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Parallel Synthesis: A New Approach for Developing Analytical Internal Standards. Application to the Analysis of Patulin by Gas Chromatography−Mass Spectrometry

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The polymer-assisted reaction of 4-(hydroxymethyl)furan-2(5H)-one (4HM2F) with 21 carboxylic acids using polystyrene-carbodiimide (PS-carbodiimide) yielded an ester library. Four of the esters, (5-oxo-2,5-dihydrofuran-3-yl)methyl acetate (IS-1), (5-oxo-2,5-dihydrofuran-3-yl)methyl butyrate (IS-2), (5-oxo-2,5-dihydrofuran-3-yl)methyl 2-methylpropanoate (IS-3), and (5-oxo-2,5-dihydrofuran-3-yl)-methyl chloroacetate (IS-4), were tested as internal standards for the quantification of patulin in apple juice by gas chromatography−mass spectrometry in the selected ion monitoring mode (GC-MS-SIM). The developed method combines an AOAC official extractive step and a GC-MS-SIM analysis. Using a chromatographic column containing trifluoropropylmethylpolysiloxane as the stationary phase and IS-1 as the internal standard, it was possible to perform an accurate and precise quantification of underivatizated patulin in apple juice at concentrations down to 6 μg/L. A detection limit of 1 μg/L was established.

KEYWORDS: Solid phase synthesis; esters; internal standard; apple juice; underivatized patulin; GC-MS-SIM; quantification

INTRODUCTION

Several approaches have been used to analyze patulin content in apple juice. To date, the most widely used method has been liquid chromatography (LC), since patulin is a polar compound (*1*, *2*). Even so, conventional LC methods with UV (ultraviolet) detection exhibited poor selectivity because interfering compounds, like 5-hydroxymethylfurfural (HMF), can simultaneously elute with patulin from a reverse phase column (*3*). Hyphenated techniques such as liquid chromatography−mass spectrometry (LC-MS) (*4*−*6*) and gas chromatography−mass spectrometry (GC-MS) (*7*−*9*) have therefore been developed and applied to the analysis of patulin residues. Whereas most GC and GC-MS procedures imply the use of patulin derivatives (*7*−*10*), adequate detection of underivatized patulin has also been achieved using the on-column injection technique (*3*, *11*).

Although LC-MS and GC-MS are intensively applied in the analysis of many xenobiotics present in complex matrices, an accurate quantitative approach for these techniques requires using internal standards (IS). Isotopically labeled analogues (ILAs) of analytes are the preferred IS for mass spectrometric analysis (*12*). However, the use of structurally related analogues can offer an alternative to expensive ILAs (*13*). Moreover, solid support techniques have recently been developed to synthesize libraries of structurally related compounds. These techniques offer important advantages with respect to conventional synthetic chemistry, allowing the relatively rapid preparation of sets of related chemical compounds.

The main purpose of the present work is to show how a parallel synthesis strategy can yield a set of esters that can then be tested as ISs for the quantitative analysis of patulin in apple juice by GC-MS-selected ion monitoring (SIM). The synthesis of the esters was carried out using a solid phase approach. Thus, carbodiimide in a solid phase support was used to obtain a wide variety of similar esters from 4-(hydroxymethyl)furan-2(5H)-one (4HM2F) and 21 different acids. One new IS obtained, (5-oxo-2,5-dihydrofuran-3-yl)methyl acetate (IS-1), allows us to considerably improve our earlier work for the analysis of patulin developed in our laboratory (*3*).

EXPERIMENTAL SECTION

Solvents and Reagents. Dichloromethane, ethyl acetate, tetrahydrofuran, and dimethylformamide (DMF), for organic residue analysis, were purchased from J. T. Baker (Deventer, Holland). Chloroform and methanol, synthesis grade, were purchased from Scharlab Chemie S. A. (Barcelona, Spain). 4HM2F was synthesized according to refs *14* and *15*. Polystyrene (PS)-carbodiimide resin was obtained from Argonaut Technologies (NET Interlab, Spain). Patulin was supplied by Sigma (Steinheim, Germany).

