of lower Met+Cys value for MHA-FA to a large extent and thus pull MHA-FA into the diet instead of DL-Met regardless the lower Met+Cys activity. That makes MHA-FA attractive not at least for price negotiations. However, while these proposed energy values were not determined in animal trials but by calculations on molecular level, it should be noticed that (supplemental) amino acids or their hydroxy analogues are fed for effective incorporation into body protein and are, therefore, not meant for oxidation. In this context it should be emphasized that methionine+cysteine are first limiting factors in many diets which consequently indicates maximized utilization. If this is taken into account, the picture would even change as all MHA-isomers need to be converted into L-methionine which is actually – indeed small – an energetic burden.

MHA-FA as acidifying agent

A further attempt to increase the relative attractiveness of MHA-FA against DL-Met in LCF is offered with the "ABC-4 Acidsaver" by Adisseo. This web application calculates how much of an acidifier can be saved just with supplementation of MHA-FA which itself is indeed a strong organic acid with a pH of one. Replacing (a certain amount of) organic acid products in feed would reveal an economic advantage. Question remains whether MHA-FA can act as acidifier in feed. When disappearance of MHA-FA and DL-Met from digesta in gnotobiotic chicken and swine was evaluated, it was concluded that intestinal microbes utilize quite some MHA-FA in contrast to DL-Met which then was not available to the host [51,52]. However, while these studies did not allow for assessing whether microbes consuming MHA-FA died, they suggest that MHA-FA would not have a double functionality. An investigation of the impact of formic acid, DL-Met, MHA-FA and gradual replacement of formic acid by MHA-FA on microbial activity in swine ileum and colon revealed no impact of MHA-FA on microbial density nor on short chain fatty acid production [53]. Data reported by Smith et al. confirm that MHA-FA does not modulate microbiota or gut characteristics in swine and broilers [54]. Locatelli et al. concluded that MHA-FA is ineffective for feed

preservation such as controlling salmonella [55]. Analogous to the dietary energy, growth responses for determining bioefficacy would include any impact on both gut microbiota and health. Moreover, validation trials challenging the recommended bioefficacy of 65% were conducted under controlled but also field conditions and did not suggest any difference between environments and, therefore, health beneficial effects of MHA-FA in monogastric farm animals [13,12,8].

MHA-FA as antioxidative agent

Oxidative stress is a relevant challenge in animal production. It is triggered by various stressors such as heat stress but also stocking density or dietary insufficiencies. The so-called reactive oxygen species (ROS) are in the center of this discussion as the organisms need to counteract those with various antioxidative strategies. Among many other compounds also sulfur containing amino acids have been reported to ameliorate oxidative stress [56]. As summarized by Magnuson et al. the sulfur group of methionine can be oxidized to sulfoxide at oxidative stress enabling methionine to counteract ROS [57]. Moreover, via the transsulfuration pathway methionine can be transformed into cysteine which in turn is a precursor of glutathione (GHS) being an effective antioxidant [57]. In their questionable review (see above) Becquet et al. emphasized the beneficial effects of MHA-FA against ROS. While they reported studies where ratios of reduced GHS to total or oxidized GHS were higher with MHA-FA than with DL-Met suggesting a higher capacity for oxidative stress defense [58–60], they did not mention other studies reporting a higher anti-oxidative efficiency for DL-Met [61,62] or studies which could not differentiate the products in this context [63,64]. A recent publication addresses the origin of ROS and it could be shown in muscle cell (myoblast) models that particularly D-MHA is transformed to L-Met in mitochondria rather than in peroxisomes resulting in higher concentration of H_2O_2, which is a strong ROS, in extracellular space and which would stimulate defense of oxidative stress [65]. Accordingly, MHA-FA metabolism itself adds to oxidative stress on cell level. Again, as pronounced earlier, application of the recommended bioefficacy of 65% for MHA-FA is successful under any environmental, nutritional and husbandry condition which therefore excludes an extra-benefit of MHA-FA under such conditions [13,32,8,12].

Summary and conclusion

To summarize, multi-exponential regression of simultaneous dose-response data allows for determination of the bioefficacy of MHA-FA relative to DL-Met as well as for validation of the method. Accordingly, a bioefficacy of 65% is recommended. This is, by the way similar to findings of Adisseo's predecessor company Rhône-Poulenc – AEG, which already 1983 concluded on a bioefficacy of 70% on product level [4]. Particularly MHA-FA producers and scientists supported by those companies introduced various statistical approaches in order to establish nutritional equivalency of MHA-FA and DL-Met. Moreover, in a couple of examples it could clearly be demonstrated that such publications and even so-called reviews ignored other research which is inconvenient for the MHA producers in this context. In addition to the discussion about bioefficacy, several attempts are made to increase the nutritional value of MHA-FA relative to DL-Met by suggesting a higher dietary energy value, acidifying properties or anti-oxidative capacity for MHA-FA. However, not one single approach can negate practical applicability of our recommended 65% bioefficacy and related savings for the user because more than 150 challenge tests under any production condition prove validity of our recommendation.

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