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Research Article

High Performance Liquid Chromatographic Method for the Determination of Patulin in Oil-based Products: Cheese Approach

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Abstract

In this study, a reversed-phase high-performance liquid chromatographic procedure has been developed for the determination of patulin in various cheese samples. The patulin was analyzed by HPLC on a Hypersil C-18 (100x4.6 mm, 3 µm) column with acetonitrile-water (10/90, v/v) as mobile phase in isocratic mode. The flow rate was 2.0 ml/min and analyte was monitored at 272 nm. Identification of patulin was achieved by comparing its retention time value and UV spectra with that of standard stored in a data bank. The detection limit was found to be 0.1 µg/L. The accuracy of the method was tested obtaining an average recovery ranging between 92.35 and 96.80%.

Keywords: Cheese; HPLC; Patulin; Turkish

Introduction

There are 1000 kinds of cheese world-wide nowadays and its production began about 8000 years ago [1]. Turkey is also rich in terms of cheese varieties with approximately 200 varieties. White cheese, kashar cheese, tulum cheese, plaited cheese, cottage cheese, mihalic cheese are traditionally popular and each have unique flavour and form [2].

Cheese production process contains generally four ingredients: milk, rennet, microorganisms and salt. The process involves some common steps such as gel formation, whey expulsion, acid production, salt addition and ripening. Variety of ingredients and processing parameters such as cook temperature and curd handling techniques have caused emerge of different type of cheese. The cheese micro flora has also critical and major role in the improvement of the unique characteristics of each cheese variety [3].

The main goal of cheese production was extend to the shelf life and preserve the nutritional value of milk by allowing acid production and/or dehydration. Lactic acid produced by starter flora provides reducing in the pH value of the milk and contributes separating water of the curd and expulsion of whey, when combined with stages of process such as cooking and stirring [4].

During ripening and storage at low temperatures, mould growth on cheese is observed and generally associated with altering characteristics of cheese such as smell, texture and flavour because of enzyme activity and volatile compounds. Although these changes are rarely favourable (e.g. blue cheese), it is mostly undesired case because of deterioration of flavour, colour and structure [5]. Besides, some kinds of moulds impose their toxic compounds to food tissue [6].

Many species of fungi produce toxic secondary metabolites which are called mycotoxins. In dairy products, mycotoxins can occur through indirect contamination or direct contamination. While indirect contamination results in consumption of contaminated feed by animals, intentional or accidental fungal growing is called direct contamination [7]. Mycotoxins have several negative effects such as carcinogenic, mutagenic and teratogenic [8,9]. Common mycotoxins synthesized by moulds are patulin, aflatoxins, fumonisin B_1 [10].

Patulin (4-hydroxy-4*H*-furol[3,2-*c*]pyran-2(6*H*)-one), commonly produced by *Penicillium expansum* is one of the most extensive mycotoxins in fruits, particularly apple and its products [11,12]. Patulin is also produced by several species of *Penicillium* [*Penicillium expansum*, *P. patulum*, *P. calavrforme*, *P. melinii*, *P. equinum*, *P. roqueforti*, etc.], *Aspergillus* (*Aspergillus clavatus*, *A. giganteus*, *A. terreus*) and *Byssochlamys* (*Byssochlamys nivea* and *B. fulva*) [13,14].

Patulin is considered as a quality parameter because of mutagenic, teratogenic and carcinogenic effects on human health. World Health Organisation (WHO) has limited acceptable level of patulin as 50µg/kg [15]. In products such as apple and its derivatives, which have low fat and high sugar content, only ethyl acetate is generally used as the main reagent, while in fatty products such as cheeses ACN, hexane and Skelly solvent B are mainly used [16-20].

This study aimed to improve a more sensitive and faster method for determination of patulin by using petroleum ether combined with centrifugation stage in extraction process in oil-based foods.

