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Selective removal of ATP degradation products from food matrices II: Rapid screening of hypoxanthine and inosine by molecularly imprinted matrix solid-phase dispersion for evaluation of fish freshness

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ABSTRACT

A water compatible molecularly imprinted polymer (MIP), synthesized using theophylline (TPH) as dummy-template and acrylamide (AM) as functional monomer, has been employed as supporting material in matrix solid-phase dispersion combined with ultra performance liquid chromatography–photodiode array detection (MSPD–UPLC–PDA) for selective determination of adenosine triphosphate (ATP) derivatives in fish samples. ATP degradation products are used as freshness index for assessment of fish quality. The solid sample was directly blended with MIP in MSPD procedure resulting in sample disruption and subsequent adsorption of the compounds on the MIP. By using *n*-hexane and ammonium hydroxide aqueous solution at pH 9 as the washing and elution solvent, respectively, satisfactory recoveries and clean chromatograms have been obtained. Good linearity for hypoxanthine (HYP) and inosine (INO) has been observed with correlation coefficients (R^2) of 0.9987 and 0.9986, respectively. The recoveries of the two ATP derivatives at three different spiked levels ranged from 106.5% to 113.4% for HYP and from 103.1% to 111.2% for INO, with average relative standard deviations lower than 4.2% in both cases. This new method, which is rapid, simple and sensitive, can be used as an alternative tool to conventional tedious methods.

1. Introduction

A large number of post-mortem reactions are initiated in fish (glycolysis, proteolysis and lipolysis) immediately after the animal is slaughtered, affecting its quality and freshness conditions. One of the most important changes consists of the formation of nucleotide and nucleoside metabolites resulting from ATP degradation [1]. ATP degradation to ADP (adenosine diphosphate) and AMP (adenosine monophosphate) takes place rapidly, with the subsequent accumulation of IMP (inosine 5′-monophosphate) [2]. The IMP is hydrolyzed by autolytic enzymes (5′-nucleotidase) to inosine (INO), which, in turn, is degraded to hypoxanthine (HYP) by autolytic and/or microbial action (nucleoside phosphorylase) [3,4]. Next, HYP will be oxidized to xanthine (XAN) and then to uric acid (UA) through a much slower reaction, due to xanthine oxidase (XO) in case of spoilage by microorganisms [5–7].

The pathway of ATP catabolism as a degradative sequence has been widely studied in different fish species [7–12] besides beef [13,14], chicken [15,16] or pork meat [2,3,17–19] and some of the above mentioned nucleotide metabolites have been proposed as freshness indexes in quality assessment [18,19]. Several analytical methods such as electrophoresis [20,21], radioimmunoassay [22], nuclear magnetic resonance spectroscopy (NMR) [23] or amperometric and voltamperometric methods [24] have been reported for quantitative determination of these compounds. Besides, in recent

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years, the use of biosensors has been introduced as an alternative [15]. In particular, a significant number of biosensors have been designed based on the enzymatic reaction catalyzed by the XO [25,26]. However these XO based biosensors have some common drawbacks such as poor stability, non-reusability, slow electron transfer and complexity of immobilization. Conversely, versatility, short analytical time and high resolution have made high performance liquid chromatography (HPLC) the most widely used technique for the analysis of nucleotides and nucleosides in biological samples [7,27,28].

Prior to HPLC determination, a sample preparation process is needed which is really the critical step of the whole analytical process. It should remove potential interferences, pre-concentrate analytes and sometimes, convert them into a suitable form for determination or separation. More to the point, it must provide a robust and reproducible method, independent of variations in the matrix sample. Nowadays, it is also very important to reduce the initial sample size, improve the selectivity of the extraction process, minimize the use of organic solvents and facilitate the automation of the procedure [29]. Solid-phase extraction (SPE) is probably the most widely used technique of sample preparation today, but sample matrix interferences co-elute with the analytes of interest due to the lack of selectivity of common sorbents used; hence, subsequent clean-up steps are required. The use of molecularly imprinted polymers (MIPs) as selective sorbent materials in SPE (MISPE) avoids this problem. MIPs allow analyte pre-concentration and elimination of sample interferences [29,30] in a single step, since they are synthetic materials with artificially generated recognition sites able to rebind a target molecule specifically, even in preference to other closely-related compounds [31,32].

As a general rule for tissue samples, an exhaustive treatment is always required before SPE. The tissue is usually homogenized and centrifuged and only the extract is passed through the MISPE cartridge. However, cell disruption is often incomplete. By contrast, molecularly imprinted matrix solid-phase dispersion (MIP–MSPD) performs simultaneous disruption, extraction and clean-up of solid, semi-solid and highly viscous samples [33–36]. Furthermore, the complete sample disruption and dispersal onto MIP particles occurs, providing an enhanced surface area for subsequent extraction step. MIP–MSPD is less time consuming and manual-intensive as well as more eco-compatible than MISPE. Experimentally, the sample is placed in a glass mortar and blended with the sorbent until complete disruption and dispersion of the sample on the solid support is attained. Then, the mixture is directly packed into an empty cartridge and analytes are eluted after a proper washing step to remove interfering compounds.

