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# Effect of stunning methods on the differentiation of frozen-thawed bullfrog meat based on the assay of β-hydroxyacyl-CoA-dehydrogenase

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**Abstract**

The effect of electrical or thermal stunning was investigated by β-hydroxyacyl-CoA-dehydrogenase (HADH) assay after extraction in phosphate buffer to segregate unfrozen (stored in crushed ice) from frozen-thawed bullfrog (*Rana catesbeiana*) *gastrocnemius* muscles. Also investigated were possible influences of the type and time of storage on the upper and lower limits of the HADH assay.

Stunning method and time of storage in crushed ice did not affect ($P > 0.05$) the HADH activity in unfrozen or frozen-thawed muscles. However, HADH values of the frozen (−18 °C) samples increased ($P > 0.05$) over the evaluated period. Nevertheless, it was possible to establish upper and lower HADH values leading to the differentiation of unfrozen from frozen-thawed samples. Samples with HADH values lower than 65 were classified as unfrozen and those with HADH values higher than 69 were classified as frozen-thawed. Samples with HADH values between 65 and 69 were considered as uncertain. Using these limits, a high level of success (96.5%) was attained in distinguishing unfrozen from frozen-thawed bullfrog meat.


*Keywords:* Stunning method; Storage type; Differentiation; Unfrozen frog meat; Thawed frog meat

## 1. Introduction

Frog meat is not only appreciated for its exquisite flavour and texture but also as a source of protein of high biological value (Vieira, 1993). Although, in Brazil, frogs are market as whole carcass, frog meat is usually commercialized as fresh or frozen legs in the international market, with unfrozen legs obtaining higher prices (Lima, Cruz, & Moura, 1999; Pavlov, Garcia de Fernando, Ordonez, & Hoz, 1994). Furthermore, countries such as the United States, Canada and France import live animals, due to the consumers' preference for fresh meat (Lima et al., 1999).

In fact, even in regard to traditional meats, many consumers prefer fresh meat despite its higher cost and lower shelf life. For this reason, selling thawed meat as unfrozen meat, especially poultry and fish, has become a common practice. However, this practice is harmful to the consumers since thawed meat must be quickly consumed, as it is more susceptible to microbial contamination and deterioration. Since the sensory properties of thawed meats are very similar to those of fresh meats, it is almost impossible to distinguish them sensorially. To avoid this type of fraud, research has been conducted on methods for sorting frozen from unfrozen meats.

Gottesmann and Hamm (1983) developed an enzymatic assay to differentiate unfrozen from frozen-thawed meat, using the β-hydroxyacyl-CoA-dehydrogenase (HADH, EC 1.1.1.35) enzyme released from the mitochondria to the sarcoplasm during the freeze-thawing

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process. In their method, the enzyme was extracted by pressing the meat between two plexiglass plates. The activity of the HADH enzyme, after extraction by this pressing method, has been successfully used in sorting frozen-thawed from unfrozen meats in different animal species (Billington, Bowie, Scotter, Walker, & Wood, 1992; Chen, Yang, & Guo, 1988; Gottesmann & Hamm, 1983; Toldrá, Torrero, & Flores, 1991). However, it was found unsatisfactory for fish meat (Gottesmann & Hamm, 1983).

More recently, Garcia de Fernando, Fernandez, Diaz, Ordóñez, and Hoz (1992) proposed a new HADH extraction procedure by immersing meat samples in phosphate buffer, which proved to be efficient in sorting unfrozen from frozen-thawed fish. Their method was further tested and validated in fish, and other seafood, by other research groups (Fernández, Mano, García de Fernando, Ordóñez, & Hoz, 1999; Hoz, Fernández, Diáz, Ordóñez, Pavlov, & Garcia de Fernando, 1993; Hoz, Yustes, Camara, Ramos, & Garcia de Fernando, 1992; Pavlov et al., 1994). Furthermore, The HADH extraction method proposed by Garcia de Fernando et al. (1992) is much easier in practice and showed better results in differentiating fresh from thawed frog (*Rana esculenta*) meat (Pavlov et al., 1994).

Brazil is a pioneer country in frog rearing, having developed and disseminated a frog farming system in countries where frogs were obtained by hunting (Lima et al., 1999). Our research group has been conducting research to improve the frog slaughtering process.

The stunning methods presently used in the Brazilian commercial frog slaughtering can be divided into two groups: thermal stunning, i.e., immersing live frogs in a cold water or salt solution bath, and electrical stunning (Albinati, 1994; Lima & Agostinho, 1988; Longo, 1986). The electrical stunning method is more recent (Albinati, 1994; Ayyappan Pillai, 1986) and was found to be more appropriate for frog slaughtering than the traditional stunning methods (Albinati, 1994). However, in a survey conducted by Lima et al. (1999), all frog slaughterhouses operating in Brazil use the thermal stunning method by immersing the frogs in tubs with water and ice.