Materials & Methods

Materials

The cheese samples (Ovma, Kashar, Konya blue cheese, Ezine, Halloumi, and Feta cheese) studied was obtained from local markets (Denizli and Konya, Turkey).

Equipment

For the analysis, a liquid chromatography (Shimadzu corporation, Kyoto, Japan) system consisting of a column oven (Shimadzu, Model

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Table 1: Linearity of standard curves and detection limits for patulin for determination of patulin in various cheese samples.

Cheese variety	Linear range (µg L^{-1})	R	r^2	Detection limit (µg L^{-1})
Ovma	1.0-100.0	0.999	99.44	0.1
Kashar	1.0-100.0	0.999	99.44	0.1
Konya Blue	1.0-100.0	0.999	99.44	0.1
Ezine	1.0-100.0	0.999	99.44	0.1
Haloumi	1.0-100.0	0.999	99.44	0.1
Feta	1.0-100.0	0.999	99.44	0.1

CTO-20A), a UV-VIS diode array detector (Shimadzu, model SPD-M20 A), a degasser (Shimadzu, Model DGU 20A), a liquid chromatography pump (Shimadzu, Model LC-20AD), and a Software Program (Shimadzu) was used to calculate peak areas. The sample (20 µL) was injected with a syringe (Hamilton Co., Reno, NV, USA) into the HPLC. The HPLC column used was a reversed-phase BDS Hypersil C_{18} (100x4.6 mm, 3µm) from Thermo scientific (USA).

Reagents

Acetonitrile (HPLC grade), hexane and Na_2CO_3 (extra pure) were obtained from Merck (Darmstadt, Germany). Water used in all the experiments was distilled. The standard of patulin was of analytical-reagent grade and obtained from Sigma (Sigma-Aldrich Chemie GmbH, Deisenhofen-Germany) and was not further purified. Stock and standard solutions of patulin were prepared in mobile phase. For preparing calibration curve, five different concentrations of each standard were used. These solutions were sonicated and stored in dark glass flasks, in order to protect them from light, and kept under refrigeration. Thus, a calibration curve was prepared for patulin. Correlation coefficients of patulin based on the concentration (µg/L) *versus* peak area (mAU) were found to be > 0.999.

Sample preparation

Sample preparation suggested by ISO (1993) and Pattono et al. 2013 was modified. Deionised water (70ml, pH 3.3) were added into cheese samples (5g). The mixture was homogenised using a homogeniser at high speed for 1 min. Hexane (30ml) was added into the mixture and then the mixture was heated at 90°C for an hour. Following the heat treatment, the mixture was filtered. The filtrate (5ml) was added into a separator funnel and the filtrate (5ml) was mixed with ethyl acetate (5ml) and the mixture was shaken for 1 min. When separation realized, the upper layer was collected. The process was repeated three times for lower layer and then the lower layer was removed. After that, Na_2CO_3 (1.4%) and ethyl acetate (5ml) were added into the last separator funnel. After shaking, separated upper layer was combined with the other upper layers and acetic acid was dropped (five drops) into the collected upper layers. The solvent was evaporated by rotary evaporator and remaining liquid was dried under nitrogen gas until dryness. The residue was dissolved in ACN solution (10:90, ACN: water, v/v). Aliquots of 20 µl were injected into the HPLC column.

Methods

The column eluate was monitored with a photodiode array detector at 272 nm for patulin. The mobile phase was filtered through a 0.45-µm membrane and degassed by sonication prior to use.