This work represents the first attempt to use MIPs as MSPD sorbent to develop a new MIP–MSPD–UPLC–PDA method for the selective extraction and determination of ATP related compounds in fish samples. Besides, it is well known that IMP contributes to the pleasant flavor of fresh fish and its degradation to INO and then to HYP is responsible for the progressive loss of the desirable flavor and the development of the stinking fishy smell [37,38]. In addition, it is accepted that HYP is accumulated owing to INO rapid degradation to HYP and its subsequent slow transformation into XAN and UA by xanthine oxidase [17,18]. Thus, HYP has been chosen to determine the freshness of fish.

2. Experimental

2.1. Material

Theophylline (TPH), INO, XAN, UA and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (Steinheim, Germany). Acrylamide (AM), ethylene glycol dimethacrylate (EGDMA), 2, 2-azobisisobutyronitrile (AIBN) and ammonium hydroxide solution (25% in water) were supplied from Fluka (Buchs, Switzerland). Ethanol (EtOH), methanol (MeOH) and hexane were obtained from Merck (Darmstadt, Germany), chloroform ($CHCl_3$) was from Scharlab (Barcelona, Spain) and acetic acid glacial (AcOH) from Panreac (Barcelona, Spain). HYP was purchased from ACROS organics (Geel, Belgium). Water used in the experiments was purified using a Milli Q Ultrapure water-purification system (Millipore, Bedford, MA, USA).

2.2. UPLC–PDA analysis

UPLC analyses were performed using an Acquity system from Waters (Milford, MA, USA) with gradient pump and automatic injector. Chromatographic experiments were carried out using a stainless steel column Acquity UPLC™ BEH C_{18}, 2.1 × 50 mm, 1.7 μm (apt to work in a 1–12 pH range, at temperatures between 20 and 90 °C, and capable of operating at pressures up to 15,000 psi). Detection was carried out using a photodiode array detector (PDA) set in the range of 200–400 nm. Output signals were monitored and integrated using a personal computer operated under the Empower 2 software (Waters). Wavelength of 250 nm for HYP analysis was selected as output PDA signals. A two solvent gradient elution was performed, with flow rate of 0.5 mL min^{-1} and injection volume of 3 μL. The mobile phase consisted of aqueous TFA (A) (0.1% TFA in deionized water, pH 2.2, v/v) and MeOH (B) gradient [39]. The gradient elution profile starts at 99% of A, was linearly increased to 70% of A in 0.70 min and then brought back to the initial conditions at 1 min.

2.3. Preparation of the molecularly imprinted polymer

The MIP having HYP recognition sites was prepared according to a previous work performed by our research group by non-covalent precipitation polymerization. The MIP has already been fully characterized [40]. TPH as dummy-template molecule (1 mmol) was dissolved in 60 mL of chloroform and subsequently, 4 mmol of AM as functional monomer was mixed until homogenization. Next, 20 mmol of EGDMA as cross-linker monomer was added to the mixture, followed by 0.5 mmol of the initiator of the polymerization, AIBN.

The pre-polymerization mixture was degassed in a sonicating bath and purged with nitrogen for 5 min. Polymerization took place in a water bath at 60 °C for 24 h. The final polymer was dried at 40 °C. TPH was removed by Soxhlet extraction with MeOH for 48 h. The complete removal of TPH from the MIP was assessed via UPLC–PDA method. A non-imprinted polymer (NIP) was similarly prepared excluding TPH from the pre-polymerization media.

2.4. Binding evaluation of TPH–AM–EDMA–MIP

Batch binding assays were carried out for evaluation of the MIPs molecular recognition behavior. Taking into account the fact that these MIPs will be used to extract HYP from fish samples, the solvent for batch rebinding assays was selected according to community legislation [41], which establishes water:EtOH (9:1) as simulant for fresh, cooled, processed salted or smoked fish.

Pre-weighed amounts (0.2 g) of cleaned MIPs were placed into glasses for 5 min incubation in ultrasonic bath at room temperature with eight water:EtOH (9:1) solutions (4 mL) of HYP (from 5.25 to 1009 μg mL^{-1}). After incubation, supernatants were removed by filtration and analyzed by UPLC–PDA at 250 nm to determine HYP residual concentrations, C (μmol L^{-1}). HYP adsorbed concentrations, q (μmol g^{-1} or μg g^{-1}), were calculated by subtracting C from the initial concentrations of HYP. Batch binding experiments were done in a similar way with blank polymers.