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# Chemical and microbial analyses of squid muscle (*Loligo plei*) during storage in ice

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**Abstract**

Chemical and microbial changes in squid muscle (*Loligo plei*) and the efficiency of chemical indices for freshness evaluation were investigated during storage in contact ice (CI) and non-contact ice (NC). Psychrotrophic bacterial counts and non-protein nitrogen, free amino acids, trimethylamine, volatile basic nitrogen (VBN), urea and free tryptophan contents were periodically determined in squid muscle. No difference ($P < 0.05$) was detected between treatments regarding the increase of psychrotrophic bacterial counts in squid muscle during storage. The leaching of soluble compounds in CI treatment drastically affected the efficiency of the chemical indices for freshness. Trimethylamine and VBN contents in squid muscle increased only after day 9 and no increase was observed in free tryptophan and urea contents in CI treatment. In the NC treatment the contents of VBN and trimethylamine slowly increased during the first days of storage whereas free tryptophan and urea contents markedly increased during the whole storage. Free tryptophan and urea can be useful freshness indices for *L. plei* in storage conditions wherein leaching have been minimized.


*Keywords:* Squid; Chemical analysis; Microbial counts; Freshness indices

## 1. Introduction

The exploitation of cephalopods has increased in recent years as a consequence of the declining stocks of commonly exploited fish species. The world demand for cephalopods increased at a rate of 15% per year between 1994 and 1996, an index greater than that related for marine fishes, indicating a specific demand for these animals (Guerra, 1996). The world total catches of cephalopods (squids, cuttlefishes and octopuses) were higher than 3.3 millions of tons between 1999 and 2001 (FAO, 2001). The most valuable cephalopod species belong to the orders *Octopoda* (octopus) and *Sepioidea* (cuttlefish), and to the *Loliginidae* family (inshore squid), within the order *Teuthoidea* (Paarup et al., 2002). Within the *Loliginidae* family, squids of the *Loligo* genera are generally regarded on the world market as more valuable, mainly due to their excellent sensory properties (Sikorski & Kolodziejska, 1986).

*Loligo plei* and *Loligo sanpaulensis* are the most abundant cephalopods on the continental ridge of southern Brazil (Haimovici & Perez, 1991) and both species represent the bulk of cephalopod catches of the offshore trawling fishery (Costa & Haimovici, 1990; Juanicó, 1980). The fishing mostly occurs during summer months, along the coasts of the states of Rio Grande do Sul, Santa Catarina, São Paulo and Rio de Janeiro. In Brazil the majority of squid is caught as a by-catch in shrimp fisheries, resulting in low quality squid. On the other hand, the catch of squid by hand jiggers reaps high quality squid. This is the method used by craft fishermen, for whom the squid catch represents an alternative financial source when the catch of shrimp is prohibited (during the shrimp spawn period).

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There are few scientific studies providing information on quality changes of *L. plei* squid after catch and during storage. This knowledge is greatly needed to develop adequate storage methods and tests for freshness evaluation. Many chemical indices for freshness evaluation of fish, crustaceans and mollusks are based on changes of non-protein nitrogen (NPN) components during the storage, such as volatile basic nitrogen (VBN) and trimethylamine (TMA). TMA and VBN acceptability limits have been proposed for some squid species (Ke, Burns, & Woyewoda, 1984). Other studies (Civera, Grassi, & Pattono, 1999; Paarup et al., 2002; Romo, Astudillo, Muñoz, & Contreras, 1996) have shown that acceptable limits are dependent on the species and storage conditions, and thus, highly variable. In addition to the VBN analysis, the determinations of free tryptophan (Romo et al., 1996) and urea (Romo et al., 1996; Otsuka, Tanaka, Nishigaky, & Miyagawa, 1992) have been evaluated as freshness indices in squid species, showing promising results.

Squids, as with most fish, are stored between layers of triturated ice during transport and commercialization. Thus, the deterioration process occurs between 0 and 1 °C with continuous melting of water from the ice. In this storage condition, the increase in the content of a soluble compound in the muscle will only be detected when the rate of its formation overcomes the loss rate, resulting in a loss of efficiency of many chemical tests for freshness evaluation. In such a case, when specific freshness indices for squid are evaluated, it becomes important to study the efficiency of tests in different storage conditions, and to verify their correlation with microbial contamination, to guarantee consumer safety.

