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A near-infrared fluorescence assay method to detect patulin in food

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ABSTRACT

Patulin (PAT) is a toxic secondary metabolite (mycotoxin) of different fungal species belonging to the genera *Penicillium*, *Aspergillus*, and *Byssochlamys*. They can grow on a large variety of food, including fruits, grains, and cheese. The amount of PAT in apple derivative products is a crucial issue because it is the measure of the quality of both the used raw products and the performed production process. Actually, all current methodologies used for the quantification of PAT are time-consuming and require skilled personnel beyond the sample pretreatment methods (e.g., high-performance liquid chromatography, mass spectrometry, and electrophoresis techniques). In this work, we present a novel fluorescence polarization approach based on the use of emergent near-infrared (NIR) fluorescence probes. The use of these fluorophores coupled to anti-PAT antibodies makes possible the detection of PAT directly in apple juice without any sample pretreatment. This methodology is based on the increase of fluorescence polarization emission of a fluorescence-labeled PAT derivative on binding to specific antibodies. A competition between PAT and the fluorescence-labeled PAT derivative allowed detecting PAT. The limit of detection of the method is 0.06 μg/L, a value that is lower than maximum residue limit of PAT fixed at 50 μg/L from European Union regulation.

Patulin (PAT)[2] is a toxic fungal secondary metabolite mycotoxin, produced by different genera of fungi such as *Aspergillus*, *Penicillium*, and *Byssochlamys*, capable of growing on a variety of foods, including fruits (in particular apples), grains, and cheese.

Several studies have demonstrated that PAT is present in a number of foods, including apple juice, apples and pears with brown rot [1], flour, and malt feed [2]. The amount of PAT in apple derivative products, such as apple juice, can be used as a measure to estimate the quality of the used raw products.

Different studies have shown genotoxic, immune-toxic, and neurotoxic effects of PAT residues on human health. Due to these severe effects on health, the assumption of PAT by diet is considered an important risk factor [3]. With the aim to prevent the negative impact of PAT residues present in apple juice and related products on health, many countries have established PAT maximum residue limits (MRLs).

The European Union (EU) has set the limit of PAT to 50 μg/L in both apple juice and cider and 25 μg/kg in solid apple as well as a special restriction of 10 μg/L in products for infants and young children [3]. The World Health Organization recommends a maximum concentration of 50 μg/L in apple juice.

Currently, different analytical methods are used to detect PAT in apples and apple juice, including thin layer chromatography (TLC) [1,4], mass spectrometry [5], colorimetry [6], micellar electrokinetic chromatography (MEKC) [7], gas chromatography mass spectrometry (GC–MS) [8,9], liquid chromatography mass spectrometry (LC–MS) [10–13], and high-performance liquid chromatography with ultraviolet detection (HPLC–UV) [14–17]. All of these methods are sensitive and highly specific, but at the same time they are time-consuming, expensive, and not suitable for routine analysis of large-scale samples. In addition, they require the use of expensive instrumentation as well as highly skilled personnel.

In this context, optical methodologies have recently become increasingly used approaches for food safety analyses.

Actually, optical methods are successfully used for nondestructive, fast, real-time monitoring of food matrices [18]. Recently, our

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[2] *Abbreviations used:* PAT, patulin; MRL, maximum residue limit; EU, European Union; GlnBP, glutamine-binding protein; NIR, near-infrared; FP, fluorescence polarization; EDC, 1-[3-(dimethylamino)-propyl]-3-ethyl-carbodiimide; BSA, bovine serum albumin; HRP, horseradish peroxidase; TMB, 3,5-tetramethylbenzidine; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Sat, Satured.

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lab contributed to increasing the knowledge about this topic, developing a competitive fluorescence immune assay and a surface plasmon resonance (SPR) assay for the detection of PAT in apple juice. Both assays were based on the use of specific antibodies against novel chemically synthesized PAT derivative compounds [19,20].

Here, we present a new method for the detection of PAT based on the use of fluorescence polarization and an ad hoc synthesized fluorescence patulin conjugate molecule, PAT–GlnBP (glutamine-binding protein), covalently labeled with a near-infrared (NIR) fluorescence probe.

In particular, a competitive immune assay has been developed to directly detect the presence of PAT in raw samples of apple juice. To do it, the PAT–GlnBP conjugate has been covalently labeled with DyLight IF800, which displays absorption and emission spectra in the NIR region of light.

The fluorescence polarization (FP) method is based on the principle that when a molecule is excited by a plane polarized light, the emission of polarized radiation from it is dependent on the lifetime of the excited state compared with the rotational time motion (Brownian motion in solution). At fixed temperature and viscosity of the molecule solution, the FP value is directly dependent on the effective molecular size of the excited molecule. In the case of small molecules with fast Brownian rotation in solution FP values are low, whereas for larger molecules (e.g., in complex with antibody) FP values are higher [21].

The choice of NIR probe is related to the significant reduction of the background signal, the low absorption in the visible region of the light, low scattering, and the requirement of an inexpensive excitation source. In addition, in some instances the presence of autofluorescence may limit the detection of weak signals of fluorophores emitting in the visible region. Because most biomolecules have very low absorption in the NIR region, the use of NIR fluorescence probes for the detection of PAT traces in apple juice samples provides a high level of performance not achievable with the use of visible emitting fluorescence dyes. In fact, bright clear images with extremely clean backgrounds and excellent sensitivity are provided with this approach.

