Quantification of the Mycotoxin Patulin by a Stable Isotope Dilution Assay

Michael Rychlik and Peter Schieberle*

Institut für Lebensmittelchemie der Technischen Universität München,
Lichtenbergstrasse 4, D-85748 Garching, Germany

Two stable isotope dilution assays for the quantification of patulin [4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one] in foods were developed using ^{13}C-labeled patulin as the internal standard. One method was performed by means of LC/MS in negative electrospray ionization mode without derivatization; the other used HRGC/HRMS after trimethylsilylation of the patulin isotopomers. In comparison with previously reported methods based on high-performance liquid chromatography with UV detection, HRGC/HRMS of the derivatized samples showed better repeatability, higher recovery rates (96% at a spike level of 200 ng/L), and a 100 times lower detection limit (12 ng/L). In contrast, LC/MS showed a much lower performance as compared to HPLC/UV or HRGC/HRMS. Using HRGC/HRMS, the mycotoxin was quantified in many different fruit products and in molded wheat bread.

Keywords: *Apple juice; patulin; Penicillium expansum; stable isotope dilution assay; wheat bread; high-resolution mass spectrometry*

INTRODUCTION

Patulin [4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one] is a toxic metabolite produced by different species of *Penicillium*, *Aspergillus*, and *Byssochlamys* (Northolt et al., 1978; Lovett and Thompson, 1978; Roland and Beuchat, 1984). The most commonly encountered fungus is *Penicillium expansum*, the causal mold of apple rot. As the contamination incidence in apple juices is relatively high (Jelinek et al., 1989), this food is thought to be the main source of patulin in the human diet. However, the mycotoxin has also been detected in berries (Lindroth et al., 1978), bread (Reiss, 1973), and meat products (Alperden et al., 1973).

Toxicologic studies have proved patulin to be acutely toxic (Burghardt, 1992) and to produce tumors in rats when injected subcutaneously (Dickens and Jones, 1961). Therefore, many countries have limited its concentration in foods to 50 μg/kg (Van Egmont, 1989).

The most common method used in patulin analysis is high-performance liquid chromatography (HPLC) coupled to UV detection (AOAC, 1997a). Food extraction is performed with ethyl acetate as the solvent, usually by liquid–liquid partitioning, but recently the use of a diphasic dialysis membrane procedure has been reported (Prieta et al., 1994). However, to reach detection limits of 1 μg/kg and recoveries up to 90%, successive cleanup by column chromatography on silica (Geipel et al., 1981) or on silica cartridges (Rovira et al., 1993) is necessary.

Gas chromatographic (GC) procedures determining patulin either as acetate (Ralls and Lane, 1977), trimethylsilyl ether (Suzuki et al., 1974), or its heptafluorobutanoic ester (Tarter and Scott, 1991) did not gain much interest because of incomplete derivatization and the lack of a suitable internal standard (Price, 1979).

Moreover, methods known in the literature are not sensitive and selective enough for, for example, physiological studies on patulin metabolism in humans. So far, trace concentrations of patulin can be detected only indirectly by monitoring its reaction with glutathione and determining glutathione concentrations (Burghardt, 1992). Therefore, many efforts have been made to establish an enzyme-linked immunosorbent assay (ELISA), but due to the instability of patulin conjugates, up to now, no ELISA method is commercially available (Riedel de Haen, personal communication).

For the analysis of trace compounds in complex matrices, such as heterocyclic amines (Holder et al., 1997), odorants (Rychlik and Grosch, 1996), or migrants (Castle et al., 1988), stable isotope dilution assays (SIDAs) in which isotopomers of the respective analytes are used as the internal standards to correct losses of analytes during cleanup have been successfully applied.

A first step toward a SIDA for patulin was made by Price (1979), who added [2H9]-TMS-patulin as an internal standard to the extract of apple juice. However, during the derivatization procedure the [2H9]-TMS groups of the internal standard and the [1H9]-TMS groups of the silylating agent exchanged, making an exact quantification impossible.

Recently, we synthesized the ^{13}C-labeled patulin (Rychlik und Schieberle, 1998). Using this isotopomer, we developed SIDAs for the quantitation of patulin in foods by means of HRGC/MS and LC/MS.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained commercially from the sources given in parentheses: *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and patulin (Aldrich, Steinheim, Germany); sodium carbonate, perfluorokerosene, and sodium sulfate (Merck, Darmstadt, Germany); Tween 80 (Serva, Heidelberg, Germany). [13C2]Patulin was synthesized as recently reported (Rychlik und Schieberle, 1998).