Therefore, the objectives of this study were to determine the chemical composition of squids from the *L. plei* species; to study the chemical and microbial changes during storage; and to evaluate the efficiency of chemical indices for freshness evaluation, comparing two storage conditions: squids refrigerated in direct contact with ice and squids packed in polyethylene bags and refrigerated in ice.

## 2. Materials and methods

### 2.1. Materials

Squids of the *L. plei* species, commercialized in a local market (Campinas, Brazil), were used in the experiments. These squids had been stored in ice for three days after the capture. The average mantle length and total weight of squids were 26.8 cm and 171.1 g, respectively. Six *L. plei* squids caught by hand jiggers (island of São Sebastião, southeast Brazil) and brought to the laboratory within 24 h of their capture were used as control. Three of these squids were frozen in carbonic acid immediately after the capture and used as control (0 h) for chemical analysis and the other three were kept in ice and used as control (day 1) for microbial analyses.

### 2.2. Experimental procedure

Four squids were separated for the first sampling and the others ($n = 48$) were randomly divided and submitted to two different storage conditions: contact ice (CI) and non-contact ice (NC). The CI storage was effected by holding the whole squids between layers of ice in an insulated container. In the NC storage the whole squids were placed in polyethylene bags (four squids in each) and the bags were manually folded in their open edge to protect the squids from the melted ice and to avoid leaching. Then, the bags were placed between layers of ice in an insulated container. In both treatments the ratio of squid:ice was 1:4. Freshwater ice was daily replaced. At 2, 4, 7, 10 and 15 days of storage four squids were randomly sampled from each treatment. Two squids were submitted to microbial counts, and the other two to chemical analysis. Because the squids had been stored in ice for three days in the local marked after capture, sampling days reflected storage times of 3, 4, 6, 9, 12 and 17 days after catch. The whole experiment was replicated twice.

### 2.3. Microbiological analysis

Psychrotrophic bacterial counts (PsyC) were made by homogenizing 25 g of mantle muscle without skin with 225 ml of saline peptone water (0.85 g NaCl and 0.1 g peptone in 100 ml water). Decimal dilutions were prepared according to the estimated contamination. Surface inoculation was made in plates containing Plate Count Agar, which were incubated at 7 °C for 10 days (Cousin, Jay, & Vasavada, 1992).

### 2.4. Chemical analyses

Contents of non-protein nitrogen (Horwitz, 1980), free amino acids (adapted from Adler-Nissen, 1979), TMA (Murray & Gibson, 1972), trimethylamine oxide (adapted from Murray & Gibson, 1972), VBN (Howgate, 1976), ammonia ($NH_3$, adapted from Adler-Nissen, 1979), urea (SIGMA kit Urea Nitrogen 535A) and free tryptophan (Contreras & Lapa-Guimarães, 1989) were determined in squid muscle extracts. These extracts were obtained by homogenizing 25 g of minced squid mantle with 75 ml of 5% trichloroacetic solution, followed by filtration through a filter paper. Contents of moisture, ash and total nitrogen (Horwitz, 1980) and total lipids (Bligh & Dyer, 1959) were determined in the

muscle of control squids, which had been frozen immediately after catch.

### 2.5. Statistical analysis

Results were evaluated by two-way ANOVA, where the main effects were treatment (CI and NC) and storage time (3, 4, 6, 9, 12 and 17 days). Simple linear correlation (Pearson correlation) analyses between microbial and chemical analyses were evaluated. A least significance level at $P < 0.05$ was used. This statistical analysis was performed using the STATISTICA software, version 6.0 (Stat Soft, Inc. 1995, Tulsa OK, USA).

## 3. Results and discussion

### 3.1. Microbial analysis

Control squids, analyzed 1 day after catch, showed a PsyC equal to $8 \times 10^2$ cfu/g of sample. In the commercialized squids, PsyC increased from $1 \times 10^4$ cfu/g at the beginning of storage to $5 \times 10^6$ and $4 \times 10^6$ cfu/g in CI and NC treatments, respectively, after 12 days of storage (Fig. 1). Although counts in CI treatment were slightly higher to those of the NC treatment from day 9, no difference ($P < 0.05$) was detected between treatments during the whole storage. These data confirm results of our previous study (Lapa-Guimarães, Felício, Silva, & Contreras, 2002).

