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Fast Protein and Metabolites (Nucleotides and Nucleosides) Liquid Chromatography Technique and Chemical Sensor for the Assessment of Fish and Meat Freshness

Artur Kuznetsov [1], Aleksandr Frorip [1], Alar Sünter [1,2], Nensi Kasvand [1], Vadim Korsakov [3], Georgii Konoplev [4], Oksana Stepanova [4], Linda Rusalepp [2], Dea Anton [2], Tõnu Püssa [2], Mati Roasto [2], Liubov Abramova [5], Andrey Kozin [5], Lauri Toom [6], Soeren Hirsch [7] and Nikolay Mukhin [7,*]

1 AS Ldiamon, 50411 Tartu, Estonia
2 Veterinary Biomedicine and Food Hygiene, Estonian University of Life Sciences, 51006 Tartu, Estonia
3 Jeko Disain OÜ, 51014 Tartu, Estonia
4 Department of Photonics, Saint Petersburg Electrotechnical University "LETI", Saint Petersburg 197022, Russia
5 Research Institute of Fisheries and Oceanography, Moscow 105187, Russia
6 Institute of Chemistry, University of Tartu, Ravila 14A, 50411 Tartu, Estonia
7 Department of Engineering, University of Applied Sciences Brandenburg, 14770 Brandenburg an der Havel, Germany
* Correspondence: nikolay.mukhin@ovgu.de

Abstract: Fast protein and metabolite liquid chromatography (FPLMC) was introduced years ago to enable the easy separation of high-molecular compounds such as proteins from small molecules and the identification of the low-molecular substances. In this paper, the method is applied for the rapid evaluation of freshness and monitoring the aging of animal meat and fish. A novel chromatographic sensor was developed with a deep UV LED-based photometric detection unit (255–265 nm), an original flow cuvette and registration scheme; the processing of a chromatogram with the sensor takes approximately 15 min. Strict isochronism between the elution of ATP metabolites, mainly hypoxanthine (Hx) and inosine monophosphate (IMP), and the time of maturation of meat or fish, was discovered. A new freshness index H* = [Hx]/[IMP] was introduced, which is proportional to the instrumental delay time in the FPMLC chromatograms: the H* index < 0.5 indicates the presence of inosine monophosphate (IMP) and the high quality of the meat or fish. Reasonably strong correlations were revealed between data obtained by FPMLC and total volatile basic nitrogen TVB-N (for fish) or volatile fatty acids VFA (for meat) content. Moreover, putative nucleotide salvage and an increase in the concentration of IMP were observed in fish after heat treatment using the FPMLC sensor and NMR technique.

Keywords: fast protein liquid chromatography; UV detection; meat freshness; fish freshness; inosine monophosphate; hypoxanthine; nucleotide salvage

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1. Introduction

Fast protein liquid chromatography (FPLC) is a form of liquid chromatography that was developed in 1982 by Pharmacia LKB (Uppsala, Sweden) for proteins as a partial alternative to high-performance liquid chromatography (HPLC) [1]. Initially, FPLC was considered exclusively as a preparative technique for the purification and fractionation of proteins and other biopolymers, but later it was widely demonstrated that the method can be successfully used in analytical tasks as a more affordable and cost-effective method than HPLC. The price of an FPLC column is approximately ten times lower than the price of a corresponding HPLC column, and the total cost of the test can be up to 30 times lower [2,3]. The positive features of FPLC include high loading capacity, biocompatible aqueous buffer systems, high flow rates, and the availability of stationary phases for most common chromatographic modes (e.g., ion exchange, gel filtration, reversed phase, and affinity chromatography) [4]. The use of FPLC as a characterization and analytical

technique can be particularly relevant in the fields where the availability of rapid and affordable analytical methods and tools is crucial, e.g., routine test in healthcare or the food industry [2,3,5–9]. In this context, FPLC can be modified and enhanced with the ability to fractionate and detect low and middle molecular weight metabolites: ATP metabolites nucleotides and nucleosides, advanced glycation end products (AGE), uric acid, and other metabolic products originating from various biological samples [5,7–9]. In our opinion, the term fast protein and metabolites liquid chromatography (FPMLC) is more appropriate for such applications and will continue be used in this paper.

The ability of FPMLC to separate and detect ATP metabolites can be particularly important in food science and technology, because relative changes in the concentrations of ATP metabolites: adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine (Ino), inosine monophosphate (IMP), and hypoxanthine (Hx) during the post-mortem catabolism of adenosine triphosphate (ATP) in muscle tissue have been proven to be a reliable indicator of the freshness of animal or fish meat. The chain of ATP transformations after animal slaughter or harvesting fish is accompanied by a gradual decrease in the molecular weights of the ATP metabolites (the molecular weights in Daltons are in parentheses) [10,11]:

$$\text{ATP}(507) \rightarrow \text{ADP}(427) \rightarrow \text{AMP}(347) \rightarrow \text{IMP}(348) \rightarrow \text{Ino}(268) \rightarrow \text{Hx}(136). \quad (1)$$

The first step of the chain is relatively fast: the biochemical processes of ATP, ADP and AMP transformation to IMP are generally limited to 24 h. As a result, muscle tissue rapidly accumulates IMP, which is partly responsible for the pleasant taste (umami) and high nutritional value of meat and fish products [12]. In the following slower step, IMP is further degrading into Ino and Hx; the process is often associated with loss of freshness. In the final stage of bacterial spoilage, Hx is converted to xanthine, uric acid, and other circle cleavage end products [10,11]. The freshness index K is calculated as the ratio the concentrations of ATP metabolites concentrations (usually presented in μmol/L) [13]:

$$\text{K} = \frac{[\text{Ino}] + [\text{Hx}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}] + [\text{IMP}] + [\text{Ino}] + [\text{Hx}]} \cdot 100\% \quad (2)$$

This index was first introduced in 1959 in Japan and has been widely used worldwide to evaluate fish freshness [14]. This correlates with fish taste and flavor: very high-grade products have a K-value of less than 10%; high-grade products have K-values lower than 20% or 30%, depending on fish species; fish with a K-value up to 50% are of medium grade; K-values larger than 50–70% are obtained for low-grade samples [15]. Multiple alternative indices based on ATP catabolism (K, K_0, K_I, H, P, G, Fr, Hx, etc.) were also proposed for various fish and shellfish species [10]. The index K_I is of particular interest, because it was specifically designed to assess freshness after more than 24 h post-mortem, when almost all ATP, ADP and AMP have already been catabolized:

$$\text{K}_\text{I} = \frac{[\text{Ino}] + [\text{Hx}]}{[\text{IMP}] + [\text{Ino}] + [\text{Hx}]} \cdot 100\%. \quad (3)$$

Starting from the 1980s, the K-value and, in some cases, the index K_I have been also applied for pork, beef, rabbit, and poultry [16–19]. Nevertheless, there are still no universal approaches to freshness evaluation based on ATP breakdown, which are equally applicable for meat and fish testing, and leading researchers in these fields work independently [15,19].

The main convenience of the K-value approach compared with universally accepted standard methods, e.g., determination of total volatile basic nitrogen (TVB-N), trimethylamine nitrogen (TMA-N) or volatile fatty acids (VFA), is the ability to detect changes in food freshness during storage at an early stage of quality declination, before any evidence of microbial spoilage appears [20,21]. Despite the obvious advantages of this approach, it is still used quite rarely in routine food quality control because conventional laboratory methods for the determination of ATP metabolites, including HPLC, nuclear magnetic

resonance (NMR) spectroscopy, and mass spectrometry, are cumbersome, time consuming, and require extremely expensive analytical instruments operated by highly qualified staff [22,23]. By their nature, these instrumental methods cannot ensure rapid on-site freshness testing, and sensory evaluation by organoleptic methods, which are strictly regulated by national standards (GOST 7631-2008 in Russia and Council Regulation (EC) No 2406/96 in EU), are still dominant in the industry. An alternative approach to the EU standard is the quick Quality Index Method (QIM) [24].

