Brazilian Journal of Microbiology (2002) 33:269-273
ISSN 1517-8382

THE DEVELOPMENT OF AN ANALYTICAL METHOD FOR TWO MYCOTOXINS, PATULIN AND VERRUCULOGEN, AND SURVEY OF THEIR PRESENCE IN COMMERCIAL TOMATO PULP

Luciane M. Kawashima; Lucia M. Valente Soares*; Pilar R. de Massaguer

Departamento de Ciência de Alimentos, Universidade Estadual de Campinas, Campinas, SP, Brasil.

Submitted: March 16, 2001; Returned to authors for corrections: July 11, 2001; Approved: July 26, 2002

ABSTRACT

The mycotoxin patulin causes gastroinstestinal distress, neurotoxic and immunotoxic effects in animals. It can be produced by several species of *Penicillium*, *Aspergillus* and *Byssochlamys* and it has been found in fruits, vegetables and cereals. Verruculogen is a toxin produced mainly by *Penicillium* and *Aspergillus* spp. and causes severe tremors in affected animals. Tomatoes are especially susceptible to fungi invasion and their products need to be investigated for possible mycotoxin contamination. A method for the determination of patulin and verruculogen in tomato products was developed involving an extraction with ethyl acetate, a cleanup by silica gel column and determination and confirmation by high performance liquid chromatography with diode array detector. The quantification limits of the method, defined as the minimum amount that allowed quantification and confirmation by the DAD detector, were 10 ng/g and 20 ng/g. The average recovery for patulin at five levels of addition (from 20 to 200 ng/g) was 75% and at the single level of 100 ng/g was 90 % .The average recovery for verruculogen at five levels of addition (from 50 to 300 ng/g) was 54% and at the single level of 100 ng/g was 52%. The processing of two tomato plants was followed during 1996, 1997, and 1998. Eighty-four samples of tomato pulp were analyzed for patulin and verruculogen. The toxins were not detected in any of the samples.

Key words: mycotoxins, patulin, verruculogen, tomato products

INTRODUCTION

The presence of undesirable fungi growth in food products has been extensively reported in foods and it is caused by a deterioration process or by contamination due to improper handling. Fungi can produce metabolites in the foods they invade that are toxic to man and animals and for that reason these compounds are called mycotoxins. Tomatoes, a soft skinned vegetable, are highly susceptible to fungal invasion at field conditions and also during storage, transportation and processing (25). Tomato products are largely consumed in Brazil and tomatoes constitute the horticultural product with the highest plant processing volume in the country and possibly in the world (4). In 1998 the consumption of tomato paste and tomato pulp in Brazil was 138 488 tons and 128 257 tons, respectively (2).

The mycotoxin patulin may be produced by several species of *Penicillium*, *Aspergillus* and *Byssochlamys*. Originally, it drew researchers attention due to its antibiotic properties. However, it was rapidly shown to be highly toxic to plants and animals (13). The LD_{50} for rats and mice ranges from 5 to 30 mg/kg b.w. (6). Patulin causes gastroinstestinal distress and neurotoxic effects in rodents (16), immunotoxic effects in mice and rabbits (32), and genotoxic effects on mammalian cells (41).

Patulin has been found in apples, pears, their juices and jams (5, 20, 22, 27, 35), grapes and grape juices (31), and beets (40). It was found in fruits that exhibited brown rot, such as bananas, pineapples, grapes, peaches, and apricots, indicating that the use of unsound fruits for processing would lead to the presence of the toxin in the products (11). A limited survey was conducted in tomato products in Germany but the toxin has not

* Corresponding author. Mailing address: FEA/UNICAMP, Caixa Postal 6121. 13081-970, Campinas, SP, Brasil. E-mail: valente@fea.unicamp.br

been detected (1). Patulin was also found in peaches, apricots, bananas, strawberries, melons, tomatoes, cucumbers and carrots inoculated with *P. expansum, P. urticae* and *Byssochlamys nivea* (11) and in barley inoculated with *Aspergillus clavatus* (18). The effect of processing on patulin has been extensively studied in apples. The toxin was shown to be stable to heat and to the presence of acids, however, alcoholic fermentation will destroy it (30).