Solid Phase Parallel Synthesis of Esters. Parallel synthesis was performed on a carousel reaction station with 12 threaded glass reaction

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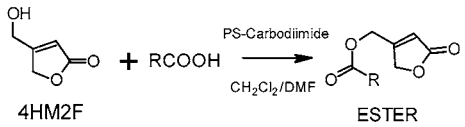

R = $C_8H_7O_2$, C_5H_3ClN, C_5H_4N, C_5H_4N, $C_4H_3N_2$, C_5H_4, CF_3, H, CHF_2, CH_3, C_3H_7, n-C_4H_9, CH_2Cl, $CHCl_2$, CH_2Br, $C_9H_9O_2$, CCl_3, C_7H_5, $C_7H_{14}NO_2$, C_8H_7, $C_4H_3N_2$

Figure 1. Synthesis of esters and the relation of 21 acids used.

tubes, 24 mm × 150 mm (Radleys Discovery Technologies, Essex, United Kingdom). One gram (1.05 mmol) of PS-carbodiimide resin was added to a dry reaction tube. Each acid (0.85 mmol) was dissolved in 7 mL of dry dichloromethane (with 1% dry DMF if required). **Figure 1** shows all different acids essayed. The solution was then added to the dry resin, and the mixture was stirred for 1 h at room temperature. Then, 75.3 mg (0.66 mmol) of 4HM2F in 3 mL of dry CH_2Cl_2 was added and the reaction mixture was stirred at room temperature overnight. The progress of the reaction was monitored by thin-layer chromatography analysis on silica gel 60 F_{254} (chloroform−methanol 5%). The reaction mixture was filtered, and the resin was washed twice with the reaction solvent. To evaluate the presence of ester and its purity, the filtrate was subsequently analyzed by GC-MS as described below. A scheme of the reaction is given in **Figure 1**.

Calibration Standards. Patulin stock solution was prepared according to the AOAC Official Method 2000.02 (*2*). Calibration standards were prepared from free patulin extract obtained according to Brause et al. (*16*). The extract solution was evaporated under vacuum conditions at 40 °C, and the final residue was redissolved in 200 μL of standard solutions containing 0.2 μg/mL of ISs and either 0.15, 0.25, 0.38, 0.50, 0.75, or 1.25 μg/mL of patulin.

Spiked Samples. A working solution of 100 μg/mL of patulin was used to prepare a set of ethyl acetate solutions containing 0.6, 1.0, 3.0, and 5.0 μg/mL of patulin and 0.8 μg/mL of the ISs (IS-1, IS-2, IS-3, and IS-4). Five milliliters of free patulin apple juice was then spiked with 50 μL of these ethyl acetate solutions. After shaking vigorously for 1 min to homogenize, samples were extracted and cleaned-up as described by Brause et al. (*16*). The final residue was dissolved in 200 μL of ethyl acetate and analyzed by GC-MS as described below. The essays were carried out in triplicate. A UV/vis UV2 spectrophotometer from Unicam was used to confirm the concentration of patulin in the stock solution.

GC-MS Analysis. Analysis was carried out using an Agilent 6890N gas chromatograph interfaced to a 5973N mass selective detector. Mass spectrometric data were collected in full-scan and SIM modes. Full scan data were used for preliminary selection of best target *m*/*z* ions and qualifiers. In other cases, the SIM mode was used to quantify patulin in apple juice in order to maximize sensitivity and selectivity. SIM was performed monitoring the ions in one group, and the dwell time applied for each ion was 50 ms with a rate of 3.03 cycles/s.

One microliter of the extract was injected using the on column mode and following a ramp pressure technique and track oven temperature programmed. A fused silica deactivated retention gap of 3m × 0.32 mm (Agilent, Anorsa, Barcelona, Spain) was connected between the injector and the analytical column using a universal deactivated press fit connector (Agilent). The carrier gas was helium at a constant flow rate of 1.5 mL/min. The columns used were a HP-5 MS (cross-linked 5% phenylmethylpolysiloxane) 30 m × 0.25 mm i.d. column, df = 0.25 μm (Agilent, Cromlab, Barcelona, Spain) and a Rtx-200 MS (crossbond trifluoropropylmethylpolysiloxane) 30 m × 0.25 mm i.d. column, df = 0.25 μm (Restek, Teknokroma Barcelona, Spain). GC temperature parameters varied slightly according to the column used. The oven temperature was programmed at 140 °C and was ramped at 10 °C/min up to a maximum of 280 °C for 10 min when a DB-5 MS was used. When a Rtx-200 MS was used, the GC temperature was programmed at 140 °C, initially ramped at 5 °C/min to 170 °C, further ramped at 15 °C/min up to 280 °C, and then held until a total run time of 24 min. The GC-MS transfer line was held at 280 °C, and the quadrupole analyzer and the ion source heaters were maintained at 150 and 230 °C, respectively.