Biosensor and microfluidic technologies have demonstrated great potential in the field of medical testing and food quality control [25–27]. The development of biosensors for the express testing of fish freshness is particularly active, as low testing time and the possibility of on-site analysis play a crucial role here. Electrochemical and enzymatic biosensors are capable of detecting marker substances such as xanthine, hypoxanthine, histamine, and uric acid; gas biosensors can detect volatile amines [19,28–31]. Moreover, multienzyme biosensors were developed for the simultaneous detection of ATP and its post-mortem breakdown products, which can directly measure the freshness indices K and K_I [19,29–36]. In addition to enzymatic biosensors, more conventional assay kits based on enzymatic reactions with microplate spectrophotometric detection are available on the market, but the price per test is quite high [37].

Undoubtedly, biosensors and microfluidic technologies can significantly simplify sampling procedures, detection efficiency, and portability of analytical systems for fish and meat freshness evaluation. At the same time, obvious difficulties in achieving high reproducibility and stability of characteristics during biosensor manufacturing and storage, the complexity of detection systems, and the high cost of disposable biosensors, comparable with the cost of some fish species [28], prevent their wider practical application as a convenient and cost-effective alternative to conventional analytical techniques and organoleptic evaluation. Prospects for the development of microfluidic biosensor chips are also inextricably linked with progress in chip technology [38] and on-wafer techniques for characterization purposes [39,40].

The obvious gap between sophisticated and expensive laboratory analytical instruments and miniaturized biosensing microfluidic devices can be filled with compact, simplified mid-range instruments based on classical methods of analysis, but which can be used on-site, outside the laboratory, do not require complex sample preparation, and are suitable for express testing. The aim of this work is the development and validation of a compact, affordable, non-disposable optical chemical sensor and analytical protocol for the rapid on-site evaluation of meat and fish freshness using FPMLC with UV photometric detection complemented with the ability to determine the relative content of the nutritional nucleotide and nucleoside originating from ATP post-mortem degradation.

2. Materials and Methods

2.1. FPLMC Optical Chemical Sensor

An optical chromatographic sensor based on FPMLC with UV photometric detection at a wavelength of 260 nm was designed and manufactured by Ldiamon AS (Tartu, Estonia) for meat and fish freshness evaluation. Previously, optical sensors based on the same principles were developed by the authors of this work for point-of-care diagnosis and screening of proteinuria, and assessment of protein loss during continuous ambulatory peritoneal dialysis; the instruments were clinically tested on urine and effluent peritoneal dialysate samples, respectively [6,7].

The chromatographic sensor is adapted for low-cost commercially available PD-10 protein desalting columns (Code No. 17-0851-01) from GE Healthcare® Bio-Sciences AB (Uppsala, Sweden), repurposed for the separation and detection of nutritional nucleotides and nucleosides. The PD-10 is a gel-filtration column filled with Sephadex G-25 Medium chromatographic gel originally designed for protein purification; the column is a single-use type according to the instruction for use, but it was demonstrated that it can be successfully regenerated and used multiple times up to several hundred tests [7]. The photometric

detection unit employs a narrow-band (255–265 nm) deep UV LED with a quartz collimator and a visible-blind UV photodetector (Figure 1a). The detection wavelength corresponds to the UV absorption maxima of the ATP and its main metabolites: ATP, ADP, and AMP (259 nm); IMP and Ino (248 nm); Hx (249 nm) [41]. The UV absorption spectrum of ATP aqueous solution (concentration 0.3 mM, cuvette thickness 10 mm) and the normalized spectrum of the UV LED emission (maximum emission 262 nm, FWHM 12 nm) measured with the AvaSpec-2048 spectrophotometer from Avantes B.V. (Apeldoorn, The Netherlands) are presented in Figure 1b. The photo of the device is presented in the Figure 2.

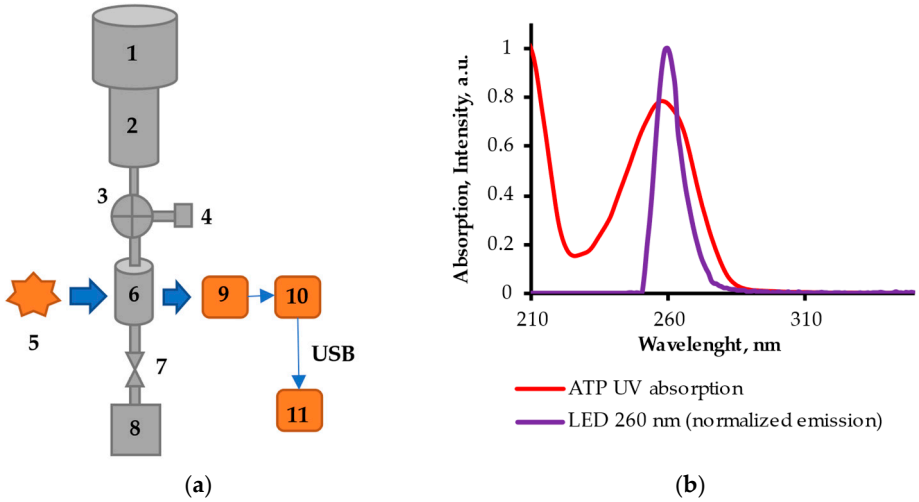


Figure 1. The general principles of the sensor operation: (**a**) Schematic diagram of the sensor; (**b**) ATP UV absorption and UV LED emission spectra (1–LabMate buffer reservoir (Code No. 18-3216-03) from GE Healthcare® Bio-Sciences AB (Uppsala, Sweden); 2–PD-10 column; 3–three-way valve; 4–service port; 5–UV LED; 6–flow cell; 7–flow rate regulator; 8–drain vessel; 9–photodetector; 10–electronic unit, 11–laptop PC).

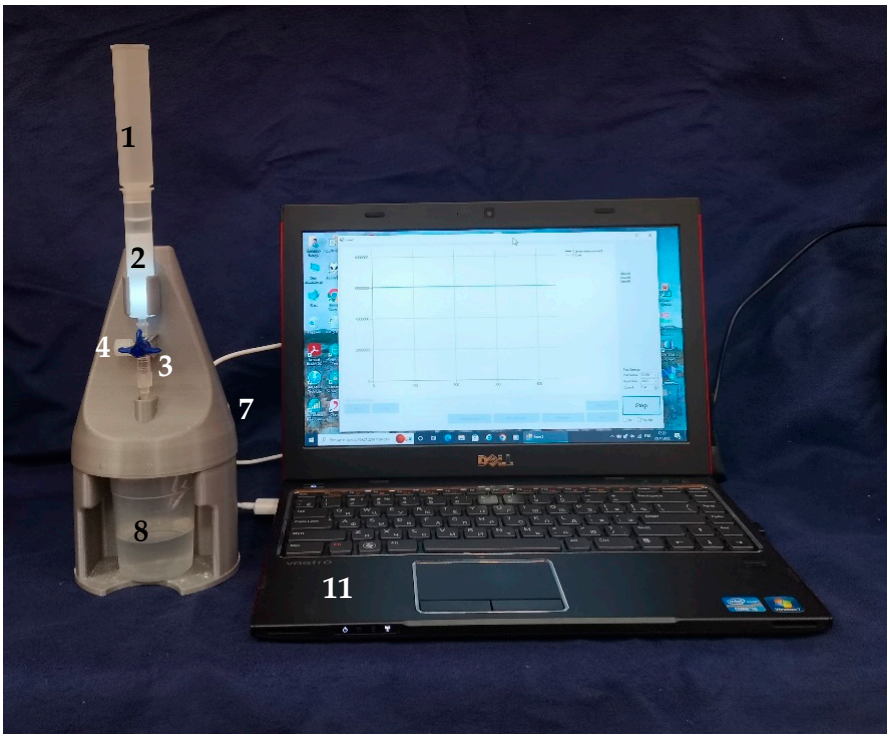


Figure 2. The photo of the sensor.