Food Samples. Commercial apple juices and fruit products were purchased in a local store. Homemade apple juices were

* Author to whom correspondence should be addressed (telephone +49-89-289 132 65; fax +49-89-289 141 83; e-mail Peter.Schieberle@lrz.tu-muenchen.de).

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produced in the years 1996 and 1997 from ripe apples grown in a local garden. The apples were pressed; the juice was then pasteurized at 78 °C (3 min) and filled into glass bottles.

In another experiment, sound apples of the variety Golden Delicious were bought in a local market, core and peel were discarded, and the apple tissue was homogenized and pressed. The juice was then heated to 100 °C for 5 min and stored at −30 °C.

Mold Cultures. Freeze-dried cultures of *P. expansum* were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and grown on plates filled with potato dextrose agar (Oxoid, Basingstoke, England). Conidia were harvested from the cultures in sterile phosphate buffer (10 mmol/L, pH 7.0) containing 0.05% Tween 80. Conidia count was determined by the plate count method on potato dextrose agar.

Infection and Incubation of Wheat Bread. Wheat bread was infected by spraying 0.5 mL of a *P. expansum* conidia suspension (5×10^5 conida/mL) on the crumb surface. The bread was then stored for 7 days at 25 °C.

Photometric Quantification of Patulin (AOAC, 1997b). Patulin standard solutions were prepared by dissolving commercial crystalline patulin (Pat; 5 mg) in chloroform (50 mL). [$^{13}C_2$]Patulin ([$^{13}C_2$]-Pat) solutions were checked for purity by thin-layer chromatography (TLC) on silica/fluorescence indicator (254 nm) using ethyl acetate/hexane (1:1 v/v) as the mobile phase.

Pure standard solutions (250 μL) were evaporated to dryness in a stream of nitrogen and dissolved in ethanol (5 mL). Absorption (*A*) of the solutions was recorded at 275 nm by a UV spectrophotometer type 550 (Perkin-Elmer, Überlingen, Germany), and patulin concentrations were calculated using the following equation (1 cm cell path length): C (μg/mL)$= A \times 10.55$ (AOAC, 1997b).

Extraction and Cleanup of Fruit Products for SIDAs. Small volumes of liquid samples (5 mL) were directly extracted with ethyl acetate (2×10 mL); pastelike samples (5 g) were homogenized with water (10 mL) prior to extraction. The solvent, ethyl acetate, was spiked with an amount of [$^{13}C_2$]-patulin so that its mass ratio to unlabeled patulin was within the range of 1:3 to 3:1. The combined organic solutions were washed with an aqueous solution of sodium carbonate (1.5 wt %, 2 mL), and the aqueous phase was extracted with an additional portion of ethyl acetate (10 mL). The organic phases were combined, dried over anhydrous sodium sulfate, and evaporated to dryness in a stream of nitrogen.

Large volumes (100 mL) were extracted with ethyl acetate (2×100 mL), which was spiked with an appropriate amount of [$^{13}C_2$]patulin. The combined organic solutions were washed with an aqueous solution of sodium carbonate (1.5 wt %, 20 mL), and the aqueous phase was extracted with ethyl acetate (50 mL). The organic phases were combined, dried over anhydrous sodium sulfate, and concentrated in a stream of nitrogen. For liquid chromatography/mass spectrometry (LC/MS) the sample was dissolved in methanol (100 μL).

For HRGC/HRMS of the silylated sample, the concentrated extract (1 mL) was purified on a Sep-Pak silica cartridge (containing 1 g of silica; Waters, Milford, MA), preconditioned with chloroform (1 mL). Three fractions were eluted with mixtures of chloroform/hexane (8:2, v/v, 1 mL, fraction 1; 5:5, v/v, 1 mL, fraction 2; 2:8, v/v, 3 mL, fraction 3). Fraction 3 containing Pat and [$^{13}C_2$]-Pat was evaporated to dryness in a stream of nitrogen and subjected to TMS derivatization.

