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The Occurrence of Non-volatile Amines in Chilled-stored Rainbow Trout (*Salmo irideus*)

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ABSTRACT

*The effect of holding freshly caught whole rainbow trout (*Salmo irideus*) at 0, 10, 20 and 30°C for a period of 6 h prior to chilled storage was studied. Whole and eviscerated samples were analysed for putrescine, cadaverine, histamine, spermidine and spermine by high-performance liquid chromatography (HPLC) of their benzoyl derivatives after 2, 4, 7, 10 and 14 days of storage at 0°C. The concentrations of putrescine, cadaverine and histamine increased during storage, while those of spermidine and spermine decreased after an initial rise during the first 4 days. Tyramine was not detected in any of the samples. In general, holding of the samples at temperatures above 0°C for 6 h prior to chilling gave rise to increased concentrations of non-volatile amines. Eviscerated fish contained lower concentrations of amines than whole samples. Evidence is provided that the concentration of putrescine, and possibly that of cadaverine and histamine, in the flesh of chilled-stored rainbow trout, can be used as a criterion for the assessment of the freshness of the fish.*

INTRODUCTION

The freshness of fish is generally assessed by sensory evaluation which relies on trained members of carefully selected panels. However, certain types of spoilage which give rise to toxicity cannot be assessed either by appearance or odour and require analytical techniques. It is well established that spoilage of fish during cold storage is due mainly to the action of enzymes of the microflora on substrates such as trimethylamine oxide and amino acids (Ritchie & Mackie, 1980). Therefore, the determination of the total volatile bases (TVB) has been used as an indicator of spoilage. However, the determination of TVB is non-specific and should be supplemented by other methods to give a precise indication of the quality of chilled-stored fish (Herzberg *et al.*, 1977; Poulter *et al.*, 1981; Dawood *et al.*, 1986*a*,*b*). It was also pointed out by Dugal (1967) that the trimethylamine content is not an adequate indicator of the quality of freshwater fish.

Non-volatile amines have been identified in decomposed fish and histamine has been suggested as an indicator of the state of decomposition (Arnold & Brown, 1978), but other authors questioned the validity of this suggestion (Mietz & Karmas, 1977; Karmas & Mietz, 1978) on account of inconsistent results even within the same species of fish. Edmunds & Eitenmiller (1975) found a large increase in histamine content in mackerel and in mullet held at 24°C for 48 h, although storage at 4°C gave rise to very low levels. A number of authors studied the influence of storage temperature on the production of histamine. Eitenmiller *et al.* (1982) reported that rapid enzyme production and histamine formation occurred in yellowfin tuna inoculated with *Proteus morganii* when stored at 24 and 30°C compared with storage at 15°C. Okuzumi *et al.* (1984) confirmed that changes in the histamine content in mackerel depend on the temperature of storage and on the species of contaminating bacteria. Shewan & Liston (1955) reported that histidine, the precursor of histamine, is easily decarboxylated at 0°C. Edmunds & Eitenmiller (1975) detected very little histamine (less than 1 μg/g) in marine fish stored for 14 days at 4°C, but they found that histamine content increased on storage at 24°C, although it never reached levels which are thought to cause poisoning (i.e. more than 1000 μg/g). Yoshinaga & Frank (1982) showed that the optimum temperature for the production of histamine is 38°C. In tropical regions, where the temperature of the water is high and where cooling and chilling facilities are inadequate, histamine content rises to toxicologically significant levels within 12 h at 38°C (Frank *et al.*, 1981). According to Arnold & Brown (1978) histamine-producing organisms are found on the skin and in the gills and intestines of many fish, and are part of the normal microflora.

Histamine appears to be the main compound on which most investigations related to fish spoilage have been based. However, Takagi *et al.* (1969, 1971) confirmed the presence of other amines, including putrescine, cadaverine and tyramine, in decomposed marine products. In investigations of tuna, rockfish, salmon, lobster and shrimp to establish a chemical index of fish decomposition, Mietz & Karmas (1977, 1978) identified the polyamines putrescine, cadaverine, spermidine and spermine, along with histamine. They reported that as decomposition progressed, histamine, putrescine and cadaverine increased, while spermidine and spermine decreased. Ienistea (1973) pointed out that diamines such as putrescine, cadaverine and spermine may facilitate the transport of histamine through the intestinal wall and increase the toxicity of the fish flesh.

