K value measurement
for the determination of Fish & Seafood freshness

Enzymatic method or HPLC method

Aim: Determination of the level of freshness of foodstuffs, especially for fish and seafood.

How do nucleotides indicate the freshness level of a Fish (& meat)?

ATP (adenosine triphosphate) is the source of energy for muscle contraction. Its content is particularly high in muscle tissue. **As soon as an animal dies**, when slaugthered for instance, cell respiration stops as well as ATP formation. After few minutes, the *rigor mortis* stage is reached and a sequential and progressive **ATP depletion** starts. This catabolism of ATP goes through a succession of reactions, as shown on the following figure.

The K value concept

In the late 1950's, a Japanese research team (Saito *et al.*) proposed a new concept, called "K value", for the indication of the freshness of fish. It is based on ATP breakdown and the subsequent formation of nucleotide by-products, namely inosine monophosphate (IMP), inosine (Ino) and, at a later stage, hypoxanthine (Hx).

K-value measures how far ATP degradation has progressed within a tissue. It is expressed as a percentage of the content of the last two final compounds of the ATP catabolic pathway, *i.e.* "Ino" and "Hx", over the total content of ATP and its degradation products, that is to say ATP, ADP, AMP, IMP, Ino and Hx.
However, because ATP decomposes very quickly to IMP in most animals, a simplified K value (generally called K_I value) was soon proposed by Karube *et al.* (1984) and is currently considered as equivalent to the original 6-parameter equation for K-value:

$$K = \frac{[Ino] + [Hx]}{[IMP] + [Ino] + [Hx]}$$

The lower the K value, the fresher the flesh.

K value is considered for several decades as **the most effective and objective indicator of the freshness of fish, as well as of meat (beef, pork, lamb and poultry)**

Because nucleotides are stable to heating and ATP depletion follows the same "universal" catabolic pathway, K value can be measured on:

any transformed products:	... and any species:
• Chilled or frozen fish	• Finfish (freshwater, seawater)
• Raw, smoked, salted, pickled or even cooked seafood	• Shellfish (*i.e.* Scallops)
	• Crustaceans (Shrimps, Prawns, Crab...)
	• Cephalopods (Squid, Octopus...)

Why and when measuring K-value?

Freshness is the most important quality criteria regarding fish and seafood products. If freshness can be quite easily checked on entire crude and chilled finfish by sensory analysis, freshness control remains puzzling when considering transformed fish.
Current CQ analytic tools, such as TVBN or TMA, are routinely used but they are based on the biochemical changes that appear when spoilage has started, and can be relevant for a medium- or a late-stage of the process.
However, since ATP depletion is the very first biochemical process that occurs once a fish has died, long before spoilage starts, K-value changes concern the early stage of fish transformation and storage and allows professionals to anticipate the beginning of spoilage (TVBN formation) and to better control the "highly" freshness level of the product. In other words, K-value is the choice freshness marker at the early stage of the process.
Most of Japanese researchers consider that raw fish can be eaten as *sashimi* when the K-value is up to than 20%.

Example: Tuna samples analysis

Analytical system: 1)Agilent 1100 series liquid chromatograph fitted with binary pump G1312A, vacuum degasser G1322A, well-plate autosampler G1367A, thermostated column compartment G1316A and multiple wavelenght and diode array detector G1315B. Run and data acquisition are controlled by Agilent ChemStation software.
2) Analytical column Zorbax Eclipse Plus C18 4.5x150mm, 3.5µm particle size and analytical guard column Zorbax Eclipse Plus-C18 4.6x12.5mM 5µm (Agilent).

HPLC conditions: Nucleotides were analyzed by a ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides* with slight modifications in the pH and concentration of buffers adjusted to ensure adequate resolution of all nucleosides.