Extraction and Cleanup of Wheat Bread Samples for SIDAs. Bread (5 g) was frozen in liquid nitrogen and ground in a blender (Privileg, Fürth, Germany). The powder was suspended in water (10 mL) and extracted with ethyl acetate (2×10 mL) spiked with an appropriate amount of [$^{13}C_2$]-patulin. The combined organic solutions were washed with an aqueous solution of sodium carbonate (1.5 wt %, 2 mL), and the aqueous phase was extracted with ethyl acetate (10 mL). The organic phases were combined, dried over anhydrous sodium sulfate, and evaporated to a volume of 0.5 mL in a stream of nitrogen. The concentrated extract was then purified on a Sep-Pak silica cartridge (Waters, Milford, MA), precon-
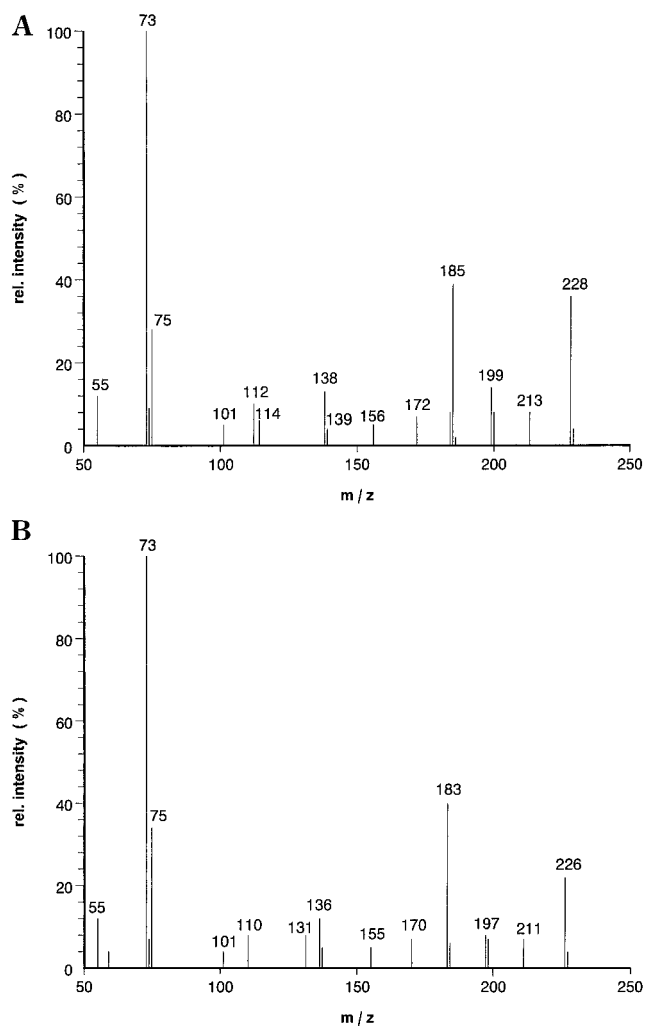

Figure 1. Mass spectrum of the TMS derivatives of labeled (A) and unlabeled patulin (B) in the electron impact mode.

ditioned with hexane (1 mL). The column was flushed with hexane (4 mL), followed by a mixture of ethyl acetate/hexane (1:1, v/v; 3 mL, fraction 1; followed by 1:1, v/v; 8 mL, fraction 2). Fraction 2, containing Pat and [$^{13}C_2$]-Pat was evaporated to dryness in a stream of nitrogen and subjected to TMS derivatization.

Calculation for Patulin Quantification by SIDAs. Patulin concentrations *C* in food quantified by SIDA were calculated using the following equation

$$C = A_{\text{Pat}} m_{^{13}\text{C−Pat}} R_f / A_{^{13}\text{C−Pat}}$$

where A_{Pat} is the area of unlabeled patulin in mass trace m/z 226 for low-resolution MS or m/z 226.0661 for high-resolution MS, $A_{^{13}\text{C−Pat}}$ is the area of ^{13}C-labeled patulin in mass trace m/z 228 for low-resolution MS or m/z 228.0728 for high-resolution MS, $m_{^{13}\text{C−Pat}}$ is the amount of added ^{13}C-labeled patulin, and R_f is the response factor (= 1.07).

Extraction and Cleanup of the Samples for HPLC/UV. Extraction and cleanup of the samples was performed as described above for LC/MS, but without addition of [$^{13}C_2$]-Pat.