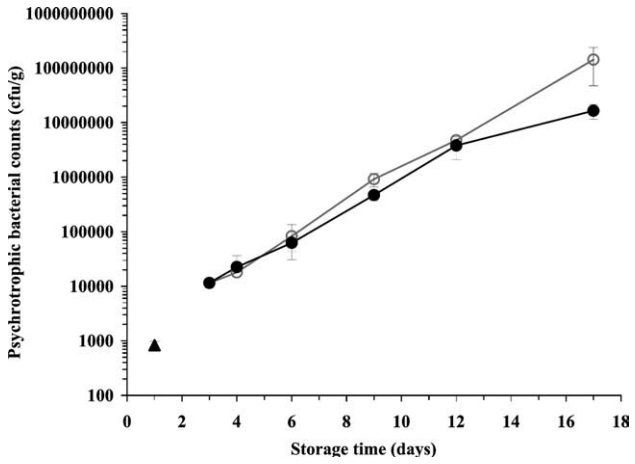


Fig. 1. Variations in psychrotrophic bacterial counts in muscle of control squids (▲) and in muscle of squids held in contact ice (○) and non-contact ice (●) treatments.

### 3.2. Chemical analysis

#### 3.2.1. Chemical composition

The chemical composition of control squid mantle is shown in Table 1. Protein, lipid and ash values compared well with those reported for other squid species (Jhaveri, Karakoltsidis, Montecalvo, & Constantinides, 1984; Krzynowek, D'Entremont, & Murphy, 1989; Sikorski & Kolodziejska, 1986). The moisture content of control squids (74.2%) was lower than those found in commercialized squids (79.6%) and reported in the literature, probably because chemical composition was assessed in squids frozen immediately after the capture, which had not had any previous contact with ice. The content of non-protein nitrogen (NPN) detected in *L. plei* was very similar to those found in mantle of the cephalopods *Illex coindetti*, *Todaropsis eblanae* and *Eledone cirrhosa* (Ruíz-Capillas, Moral, Morales, & Montero, 2002). The content of nitrogen from free amino acids (FAA-N) in *L. plei* represented 36.8% of NPN, and the NPN fraction represented 31.7% of the total nitrogen (TN). Sikorski and Kolodziejska (1986) reported that NPN fraction represents about 37% of TN in squids, a value higher than that found in this study.

#### 3.2.2. Changes in non-protein nitrogen and free amino acid nitrogen

Commercialized squids presented a reduction of 15.4% of the free amino acid nitrogen (FAA-N) and of 33.5% of non-protein nitrogen (NPN) in comparison with the control sample (Fig. 2). In squids submitted to CI treatment, the decrease of NPN continued along the storage, and 69.7% of NPN and 64.8% of the FAA-N of the muscle had been lost at day 9. The effect of leaching on the NPN content during the iced storage of squids is well known. Raghunath (1984) verified that the NPN content decreased in the mantle of squid (*Loligo duvauceli)* while the NPN concentration increased in water derived from ice during 8 h of storage. Romo et al. (1996) reported that squids of the *Dosidicus gigas* species had lost 40% of FAA after 72 h of storage in ice.

Changes in NPN and FAA-N contents in *L. plei* were less during storage in the NC treatment (Fig. 2). The NPN content remained stable after initially increasing,

Table 1
Chemical composition of squid mantle muscle (*Loligo plei*)[a]

| Moisture (%) | Lipids (%) | Ash (%) | TN (%) | NPN (%) | FAA-N (mg/100 g) | Protein (%)[b] |
|---|---|---|---|---|---|---|
| 74.2 (0.48) | 2.0 (0.04) | 1.7 (0.44) | 3.4 (0.20) | 1.1 (0.09) | 401 (5.27) | 14.4 |

TN, total nitrogen; NPN, non-protein nitrogen; FAA-N, nitrogen of free amino acids.
[a] Values represent average (standard deviation) of three repetitions.
[b] Calculated as: (TN − NPN) × 6.25.