Ion abundance ratios and retention times were applied as criteria for identification of patulin in samples. The ions selected for patulin identification were as follows: *m*/*z* 110 $(M - C_2H_4O)^+$, which was used as the target ion; and 126 $(M - CO)^+$, 136 $(M - H_2O)^+$, and 154 (M^+), which were used as qualifier ions. The specificity of the chromatographic method coupled to a MS detector was readily demonstrated by establishing the ratios for patulin identification, *m*/*z* 126/110 = 55.0 ± 5%, *m*/*z* 136/110 = 34.0 ± 5%, and *m*/*z* 154/110= 27.0 ± 5% (mean ± RSD %, $n = 14$). Those ratios were calculated from the different mass spectra obtained analyzing spiked samples with 100 μg/L of patulin in full scan mode (40−400 amu). To confirm the presence of patulin in samples analyzed in SIM mode, the specified ratios were calculated by integrating the individual ion chromatograms. The ion selected for ester quantification was *m*/*z* 96 $(C_5H_4O_2)^+$.

Statistical Analysis. Calibration curves were generated in EXCEL using least-squares linear regression analysis. We established the utility of esters as ISs by predicting the concentration of spiked samples throughout the calibration range for three different levels of patulin. Predictions were made on the basis of the fitted line and the estimated standard deviation (S_x) of a predicted value for x_i (*17*). The calculation of the limit of detection (LOD) was based on the residual standard deviation of the regression line ($S_{y/x}$) and the slope (b), LOD = $(3.3 \times S_{y/x})/b$.

RESULTS AND DISCUSSION

Preparation of Putative IS. Table 1 summarizes the results of the solid phase syntheses carried out as indicated in **Figure 1**. The efficiency of the process and the chromatographic behavior in the GC-MS analysis is shown for each synthesized ester. The first six compounds listed in **Table 1** were discarded due to the low yields of their synthesis (<50%). The yield of the synthesis for the other esters was 80−85%. One microliter of a 100 μg/mL ethyl acetate solution of each of the chosen esters was injected into the GC-MS to determine its relative retention time (rR_t) to patulin. The predominant fragments from their mass spectra obtained in scan mode (scan range 40−400 amu) were also determined. Compounds with rR_t values similar to that of patulin were then selected for the recovery assays. All of the selected compounds exhibited an *m*/*z* 96 fragment $(C_5H_4O_2)^+$ (**Table 1**), which was very similar to *m*/*z* 110 $(C_5H_2O_3)^+$, the main fragment selected for patulin. Thus, in the absence of sources of interference, MS performance should affect fragments in the same way.

Recovery studies were carried out by spiking 5 mL of apple juice with 50 μL of a mixture of selected esters, each in a concentration of 0.8 μg/mL. The samples were then extracted and cleaned-up as described in the Experimental Section (*16*). **Table 2** shows the recovery efficiency of the esters (Q_{is}) throughout the selected extraction method. Q_{is} was calculated for each ester according to the following equation:

$$Q_{is} = \bar{A}_{IS-spk} \times 100/\bar{A}_{IS-std}$$

where $\bar{A}_{IS-spk}$ is the mean chromatographic peak area of *m*/*z* 96 corresponding to each ester from the spiked samples and $\bar{A}_{IS-std}$ is the expected mean value for each ester. The esters with recovery rates greater than 90%, IS1, IS2, IS3, and IS4, were

Table 1. Synthesized Compounds Sorted by Ascending Order of Relative Retention Time with Respect to Patulin, Yields > 50%, and Reference Ions