Verruculogen is a toxin produced by *Penicillium* spp., such as strains of *P. verruculosum* Peyronel, *P. paraherquei*, *P. piscarium* Westling, and *P. janthinellum*; and by *Aspergillus* spp., such as strains of *A. caespitosus*, *A. fumigatus*. *Neosartorya fischeri* has also been cited as a verruculogen producer. The latter fungus species is also termoresistant and may survive heat treatment during vegetable and fruit processing (37). Verruculogen elicits severe tremorgenic response in animals (7) and causes a drop in the levels of the γ-aminobutiric acid in the central nervous system accompanied by loss of the GABA inhibitory function in mice dosed with the toxin (8).

Tomato products in Brazil were investigated for alternariol, alternariol monomethylether, tenuazonic acid (TEA), and cyclopiazonic acid (CPA) (24). TEA and CPA were found in the samples indicating the use of tomatoes with undesirable levels of fungi invasion. Other potentially important mycotoxins for tomato products remain to be searched for. No survey of patulin and verruculogen has been conducted so far on Brazilian tomato products.

Patulin has been determined in apple juice by thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC). For sample extraction there is an almost universal preference for ethyl acetate. The cleanup step may involve one of the following techniques: (a) extraction with a sodium carbonate solution; (b) chromatography with Extrelut, Florisil, silica, or octadecilsilyl columns, singly or in combination of two of these columns; (c) dialysis. Confirmation of identity has been accomplished by derivatization reaction with acetic anhydride, mass spectrometry, and diode array detectors. Detection limits claimed range from 4-10 ng/g for TLC, 10-50 ng/g for GC, and 1 – 44 ng/g for HPLC (14, 15, 17, 19, 22, 23, 28, 34, 36, 38). Tomato products have been analyzed by extraction with ethyl acetate, followed by a Florisil column and two dimensional TLC with visualization by derivative formation with chloro-o-dianisidine. The reported limit of detection was 40 ng/g (1).

There are no reports on determination of verruculogen in foods. A few workers have only described its isolation from culture media (9, 26, 29).

The present work aimed at developing a method to determine patulin and verruculogen in tomato products and surveying Brazilian tomato products for the possible presence of these toxins.

MATERIALS AND METHODS

Samples

Three packages of tomato pulp were collected at random from each of 14 batches during the 1996/97 tomato harvest and also outside harvest time at two tomato processing plants located in the states of São Paulo and Goiás. The sampling was repeated during 1997/98 when 14 batches were sampled. The processed tomato pulp was packaged in Tetrabrik cartoons (Tetrapak) and had 8.1º Brix and pH 4.3.

Sample extraction and cleanup

The tomato pulp samples were treated with commercial pectinase (Pectinem Ultra SPL, Ovo Nordisk Fermented Ltd.). Three hundred µL of pectinase (previously diluted to 1:10.000) were added to 20 g sample, heated to 40ºC during 15 minutes and filtered through qualitative filter paper. The residue in the filter paper was pressed to remove excess water as was needed. A 5 g aliquot was collected and extracted with 10mL ethyl acetate followed by a second extraction with 5 mL ethyl acetate. The ethyl acetate phases were collected, dried with sodium sulfate and transferred to a silica gel column (Silica gel 60, 70-230 mesh, Merck), prepared in a glass tube with 4-4.5 cm i.d. and filled to a 6 cm height and topped with 1 cm anhydrous sodium sulfate. The toxins were eluted from the column with 15 mL ethyl acetate and the eluate dried under a gentle nitrogen stream at 40ºC.