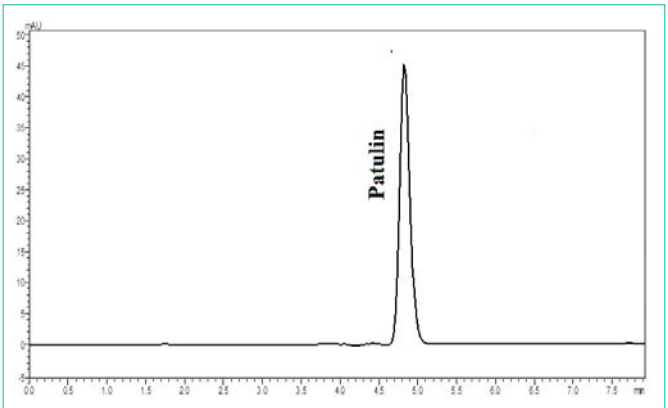

Figure 1: Typical HPLC chromatogram of 50 ppb standard patulin.

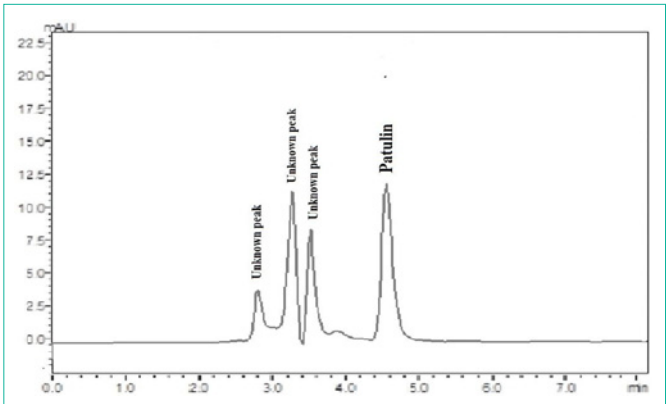

Figure 2: Separation of patulin by isocratic elution with acetonitrile-water (10:90; v/v).

The used elution solvents were ACN solution (10:90, ACN: water, v/v). The chromatographic data on the peaks were integrated up to 8 min. The flow-rate was 2ml/ min. The column was operated at room temperature (25°C). The sample injection volume was 20 µl. Identification of compound was achieved by comparing its retention time value and UV spectra with that of standard stored in a data bank. Concentration of patulin was calculated from integrated areas of the sample and the corresponding standard.

Recovery of patulin

Cheese samples containing known amounts of patulin were spiked with the two addition levels (Table 1) of standard patulin to determine the recovery. Five determinations were carried out for each addition level.

Calculation method

The patulin content of cheese samples were expressed in µg/kg. A Minitab 16 Statistical Software was used for the processing of the results of analyses.

Results and Discussion

A new method using heat treatment was tried for the determination of patulin in oil based product (cheese approach). Extraction of patulin in oil-based products is very problematic. This new extraction method has been developed because of the lack of reliable results from the previously used extraction method [17]

developed for determining patulin in cheese. It is very important to remove the oil from oil-based products such as cheese in order to get correct results. Otherwise, oil residue remains in the evaporation tube when the solvent is evaporated in the last stage of extraction. This modified extraction method eliminates the mentioned problems and introduces a new approach to patulin extraction in oil-based products. The first advantage of this method is to provide easiness removing of oil from the sample by increasing viscosity of oil with heat treatment. Hereby, oil separation yield of hexane is enhanced. Also, any solvent having low polarity is not used except for hexane until the end of entire oil removing when compared to the previous method. In this way, it is avoided other solvents that may influence the extraction. Likewise, hexane is used for the removing of oil from sample in the previous method. In the previous method, hexane is mixed with sample homogenised with acetonitrile and the solution is shaken. However, an amount of patulin may pass to the hexane phase by adsorbing to oil molecules as a result of shaking. This increases the loss of patulin and decreases precision. This new method also minimizes this possibility as any shaking process is not carried out.

As known, each component has a wavelength that gives maximum absorbance such as patulin. Therefore, the detection wavelength was set at max absorption wavelength of patulin for higher sensitivity. Separations of standard patulin and patulin in Kashar cheese sample by isocratic elution are shown in (Figures 1 & 2), respectively. Patulin is separated well and a good separation achieved in 5 min. Several unknown peaks were also detected in the chromatogram. However, no interference between the patulin and the unknown peaks were observed.