Most frogs raised in Brazil belong to the bullfrog (*Rana catesbeiana*) species, which has shown the best adaptation capacity and productivity (Longo, 1986; Mello, 1995). During hibernation periods, bullfrog (*R. catesbeiana*) and wood frog (*Rana sylvatica*) are able to produce glucose as a cryoprotective agent in order to protect their energetic metabolism, as shown by the increase of their blood and liver glucose concentrations (Costanzo & Lee, 1993; Steiner, Petenusci, Brentegani, & Branco, 2000; Storey & Storey, 1984, 1985). Therefore, with the likelihood of cryo-protector production stimulation during thermal stunning, differentiating unfrozen from frozen frog muscles by HADH activity may lead to a mistaken interpretation of the storage conditions of this meat. In this regard, thermally stunned frogs could generate meats with similar HADH activity from both the unfrozen and frozen-thawed samples.

Thus, this research was undertaken to evaluate the effects of the two major frog stunning methods on the phosphate buffer-extracted HADH assay, and to show how this could influence the differentiation of unfrozen from frozen-thawed bullfrog meat.

## 2. Material and methods

### 2.1. Animals and slaughtering

One hundred and thirty six bullfrogs (*R. catesbeiana*), of $217 \pm 41$ g live weight, were harvested at the frog farm system of Universidade Federal de Viçosa and maintained off feed for 24 h (Moura, Gomide, & Ramos, 2001), then separated into two groups, weighed and slaughtered. One group was slaughtered after stunning by immersion in water and ice (1:1) for 15 min (thermal stunning) and the other after electrical stunning (60 V, 60 Hz) for seven seconds (Albinati, 1994). Afterwards, bleeding was accomplished by cutting the frog's cardiac veins (Albinati, 1994) and their carcasses were eviscerated by the alternative method proposed by Loaiza (1996). The *gastrocnemius* muscles were removed and placed in labelled plastic bags.

### 2.2. Sample storage

To evaluate whether time of frozen storage could lead to incorrect results due to a possible decrease in HADH activity, 28 animals from each stunning group had one of the *gastrocnemius* muscles excised and frozen. After storage (−18 °C) for 2, 30, 65 and 90 days, the frozen samples were thawed overnight at 4 °C and the HADH activity was determined. The effect of the storage time in crushed ice on the HADH activity of unfrozen and frozen-thawed muscles was evaluated in the remaining 80 animals. One *gastrocnemius* muscle, from each animal, was excised, frozen at −18 °C, and stored for two days at the same temperature and thawed overnight at 4 °C before storage in crushed ice. The other *gastrocnemius* muscle, from each animal, was directly stored in crushed ice. Sampling was performed after zero, two, four and six days of storage in crushed ice.

### 2.3. Assay for HADH activity

Portions of the *gastrocnemius* samples (around 2 g) were weighed, transferred to glass tubes and immersed in two volumes (around 4 ml) of 0.1 M phosphate buffer (pH 6.0). No superficial drippings (minimal due to the high WHC of bullfrog meat) were intentionally removed nor included before muscle cutting and weighing. The

tubes were then kept in a water bath at 25 °C for 15 min and the buffered meat juice was filtered through Whatman paper No. 2. The HADH activity was measured according to the method reported by Garcia de Fernando et al. (1992), slightly modified. The filtrate was assayed for HADH activity by mixing 34 μl of the filtrate in a quartz cuvette with 70 μl of EDTA (34.4 mM) and 860 μl of 0.1 M phosphate buffer (pH 6.0). The mixture was maintained at room temperature for 3 min before the addition of 40 μl of NADH (1.5 mM) and 20 μl of acetoacetyl-CoA (5.9 mM). Immediately thereafter, the absorbance was measured every 0.5 min up to 3 min, at 340 nm. The HADH value was calculated by multiplying the slope of the linear regression obtained from the spectophotometric measures by $-10^4$.

### 2.4. Statistical analysis

The four frozen storage data sets were submitted to analysis of variance in a split-plot scheme, with stunning (electro and thermal) method as the main plot and the frozen storage time as the sub-plot, in a complete randomized design. Data for crushed ice storage were submitted to analysis of variance in a split-split-plot scheme, with stunning (electro and thermal) method as the plot, treatment (frozen and unfrozen) as the sub-plot, and crushed ice storage time as the sub-sub-plot, in a complete randomized design. The variance and regression analysis were conducted using the SAS® System for Windows™, v. 8.0 (SAS Institute Inc.), with a significance level of 5%. The 95% confidence limits for the mean were used to establish the upper and lower limits, leading to distinguishing the unfrozen from the frozen-thawed state, under the work conditions.

## 3. Results and discussion

Bullfrog *gastrocnemius* HADH activity values were unaffected by stunning methods ($P > 0.05$), suggesting that thermal stunning did not induce any significant difference in the amount of cryoprotectants produced in its muscles. This may be due to the short time the animals were subjected to cold temperatures with this stunning method, compared to the time that frogs survive in cold weather. In this case there may not have been enough time to induce an increase in muscle glucose level, as has been shown in frog's liver and blood (Costanzo & Lee, 1993; Steiner et al., 2000; Storey & Storey, 1984, 1985). Therefore, there would not be enough muscle cryoprotectant to allow minimization of mitochondrial membrane rupture, due to the prevention of formation of large ice crystals by glucose molecules, which would diminish the amount of HADH released in the sarcoplasm. On the contrary, initial results (unpublished data) we have obtained with another set of bullfrog samples show that thermally stunned frogs have lower (0.86 μmol $g^{-1}$) initial post-slaughter muscle glucose levels than electrically stunned frogs (1.33 μmol $g^{-1}$).