High performance liquid chromatography

The dried extract was dissolved in 500 mL 10% acetonitrile-water and filtered through a filter with 0.45 µm diameter pores (Millipore Corp.). The samples and standards were injected using a 20 µL loop into a isocratic liquid chromatograph with diode array detector (Hewlett Packard, model 1050) with a data processing HP Chemstation LC 3D System. Individual stock solutions were prepared for the patulin and verruculogen standards (Sigma) in ethanol and the concentrations checked by ultraviolet spectrofotometry. The working solutions were prepared by diluting the stock solutions with 10% acetonitrile-water.

For the patulin determination the wavelength was set at 276 nm. The analytical column was a reverse phase C18, 5µm, 4.6 x 150 mm (Microsorb MV, Varian). The mobile phase was 10% acetonitrile-water at 1mL/min. Under these conditions patulin has a retention time of 4.1 minutes. For the verruculogen determination the wavelength was set at 225 nm. The analytical column was a reverse phase C18, Spherisorb ODS-2, 5µm, 4.6 x 250 mm (Sigma). The mobile phase was 50% acetonitrile-water at 1 mL/min. Under these conditions verruculogen elutes at 10.3 minutes. Quantification was accomplished by external standards at 5 concentration levels. Confirmation of identity was obtained through comparison of the presumptive peaks spectra and the standards spectra obtained under the same chromatographic conditions.

Analytical quality control

Quantification limits of the method were taken as the minimum amount of the toxin detected in the product that allowed for confirmation by the diode array detector by yielding a clearly recognizable spectrum. The recovery of the method for patulin and verruculogen were tested at five levels of addition (from 20 to 200 ng/g and from 50 to 300 ng/g, respectively). The detection limits of the pure toxins by the DAD detector were measured as three times the baseline standard variation under the same conditions employed for the tomato pulp samples. A blank and a recovery test for each toxin (a sample spiked at 100 ng/g with patulin and verruculogen) were added to each series 10 of samples analyzed.

RESULTS AND DISCUSSION

Evaluation of analytical methods for patulin and verruculogen in tomato products

Four extraction and cleanup systems described for patulin in apple products (3, 21, 33, 39) and one described for tomato products (1) were tested as described by the authors and also in combination. The results were found inadequate. The best extraction solvent proved to be ethyl acetate, a popular solvent for the extraction of patulin from apple products. The cleanup system that worked best for tomato products was the silica gel column, not previously described for this kind of food product.

Hydroximethylfurfural, a compound produced when sugars are heated under acidic conditions during food processing, is a common interferent to patulin during liquid chromatography. The appropriate mobile phase and column for separating both compounds were searched for among the mobile phases recommended by AOAC for patulin (3) and two brands and sizes of chromatographic columns were tested. The best combination found is the one described in the Materials and Methods section.

For the extraction and cleanup of verruculogen in tomato products there were no methods to act as a guide or a starting point. Thus, extractions used for cultures of verruculogen producing fungi were tested (10, 12, 26) but no adequate results were reached. Luckily, it was found that the same extraction solvent and cleanup system that worked for patulin in tomato products also worked for verruculogen. Chromatographic conditions mentioned for culture extracts were tried (26) but the verruculogen peak was not resolved. For the liquid chromatography step the mobile phase had to be modified to 50% acetonitrile-water in order to elute the verruculogen. On the other hand a shorter chromatographic column (15 cm) yielded better results than the longer one used for patulin (25 cm). A gradient solvent system might have solved these difficulties and eluted both compounds in the same run but the use of an isocratic pump precluded this. Under these circumstances it was chosen to chromatograph verruculogen and patulin under separate runs in order to change columns and mobile phases as described under the Materials and Method section. However, although the two toxins were quantified and confirmed under separate chromatographic conditions the extraction and cleanup step is common to both and is greatly simplified when compared to the patulin methods previously described for apple products and the one described for tomato products. The common extraction and cleanup for the two toxins also saves a great amount of time for the analyst and reduces reagents costs.