The sensor is a gravity flow chromatograph instrument: reservoir 1 is filled with TRIS buffer (pH 8.0), which flows via column 2 into flow cell 6 of the photometric detection unit into drain vessel 8. A mechanical regulator 7 is necessary to adjust buffer flow rate: in normal operation, it is about 2 mL/min when the buffer reservoir is full and about 1 mL/min when the reservoir is almost empty. The three-way valve 3 can be set to three positions: (a) the column is locked (standby mode), (b) the column is connected to the flow cell (chromatogram-recording or column-regeneration modes), and (c) the flow cell is connected to the service port 4 for manual cleansing or air bubble removal (a Luer-Lock syringe filled with buffer solution must be connected to the service port for this operation). A more detailed description of the device can be found in Kuznetsov et al., 2022 [7].

2.2. Chemicals

TRIS buffer (pH 8.0) contained 10 mM of tris(hydroxymethyl)aminomethane (TRIS), 150 mM of sodium chloride (NaCl), and 2 mM of ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2); washing solution (pH 13.0) was 200 mM of sodium hydroxide (NaOH). TRIS (Product No. GE17-1321-01), EDTA (Product No. ED), sodium hydroxide (Product No. S5881), and sodium chloride (Product No. S9888) were purchased from Sigma-Aldrich (Darmstadt, Germany).

Standards of bovine serum albumin (BSA), ATP, IMP, Ino and Hx were also from Sigma-Aldrich., TRIS buffer, acetonitrile of ultra lc grade for LC-MS was from Romil PC (Cambridge, UK); ultrapure water was prepared by EASYpure RF, Barnstead type.

2.3. Sample Preparation

As samples of animal products for freshness testing with the FPMLC device, porcine *longissimus dorsi* muscle (striploin) obtained from a slaughterhouse within 24 h post-slaughter and completely fresh or within 24 h after catch farmed fish fillets of Trout (*Oncorhynchus mykiss*), Carp (*Cyprinus carpio*) were used. To analyze the effects, the thermal processing and putative nucleotide salvage defrosted fish fillets of Alaska pollack (*Theragra chalcogramma)*, Hake (*Merluccius)*, Norway haddock (*Sebastes)*, Pangasius (*Pangasianodon hypophthalmus)*, Wolffish (*Anarhichas)* from a local supermarket were used as samples of consumer products. Alaska pollack was frozen on 10 January 2021and the best before day (BBD) was 2 August 2023; Wolffish was frozen on 22 September 2021and the BBD was 22 September 2022; the experiments with Wolffish were carried out late August 2022; the other fish fillets had no data on the packaging.

The muscle tissue was finely minced with a meat grinder or cut into small pieces, approximately, of 2 mm× 2 mm × 2 mm, two grams of the ground meat (pH = 5.6) were placed into a 15 mL test tube with 6 mL of TRIS buffer (pH = 8.0). The mixture was shaken for 10–15 min in a rotator Biosan Multi RS60 (BioSan, Riga, Latvia), and used a syringe filter Whatman® GF/B (Product No. Z242195) from Merck KGaA (Darmstadt, Germany). For thermal processing, the samples were put in a microwave oven for 1–2 min at 400 W or steam cooked at +100 °C for 15–60 min; in this case, the fish fillets were cut into slightly larger pieces of 5 mm× 5 mm × 2 mm.

When working in the fields, a simplified version of the just-described sample preparation protocol is possible; for instance, when a rotator is not available, the tube with sample pieces can be manually shaken.

2.4. pH Determination

pH values of the samples were determined in homogenates composed of 1 g of sample and 9 mL of distilled water. Readings were taken with Consort C833 digital pH-meter (Consort, Turnhout, Belgium) at room temperature, and the pH meter calibration was regularly checked.

*2.5. FPMLC Measurement Protocol and New Freshness Index H**

The FPMLC device was operated in the same mode as previously described in [42,43]. A total of 6 drops (200–300 µL) of the extract prepared according to the procedure described in Section 2.3 were placed directly on the gel surface in the PD-10 column previously washed with the NaOH wash solution (pH 13.0) and regenerated with 25 mL of TRIS buffer (pH 8.0). After the sample was completely absorbed by the gel, another 25 mL of TRIS buffer was added to the reservoir, and chromatograms were measured according to the procedure described in the work [7].

Gel filtration separates the molecules in the extract based on their size: proteins and other macromolecules larger than the pores of the gel matrix (the exclusion limit of the PD-10 column is M_r = 5000 Da) are quickly eluted with the void buffer volume outside the matrix, while smaller molecules, e.g., nucleotides and nucleosides, enter the pores and elute later at different times depending on the molecular weight [44,45]. The photometric detection unit of the sensor continuously measures the UV transmittance $T(t)$ of the eluate at a wavelength of 260 nm and records it at a time interval of 1 s; the optical transmission signal $T(t)$ is then converted into the optical density of the eluate (OD); the recording time of one chromatogram is about 15 min.

FPMLC chromatograms of both fish and mammalian meat samples consist of two main parts: a sharp protein peak and a broad post-protein band (Figure 3), which is formed by the merger of individual peaks of the main nucleotide and nucleoside actors. During meat storage, ATP is broken down by the enzymes into metabolites with a lower molecular weight according to the chain of transformations presented in (1), as a result of which, the retention time of the metabolites increases. The main parameter of FPMLC used in the measurements is the interval between the retention times of the broad band maxima of the metabolites (FPMLC fraction II) and the sharp protein peak (fraction I) on the FPMLC chromatograms (Figure 3), hereafter referred to as the index Time. This parameter directly corresponds to the relative content of different ATP metabolites and increases gradually during meat storage.

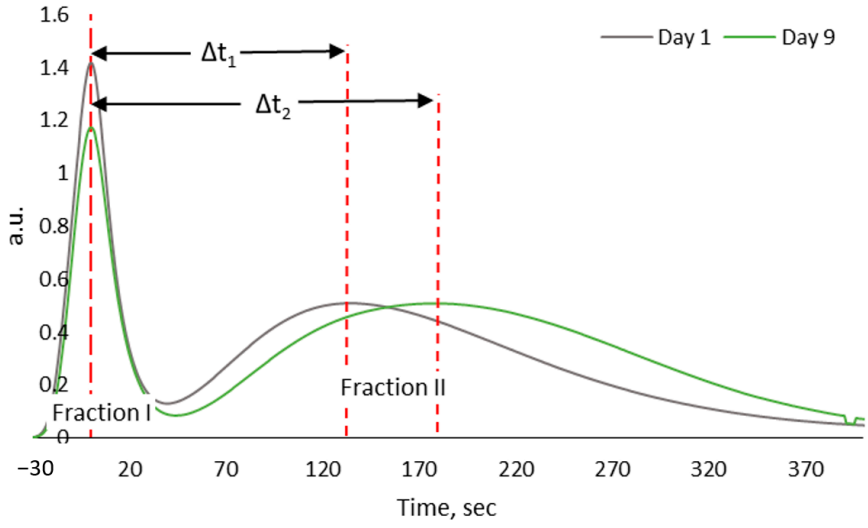


Figure 3. Comparison of two FPMLC chromatograms obtained on days 1 and 9 of a horse meat sample storage.

The first stage of this process, often described as absolute freshness, when ATP, ADP and AMP are still detected in a sample in significant amounts, lasts only several hours post-mortem; thus, fresh meat or fish are extremely rare in the food industry. In the following stages, IMP, Ino, and Hx become dominant and determine the shape of chromatograms and the index Time. In the final stage of massive bacterial contamination and obvious spoilage, Hx is mainly detected, and the maximum retention time for FPMLC fraction II is reached.

To establish the relationship between FPLMC data and the relative content of the ATP metabolites, Equation (3) can be further simplified, and a new freshness index H*, proportional to the instrumental lag time, can be introduced:

$$H* = \frac{[Hx]}{[IMP]}. \quad (4)$$

This assumption is based on the anticorrelation between the concentrations of inosine monophosphate [IMP] and hypoxanthine [Hx]. The anticorrelation phenomenon makes it possible to level out random deviations in the concentrations of Hx and IMP and increase the accuracy of measurements to the value Stdev/Mean ≈ 3%. H* index < 0.5 indicates the presence of IMP and high quality of the meat/fish.