* D. Di Pierro, B. Tavazzi, C. Federico Perno, M. Bartolini, E. Balestra, R. Calio`, B. Giardina, G. Lazzarino (1995) **An Ion-Pairing High-Performance Liquid Chromatographic Method for the Direct Simultaneous Determination of Nucleotides, Deoxynucleotides, Nicotinic Coenzymes, Oxypurines, Nucleosides, and Bases in Perchloric Acid Cell Extracts** *Analytical Biochemistry* 231, 407–412

HPLC calibration

Stock solutions of Hypoxanthine (Hx, from Serva), Inosine, (IR, from Sigma-Aldrich) and Inosine Monophosphate (IMP from MP Biochemicals) at 100mM were freshly prepared. 6 concentrations were used for HPLC calibration: 50, 100, 250, 500, 750 and 1,000µM (Fig. 1).
The area of individual peaks was measured using ChemStation software (Agilent). The data were used
a) for the identification of Hx, IR and IMP peaks in fish extracts;
b) for the calculation of nucleotide concentration in fish extracts.

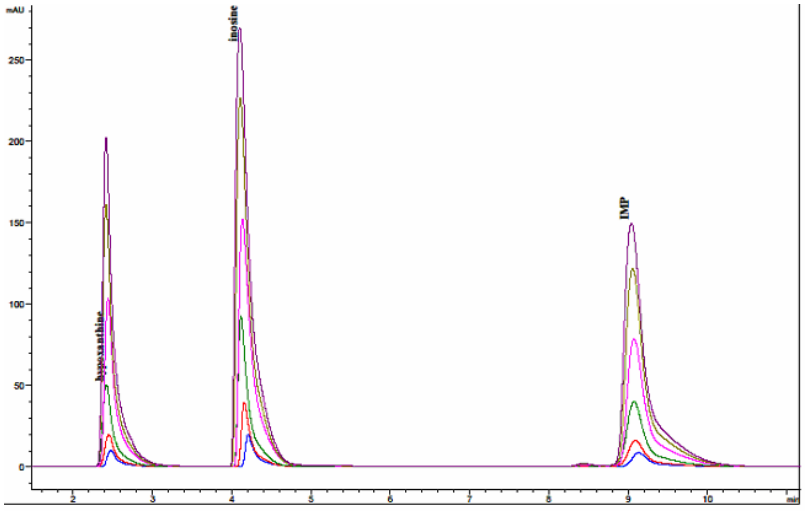

Fig. 1. Superposed chromatograms of Hx, IR, IMP used for calibration curves.

Sample preparation: For each sample of frozen Tuna, a slice of about 1g (done in duplicate) of raw material was cut off, thawed by boiling for 15min, and further used for nucleotide extraction. Sample deproteinisation was done by addition of TCA (trichloroacetic acid) to 10% TCA and centrifugation at 10,000g, 20min. TCA was back-extracted with water-saturated diethyl ether (3 times), and diethyl ether was further dried off from the extracts.

Quantification results: Linear calibration curves were obtained for Hx, IR, IMP over calibration range of 50-100µM. Determination coefficient values were of R^2 0.9991 for all three nucleotides. The regression formulas were used for calculation of Hx, IR and IMP and K value (defined as **(IR+Hx) / (IMP+IR+Hx) concentrations ratio**) in Tuna extracts.

Table 1 illustrates high reproducibility of results obtained with two independently produced extracts, as also shown on Figures 2 and 3.

Results:

Samples	Hx (µM)	IR (µM)	IMP (µM)	$K = \frac{[IR] + [Hx]}{[IMP] + [IR] + [Hx]}$
1a	8,78	66,80	746,20	9%
1b	9,18	74,58	778,95	10%
2a	50,20	175,66	356,34	39%
2b	73,54	214,54	453,89	39%

Table 1. Concentration of Hx, IR, IMP and K-values in two samples, 1 and 2, analysed in duplicate (a and b)