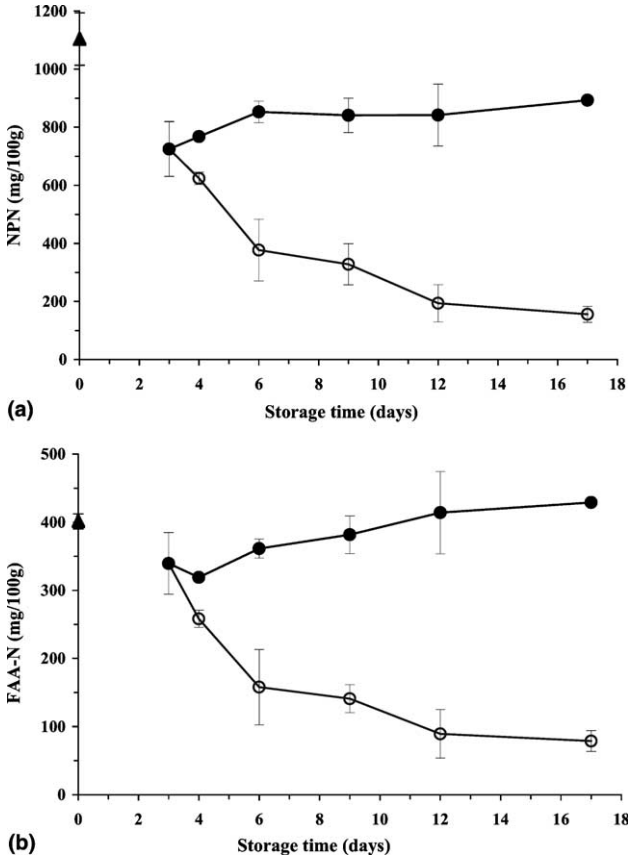


Fig. 2. Variations in contents of non-protein nitrogen (NPN) (a) and nitrogen of free aminoacids (FAA-N) (b) in muscle of control squids (▲) and in muscle of squids held in contact ice (○) and non-contact ice (●) treatments.

whereas the content of FAA-N showed an initial decrease, and after day 4 a tendency of this compound to increase in the squid muscle. Otsuka et al. (1992) also reported that the total content of FAA in squids of *Doryteuthis bleekeri* species stored in plastic bags in ice decreased after the first day, but afterwards remained stable until 15 days of storage.

The NPN determination as a freshness index for fish has little utility, because results are highly dependent on the species being studied, environmental factors and enzymatic activity. According Contreras (1994) the variations of NPN during the storage are dependent on the species, being related to their autolytic capacity and to their anatomical characteristics. In the present study, NPN and its FAA-N fraction were useful indices to verify the leaching occurrence, because they are soluble compounds that are present in high quantity in fresh squids. The results indicated the occurrence of an intense leaching during the storage in the CI treatment, as a consequence of the direct contact of squids with the ice. The peculiar format of squid body, allowing water contact with the outer and inner part of the mantle, generates a great contact surface, which facilitates the extraction of these soluble compounds from the muscle. Considering this aspect, the storage of squids without direct contact with ice would be a good option to avoid the loss of the NPN fraction.

The increase of NPN and of FAA-N contents during the storage can be a consequence of endogenous or microbial proteolytic enzymatic activity. In this study, the increase of FAA-N content in the squid muscle observed in the NC treatment was correlated ($P < 0.05$) with the increase of PsyC (Table 2).

### 3.2.3. Changes in volatile basic nitrogen, ammonia, trimethylamine and trimethylamine oxide

The muscle of control squids showed 9.0 mg of $NH_3$-N and 15.7 mg of VBN/100 g, with the $NH_3$-N representing 57.3% of VBN. The value for VBN is similar to those detected in other cephalopods 24–36 h after capture (Ruíz-Capillas et al., 2002). In commercialized squids the muscle presented 17.1 mg of $NH_3$-N/100 g, which represented 88.6% of VBN. In the CI treatment a decrease of VBN content occurred at the beginning of the storage, indicating that the loss by leaching was higher than the VBN production for endogenous or microbial proteolytic enzymatic systems (Fig. 3). Only after the day 12 did the VBN content overcome the initial concentration. In the NC treatment a progressive increase of the VBN content occurred since the beginning of storage, and a more intense formation after day 12, reaching 114 mg/100 g at day 17. The contents of

Table 2
Coeffients of Pearson correlation ($r$) between results of the chemical and microbiological analyses performed in squids (*Loligo plei*) held in the contact ice (CI) and non-contact ice (NC) treatments

| Microbiological analysis | Treatment | Chemical analysis | | | | | |
|---|---|---|---|---|---|---|---|
| | | NPN | FAA-N | VBN | TMA | Free tryptophan | Urea |
| Psychrotrophic bacterial counts | CI | $r = -0.481^{ns}$ | $r = -0.401^{ns}$ | $r = 0.718^{**}$ | $r = 0.992^{***}$ | $r = 0.049^{ns}$ | $r = -0.310^{ns}$ |
| | NC | $r = 0.511^{ns}$ | $r = 0.668^{*}$ | $r = 0.912^{***}$ | $r = 0.844^{***}$ | $r = 0.696^{*}$ | $r = 0.706^{*}$ |

$^{ns}$Not significant.
NPN, non-protein nitrogen; FAA-N, nitrogen of free aminoacids; VBN, volatile base nitrogen; TMA, trimethylamine.
$^{*}$ $P < 0.05$.
$^{**}$ $P < 0.001$.
$^{***}$ $P < 0.0001$.