2.6. Validation Methods

Results obtained by FPLMC were validated by direct identification and quantification of ATP metabolites by LC-DAD MS or NMR spectroscopy, and by comparison with widely recognized standard meat (VFA) and fish (TVB-N) quality control test methods.

2.6.1. LC-DAD MS

Liquid chromatographic analysis of the broad band of metabolites fraction II from FPMLC was carried out on a 1290 Infinity system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6450 Q-ToF mass spectrometer equipped with a Jetstream ESI source. Samples were subjected to a Zorbax 300SB-C18 column 2.1 × 150 mm; 5 µm (Agilent Technologies), and maintained at 40 °C. A gradient of 0.1% of formic acid in water (A) and 5% of water in acetonitrile (B) was used to separate the compounds as follows: 0.0 min 1% B, 3.0 min 1% B, 3.01 min 99% B, 11.1 min 99% B, 11.01 min 1% B, and a regeneration time of 8 min. The eluent flow rate was 0.3 mL/min and the injection volume was 2.5 µL. The mass-spectrometer was operated in the negative ion mode in the mass-to-charge ratio (m/z) range of 100–1000 Da. UV absorbance was measured at λ = 250 nm. Data acquisition and initial data processing were performed with MassHunter software (Agilent Technologies).

Identification of IMP, Ino and Hx in the fraction II of the FPMLC eluate samples was performed by comparing MS/MS and UV spectra of these substances with respective parameters of analytical standards. IMP, Ino, and Hx were quantified by UV absorption at 250 nm using the external calibration curve method. Methanolic standard solutions were prepared at concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 µM for analytical standards of all three metabolites. Calibration curves were characterized by a high correlation coefficient (R^2 = 1) [46].

2.6.2. NMR Spectroscopy

The water-soluble polar metabolites of fish samples were extracted with 7.5% trichloroacetic acid (TCA) solution, as described in [47,48]. For this purpose, 25 g of fish muscle was added to 50 mL of 7.5% TCA and homogenized with a vertical homogenizer. The homogenate was filtered through a paper filter and the filtrate was neutralized with 9 M solution of KOH up to a pH of 7.8. The solution was filtered through a regular paper filter and stored at minus 40 °C.

NMR measurements were performed using a Bruker Avance III 700 NMR spectrometer equipped with a 5 mm BBO probe. The ^{1}H Larmor frequency was 700.08 MHz. The ^{1}H NMR spectra were measured at 298 K with solvent suppression using the noesypr1d pulse sequence. The acquisition time was 3.67 s; the recycle delay was set to 6.00 s. For every ^{1}H NMR spectra, 1520 scans were collected. NMR solutions were prepared by adding approximately 20% of D_2O containing 0.05% (w/v) sodium 2,2,3,3-tetradeuterio-3-trimethylsilylpropanoate (TSP-d_4) to the aqueous samples. All spectra were referenced to TSP-d_4 (0 ppm). Corrections of phase and baseline were performed with the Bruker Topspin 3.6.2 (Bruker, Rheinstetten, Germany). The free induction decays (FIDs) were multiplied by a line-broadening function of 0.3 Hz prior to Fourier transformation.

2.6.3. TVB-N and VFA Determination

The evaluation of VFA (for pork) and TVB-N (for fish) was carried out the at National Centre for Laboratory Research and Risk Assessment (before 1 January 2023–Estonian Veterinary and Food Laboratory, Tartu, Estonia, https://labris.agri.ee/en, accessed on 2 January 2023), according to the requirements of standard EVS-EN ISO/IEC 1705:2017 and Laboratory of Fish Products Quality Control, Russian Federal Research Institute of Fisheries and Oceanography (Moscow, Russia, http://vniro.ru/en/, accessed on 2 January 2023) independently. The measurements of TVB-N were conducted according to the method EU 2074/2016 (Estonia) or GOST 7636-85 (Russia); VFA content has been measured according to GOST 23392-78-2/1980.

3. Results

3.1. FPLMC Sensor Calibration

As a general rule in gel-filtration chromatography, the elution time of chemical substances with a molecular weight higher than the column exclusion limit depends on the molecular weight. For ATP metabolites, this means that the lowest elution time belongs to ATP, which has the largest molecular weight (M = 507) among the substances in the transformation chain (1), and the highest elution time to Hx, which has the lowest molecular weight (M = 136).

On the contrary, all proteins and other high molecular weight substances, such as free RNA and DNA, are synchronously eluted shortly after the process is started, as larger molecules cannot penetrate the gel pores. Since various water-soluble proteins are always present in animal and fish products, the first protein peak could be used as a reference point (zero on the timescale) from which elution times of nucleotides and nucleosides are counted.

To calibrate the FPMLC device, 100 µL of 1 g/l BSA and 100 µL of ATP, IMP, Ino, or Hx standard solutions were applied to the PD-10 column, and respective chromatograms were recorded (Figure 4) according to the procedure described in [7,42,43]. The mean time intervals between the BSA peak and the peaks of ATP, IMP, Ino, and Hx are presented in the Table 1: as predicted, the elution time monotonously increases in inverse proportion to the decrease in the molecular weight.

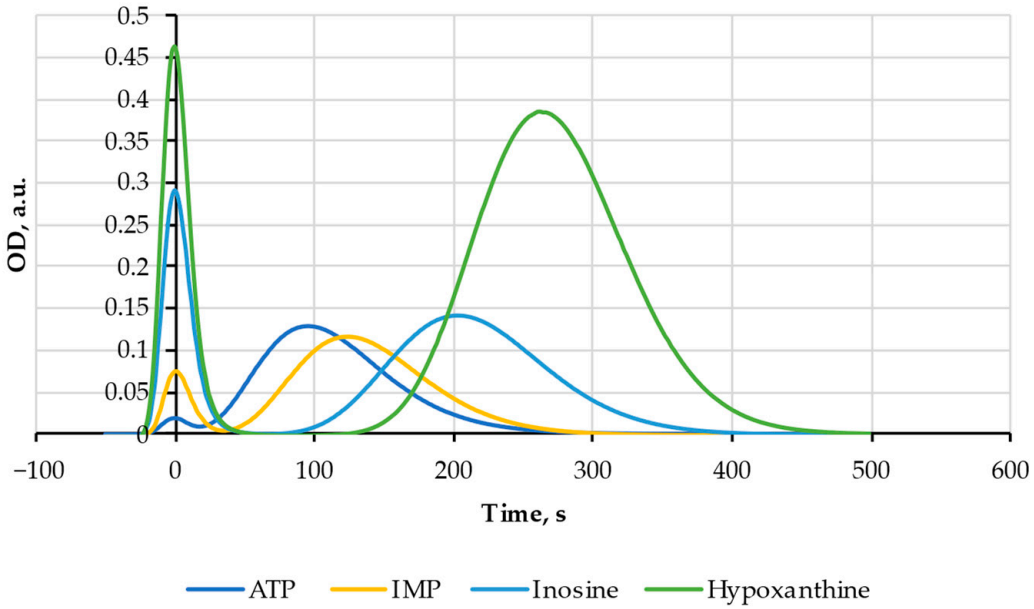


Figure 4. FPLMC sensor calibration with BSA and the nutritional nucleotides and nucleosides standards.