HPLC was performed by injecting 10 μL of the extract onto a Nucleosil RP18 column (250 × 4.0 mm i.d., 5 μm, Macherey-Nagel, Düren, Germany) mounted in an HPLC type 420 (Kontron, Neufahrn, Germany). Gradient elution was performed at a flow rate of 1 mL/min. After the column had been flushed for 5 min with acetonitrile/water (5:95 by vol), a linear gradient was programmed within 20 min to acetonitrile/water (95:5 by vol). The effluent was monitored at 275 nm using an HPLC detector 432 (Kontron, Neufahrn, Germany).
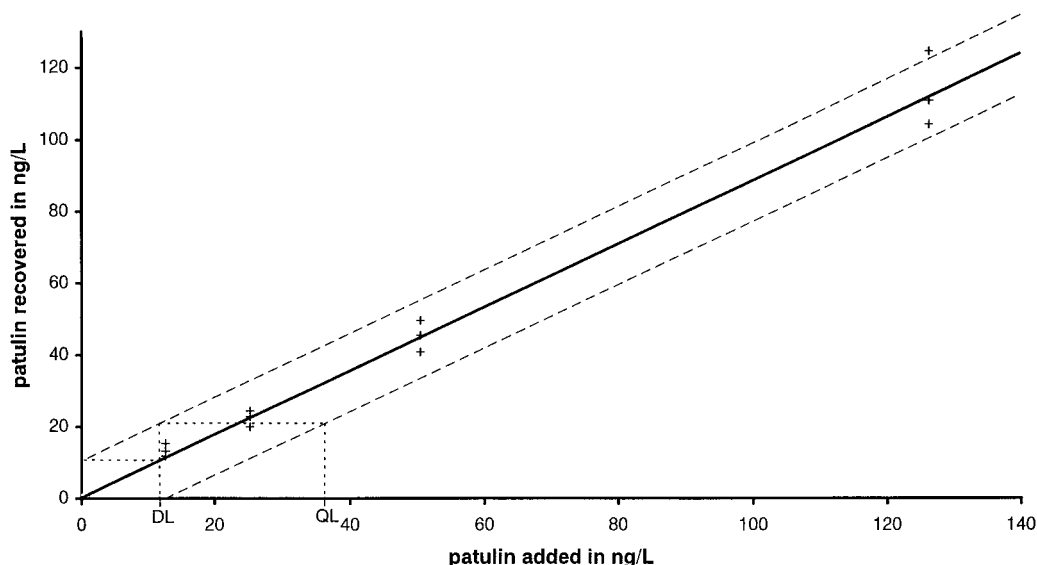
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Figure 2. Determination (- - -) of the DL and QL of patulin in apple juice by GC/MS; (− − −) 95% confidence limit of the calibration line (−).

Determination of Detection and Quantification Limits. To a patulin-free apple juice the following amounts of patulin were added: 13, 25, 50, and 130 ng/L for HRGC/HRMS (SIDA); 19, 40, 93, and 190 μg/L for LC/MS (SIDA); and 1, 2, 4, and 10 μg/L for HPLC/UV. Each sample was analyzed in triplicates. Detection limit (DL) and quantification limit (QL) were calculated according to the method of Hädrich and Vogelgesang (1996): DL is the addition value referring to the 95% confidence limit of the calibration line at the zero addition level. QL is the addition level which lowers the 95% confidence limit to meet the upper 95% confidence limit of the addition level at the DL.

Preparation of TMS Derivatives. Fifty microliters of BSTFA was added to the purified fractions, and the mixture was heated for 10 min at 80 °C in a closed vial. After cooling to room temperature, the solution was evaporated to dryness in a stream of nitrogen. After addition of 100 μL of hexane, the samples were ready for HRGC/HRMS.

Gas Chromatography/High-Resolution Mass Spectrometry (HRGC/HRMS). HRGC was performed by means of a type 5300 gas chromatograph (Carlo Erba, Hofheim, Germany) using a capillary DB-5 (30 m × 0.32 mm fused silica capillary, film thickness of the stationary phase d_f = 0.25 μm; Fisons Instruments, Mainz, Germany).

The samples were applied by the cold on-column technique at 60 °C. One minute after injection of the sample, the temperature of the oven was raised to 250 °C at a rate of 10 °C/min. The flow rate of the carrier gas helium was 2 mL/min.