The purpose of this study was to investigate the production of non-volatile amines, as an index of loss of quality, in freshwater fish held at temperatures up to 30°C for 6 h prior to chilled storage, thus simulating conditions prevailing in warm climates where considerable time may elapse before the catch is packed in ice. The effect of the pre-storage temperature was subsequently studied during storage at 0°C, in whole as well as in eviscerated fish, for a period of up to 14 days.

MATERIALS AND METHODS

Materials

Putrescine, cadaverine, histamine, spermidine and spermine were obtained as their hydrochloride salts from the Sigma Chemical Company. These were dissolved in water to give standard solutions containing 120 μg/ml. Benzoyl chloride and all other reagents were of analytical grade.

Methods

Preparation of samples

Rainbow trout were obtained from Scot Trout Ltd (Scotland). The fish were divided into four groups and each group was held for 6 h at 0, 10, 20 and 30°C, respectively. Fish that had been held at the indicated temperatures were divided into two subgroups; one subgroup consisted of entire (whole) fish, while the other consisted of fish from which the heads and the viscera had been removed, followed by thorough washing. The fish were then packed in polyethylene bags and stored at 0°C without delay. From each treatment of 6 hours' duration, four fish were taken for testing immediately

(zero time) and after 2, 4, 7, 10 and 14 days. For analysis the fish were filleted and passed through a mincer.

Extraction of amines

The amines were extracted by an adaptation of the method described by Mietz & Karmas (1978). Minced flesh (50 g) was mixed with 75 ml of trichloroacetic acid (TCA, 5 g/100 ml water) in a blender for 2 min, then centrifuged at 2000 × *g* for 10 min. The supernatant was filtered through glass wool. This treatment was repeated twice more and the combined supernatants were made up to 250 ml with 5% TCA. From this solution 10 ml (≡2 g sample) were transferred in duplicate into glass-stoppered tubes (25 ml capacity), to which 4 g NaCl, 1 ml NaOH (50 g/100 ml) and 5 ml chloroform:butan-1-ol (1:1) were added, and shaken vigorously for 2 min. After centrifugation for 5 min, the organic (upper) layer was transferred into a separating funnel. The extraction was repeated with 2 × 5 ml portions of chloroform:butan-1-ol. To the combined extracts in the separating funnel, 15 ml of *n*-heptane were added and shaken with 3 × 1 ml portions of 0·2 M HCl. Each portion of 0·2 M HCl was transferred into a Pyrex tube (10 ml capacity, provided with a screw-cap fitted with PTFE-faced rubber disc) with the aid of 1 ml of water. The tubes were immersed in water at 80°C and the combined extracts were evaporated to dryness in a current of air.

Preparation of benzoyl derivatives

The derivatives were prepared according to Redmond & Tseng (1979). To the dry residue (from the previous section) 2 M NaOH (1 ml) was added, followed by 5 μl of benzoyl chloride, mixed on a vortex mixer and allowed to stand for 20 min. Saturated NaCl solution (2 ml) was then added, followed by extraction with 2 ml of diethyl ether. After centrifugation, the upper organic layer was transferred into a clean tube and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 100 μl of methanol, filtered through a Millipore filter (catalogue No. SJHVL04NS, 0·45 μm pore size) and 5-μl aliquots were injected for HPLC analysis. A standard mixture containing the pure amines (equivalent to approx. 10 μg of each amine in the hydrochloride form) was similarly treated for the preparation of the benzoyl derivatives.

High-performance liquid chromatography (HPLC)

The instrument used was a Varian LC 5000 fitted with a manually-operated (Valco) loop injection system and a 4·6 × 250 mm Brownlee RP-8 column (5 μm), protected by a 40-mm guard column packed with the same material. A Varian UV 50 variable wavelength detector was used at 254 nm and at a bandwidth of 8 nm. The detector signal (absorbance = 0·05 full-scale

deflection) was transmitted to a chart recorder and to an electronic integrator (Pye Unicam DP 88). The mobile phase was an isocratic mixture of methanol:water (62:38 by volume) and the flow rate 1·1 ml/min at room temperature.