The method developed for determination and confirmation of patulin and verruculogen in tomato products was then evaluated with recovery and reproducibility tests. The average recovery for patulin at five levels of addition (from 20 to 200 ng/g) was 75% (Table 1) and at the single level of 100 ng/g was 90% (Table 2). The average recovery for verruculogen at five levels of addition (from 50 to 300 ng/g) was 54% (Table 1) and at the single level of 100 ng/g was 52% (Table 2). The relative standard

Table 1. Recovery of patulin and verruculogen added to tomato pulp at five different levels.

Level of addition of patulin (ng/g)	Recovery (%)	Level of addition of verruculogen (ng/g)	Recovery (%)
20	43.2	50	31.5
50	66.5	75	60.0
100	78.5	100	54.3
150	93.2	200	58.7
200	92.6	300	64.0
Average	74.8	Average	53.7
sd	20.9	sd	12.9
RSD (%)	28.0	RSD (%)	24.0

Table 2. Recoveries for patulin and verruculogen added to tomato pulp samples at the single level of 100 ng/g.

Series nº	Patulin recovery (%)*	Verruculogen recovery (%)*
1	97.2	52.4
2	85.0	65.9
3	92.7	58.7
4	95.3	60.1
5	81.0	62.3
6	95.3	62.6
7	82.2	54.0
8	91.4	48.3
9	92.1	50.0
10	93.3	52.2
Average	90.6	56.7
sd	5.7	6.0
RSD (%)	6.3	10.6

deviation for 10 repetitions was 6.3 and 10.6 % for patulin and verruculogen, respectively (Table 2).

The detection limits of the DAD detector for pure standards were 6 ng/g for patulin and 3 ng/g for verruculogen. The limit of quantification was determined as the smallest quantity of the toxin that allowed confirmation by the spectrum obtained. The limit of quantification for patulin was 10 ng/g and for verruculogen was 20 ng/g. No references have been found for limits of detection for verruculogen in any food product. For patulin in tomato products there is a recent communication from the Ministry for Agriculture, Food, and Fisheries, UK, reporting a limit of detection of 20 ng/g and recoveries of 37-61% for patulin in tomato products (21).

The method developed proved to be simple due to the limited number of steps employed and the ready availability of reagents and adsorbents used when compared to other methods described for patulin in the literature with the added advantage of allowing the determination of a second mycotoxin, verruculogen. The chromatograms (Figure 1) showed no interferences at the point the toxins eluted and this was further demonstrated by the fact the spectra of the pure compounds (Figure 2) obtained during the chromatographic runs were identical with the spectra of toxins spiked into the tomato pulp samples.

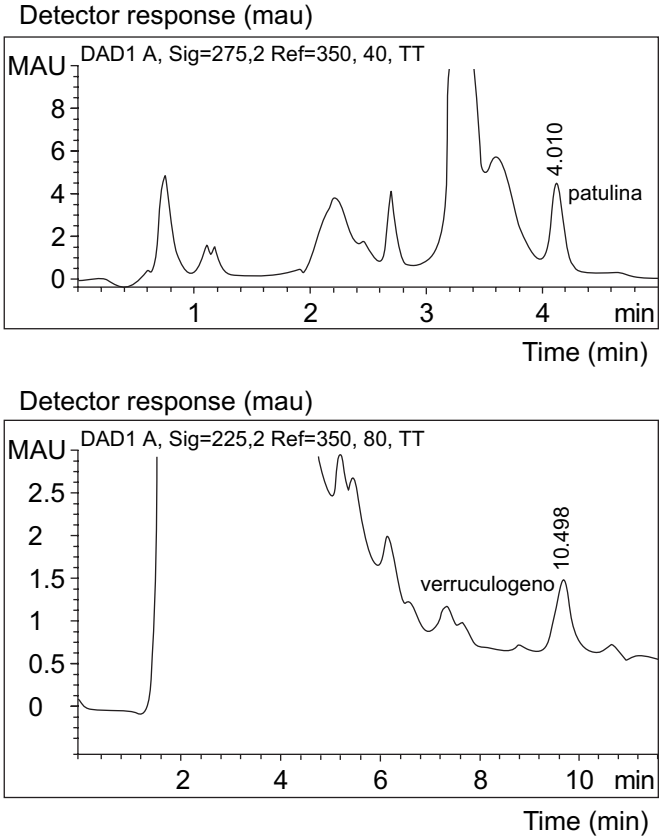

Figura 1. Chromatograms of tomato pulp samples spiked with 100 ng/g patulin and 100 ng/g of verruculogen.