Mass spectra were recorded by means of an MAT 95 S (Finnigan MAT, Bremen, Germany) coupled to capillary DB-5. Multiple ion detection (MID) was performed with a resolution of 5000 and by using perfluorokerosene for calibration. Mass traces for [$^{13}C_2$]-Pat and Pat were m/z 228.0728 and 226.0661, respectively; lock mass was m/z 218.9856, and calibration mass was m/z 230.9856. Ionization energy in the electron impact mode was 70 eV.

Liquid Chromatography/Mass Spectrometry (LC/MS). LC/MS spectra were recorded by means of an LCQ (Finnigan MAT) coupled to a spectra series high-performance liquid chromatograph (Thermo Separation Products, San Jose, CA) equipped with a Nucleosil RP18 column (250 × 2.0 mm i.d., 5 μm). Three microliters of the sample solutions was chromatographed using gradient elution and a flow rate of 0.4 mL/min. After the column had been flushed for 5 min with acetonitrile/water (5:95 by vol), a linear gradient was programmed within 20 min to acetonitrile/water (95:5 by vol). The mass spectrometer operated in the negative electrospray mode with a spray needle voltage of −4.5 kV and a spray current of 0.2 μA. The temperature of the capillary was 220 °C, and the capillary

Table 1. Patulin Concentrations in Fruit Products Quantified in SIDAs Using HRGC/HRMS

product	no. of samples	patulin concn (range in μg/L)
apple juices, commercial products	10	5.7−26.0
apple juices, homemade	2	11.4−23.9
apple juice, specially prepared[a]	1	<0.02
apple−acerola juice, commercial product	1	0.7
grape juice, commercial products	2	4.9−5.2
sour cherry juice, commercial product	1	0.2
blackcurrant juice, commercial product	1	0.1
orange juice, commercial product	1	0.1
plum pulp, commercial product	1	0.8[b]
apple pulp, commercial product	1	<0.02[b]
raspberry sirup, commercial product	1	<0.02[b]

[a] Peel and core were removed before the apples were pressed. [b] Values in μg/kg.

voltage was −6 V. The sheath and auxiliary gas nitrogen nebulized the effluent with flows of 2.3 and 3.0 mL/min, respectively. The ion trap was operated at a helium pressure of 10^{-3} Torr.

RESULTS AND DISCUSSION

Quantification by a SIDA Using HRGC/HRMS. Patulin (Pat) is not volatile enough for HRGC analysis. To increase its volatility, a derivatization of Pat and [$^{13}C_2$]-Pat was performed using BSTFA. The mass spectra of the labeled (M⁺, m/z 228) and unlabeled derivatives (M⁺, m/z 226) obtained in the electron impact (EI) mode are shown in Figure 1, parts A and B, respectively.

For both derivatives, the molecular masses were obtained (m/z 226, Pat; m/z 228, [$^{13}C_2$-Pat]). However, although the intensities were low compared to the that of the TMS fragment, m/z 73, mass chromatography of the ion intensities was used in the SIDA experiments. Increasing the sensitivity by performing mass chromatography in the chemical ionization (CI) mode was not possible, because the calibration standard perfluorokerosene could not be ionized with isobutane as reagent gas.