RESULTS AND DISCUSSION

A chromatogram of the standard mixture of benzoylated amines is shown in Fig. 1. The use of methanol:water (62:38 by volume, 1·1 ml/min), as an eluent, ensured the fairly rapid elution of tyramine (23·4 min), which has not been reported by Redmond & Tseng (1979). No loss of resolution was noticed with this eluent. The derivatives were found to be stable and required no special precautions for storage, in accord with the claims of the

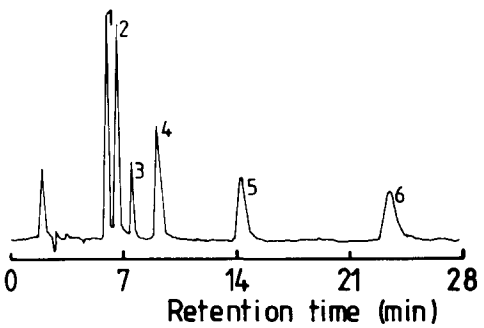

Fig. 1. HPLC chromatogram of the benzoyl derivatives of a standard mixture of non-volatile amines: 1, putrescine; 2, cadaverine; 3, histamine; 4, spermidine; 5, spermine; 6, tyramine.

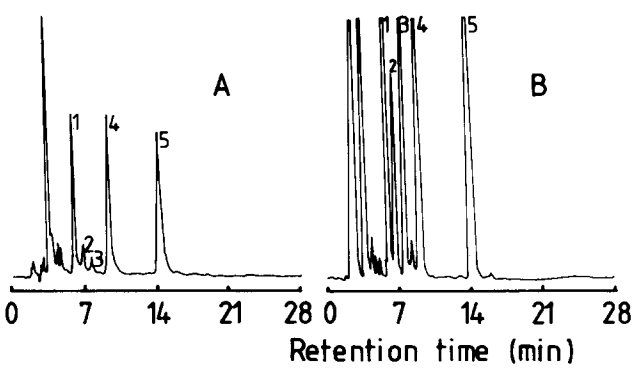

Fig. 2. HPLC chromatograms of the benzoyl derivatives of non-volatile amines extracted from rainbow trout: A, first day of catch; B, advanced decomposition. Peak identities as in Fig. 1.

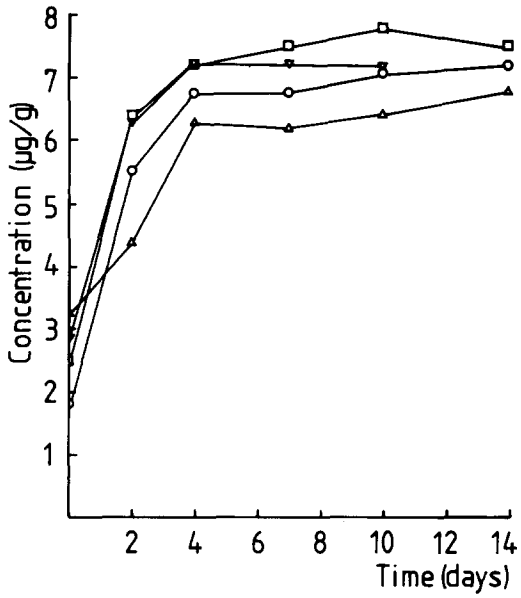

Fig. 3a. Putrescine content of whole trout as a function of storage time at 0°C, after an initial 6-h hold at: ○, 0°C; □, 10°C; △, 20°C; ▽, 30°C.

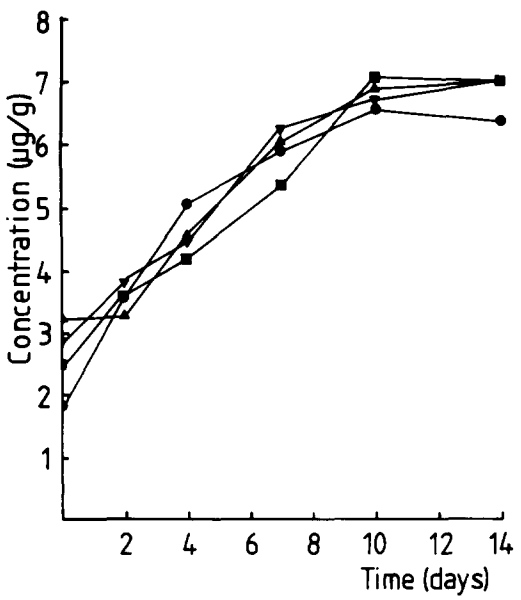

Fig. 3b. Putrescine content of eviscerated and washed trout as a function of storage time at 0°C, after an initial 6-h hold of the whole fish at: ●, 0°C; ■, 10°C; ▲, 20°C; ▼, 30°C.

aforementioned authors. Chromatograms of fresh and decomposed trout are shown in Fig. 2. In spite of the advanced decomposition, no tyramine could be detected in any of the samples. The two unidentified peaks in the region of 3 to 5 min were found to vary in size according to the storage conditions and may be of use in the assessment of spoiled samples; attempts will be made towards their identification.