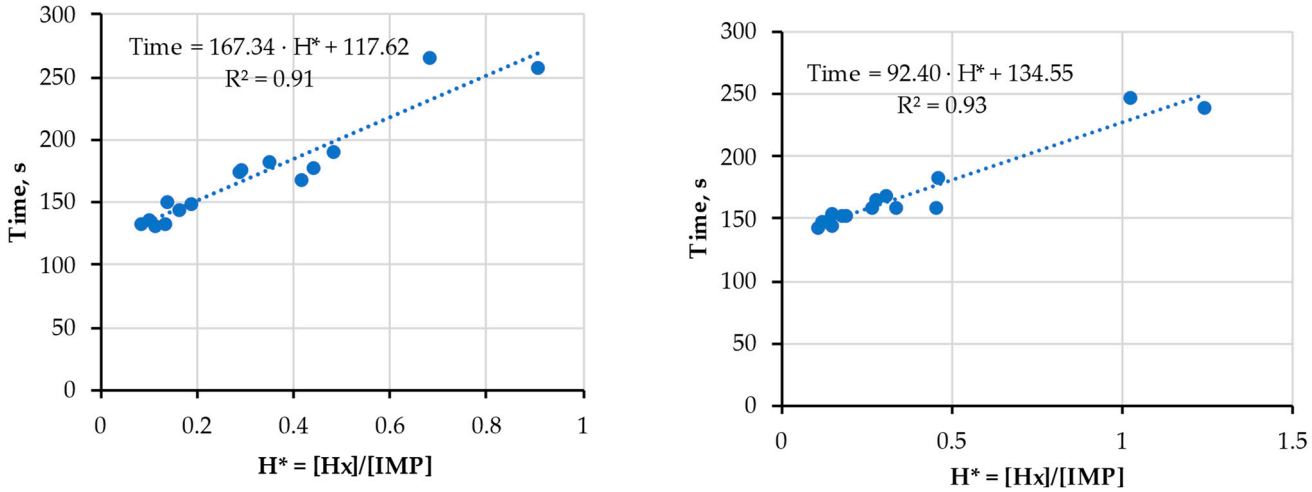
Table 1. Elution times of the main nutritional nucleotides and nucleosides.

Substance	Molecular Weight, Da	Elution Time, s
ATP	507	95
IMP	348	130
Ino	268	200
Hx	136	275

In case of routine measurements, this procedure should be repeated regularly, at least with hypoxanthine, and corresponding to the final point on the timescale; the time between BSA and Hx peaks should be kept constant near 275 ± 5 s by adjusting the buffer flow rate if necessary.

3.2. Verification of Compatibility of New Index H and FPMLC Data*

The next step was to establish the correlation between the independently obtained new freshness index H* = [Hx]/[IMP] (LC-DAD MS technique) and the index Time (FPMLC technique). This experiment can be also considered as a verification procedure of the FPMLC method by the LC-DAD MS technique [46]. Figure 5 shows the interdependences of the indices Time and H* measured during the tests of pork, both for raw samples stored aerobically (Figure 5a) and for the same samples after cooking (Figure 5b). The largest values exceeding the threshold H* >> 1 were excluded from the datasets for the diagrams in Figure 5.



(**a**) (**b**)

Figure 5. Correlation between the index H* and the index Time for raw (**a**) and cooked (**b**) minced pork stored or previously stored aerobically.

The correlations in Figure 5a,b are similar, but still have important peculiarities. In case of H* ≈ 0, the meat is absolutely fresh and the value of Time is correspondingly the smallest. For raw meat (Figure 5a), this value derived from the correlation formula is 117.6 s. The Special Time measurement within four hours after slaughter gave Time = 114 ± 5.7 s for another pork in pieces. The good agreement between the extrapolated and directly measured values strengthens confidence in the validity and perfect compatibility of the H* and Time indices.

3.3. Results of Validation of FPMLC Data by VFA and TVB-N Methods

The test results obtained with the FPMLC device were verified by the TVB-N method with farmed trout and carp samples during storage from the day 0 to day 14 in aerobic conditions at +4 °C. Figure 6 shows the dynamics of the average index Time and TVB-N

values obtained with farmed trout caught in spring and autumn seasons. It can be observed that both curves are close in shape and correlate with the coefficient r = 0.85. The onset of the strong TVB-N increase observed on day 11 and later on correlates well with the published data [37,49–51]. Moreover, the value TVB-N at day 7 in Figure 6 (the very onset of bacterial contamination according to the FAO scheme [52]) of 16 ± 2.83 mg/100g agrees very well with the average value that can be deduced from the data in [37,49–51], i.e., 16.25 ± 4.2 mg/100g. This means that quite typical trout fish have been used.

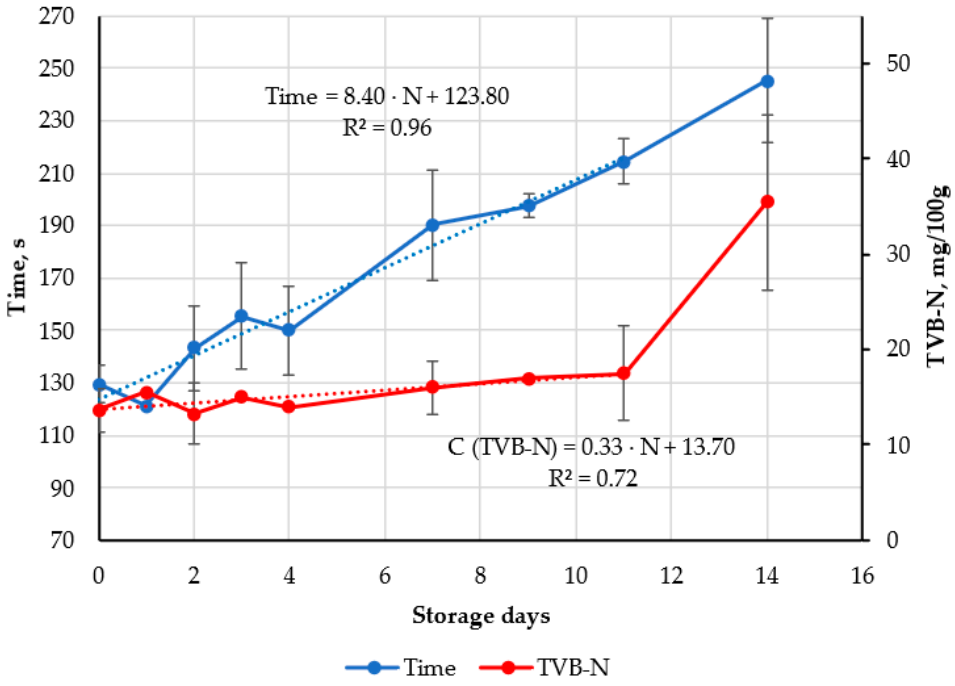


Figure 6. Averaged index Time obtained with FPMLC device and TVB-N content values for farmed trout during storage.

A similar comparison of Time and TVB-N values was conducted for carp and a correlation r = 0.8 was obtained. This, it can be concluded that the FPMLC device can provide relevant data for freshness, at least, of the well-known trout and carp fish.

The compatibility of the freshness test results with the FPMLC device with widely accepted meat quality control method based on VFA content was evaluated for porcine *longissimus dorsi* muscle (loin), obtained from a slaughterhouse the day after slaughter. The fillet sample was minced by means of a manual grinder with a sieve with 3 mm orifices. Minced meat was stored aerobically (in a plastic box) at +4 °C for 14 days. Measurements with the FPMLC device and by the VFA method were performed twice in each measurement during the 14-day period synchronously, with an accuracy not worse than 2 h. The relative standard deviations (Stdev/Mean) of the Time index and TVB-N content were in the interval of 0−5% usually around 2.5%. Bacterial contamination (CFU/g) was also estimated, but microbiological tests were synchronized more freely (within the same day's work shift) with the FPLMC and VFA measurements, due to the fixed working schedule of the microbiological laboratory.

The evolution of the Time index and VFA content during the storage period is presented in the Figure 7: the data demonstrate that VFA content increased slowly during the first 9 days, then both the Time index and VFA jump sharply, accompanied by an increase in bacterial contamination to 1.6×10^8 CFU/g from the initial level 7.2×10^3 CFU/g and pH 7.0 from the initial value of pH 5.6.