Finally, it is noteworthy that the use of NIR dyes is also currently part of emerging technologies related to several applications in biomedical and material applications [22]. The minimal interference in absorption and fluorescence emission from biological samples allows for the use of this class of fluorescence dyes for in vivo analyses of biologically relevant molecules [22–24].

Materials and methods

Reagents

All reagents were of the highest commercially available quality and used as received. 1-[3-(Dimethylamino)-propyl]-3-ethyl-carbodiimide (EDC), bovine serum albumin (BSA), and carboxymethoxylamine hemihydrochloride were used. The fluorescent probe DyLight IF800 was purchased from DyLight. Goat polyclonal to rabbit IgG–HRP (horseradish peroxidase) conjugate (secondary antibody) was obtained from Abcam. Affinity resin EAH Sepharose 4B was purchased from Amersham Biosciences. Nitrocellulose transfer membrane Protran was obtained from Schleicher & Schuell, and ECL (enhanced chemiluminescence) detection reagents from Amersham Biosciences were used in dot blot and Western blot experiments. Microplates (96-well, LockWell MaxiSorp) from Nunc, 3,5-tetramethylbenzidine (TMB) enzyme substrate from Sigma, and a microplate reader (Multiskan EX) from Thermo Scientific were used for enzyme-linked immunosorbent assay (ELISA) experiments. Ultraviolet (UV) measurements (detection at 278 nm) were carried out on a Varian Cary 50 Bio spectrophotometer.

Synthesis of PAT–Sat–BSA conjugate

A high stable patulin derivate lacking the highly reactive C7–C7a double bond, PAT–Sat, was synthesized from L-arabinose and conjugated to BSA as described by De Champdorè and coworkers [19]. In brief, a solution of PAT–Sat (1.5 mg, 0.0054 mmol) in Tris–dioxane (1:1, v/v, 0.4 ml, pH 8.0) was added to 20 µl (1.0 mg, 0.0054 mmol) of an EDC solution in H_2O (50 mg/ml) and 0.5 ml of a BSA solution (8 mg/ml) in phosphate-buffered saline (PBS, 0.1 M) at pH 7.4. After 2 h at room temperature, the reaction mixture was dialyzed against PBS (0.01 M) and NaCl (0.01 M) at pH 7.4 (0.5 L) for 3 days with daily buffer changes. The conjugate concentration determined spectrophotometrically at $\lambda = 278$ nm was 4.2 mg/ml.

Antibody production and IgG purification

The antibodies used in this work were produced by and purchased from COVALAB (Villeurbanne, France). The company used as antigen the conjugate BSA–PAT produced. From the serum provided from COVALAB, a 2.0-ml sample of rabbit serum was applied to a protein A column of the PURE1A Protein A Antibody Purification Kit (Sigma), and the IgG fraction was purified according to the manufacturer's instructions. The IgG fractions were eluted with glycine–HCl (0.1 M) at pH 2.8 and immediately buffered in Tris–HCl (1.0 M) at pH 9.0. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out to evaluate the purity of the IgG sample, and the concentration was determined spectrophotometrically at $\lambda = 278$ nm.

Affinity column preparation

The affinity column was obtained by conjugating derivative PAT–Sat to EAH Sepharose 4B as follows. A 1.0-ml sample of resin was washed with H_2O at pH 4.5 (20 ml), with NaCl (0.5 M) (20 ml), and again with H_2O at pH 4.5 (20 ml) and finally suspended in 2.0 ml of H_2O. The Sepharose resin was added to a solution of PAT–Sat (5 mg in 0.5 ml of H_2O at pH 4.5), and the resulting suspension was gently shaken. The slurry was cooled to 0 °C, and EDC was added in two steps to a final concentration of 0.1 M (52 mg). After 12 h at 4 °C the reaction mixture was taken to room temperature, and after an additional 4 h the resin was extensively washed with H_2O at pH 4.5 and then treated with 1.0 ml of AcOH (0.1 M) and 38 mg of EDC for 1 h at room temperature. The suspension was washed with H_2O at pH 4.5 (20 ml), acetate buffer (0.1 M) containing NaCl (0.5 M) (20 ml) at pH 4.0, and PBS (0.1 M) containing NaCl (0.3 M) at pH 7.4 (20 ml) and finally packed into a polystyrene column (2 ml) (Bio-Rad).

Antibody purification by affinity chromatography

For the affinity chromatography purification, a 2.0-ml sample of IgG, obtained from serum, was applied dropwise to the affinity column prepared as described above. To eliminate unspecific antibodies, the column, before elution, was washed with three high-salt buffers: (i) PBS (0.01 M) and NaCl (0.1 M) at pH 7.0 (20 ml); (ii) PBS (0.01 M) and NaCl (0.5 M) at pH 7.0 (20 ml); and (iii) PBS (0.01 M) and NaCl (1.0 M) at pH 7.0 (20 ml). For the elution step, glycine–HCl (0.1 M) at pH 2.7 (2.5 ml) was applied to the column, and the eluate was collected and monitored by absorbance measurements at 278 nm. The fractions containing the antibodies were collected, concentrated by Centricon YM-3 membrane to a volume of 1.0 ml, and dialyzed against PBS (0.1 M) and NaCl