Analytical data for the non-volatile amines obtained over a period of storage of up to 14 days are given in Figs 3 to 7. At zero time, the concentration (μg/mg flesh) of putrescine and cadaverine was related to the temperature at which the whole fish was stored for 6 h prior to chilling, suggesting that high temperatures favoured decomposition of the fish (Figs 3a and 4a). The concentration of putrescine was also approximately three times higher than that of cadaverine. The concentrations of both diamines increased rapidly by a factor of approximately 3 during the first 4 days of chilled storage, then they remained practically constant. In eviscerated fish, putrescine increased relatively slowly over a period of 10 days (Fig. 3b), while cadaverine increased only slightly (Fig. 4b). The low levels of putrescine and

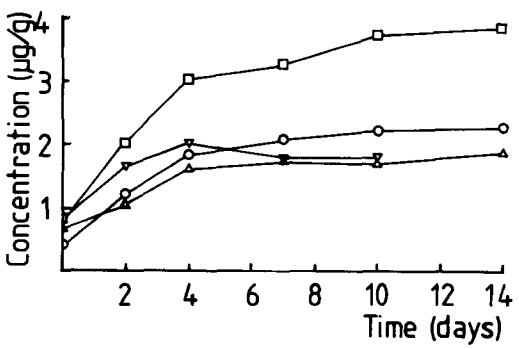

Fig. 4a. Cadaverine content of whole trout. Conditions as in Fig. 3a.

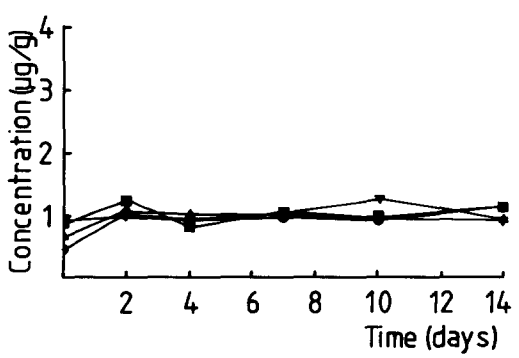

Fig. 4b. Cadaverine content of eviscerated trout. Conditions as in Fig. 3b.

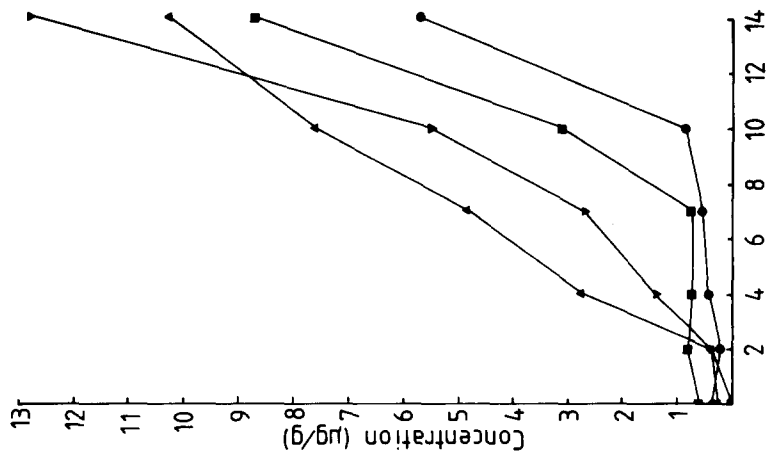

Fig. 5b. Histamine content of eviscerated trout. Conditions as in Fig. 3b.

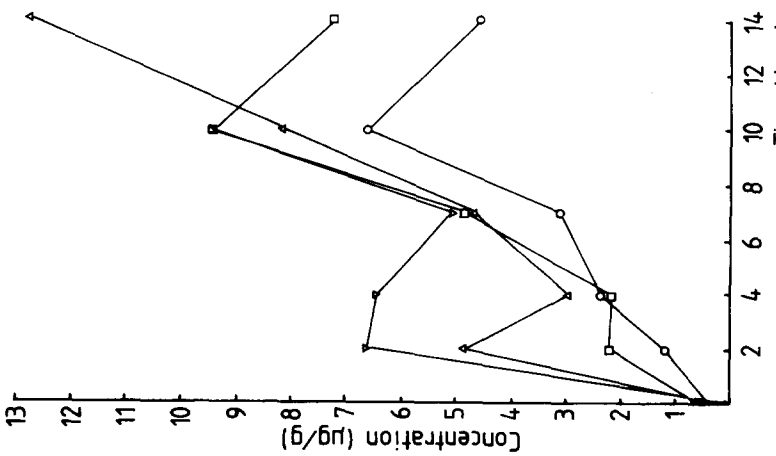

Fig. 5a. Histamine content of whole trout. Conditions as in Fig. 3a.