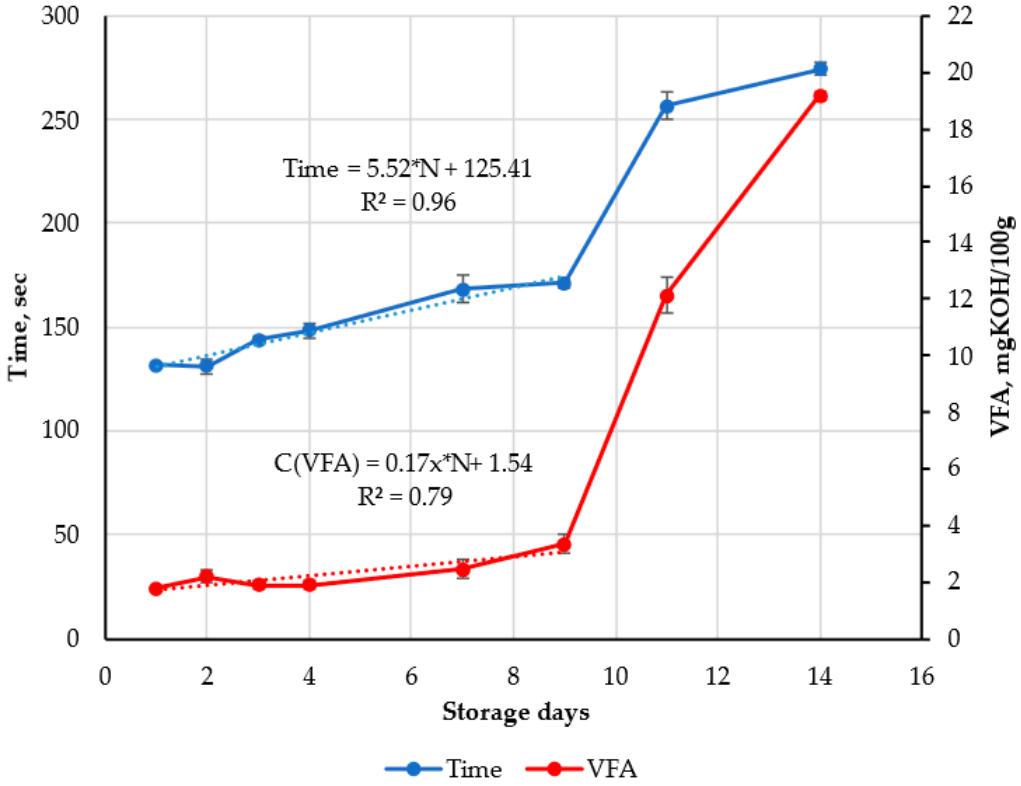


Figure 7. Temporal change of the index Time obtained with FPMLC device and VFA content during storage of minced pork at +4 °C under aerobic conditions.

Figure 7 shows the strictly parallel trajectories of the Time and VFA curves during the first 9 days of storage, which is confirmed by the high correlation coefficient r = 0.96 between these two parameters. The results obtained by the FPLMS method are quite compatible with the data of microbiological safety, which prove that the product is safe for consumption until the 9th day of storage at +4 °C. After the 11th day, the minced meat had an unpleasant off-smell of spoilage and a bad appearance, which made it unacceptable for culinary use.

A more detailed comparison of the curves at the initial stage of storage highlights a significant fact: when the meat is still in the autolysis phase (bacterial contamination on the 7th day was 2×10^6 CFU/g, which is below the threshold level 10^7 [53]) the trajectories of the curves are parallel, but the irregularities of the Time index are much less obvious than for VFA data, and the Time index correlates much better with the duration of storage (R^2 = 0.96) than VFA content (R^2 = 0.79)

It can be argued that for fresh pork, the Time is more preferable as a freshness index than VFA content, and testing meat with the FPMLC device is much faster, cheaper, and simpler than the complicated determination of VFA. We expect that after further research, this conclusion may become more general and be extended to the meat of other animals.

3.4. Effects of Heat Treatment and Putative Nucleotide Salvage

There is a number of evidences for a peculiar effect resulting in an increase in AMP and IMP content in animal meat after heat treatment (Nucleotide salvage) [54–58]. We had not found any published research data on a similar effect in fish meat and, hence, conducted a series of experiments with defrosted fish fillets (Alaska pollack, Hake, Norway haddock, Pangasius, Wolffish) from a local supermarket (Tartu, Estonia). All samples had a Time index about 200 s, corresponding to the Phase 3 of FAO UN Quality Score (see also Discussion) [52]. For heat treatment, the samples were placed in a microwave oven for 1–2 min at 400W or steam cooked at +100 °C for 15–60 min; fish fillets were cut into pieces of 5 × 5 × 2 mm.

For all fish samples, we observed the shift of the second metabolite peak (FPMLC fraction II) in the chromatograms towards lower retention times and a consequent decrease in the Time index, i.e., nucleotide relative content after thermal processing changed to become more to that of fresh fish. The shifts of the Time index ΔTime after 15–60 min of steam cooking at 100 °C are presented in Table 2.

Table 2. Decrease in the Time index values for various fish species after 15–60 min of steam cooking at 100 °C.

Fish Specie	ΔTime, s	Note
Alaska pollack	−21	–
Pangasius	−17	–
Norway haddock	−18	Two peaks at 130 and 260 s emerged after treatment
Wolffish	−37	Unstable shifts of the broad band ±10 s
Hake	−30	–

The chromatograms of Alaska pollack before (raw) and after cooking (steam) at 100 °C for 60 min are shown in Figure 8, and the difference between the two chromatograms (raw-steam) is also presented. Two extrema (besides protein) are clearly observed for the difference curve (raw-steam): one maximum at 135 s, which probably corresponds to an increase in the IMP concentration after thermal treatment, and another minimum at 282 s, which could be related to a decrease in Hx concentration.

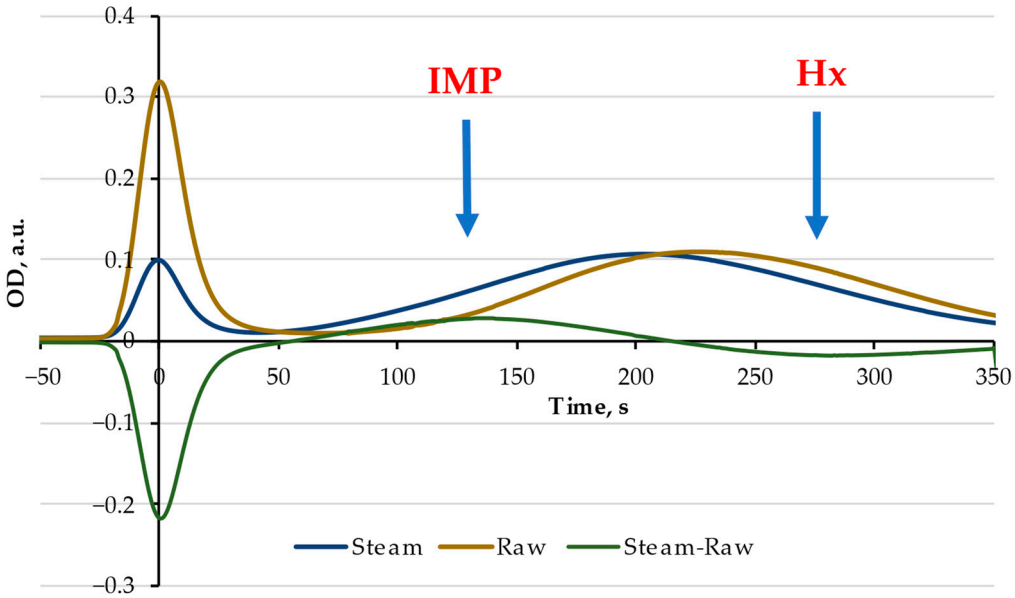


Figure 8. Chromatograms of Alaska Pollock samples before (Raw) and after (Steam) cooking at 100 °C for 60 min, and difference of the two chromatograms (Steam–Raw).

To verify this assumption, NMR spectra of two samples of raw and steam cooked Alaska Pollock samples, previously used for FPMLC testing, were measured. The measurements were carried out according to 2.6.2. The preliminary assignment of the main signals has been conducted in agreement with the data of [59–61], for strict identification spiking tricks with IMP and Inosine have been performed.