To determine the response factor for mass chromatography in the high-resolution mode, mixtures of
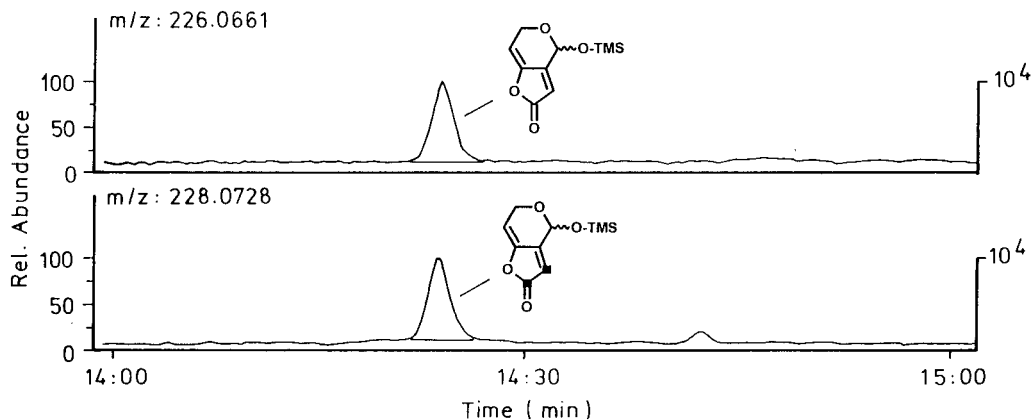
Figure 3. HRGC/high-resolution mass chromatogram of an apple–acerola juice containing 0.7 μg/L patulin. The internal standard [$^{13}C_2$]-TMS-patulin is detected in mass trace *m/z* 228.0728, unlabeled TMS-patulin in trace *m/z* 226.0661.
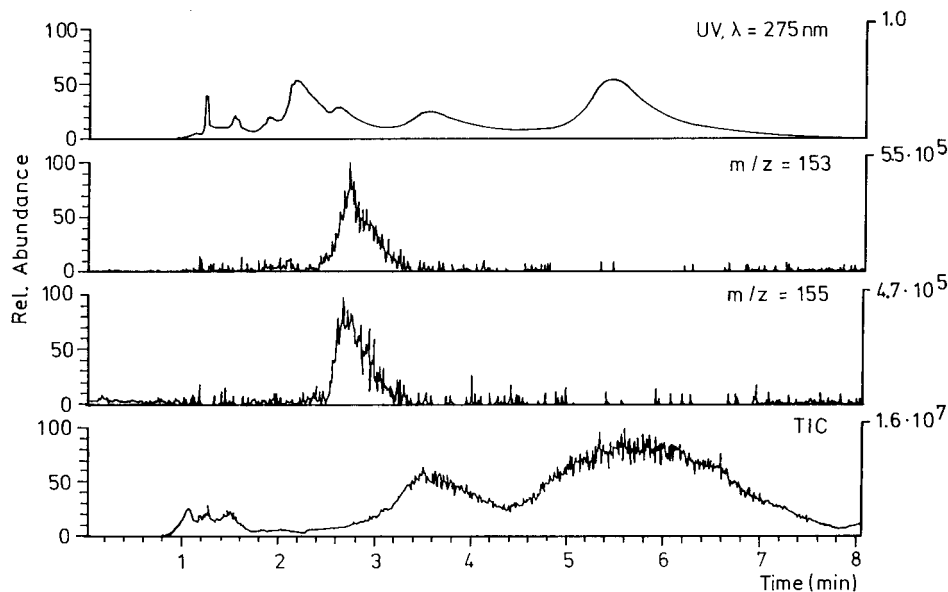

Figure 4. LC/mass chromatogram of an apple juice containing 26 μg/L patulin. The internal standard [$^{13}C_2$]patulin is detected in mass trace *m/z* 155, unlabeled patulin in trace *m/z* 153.

Table 2. Patulin Concentrations in Molded Foods Quantified in SIDAs Using HRGC/HRMS

product	patulin concn (range in μg/kg)
wheat bread, spontaneously molded	
sample 1	45
sample 2	<1.1
wheat bread, infected with *P. expansum* and stored at 25 °C for 7 days	750–21000[a]

[a] Five infected wheat bread slices were analyzed.

patulin and [$^{13}C_2$]-Pat in ratios from 3:1 and 1:3 were silylated and analyzed by HRMS. Analysis in triplicates gave a response factor of 1.07 ± 6%. This result showed that silylation and mass chromatography did not discriminate between the two isotopomers.

For patulin quantification in fruit products, [$^{13}C_2$]-Pat was dissolved in the extraction solvent; the successive extraction and cleanup procedure was similar to procedures commonly used in the HPLC methods (AOAC, 1997a). Sample sizes above 100 g required further cleanup on silica cartridges.

To determine the DL and the QL by the calibration line method, patulin-free apple juice prepared from peeled, sound apples was spiked with increasing amounts of pure patulin (from 13 to 130 ng/L) prior to analysis.

By HRGC/HRMS a DL of 12 ng of patulin/L and a QL of 35 ng/L were calculated by means of a standard curve (Figure 2). Recovery at a spike level of 200 ng/L and relative standard deviation (RSD) were 96% and 9%, respectively. Due to lower selectivity HRGC/MS in the low-resolution scanning MS mode resulted in a higher QL of 500 ng/L.