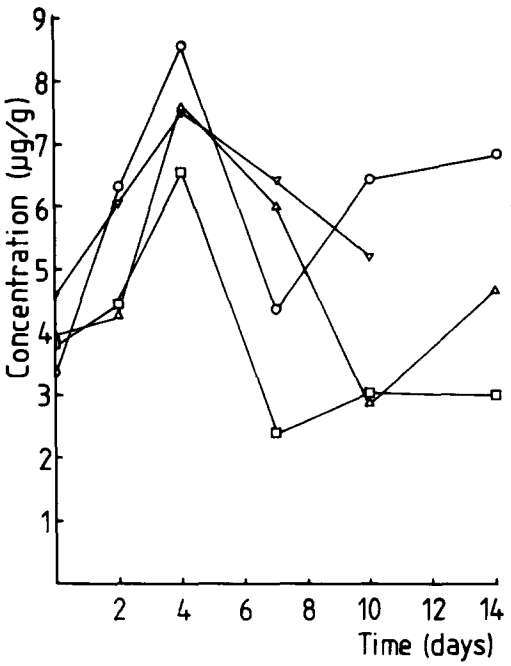

Fig. 6a. Spermidine content of whole trout. Conditions as in Fig. 3a.

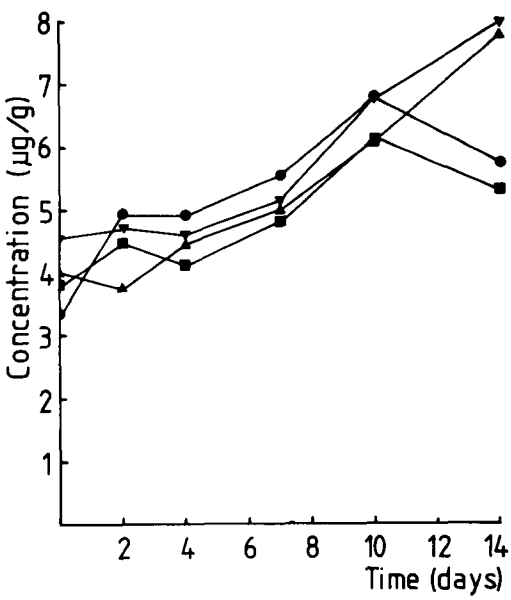

Fig. 6b. Spermidine content of eviscerated trout. Conditions as in Fig. 3b.

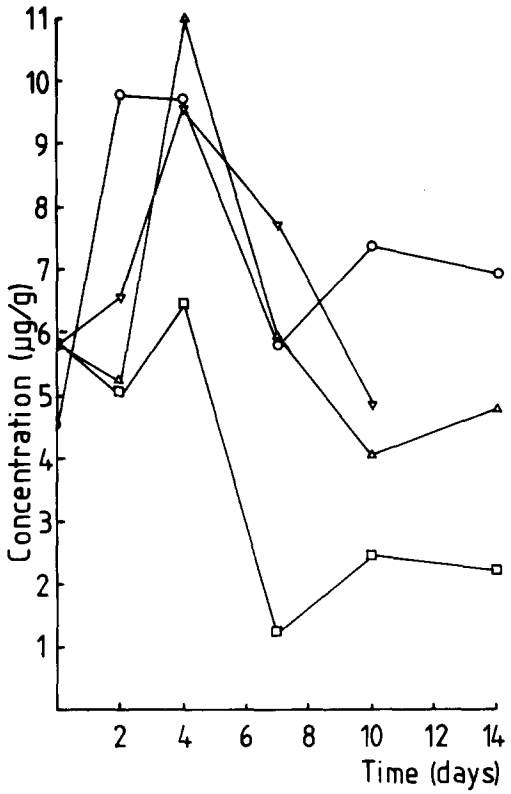

Fig. 7a. Spermine content of whole trout. Conditions as in Fig. 3a.

