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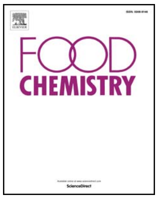

Analytical Methods

Development and validation of a HILIC-UV method for the determination of nucleotides in fish samples

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ABSTRACT

The aim of this work was the development of a simple, novel and accurate method for the determination of adenosine triphosphate (ATP) and its first five catabolites: adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx), in fish tissue, based on hydrophilic interaction liquid chromatography (HILIC). For this purpose, a stationary phase for polar and hydrophilic compounds (ZIC-pHILIC) was used. The effect of different chromatographic parameters and the molecular mechanism based on the van't Hoff plot were examined. The *t*-test and Dixon's Q-test were applied in order to examine statistical differences and outlier values. The recovery of the method ranged between 82.7% and 127% and the %RSD values were lower than 10% for all analytes determined. The method was applied in frozen sea bream samples stored at 0–4 °C. The K_i-, *G*-, *H*- and *F* values were calculated for the estimation of the level of fish freshness.

1. Introduction

After organism's death, vital processes stop and then autolytic and bacterial processes take place. The monitoring of these processes in fish constitutes a means of their biochemical and bacterial freshness assessment. Degradation of the nucleotide ATP has been one of the first processes identified (Alasalvar, Taylor, Öksüz, Shahidi, & Alexis, 2002; Belitz, Grosch, & Schieberle, 2009, chaps. 12 & 13; Dalgaard, 2000; Rehbein & Oehlenschläger, 2009, chap. 4; Wilis, Proctor, & McLoughlin, 2004). In fish tissue, after death, ATP production rapidly decreases leading in general within 1–6 h to skeletal muscle stiffening (*rigour mortis*) (Rehbein & Oehlenschläger, 2009, chap. 4). Degradation of ATP is an autolytic change coinciding with muscle softening during resolution of rigour and eventually with the perceived decrease in fish freshness. ATP degrades to Hx according to the following series: ATP → ADP → AMP → IMP → Ino → Hx. In general, ATP degradation to IMP is rapid, whereas degradation of IMP to Ino and Hx proceeds at a slower rate (Kuda, Fujita, Goto, & Yano, 2007; Manju, Srinivasa Gopal, Jose, Ravishankar, & Kumar, 2007; Rehbein & Oehlenschläger, 2009, chap. 4; Wilis et al., 2004).

The *K* value, the most widely used biochemical index of fish spoilage, permits an early detection of the decrease in fish freshness based on the concentration of ATP and degradation products in fish tissue (Alasalvar et al., 2002; Wilis et al., 2004). According to fish species, the K_i value and other catabolite ratia (*G*-, *H*-, *P*- or *F* value), which do not require determination of the adenino nucleotides can be used as well (Alasalvar et al., 2001; Dalgaard, 2000; Rehbein & Oehlenschläger, 2009, chap. 4). Thus, method development for efficient analysis of ATP and its five catabolites in fish tissue are highly required. The most frequently used chromatographic technique so far for the separation of these compounds has been reversed-phase liquid chromatography coupled to visible/ultraviolet spectrometry (RPLC-UV) (Daniel et al., 2014; Ryder, 1985; Özoğul, Gökbulut, Özyurt, Özoğul, & Dural, 2005). However, RPLC has not been efficient in retention of very polar compounds (Buszewski & Noga, 2012; Greco & Letzel, 2013). On the other side, hydrophilic interaction liquid chromatography (HILIC), using a polar stationary phase and a relatively hydrophobic mobile phase, has shown very promising results for the determination of very polar compounds in complicated matrices. In this context, many advantages, such as high sensitivity and allowance of higher mobile phase flow rates tend to establish it as a very good alternative to RPLC. However, the retention mechanism of HILIC has not been yet clarified and therefore each application presupposes a careful investigation of the HILIC parameters (Appelblad, Jonsson, Pontén, Viklund, & Jiang, 2008; Boersema, Mohammed, & Heck, 2008; Buszewski & Noga, 2012; Greco & Letzel, 2013; Hemström & Irgum, 2006).

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HILIC has been successfully applied for determination of nucleotides in meat and in infant food supplements, but to our best knowledge, no HILIC method has been published for the determination of nucleotides in fish (Inoue & Dowell, 2012; Inoue, Obara, Hino, & Oka, 2010; Mora, Hernàndez-Càzares, Aristoy, & Toldrà, 2010; Mora, Hernández-Cázares, Aristoy, Toldrá, & Reig, 2011). Determination of nucleotides in fish matrices is of high difficulty since within 24 h post-mortem, the ATP, ADP and AMP levels are extremely low (Alasalvar et al., 2001; Ozoğul, Taylor, Quantick, & Ozoğul, 2000). The already published HILIC methods cannot be easily performed in fish tissue, where a method of higher accuracy is required.

Aim of this work was development and validation of a fast and accurate analytical method for the determination of ATP and its five catabolites in fish tissues to monitor fish freshness early upon fish death. A HILIC method was developed upon optimization of mobile phase pH and pH adjustment solution, (%) percentage of ACN in working solution, column temperature and program of gradient elution. Retention behaviour of the analytes was studied using the van't Hoff equation. The method was applied in cultured sea bream extracts that had been stored post-mortem at 0–4 °C for different amounts of time. The K_i-, G-, H- and F values were calculated for each sample and plotted against fish storage time.