Survey of patulin and verruculogen in tomato products

The processing of two tomato plants were followed from 1996 to 1998 and 84 samples of tomato pulp were analyzed for patulin and verruculogen. The toxins were not detected in any of the samples.

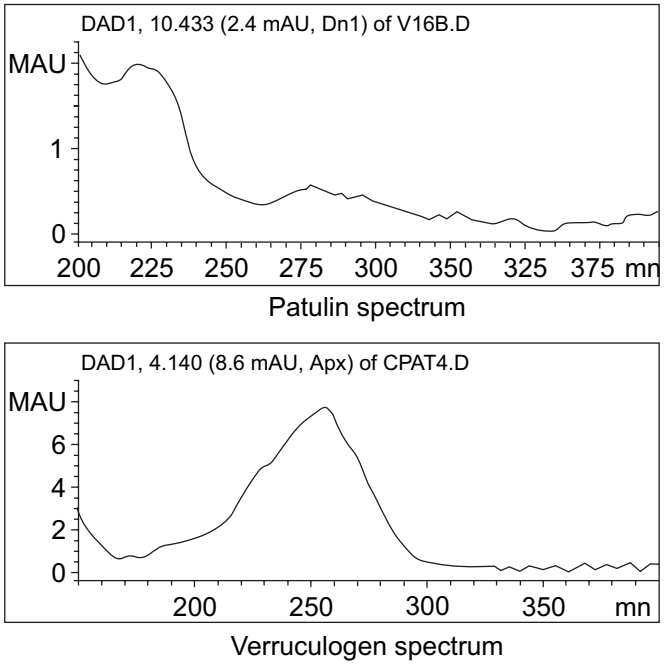

Figure 2. Patulin and verruculogen spectra taken during chromatographic run of standards.

ACKNOWLEDGEMENTS

The authors thank the Fundação de Amparo à Pesquisa do Estado de São Paulo for the financial support and the Coordenadoria para Aperfeiçoamento de Pessoal de Nível Superior for the graduate scholarship to the first author.

RESUMO

Desenvolvimento de um método analítico para duas micotoxinas, patulina e verruculogeno, e levantamento da sua incidência em polpa comercial de tomate

A micotoxina patulina causa distúrbios gastrointestinais e efeitos neurotóxicos e imunotóxicos em animais. Pode ser produzida por várias espécies de *Penicillium*, *Aspergillus* e

Byssochlamys e tem sido encontrada em frutas, verduras e cereais. Verruculogeno é uma toxina produzida principalmente por espécies de *Penicillium* e *Aspergillus* e causa fortes tremores em animais afetados. Tomates são especialmente susceptíveis a invasão fúngica e seus produtos precisam ser investigados com relação a possíveis contaminações por micotoxinas. Um método para determinação de patulina e verruculogeno em produtos de tomate foi desenvolvido envolvendo uma extração com acetato de etila, uma limpeza em coluna de sílica gel e determinação e confirmação por cromatografia de alta eficiência com detector de arranjo de diodos. Os limites de quantificação foram de 10 ng/g e 20 ng/g e as recuperações medias foram de 89% e 65% para patulina e verruculogeno, respectivamente. O processamento de duas fábricas de produtos de tomate foi amostrado durante 1996, 1997 e 1998. Oitenta e quatro amostras de polpa de tomate foram analisadas para patulina e verruculogeno. As toxinas não foram detectadas em nenhuma das amostras.