Analytical characteristic of the HPLC method

Linearity and detection limits: Linearity of standard curve and detection limit of proposed method for determination of patulin in cheese samples is shown in (Table 1). The *"R"* value of patulin was determined as 0.999. Coefficients of determination (r^2) was determined as 99.44% for patulin. The detection limit for patulin, based on "S/N" (signal/noise) of 3, was 0.1 µg/l.

Recovery

The reliability of the method was confirmed by recovery experiments using standard addition procedure. Thus, 4, 5, 20, 5, 6 and 7 µg/L concentrations of patulin standards were added to the Ovma, Kashar, Konya Blue, Ezine, Haloumi and Feta cheese samples, respectively. Six determinations were carried out for each addition level. Recovery of method for determination of patulin in various cheese samples is shown in (Table 2). The average percentage recoveries of patulin in Ovma, Kashar, Konya Blue, Ezine, Haloumi and Feta cheese samples were found to be 92.35±0.45%, 94.60±0.52%, 96.80±0.41% 95.00±0.65%, 95.70±0.60% and 96.00±0.720%, respectively.

Precision

Six determinations of the same cheese sample were performed using the same reagents and apparatus to evaluate the method precision in cheese. The precision of the method for determination of patulin in cheese samples is shown in (Table 3). The method precision was evaluated using the same reagents and apparatus under the same experimental conditions with three determinations of the same cheese. In addition, intra- and inter-day tests were applied for the calculation of precision and the results were expressed as relative standard deviation (RSD, %). The evidence of good precision for HPLC is low RSD value that determined 1.46%, 1.30%, 0.95%, 1.40%, 1.74% and 1.80% for patulin in Ovma, Kashar, Konya Blue, Ezine, Haloumi and Feta cheese samples, respectively. As known, the low RSD value also shows non-variability of the data.

Table 2: Recovery of method for determination of patulin in various cheese samples.

Cheese variety	Initial content (mg/kg)	Content after addition (mg/kg)	Recovery (%)
			Mean ± S.D[g]
Ovma	4.0 ± 0.3	7.69 ± 0.36 [a]	92.35 ± 0.45
Kashar	5.4 ± 0.2	10.13 ± 0.28 [b]	94.60 ± 0.52
Konya Blue	22.6 ± 0.5	41.96 ± 0.48 [c]	96.80 ± 0.41
Ezine	4.7 ± 0.4	9.45 ± 0.30 [d]	95.00 ± 0.65
Haloumi	5.8 ± 0.6	11.54 ± 0.36 [e]	95.70 ± 0.60
Feta	6.7 ± 0.3	13.42 ± 0.45 [f]	96.00 ± 0.72

[a] 4 µg/kg patulin addition; [b] 5 µg/kg patulin addition; [c] 20 µg/kg patulin addition; [d] 5 µg/kg patulin addition; [e] 6 µg/kg patulin addition; [f] 7 µg/kg patulin addition; [g] Mean ± standard deviation

Table 3: Precision of method for patulin determination in various cheese samples.

Cheese variety	Mean ± S.D.[a] (mg/kg)	R.S.D. (%)
Ovma	4.0 ± 0.3	1.46
Kashar	5.4 ± 0.2	1.30
Konya Blue	22.6 ± 0.5	0.95
Ezine	4.7 ± 0.4	1.40
Haloumi	5.8 ± 0.6	1.74
Feta	6.7 ± 0.3	1.80

[a]Mean ± standard deviation

Conclusion

This study purposes a new extraction method for the separation and quantification of patulin in oil based products as cheese approach. Heat treated method proved to be an effective tool for performing adequate separation of the patulin while HPLC provided a fast, accurate and reliable method for the determination of patulin with recoveries ranging from 92.35 to 96.80%. The isocratic eluent system used for patulin yielded high detection limits and good resolution within a minimum analysis time of 5 min.

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