Since HADH activity values were not affected ($P > 0.05$) by storage time in crushed ice or by stunning methods, their means were pooled to establish the upper and lower HADH limits leading to the differentiation of unfrozen from frozen-thawed bullfrog meat (Table 1).

The means in Table 1 are different from those reported by Pavlov et al. (1994) for legs (bone-in) of *R. esculenta*. The HADH values obtained for unfrozen bullfrog meat were higher than those reported by these authors, while those obtained for frozen-thawed bullfrog meats were lower. These differences may be due to variations between frog species as well as type of muscle analyzed in each experiment.

Using the average HADH activity (Table 1), and considering a 95% confidence interval, as well as the fact that the samples were normally distributed with different standard deviations, bullfrog meat may be classified as unfrozen when sample HADH values are lower than 65. For HADH values higher than 69 the bullfrog meat should be classified as frozen. Samples showing HADH values ranging from 65 to 69 should be considered as uncertain. With this criterion, 96.5% of the experimental samples were correctly classified, 2.8% were erroneously classified (1.4% fresh samples were classified as frozen and 1.4% thawed samples were classified as unfrozen) and 0.7% was considered uncertain.

The correct classification of bullfrog meat obtained with the HADH limits proposed in this study is very similar to those reported in the literature. In fish meat,

Table 1
HADH activity (means ± standard deviation) of unfrozen and frozen-thawed bullfrog *gastrocnemius* meat

| Treatment | HADH Value | | |
|---|---|---|---|
| | Eletrical stunning | Thermal stunning | Overall means |
| Unfrozen[a] | $42.9 \pm 10.9^{x}$ | $46.7 \pm 18.0^{x}$ | $44.6 \pm 14.9$ |
| Frozen-thawed[a] | $119.9 \pm 31.4^{y}$ | $110.1 \pm 28.2^{y}$ | $114.5 \pm 29.9$ |

$n = 40$ samples for each treatment.
Different letters ($x$, $y$) within a column of the same stunning method are different ($P > 0.05$).
[a] No significance differences ($P > 0.05$) were found within rows.

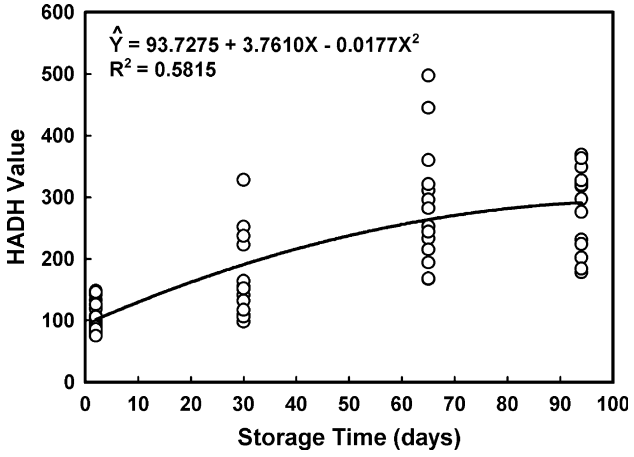


Fig. 1. Change in HADH activity of bullfrog *gastrocnemius* muscle during frozen storage at −18 °C.

correct classification was obtained in 95% (Hoz et al., 1992) and 84% (Hoz et al., 1993) of the samples. Pavlov et al. (1994) established HADH limits that allowed 100% correct classification of *R. esculenta* meats.

Contrary to Pavlov et al. (1994), who did not find significant difference in the HADH activity of *R. esculenta* samples between two and thirty days of storage, the storage time at −18 °C had ($P > 0.05$) a positive quadratic effect on the bullfrog meat HADH activity (Fig. 1). Hoz et al. (1993) did not find any significant difference in the HADH activity of kuruma prawn (*Penaeus japonicus*) meats between one and six months of storage at −18 °C.

Despite the increase in HADH activity with frozen storage time, it was still possible to differentiate between unfrozen and frozen-thawed samples, since unfrozen samples always had smaller ($P > 0.05$) HADH activity values than those of frozen-thawed samples. Thus, longer frozen storage times would only make it easier to differentiate between frozen and unfrozen samples.

## 4. Conclusions

Stunning method and time of storage in crushed ice do not interfere in the HADH activity of bullfrog *gastrocnemius* muscles, while time of frozen storage positively affects HADH values.

Even for samples previously kept on frozen storage it was possible to establish acceptable limits leading to a high level of success in distinguishing fresh from frozen-thawed bullfrog meat by measuring the HADH activity, of bullfrog *gastrocnemius* muscle, extracted by immersion in phosphate buffer.

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