Palavras-chave: micotoxinas, patulina, verruculogeno, produtos de tomate.

REFERENCES

1. Acar, J.; Klaushofer, H. Occurrence and determination of patulin in commercially tomato pastes. *Ernaehrung*, 8: 323-326, 1984.
2. Anonymous, BrazilTrend'99 – Trends in the packaged goods market. Datamark, São Paulo, 1999, 197p.
3. AOAC International. Official Methods of Analysis of AOAC International. AOAC Int., Gaithersburg, 16th ed., 3rd rev., 1997.
4. Bernhardt, L.W. *Processamento de hortaliças*. ITAL, Campinas, 1994, 70p.
5. Burda, K. Incidence of in apple, pear, and mixed fruit products marketed in New South Wales. *J. Food Protec*., 55: 796-798, 1992.
6. Busby Jr., W.F.; Wogan, G.N. Patulin and penicillic acid. *In*: Shank, R.C. (ed). *Mycotoxins and N-nitroso compounds: Environmental risks.* Vol II. CRC Press, Boca Raton, 1981, p.121-127.
7. Cole, R.G.; Cox, R.H. *Handbook of toxic fungal metabolites*. Academic Press, New York, 1981, 937p.
8. Cole, R.J.; Kirksay, J.W. The mycotoxin verruculogen: a 6-o-methylindole. *J. Agric. Food Chem.*, 21: 927-929, 1973.
9. El-Banna, A.; Pitt, J.I.; Leistner, L. Production of mycotoxins by Penicillium species. *System. Appl. Microbiol*., 10: 42-46, 1987.
10. Filtenborg, O.; Frisvad, J.C.; Svendsen, J.A. Simple screening method for moulds producing intracellular mycotoxins in pure cultures. *Appl. Envir. Microbiol*., 45: 581-585, 1983.
11. Frank, H.K.; Orth, R.; Figge, A. Patulin in Lebensmitteln pllanzlicher Herkunft. *Z. Lebensm. Unter.-Forsch*., 163: 111-114, 1977.
12. Frisvad, J.C. High-performance liquid chromatographic determination of profiles of mycotoxins and other secondary metabolites. *J. Chromatogr*., 392: 333-347, 1987.
13. Gaucher, G.M. Mycotoxins – Their biosynthesis in fungi: Patulin and related carcinogenic lactones. *J. Food Protec*., 42: 810-814,1979.
14. Gökmen, V.; Acar, J. Simultaneous determination of 5-hidroxymethylfurfural and patulin in apple juice by reversed-phase liquid chromatography. *J. Chromatogr. A*, 847: 69-74, 1999.
15. Herry, M.P.; Lemétayer, N. Liquid chromatographic determination of patulin in French apple ciders. *J. AOAC Int.*, 79: 1107-110, 1996.
16. Hopkins, J. The toxicological hazards of patulin. *Food Chem. Toxicol*., 31: 455-459. 1993.
17. Lehman, D.; Burkard, W. Bestimung von Patulin in Obstsäften mit Hochleitstungs-Flüssigkeitschromatographie nac Festphasenextracktion. *De. Lebens.-Runds*., 86: 216-217, 1990.
18. Lopez-Diaz, T.; Flanningan, B. Production of patulin and cytochalasin E by Aspergillus clavatus during malting of barley and wheat. *Int. J. Food Microbiol*., 35: 129-136, 1997.
19. Llovera, M.; Viladrich, R.; Torres, M.; Canela, R. Analysis of underivatizated patulin by FC-MS technique. *J. Food Protec*., 2: 202-205, 1999.
20. Machinski Jr., M.; Midio, M. Incidencia de patulina en jugo de manzana industrializado. *Alimentaria*, 276: 61-64, 1996.
21. Ministry of Agriculture, Food and Fisheries (MAFF, UK). Survey of fruit juices and tomato products for patulin. Food Surveillance Information Sheet, 63 (June), 1995. In: http://www.maff.gov.uk/food/infsheet/1995/no63/63patul.htm.