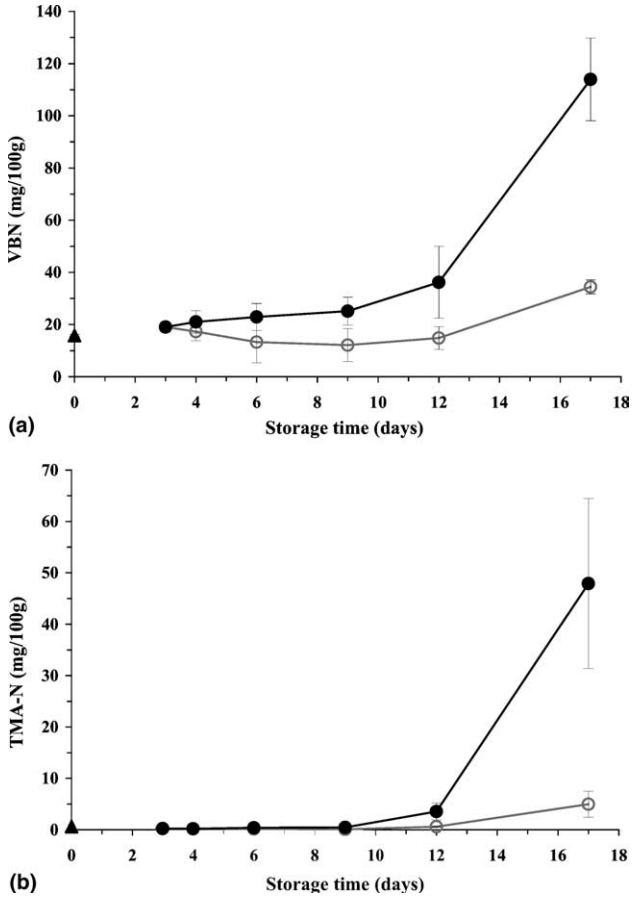


Fig. 3. Variations in contents of volatile basic nitrogen (VBN) (a) and trimethylamine (TMA-N) (b) in muscle of control squids (▲) and in squids held in contact ice (○) and non-contact ice (●) treatments.

VBN in squids submitted to the NC treatment became higher ($P < 0.01$) than those of the CI treatment from day 6 of storage. These results are similar to those found for other species of squids and cephalopods, in which the variations in VBN contents were small at the beginning of the storage. VBN has been considered useful as a spoilage indicator, having little use as a freshness index (Civera et al., 1999; Ohashi, Okamoto, Ozawa, & Fugita, 1991; Yamanaka, Shiomi, & Kikuchi, 1987).

The potential for TMA-N formation in *L. plei* is elevated, since a high content (193 mg/100 g) of trimethylamine oxide-nitrogen (TMAO-N) was determined in the muscle of control squids. A lower content of TMAO-N (82.6 mg/100 g) was found in the commercialized squids, probably as a result of the loss by leaching during the previous storage in the local market. Contreras (1994) determined a content of 224 mg of TMAO-N in fresh squids of the *Loligo* genera, a value similar to that detected in control squids in the present study. However, lower TMAO-N contents have been found in different squid species and octopus in other studies (Paarup et al., 2002; Ruíz-Capillas et al., 2002). Despite the high TMA-O values, the TMA-N formation in *L. plei* was slow in both storage conditions (Fig. 3). Values greater than 1 mg of TMA-N/100 g of muscle of squids were detected only after day 9 in NC treatment and after day 12 in CI treatment.

Variations of VBN and TMA-N contents were well correlated ($P < 0.05$) to the increase of PsyC in both storage conditions, in spite of the leaching occurring during CI treatment (Table 2).