In fact, only Inosine and Hypoxanthine lines were detected in raw pollock meat (Figure 9a), but the presence of IMP was also observed in the cooked meat (Figure 9b). The singlet at 8.58 and the doublet near it at 6.15 ppm belong to IMP, as observed in the spectrum with added IMP. Moreover, after the addition of IMP, another line of IMP was

revealed: this line overlaps very closely with the Inosine line at 8.24 ppm and remains overshadowed in the unspiked liquid. Thus, the formation of IMP (Figure 9c) in the course of the heating of this pollock fish has been registered. Such a manifestation of the possible nucleotide salvage or nucleotide synthesis in cooked fish (pollock) was observed, to the best of our knowledge, for the first time. Further experiments are under way to confirm this hypothesis. The FPMLC sensor is proved to be a very useful instrument in this work for the rapid pre-selection of samples for more precise but expensive and cumbersome evaluation methods as NMR, HPLC, MS, etc.

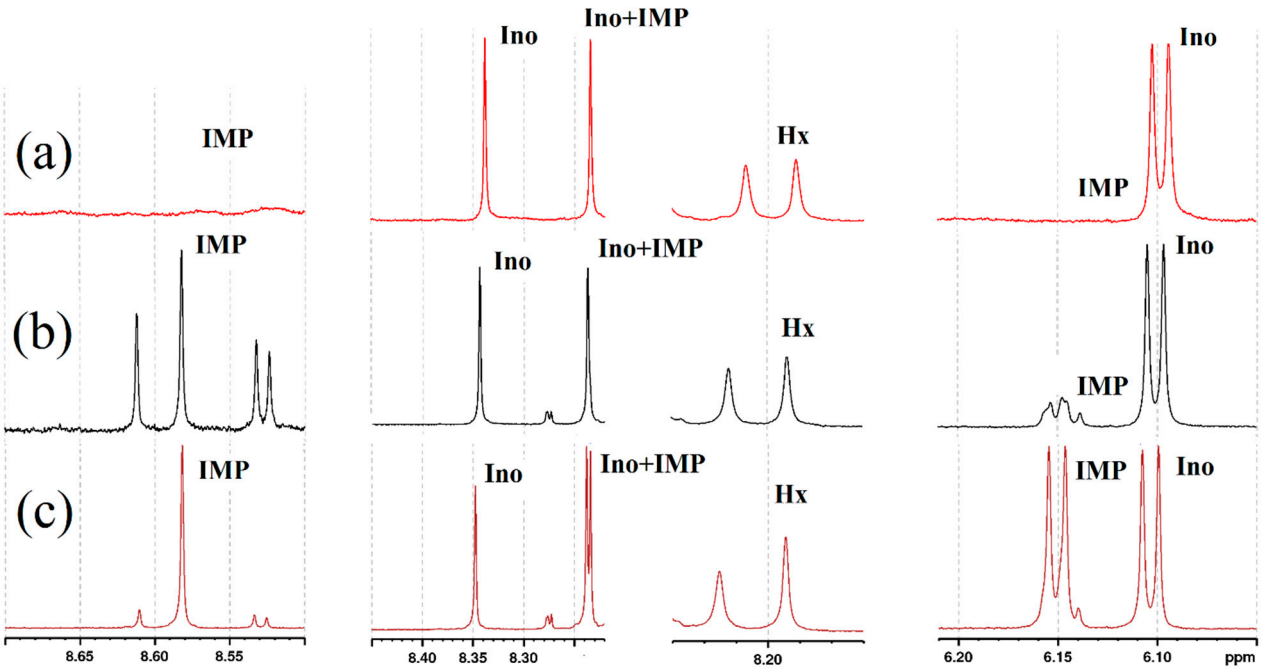


Figure 9. The low-field region of ^{1}H NMR spectra of Alaska Pollock samples: (**a**) before thermal treatment, (**b**) after steam cooking at 100 °C for 60 min, and (**c**) after steam cooking with added IMP and Ino.

4. Discussion

The results obtained for in-house-validation by TVB-N and VFA standard methods show that the FPMLC sensor can be used equally successfully to evaluate the freshness of both fish and animal meat. This conclusion is not surprising, since in both cases, freshness in this study is determined by the formulas (2) and (3) based on the main metabolites of ATP as the variables. These metabolites are absolutely the same in fish and meat, which reflects the common features of the ATP post-mortem catabolism.

The classic freshness indices K, K_I and some others have a certain drawback. Their limit value is 1 (or 100%) and approaches this limit slowly and asymptotically as the sample deteriorates. As a result, the difference between the index values for a product still acceptable for consumption and an irreversibly spoiled product can be as little as 10% or less. This situation means a low sensitivity and too late warning of imminent danger, for example, in the form of intense bacterial contamination.

The index H* = [Hx]/[IMP] proposed in this work takes into account only Inosine monophosphate IMP and Hypoxanthine Hx, but these metabolites are the most important determinants during the loss of freshness. There are no known examples of IMP content increasing with the aging of fish or meat; outside the first day, there is always a decrease in IMP. This means that H* can take values even as H* >> 1. A similar situation also occurred in this study when a rather early critical bacterial contamination (1.6×10^8 CFU/g) took place on day 9 of the experiment with minced pork (Figure 7). This resulted in dramatic growth in the Time index from 172 to 257 sec in the interval of 9–11 days of storage, accompanied

by a sharp increase in H* index from 0.44 to 0.91, reflecting the rapid deterioration of the freshness status.

Our proposal for the H* index builds on previous recommendations to improve the freshness index situation. In particular, in [62], the K* index was proposed, which differs from the K index in that it does not take into account inosine at all due to its change during the loss of freshness with an inconsistent trend—at first a slow rise in concentration followed by a slow decline. The authors of the work [62] came to the conclusion that the K* index can better predict the shelf life of refrigerated striped bass than the K or K_I indices.

Since the early 1960s, the use of hypoxanthine [Hx] as an independent index of fish spoilage has been proposed, as it is the longest-lived metabolite with a constant upward trend over time [63,64], and it was also noted that hypoxanthine concentration [Hx] increased during storage almost linearly [65]. However, the growth rate during shelf-life was too low to be useful as an index of freshness. In this regard, the H* index does not suffer from the noted drawback, since at [IMP] $\rightarrow$ 0, which always happens during prolonged storage, the ratio [Hx]/[IMP] $\rightarrow \infty$.

It can also be noted that the H* index has a strict linear relationship with Time readings of the FPMLC sensor in the most important and initial freshness region (Figure 5) and, thus, an important rule in instrumentation, i.e., linear response to a stimulus, is fulfilled. Of significant interest is the comparison of the technical parameters and capabilities of the device with the organoleptic characteristics of foods. A good pattern to achieve this goal is the Phases diagram of Quality score of the FAO of UN [52] (Figure 10). This diagram was elaborated on during the organoleptic examination of cod fishes cooked in steam. To the best of our knowledge, this is the most valuable scheme of the subject under consideration.

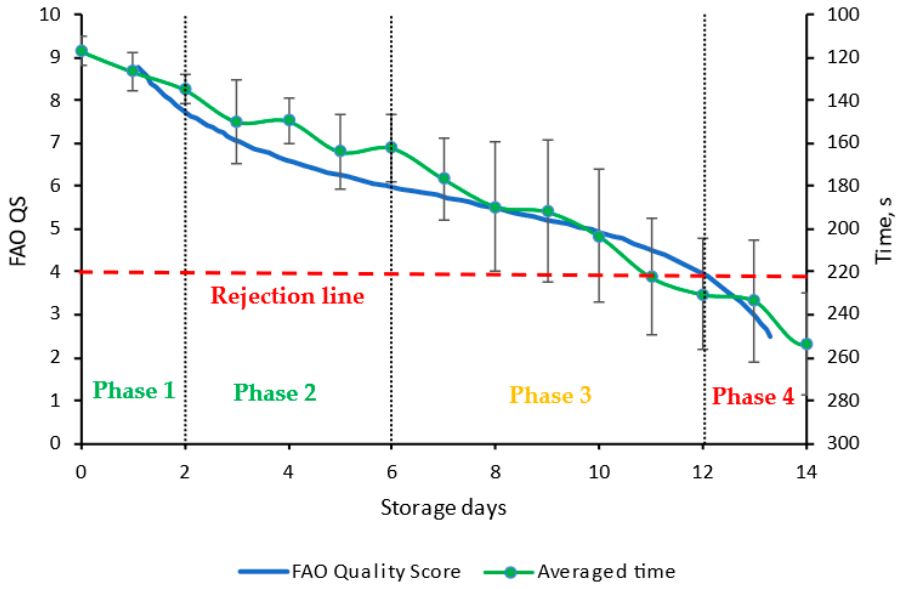


Figure 10. Comparison of UN FAO Quality Score curve and mean values of Time obtained with a number of fish and meat samples stored aerobically at +2–4 °C. Phase 1: The fish is very fresh and has a sweet, algal, and delicate taste. The taste can be very slightly metallic; Phase 2: There is a loss of the characteristic odor and taste. The flesh becomes neutral but has no off-flavors. The texture is still pleasant; Phase 3: There is sign of spoilage and a range of volatile, unpleasant-smelling substances is produced, depending on the fish species and type of spoilage (aerobic or anaerobic); Phase 4: The fish can be characterized as spoiled and putrid [52].