22. Meyer, R.A. Zur Bestimung von Patulin in Lebensmitteln – Vorschlag einer Standardmethode. *Nahrung*, 26: 337-342, 1982.
23. Möller, T.E.; Josefsson, E. Rapid high pressure liquid chromatography of patulin in apple juice. *J. Assoc. Off. Anal. Chem*., 63: 1055-1056, 1980.
24. Motta, S.; Valente Soares, L.M. Survey of Brazilian tomato products for alternariol, alternariol monomethyl ether, tenuazonic acid, and cyclopiazonic acid. *Food Add. Contam*., in press.
25. Moss, M.O. Conditions and factors influencing mycotoxin formation in the field and during the storage of food. *Chem. Ind*., 533-536, 1984.
26. Nielsen, P.V.; Beuchat, L.R.; Frisvad, J.C. Growth of and Fumitremorgin production by *Neosartorya fischeri* as affected by temperature, light, and water activity. *Appl. Envir. Microb*., 54: 1504-1510, 1988.
27. Plessi, M.; Bertelli, D.; Monzani, A. Valutazione del contenuto di patulina in prodoti per la prima infanzia a base di mela. *Riv. Scienza Alimentazione*, 27: 237-243, 1998.
28. Roach, J.A.G.; White, K.D.; Trucksess, M.W.; Thomas, F.S. Capillary gas chromatography/mass spectrometry with chemical ionization and negative ion detection for confirmation of identity of patulin in apple juice. *J. AOAC Int*., 83: 104-112, 2000.
29. Schroeder, H.W.; Cole, R.J.; Hein Jr., H.; Kirksey, J.W. Tremorgenic mycotoxins from *Aspergillus caespitosus*. *Appl. Microbiol*., 29: 857-858, 1975.
30. Scott, P.M. Effects of food processing on mycotoxins. *J. Food Protect*., 47: 489-499, 1984.
31. Scott, P.M.; Fuleki, T.; Harwig, J. Patulin content of juice and wine produced from moldy grapes. *J. Agric. Food Chem*., 25: 434-436, 1977.
32. Sharma, R.P. Immunotoxicity of Mycotoxins. *J. Dairy Sci*., 76: 892-897,1993.
33. Siriwardana, M.G., Lafont, P. Méthode densitométrique de dosage de la patuline dans les jus de fruits. *J. Chromatogr*., 173: 425-428, 1979.
34. Stray, H. High pressure liquid chromatographic determination of patulin in apple juice. *J. Assoc. Off. Anal. Chem*., 61: 1359-1362, 1978.
35. Sylos, C.M.; Rodriguez-Amaya, D.B. Incidence of patulin in fruits and fruti juices marketed in Campinas, Brazil. *Food Add. Contam*., 16: 71-74, 1999.
36. Tarter, E.J.; Scott, P.M. Determination of patulin by capillary gas chromatography of the heptafluorobutyrate derivative. *J. Chromatogr*., 538: 441-446, 1991.
37. Tournas, V. Heat-resistant fungi of importance to food and beverage industry. *C. Rev. Microbiol*., 20(2): 243-263, 1994.
38. Ware, G.M.; Thorpe, C.W.; Pohland, A.E. Liquid chromatographic method for determination of patulin in apple juice. *J. Assoc. Off. Anal. Chem*., 57: 1111-1113, 1974.
39. Wilson, R.D. Surveying some apple juices for patulin. *Food Technol. New Zealand*, 1981, 27-31.
40. Wisniewska, H.; Pirskorska-Pliszczynska, J. Natural occurrence of patulin in fodder beetroots. *Bull. Vet. Inst. Pulawy*, 25: 1-4, 1982.
41. Wouters, M.F.A.; Speijers, G.J.A. Patulin. *In: Toxicological evaluation of certain food additives and contaminants.* World Health Organization, Genebra, 1996, p.337-402.