### 3.2.4. Changes in free tryptophan and urea

Although the content of FAA is high in squids of the *L. plei* species, free tryptophan was detected in concentrations of barely 2.1 mg/100 g in control squids. Therefore, the increase of this amino acid during the storage could be a good indicator for proteolysis. The commercialized squids showed contents of 6.25 mg of free tryptophan/100 g. This content remained stable during the storage in the CI treatment. On the contrary, a progressive increase of free tryptophan contents occurred in squids held in the NC treatment. A level of 22.9 mg/100 g was determined at the end of the storage, which represents around 10 times as many free tryptophan as that found in control squid (Fig. 4). Free tryptophan levels in squids held in NC treatment were higher

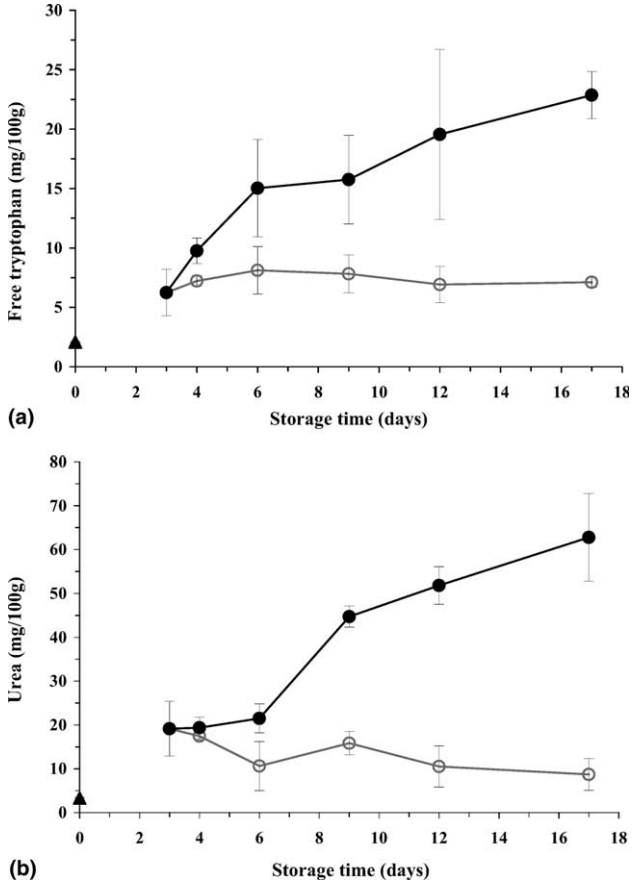


Fig. 4. Variations in contents of free tryptophan (a) and urea (b) in muscle of control squids (▲) and in squids held in contact ice (○) and non-contact ice (●) treatments.

($P < 0.01$) than in squids held in CI treatment after 4 days of storage. Romo et al. (1996) reported that free tryptophan formation was progressive and relatively intense in *D. gigas*, because it manifested despite the leaching.

The *L. plei* control sample presented 3.3 mg of urea/100 g, whereas in the commercialized squids the urea content was more elevated and equal to 19.1 mg/100 g, a value similar to that determined in *Todaropsis eblanae* 48 h after capture (Paarup et al., 2002). After initial variations, the urea levels lightly decreased in squid muscle from the day 9 of storage in the CI treatment, therefore the loss of urea overcame its production. On the contrary, in squids held in NC treatment the urea levels increased along the storage, and a more intense formation was observed after day 6, reaching 62.8 mg/100 g in day 17. These results agree with those obtained by Otsuka et al. (1992), who showed that the urea could be a good freshness index for squids of *Doryteuthis bleekeri* species stored in plastic bags in ice, because the content of this compound increased gradually during the storage.

Positive and significant correlations between the increase of PsyC and the formation of urea and free tryptophan were detected only in the NC treatment (Table 2).

## 4. Conclusions

The intense leaching of soluble compounds, which occurs during the storage of squids in direct contact with ice, drastically affects the efficiency of the chemical indices for freshness evaluation. The holding of squids in plastic bags during iced storage could introduce advantages concerning NPN and FAA preservation, without significantly altering the psychrotrophic bacterial counts. However, further investigations will be necessary to evaluate the growth of specific spoilage bacteria and pathogenic bacteria and to verify the implications of the use of each storage method regarding to consumer safety. TMA and VBN, the chemical indices traditionally used, showed no increase during the first days of storage in CI treatment and slow increase in NC treatment, serving little to estimate *L. plei* freshness and remaining shelf life. Free tryptophan and urea, on the contrary, could be useful freshness indices for *L. plei* when the leaching of soluble compounds is minimized, because their contents progressively increased from the beginning of storage in NC treatment. The storage treatment significantly affected all chemical indices evaluated but did not affect the PsyC in *L. plei*. Relatively low levels of TMA, VBN, free tryptophan and urea were found when the microbial contamination was already considerably high in squids held in CI treatment, showing that low contents of these compounds do not always correspond to squids of high quality. Therefore, acceptability limits for these chemical indices in *L. plei*, compatible with human consumption, will be different according to the storage condition.

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