2. Experimental

2.1. Chemicals and reagents

Ultra-pure water was provided by a MilliQ purification system (Millipore Direct-Q UV, Bedford, MA, USA). Acetonitrile (ACN) was obtained from Fischer Scientific (FairLawn, New Jersey) and ammonium acetate, as well as perchloric acid from Merck (Darmstadt, Germany). Finally, the standards of nucleotides ATP, ADP, AMP, IMP, Hx and Ino were purchased from Sigma-Aldrich (St. Louis, USA). All used chemical reagents were of HPLC grade.

2.2. Instrumentation

Chromatography was performed with an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA), which consisted of a quaternary pump (1100 G1311A Quat Pump), a manual injector (G1328), a thermostated column compartment (G1316A) and a vacuum degasser (G1379A0). A 20 μL-loop was used for manual injections. The chromatographic separation was performed with a ZIC-pHILIC column (2.1 mm i.d. × 150 mm, 5 μm, SeQuant, Sweden). Ultraviolet detection was made by a variable wavelength detector (1100 HPLC G1314A), which was used at a single wavelength of 254 nm.

2.3. Sample preparation

Cultured sea bream (*Sparus aurata*) samples were used. After capture, the fish samples were stored in dry ice and transferred to the laboratory. Upon arrival, the samples were either immediately analysed or stored in a freezer at −20 °C for an artificial deterioration and later analysis. The duration of the sample preparation should be the shortest possible for the prevention of analyte degradation.

For sample analysis, 10 ± 0.1 g of minced white muscle of fish was weighted and homogenized with 90.0 mL 0.6 M perchloric acid, for the sample deproteinization and the extraction of ATP and derivatives. The obtained homogenate was filtered, and the pH of the delivered aqueous extract was adjusted to 5.0 by dilution with an equal amount of 1 M ammonium acetate. Then, after filtration with a 0.2 μm filter and further dilution with ACN to the selected ACN percentage, the solution was ready for injection into the chromatographic system. Finally, the extracts, that would not be immediately analysed, were preserved in the freezer at a temperature of −20 °C, so that sample deterioration is prevented.

Table 1
Gradient elution program for the simultaneous determination of nucleotides.

Time (min)	ACN (%v/v)	H_2O (%v/v)	Ammonium acetate (% v/v)	Flow rate (mL/min)
0	85	10	5	0.4
5	70	10	20	0.4
8	70	10	20	0.4
10	45	10	45	0.4
17	45	10	45	0.4

2.4. Standards preparation

All stock standard solutions were prepared in 5 mL final volume in 5 mL-volumetric flasks by dissolution of the appropriate mass of solid compound in ultra-pure water. All solid compounds and standard solutions were preserved in the freezer at −20 °C. The final working solutions were diluted in acetonitrile at a ACN/standard solution ratio of (70:30)% v/v, which was selected upon optimization.

2.5. Description of the chromatographic method

The HILIC separation was performed using a ZIC-pHILIC column (2.1 mm i.d. × 150 mm, 5 μm, SeQuant, Sweden). Each sample was injected manually with a 20 μL-injection loop. The final chromatographic conditions are presented in Table 1. The developed HILIC-UV method was applied on extracts from sea breams that had been stored for different amounts of time at 0–4 °C. The obtained results were compared to the corresponding results of a RPLC-UV method that had been validated in the same lab for the determination of ATP, ADP, AMP, IMP, Ino and Hx in sea bream. The RPLC-UV method with the use of a C_{18} column was used as described in the literature (Ozoğul, Taylor, Quantick, & Ozoğul, 2000).

2.6. Selection of an appropriate stationary phase

A wide variety of stationary phases are available on the market since any polar phase, which can retain water, could be used in HILIC mode. The first step for the development of a successful HILIC method is the selection of the most suitable, for the selected analytes, stationary phase (Buszewski & Noga, 2012; Greco & Letzel, 2013; Hemström & Irgum, 2006). In this study, for the separation of ATP and its five first catabolites a ZIC-pHILIC (2.1 mm i.d. × 150 mm, 5 μm, SeQuant, Sweden) column was selected.

ZIC-pHILIC is a zwitterionic stationary phase, which consists of sulfoalkylbetaine groups densely bonded on a polymer surface. The polymer is made of poly(ether ether ketone) named PEEK and the functional group of sulfoalkylbetaine consists of the positively charged quaternary ammonium and the negatively charged sulphonate group in a 1:1 ratio, separated by 3 atoms of carbon. The presence of oppositely charged groups in equal amounts should impart a zero net surface charge, but in fact the sulphonate group in external position imparts a significant net positive charge due to steric factors. Therefore, the interaction of analytes with the quaternary ammonium is practically negligible. On the other side, the analytes are repelled or attracted by the negatively charged sulphonate group through weak electrostatic interactions (Appelblad et al., 2008; Greco & Letzel, 2013). Consequently, the stationary phase repels the negatively charged nucleotides, a mechanism known as electrostatic repulsion hydrophilic interaction chromatography (ERLIC) (Alpert, 2008). In the present study, the analytes and especially the phosphorylated ones, IMP, AMP, ADP and ATP, demonstrate a high negative charge. Therefore, in order to avoid ERLIC, but also achieve shorter/reasonable retention times for all analytes, a high concentration of salt solution (100 mM ammonium acetate) was used in the mobile phase.

There are many advantages for the ZIC-pHILIC column. Firstly,