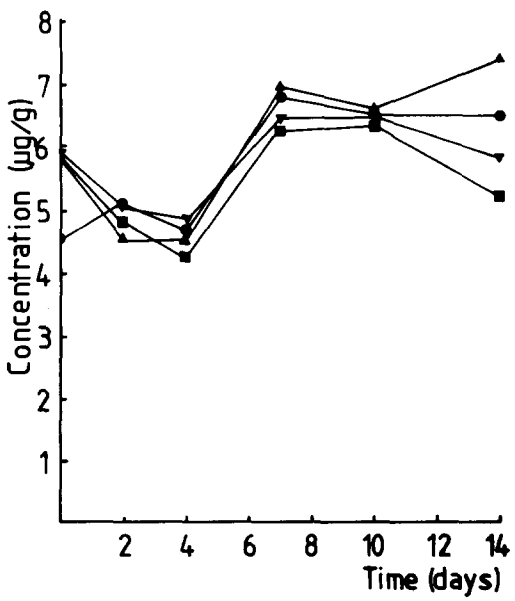

Fig. 7b. Spermine content of eviscerated trout. Conditions as in Fig. 3b.

cadaverine in eviscerated fish can be related most probably to diminished enzymic activity due to the elimination of the contaminating microflora, while the rapid formation of putrescine in the whole fish can be attributed to high enzymic activity, leading to decarboxylation of glutamic acid and arginine. Karmas & Mietz (1978) suggested that additional putrescine is formed from the breakdown of the two polyamines, viz. spermidine and spermine. Cadaverine was low in all samples at zero time and especially in the samples chilled immediately on receipt. Higher levels were found in samples of whole fish held at 10, 20 and 30°C before storage at 0°C, suggesting bacterial decarboxylation of lysine, an amino acid which is abundant in fish.

Histamine, the product of decarboxylation of histidine, was highest in fish held at 10°C (Fig. 5a), while only traces were found in fish held at 30°C for 6 h prior to chilling. However, after 14 days' storage, all samples contained increased levels of histamine. It should be noted that these levels were well below the 1 mg/g limit, a concentration which is considered sufficiently high to cause poisoning (Arnold & Brown, 1978). Differences in the rate of production of histamine under varying conditions are probably due to variations in the numbers of the contaminating micro-organisms. Salguero & Mackie (1979) suggested that histidine is higher in the viscera and that their removal is advisable. Ienistea (1971) reported that histamine formation is not related to the total bacterial flora but rather to a group of bacteria which synthesise histidine decarboxylase. In eviscerated fish (Fig. 5b) the increase in histamine content was related to the temperature at which the whole fish was held prior to chilling. Pre-chilling temperatures of 0 and 10°C resulted in the delay of histamine production by 6 and 10 days, respectively.

The development of the polyamines spermidine and spermine differed from that of the other amines. Their high initial concentration increased (Figs 6a and 7a) up to the fourth day of chilled storage, especially in the whole fish, and then declined in accord with the findings of Karmas & Mietz (1978), although it never reached zero. A more consistent pattern in the concentration of the two polyamines was found in eviscerated fish (Figs 6b and 7b) and this suggests that microbial action in whole fish must be responsible for the observed variations.

In general, the formation of non-volatile amines during the period of 14 days in chilled storage, as a function of the holding temperature prior to chilling, was in good agreement with sensory data reported for the whole fish (Dawood *et al.*, 1986*a*). Furthermore, the levels of the amines were not correlated with the numbers of micro-organisms per unit area of the fish skin, since these numbers increased moderately with time in chilled storage, although they were strongly dependent on the holding temperature before

chilling. It is likely that the non-volatile amines in the flesh of the fish depend mostly on the intestinal microflora, which was not investigated.

It is also noteworthy that, although the formation of hypoxanthine appears to be a good chemical indicator of loss of quality for trout (Dawood *et al.*, 1986*b*), HPLC analysis of the non-volatile amines provides additional and, possibly, more complete information with regard to the overall acceptability of the fish.

CONCLUSIONS

The results of the present work indicate that the concentrations of putrescine and cadaverine in rainbow trout increase by a factor of approximately 3 after 4 days' storage of the whole fish at 0°C. The temperature at which the fish is held prior to chilling has a relatively minor effect. The determination of these two diamines is, therefore, suggested as a reliable indicator of the extent of spoilage of the fish.

The concentration of histamine is sensitive to the temperature of storage prior to chilling. Evisceration of the fish leads to low levels of histamine during chilled storage, particularly during the first 7 days. In spite of the relatively rapid increase in histamine content during chilled storage of the whole fish, the concentrations measured in the variously treated samples of rainbow trout were well below those reported to cause poisoning.

Further work is required in order to establish whether the development of putrescine, and possibly cadaverine and histamine, can be used as an absolute indicator of spoilage in other species of freshwater fish.

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