Patulin Concentrations in Apple Juices. Application of the HRGC/HRMS–SIDA to commercial and homemade fruit products gave the results detailed in Table 1. In all apple juices analyzed, patulin concentrations were >0.03 μg/L with the exception of the juice made from peeled, cored, and sound apples. In agreement with the results of Geipel et al. (1981) and Ehlers (1986), most of the samples showed concentrations of the mycotoxin <20 μg/L, but in contrast to the results of the latter author, who found patulin concentrations >50 μg/L in 25% of all samples, no samples with such high contents were detected in our studies.

The results confirm the high contamination incidence of the mycotoxin in apple juices, thus proving that in commercial production, infected apples are not effectively removed from the raw material. Moreover, there were procedures reported to reduce patulin concentrations, such as trimming (Lovett et al., 1975) or washing of apples (Sydenham et al., 1995), but either
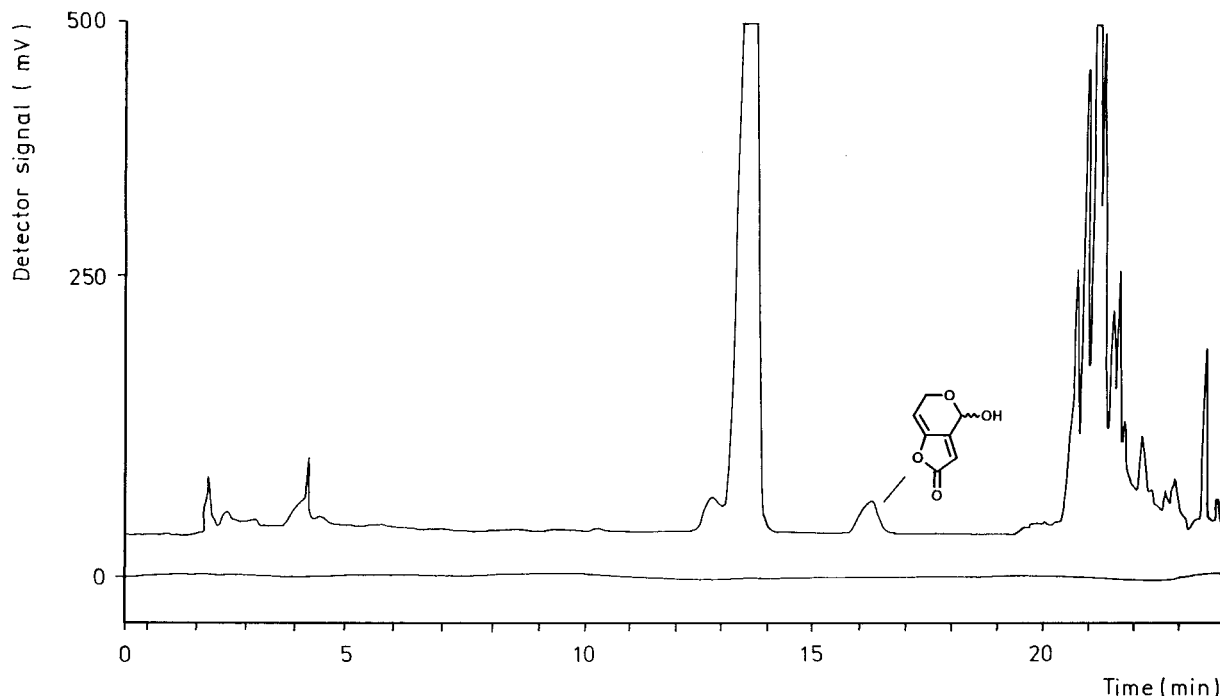
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Figure 5. HPLC/UV of an apple juice containing 26 μg/L patulin.

these methods were not applied to juice production or are not effective enough.

Remarkably, one sample of an apple–acerola juice showed a patulin concentration <1 μg/L (HRGC/HR mass chromatograms shown in Figure 3). A possible reason for the low patulin content may be ascorbic acid, which is known to reduce patulin concentrations in apple juices (Brackett and Marth, 1979) and is found in acerola fruits in concentrations between 1 and 2% (Souci et al., 1994).

Patulin Concentrations in Other Fruit Products. Besides apples, grapes are another fruit that is likely to be infected by *P. expansum*. Therefore, we quantified patulin in two grape juices and detected concentrations of ~5 μg/L (Table 1), which are similar to data reported by Altmeyer et al. (1982).

In other fruit products, the concentrations of the mycotoxin were lower than 1 μg/L or 1 μg/kg, respectively (Table 1), and, therefore, have undoubtedly not been detected in the previous publications using less sensitive methods.