We have carried out a number of Time index measurements with fish and meat in exactly the same mode as the other experiments in this study but precisely knowing the day 0 or even the hour 0 (exact day and time of animal slaughter or fish harvest). For this purpose, the work has been conducted in a slaughterhouse with absolutely fresh pork

and beef to obtain the corresponding values of Time for the Phase 1. Farmed trout and carp were used as fish samples. The obtained Time data is placed at the FAO diagram in a randomized mode, i.e., without indicating whether it was fish or animal meat (carp data are given only for the days 0 and 1). It can be observed that the series of Time points are fairly anticorrelated with the FAO curve, with a very high coefficient of r = −0.97.

The technical data obtained with the FPMLC sensor, compiled with the Phase diagram of UN FAO [52] and the experiences of ordinary food consumers, are presented in Table 3. The data therein cannot be used as an instruction or guidelines; they are only for orientation in the ocean of information about foods in the form of animal meat and fish as well. We hope that Table 3 together with the data in 3.4 encourage the researchers to look for phenomena occurring during the thermal treating of raw products in relation to the phenomenon of nucleotides salvage, which can enhance the usefulness of foods that stand near the end of shelf life. This, in turn could help consumers and retailers to reduce food waste and bring it down below the current level of 30%. The approach of this study and the FPMLC method and device can be of help in this work.

Table 3. Correlation between the consumer properties of meat and fish, Time and H* indices, and bacterial contamination CFU/g.

CFU/g Unsterile Pork, Trout Initial pH 5.5–5.8	Time, s	Index K	Index H*	Characteristic and Accordance to FAO Phases	Objects	Suits for	Some Other Characteristics
– [1]	<130	<0.3	H* < 0.1	Absolutely fresh Phase 1	All rapidly frozen fish or meat	Many purposes. Aging can be needed	Quickly (in hours) ending condition in fridge at +4 °C
$3 \times 10^3 \div 10^4$	130÷160	0.3 < K < 0.5	0.1 < H* < 0.2	Fresh Phase 2	High quality meat and fish	Haute cuisine	Rich in endogenous IMP; no need for artificial E630
$\geq 10^4 \div 10^5$	160÷190	0.5 < K < 0.75	0.2 < H* < 0.45	Rather fresh Status "Use before" The first half of Phase 3	Stored or thawed meat and fish	Ordinary consumption	Often coincides with end of bacterial lag phase
$\geq 10^5 \div 10^7$ [2]	190 ÷210	K ≈ 0.8	H* ≈ 0.5	Start or middle point of exponential bacterial growth Ending of Phase 3	Can be all kinds of samples	Standard shelf-life end	May be in quickly changing condition
$\geq 10^7 \div 10^8$	210÷240	0.8 < K < 0.95	0.8 < H*≈ 0.9	Heavy contamination but may not be spoiled irreversibly Overlapping of Phases 3 and 4	Often reduced-price foods or aged meat	Intense temperature treatment needed	Bacterial stationary phase: oscillating contamination and crash
$>10^8$	>240	K ≈ 1	1 ≈ H* >> 1	Phase 4	Spoiled	Rejection	Smell, bad appearance

[1] Not counted. [2] Contamination level 10^7 is often called "Borderline" (see, e.g., [53]).

In addition to freshness control, the developed device and analytical technique are well in line with the modern attitude towards the nutritional nucleotides, which is also gaining interest as immunity boosters [66–68]. A perspective that in the near future there will be introduced a nutritive index of nucleotides [68] and a "green transition" to a healthy

umamiization of foods achieved [12] makes the matter highly interesting. For this purpose, however, simple and reliable instruments for assessing the quality of foods and just the degree of their umamization should be available.

5. Concluding Remarks

It was demonstrated that sensing technology based on fast protein and metabolites liquid chromatography (FPLMC) provides rapid on-site assessment of animal meat and fish freshness with low-cost, affordable, multiple use, easily operated optical chemical sensors. In was demonstrated that direct readings of FPMLC sensor (the index Time, difference between the retention times of proteins and nutritional nucleotide and nucleoside pools, mainly hypoxanthine and inosine monophosphate), and the new freshness index H* = [Hx]/[IMP] strongly correlated with TVB-N (for fish) and VFA (for meat) content, which are considered reliable indicators of product spoilage. Moreover, it was demonstrated that the indices Time and H* more accurately reflect freshness deterioration at the early stages of product storage than conventional TVB-N and VFA, which are slightly sluggish and strongly react only at the late stages, when the sample is approaching close to spoilage, failing to detect the changes in muscle tissue during autolysis (Figures 6 and 7). The developed approach is free of this drawback: the averaged data obtained with the FPMLC sensor are in good concordance with the Phases diagram of Quality score of the FAO of UN based on organoleptic examination and consumer properties of fish. In addition to the routine control of meat and fish quality, the relative content of dietary nucleotides and nucleosides determined with the FPMLC sensor, especially inosine monophosphate, is extremely important for product taste (umami taste) and nutritional value. In this context, a quite peculiar effect of nucleotide salvage has been observed in fish muscle tissue and validated by NMR spectroscopy.

It should be emphasized, that most, if not all, portable low-cost instruments for the evaluation of meat and fish freshness are of indicator-type disposable sensors envisaged mainly for distinguishing between the low and high bacterial contamination, while more sophisticated tools, e.g., multienzyme biosensors, electronic noses and tongues, or near-infrared (NIR) spectroscopic analytical systems are expensive, single-use (biosensors), sensitive to contaminated laboratory environment (electronic nose), or dependent on complex object-specific chemometrics algorithms (NIR). It is very difficult, if not impossible, to monitor with simple and affordable sensors the stages of autolysis, discriminate between the Phase 1 and Phase 2 (Figure 10) and predict the residual shelf life. This problem can be easily solved with the FPMLC sensor due to its high precision (the relative standard deviation of the main instrumental parameter Time is less than 5%).

In a comprehensive review [22], as many as 41 freshness evaluation methods, including the FPLC approach presented in the current article (based on a brief previous description in a conference paper [42]), were discussed. The authors points to the obvious fact that this method still needs universal acceptance and more research to be conducted, but at the same time, mention seven positive features compared to other techniques:

- Quickest determination of fish freshness available until now,
- Can be used in labs as well as in retail chains,
- Environment friendly,
- Low cost and reliable,
- Less qualified staff can also work,
- Non-destructive and less time consuming, and
- Quickly determine shelf life of almost all varieties of fish.

The main point is that the FPMLC technique and sensor are oriented towards the future, towards the development and introduction into practice of the nutritive nucleotide index (NNI) [12,66]. Indeed, the Time values can be easily recalibrated into the values of NNI for raw or ready-to-eat foods. In any case, the FPMLC method invented and developed here would be in a more strict and direct functional connection with nucleotides' presence in subjects than the methods based on the correlation between another parameters

as it is, e.g., in the classical methods of TVB-N, TMA-N, VFA, etc. Moreover, the FPMLC sensors are potentially capable of the simultaneous qualitative determination of protein and nucleotides content provided a dual-wavelength UV detection unit is installed, and the development of such devices is underway.

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