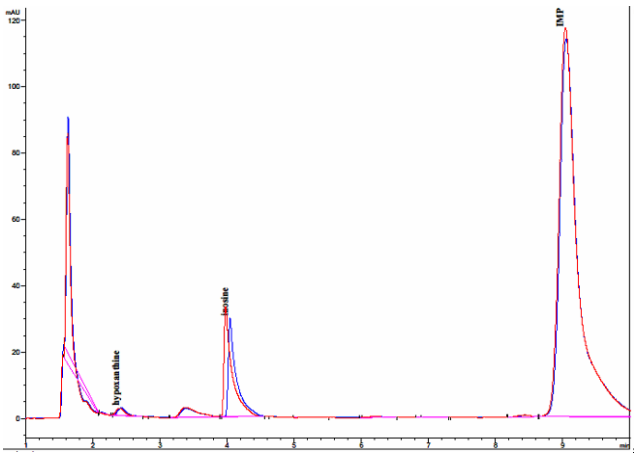

Fig. 2 Superposed chromatograms of two independent extracts of sample 1 **(K-value 10%)**

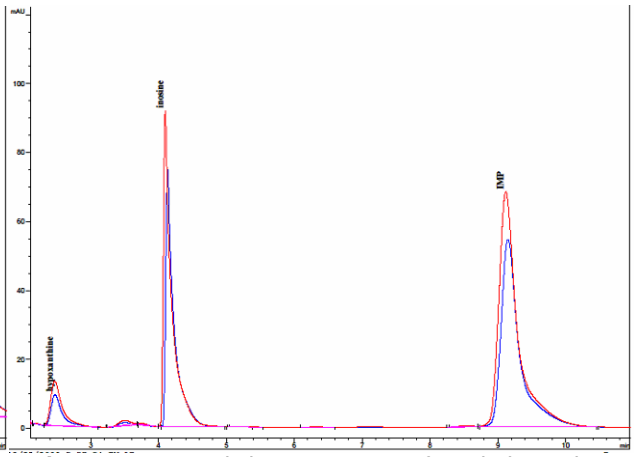

Fig. 3 Superposed chromatograms of two independent extracts of sample 2 (**K-value 39%**).

NOVOCIB proposes to measure the K-value of your fish samples by HPLC.
We are currently developing a very innovative and reliable system based on an enzymatic procedure that will soon be proposed as a "do it yourself" kit.

How to do in practice

Samples should be kept frozen for delivery, preferably in dry ice. However, given that nucleotide contents may change in raw fish even at very low temperature, it is recommended to heat the meat sample in boiling water for few minutes and to keep it clean before sending it at frozen temperature: enzymes responsible for nucleotide catabolism will be inactivated and the K-value be unaffected by the transport. Prepared this way, your samples will be perfectly adapted to a nucleotide extraction and the subsequent analysis of the K-value.

For any technical questions, please feel free to contact us.

Please, note that our enzymatic assay for K-value determination will soon be available as a kit. Do not hesitate to contact us if you want to be informed about its expected launching.

Applications:

Freshness of Finfish, Shellfish (scallop), Crustaceans (shrimp, prawn, crab…), Cephalopods (squid, octopus…)
Raw, chilled, frozen, smoked, salted, pickled, cooked seafood products
K-value can be used on other meat-based foodstuffs: beef, lamb, pork, chicken…

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K. Sasaki *et al.* (2007): **Changes in the amounts of water-soluble umami-related substances in porcine longissimus and biceps femoris muscles during moist heat cooking**, *Meat Science* 77(2), 167–172
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M. Dondero *et al.* (2004): **Changes in quality of vacuum-packed cold-smoked salmon (*Salmo salar*) as a function of storage temperature**, *Food Chem.* 87(4), 543–550
V.P. Lougovois *et al.* (2003): **Comparison of selected methods of assessing freshness quality and remaining storage life of iced gilthead sea bream (*Sparus aurata*)**, *Food Resear. Intern.* 36(6), 551–560
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R. Mendes *et al.* (2001): **Changes in baseline levels of nucleotides during ice storage of fish and crustaceans from the Portuguese coast**, *Eur. Food Res. Technol.* 212(2), 141–146