Patulin Concentration in Molded Wheat Bread. Because many foods are ideal substrates for molds, fungal infection and mycotoxin production can cause serious problems for the food industry and trade. Therefore, we quantified patulin in wheat bread that was either spontaneously molded or infected with pure cultures of *P. expansum*. Both samples were incubated for 7 days at 25 °C. Although the bread infected with *P. expansum* always showed high patulin concentrations (Table 2), the spontaneously growing molds on wheat bread produced the mycotoxin in only one case. Because the widespread fungus *P. expansum* can grow on wheat bread and produce patulin, a contamination with the mycotoxin in molded bread can never be excluded.

LC/MS. In LC/mass chromatography experiments extraction and cleanup procedures were similar to those in the HRGC/HRMS method without the additional cleanup on a silica cartridge and derivatization. The ratio of patulin and its isotopomer was determined by

Table 3. Performance Characteristics of SIDAs Based on HRGC/HRMS or LC/MS and of HPLC/UV Detection

parameter	SIDAs		HPLC/UV detection
	HRGC/HRMS	LC/MS	
DL	12 ng/L	20.3 μg/L	1.3 μg/L
QL	35 ng/L	63 μg/L	4 μg/L
corresponding sample vol	100 mL	100 mL	20 mL
RSD	9%	28%	18%
(no. of samples)	4	4	5
recovery [a]	96%	94%	90%
at spike levels of	200 ng/L	100 μg/L	10 μg/L

[a] Average of triplicates.

monitoring the quasimolecular ions *m/z* 153 and 155, respectively, obtained in the negative electrospray ionization mode (Rychlik and Schieberle, 1998).

Separation of the mycotoxin from matrix compounds was achieved by HPLC prior to MS running in the negative electrospray ionization mode (ESI). Due to patulin's low degree of dissociation, the sensitivity of detection in ESI was very low. This resulted in a high DL of 20.3 μg/L of the mycotoxin in apple juices and bad peak shapes in the mass chromatograms (Figure 4). For that reason repeatability was bad and an RSD of 28% was calculated (Table 3). The DL could not be reduced by addition of ionizing agents such as ammonium acetate or formic acid to the mobile phase. Ammonium acetate, on the one hand, had no effect in either positive or negative ESI mode. On the other hand, formic acid increased sensitivity by ~50% in positive ESI mode, but this mode showed still a higher DL than the negative mode. In atmospheric pressure chemical ionization (APCI) mode, the signal of the quasimolecular ion was as intensive as in negative ESI mode. The APCI mass spectra were shown recently (Rychlik and Schieberle, 1998).

Comparison of SIDAs with HPLC/UV Detection. To compare the newly developed SIDAs with the commonly used HPLC method with UV detection, an apple juice high in patulin was quantified by means of the

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Table 4. Comparison of Quantitative Results Obtained by SIDAs Based on HRGC/HRMS or LC/MS and HPLC/UV Detection

	SIDAs		HPLC/UV detection
sample	HRGC/HRMS	LC/MS	
commercial apple juice high in patulin	26.0 μg/L	>20.3 μg/L[a]	23.4 μg/L

[a] Content between DL and QL.

three methods. The data obtained (Table 4) showed only minor differences. HRGC/HRMS had the best recovery rate and gave the highest content of 26 μg/L.

The amount determined by LC/MS lies between the DL and QL, so that this method could only confirm a positive patulin content without giving an exact quantitative result.

HPLC with UV detection (chromatogram shown in Figure 5) showed a slightly lower content of 23.4 μg/L. This is due to the fact that this method showed the lowest recovery in the model experiments (Table 3). The DL and QL of 1.3 and 4 μg/L, respectively, agreed with those of the reported methods (Official methods for patulin analysis, 1984). Thus, regarding sensitivity as well as repeatability, HPLC/UV shows better data than LC/MS, but HRGC/HRMS gives by far the most exact results. However, when selectivity is compared, both MS methods are more specific than UV detection and, therefore, more reliable.

Conclusions. A SIDA using ^{13}C-labeled patulin as the internal standard and HRGC/HRMS as the detection method was shown to be 100 times more sensitive than the commonly used HPLC methods with UV detection.

The sensitivity and selectivity of the SIDA should make this method useful for physiological examinations, for example, in metabolism studies in the human body.

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