compound name	yield > 50%	rR_t[a] (min)	reference ions (*m*/*z*)
(5-oxo-2,5-dihydrofuran-3-yl)methyl (2*E*)-3-(3,4-dihydroxyphenyl)acrylate			
(5-oxo-2,5-dihydrofuran-3-yl)methyl 2-chloronicotinate			
(5-oxo-2,5-dihydrofuran-3-yl)methyl isonicotinate			
(5-oxo-2,5-dihydrofuran-3-yl)methyl nicotinate			
(5-oxo-2,5-dihydrofuran-3-yl)methyl pyrazine-2-carboxylate			
(5-oxo-2,5-dihydrofuran-3-yl)methyl pyridine-2-carboxylate			
(5-oxo-2,5-dihydrofuran-3-yl)methyl trifluoroacetate	+	0.63	69/96
(5-oxo-2,5-dihydrofuran-3-yl)methyl formate	+	0.75	96
(5-oxo-2,5-dihydrofuran-3-yl)methyl difluoroacetate	+	0.82	96
(5-oxo-2,5-dihydrofuran-3-yl)methyl acetate	+	0.86	68/96
(5-oxo-2,5-dihydrofuran-3-yl)methyl 2-methylpropanoate	+	1.01	71/96
patulin (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)one)		1.00	110/126/136/154
(5-oxo-2,5-dihydrofuran-3-yl)methyl butyrate	+	1.04	71/96
(5-oxo-2,5-dihydrofuran-3-yl)methyl chloroacetate	+	1.17	85/96
(5-oxo-2,5-dihydrofuran-3-yl)methyl dichloroacetate	+	1.20	67/96
(5-oxo-2,5-dihydrofuran-3-yl)methyl bromoacetate	+	1.21	96/123
(5-oxo-2,5-dihydrofuran-3-yl)methyl (2*E*)-3-(4-hydroxy-3-methoxyphenyl)acrylate	+	1.26	203/218
(5-oxo-2,5-dihydrofuran-3-yl)methyl trichloroacetate	+	1.28	96/119
(5-oxo-2,5-dihydrofuran-3-yl)methyl octanoate	+	1.47	96/127/240
(5-oxo-2,5-dihydrofuran-3-yl)methyl *N*-(*tert*-butoxycarbonyl)-β-alaninate	+	1.68	98/212
(5-oxo-2,5-dihydrofuran-3-yl)methyl (2*E*)-3-phenylacrylate	+	1.76	131/244
(5-oxo-2,5-dihydrofuran-3-yl)methyl 1-benzofuran-2-carboxylate	+	1.85	145/162/258

[a] Relative retention time with respect to patulin.

Table 2. Extraction Efficiencies of Selected Esters Determined by the Extractive Method

esters tested for recovery assays	Q_{IS}% mean ± RSD % ($n = 10$)
(5-oxo-2,5-dihydrofuran-3-yl)methyl formate	26.1 ± 30
(5-oxo-2,5-dihydrofuran-3-yl)methyl acetate (IS1)	93.5 ± 16
(5-oxo-2,5-dihydrofuran-3-yl)methyl 2-methylpropanoate (IS3)	90.1 ± 20
(5-oxo-2,5-dihydrofuran-3-yl)methyl butyrate (IS2)	101.5 ± 19
(5-oxo-2,5-dihydrofuran-3-yl)methyl chloroacetate (IS4)	108.7 ± 21
(5-oxo-2,5-dihydrofuran-3-yl)methyl bromoacetate	37.5 ± 22

chosen as putative ISs. Characterization data such as high-resolution (HR) mass spectra, ^{1}H NMR, and ^{13}C NMR were obtained. Elemental compositions deduced from the accurate measurement of the molecular mass fragment obtained confirmed the expected empirical formulas. HR mass spectra obtained were also in agreement with those obtained using a quadrupole analyzer (see Supporting Information).

GC-MS Conditions for Patulin Analysis. First, the column injection program was studied in order to prevent peak width broadening and achieve sharp peaks. The oven temperature program was optimized by comparing the TIC (total ion chromatogram) area of patulin at different initial temperatures and at maximum inlet pressure. The results obtained indicated that the optimum initial oven temperature was 140 °C (**Figure 2**). Moreover, this high temperature appeared to reduce the accumulation of low volatility compounds from the apple juice in the retention gap. It was consequently possible to carry out a large number of analyses without cutting or replacing the retention gap.

Matrix-induced response enhancement was observed when standards prepared in blank matrix extract were compared with those prepared in solvent solution (*18*−*20*). It was then necessary to use matrix-matched calibration to achieve an accurate quantification of patulin. The calibration solutions were prepared from extracts of blank samples to counteract the matrix effect. This was considered an effective way to avoid potential errors deriving from the matrix effects in the quantification process.

Influence of the Stationary Phase upon IS Behavior. Preliminary chromatographic analyses were carried out using cross-linked 5% phenylmethylpolysiloxane stationary phase.

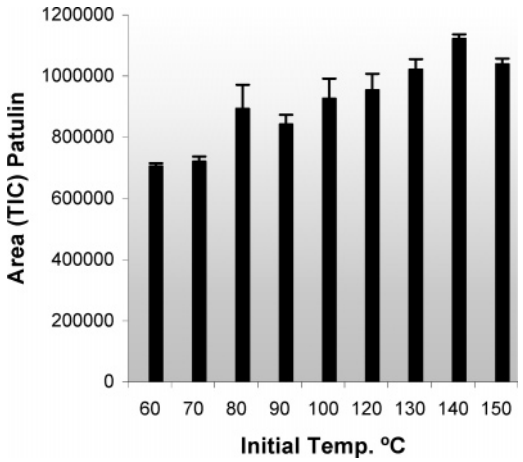

Figure 2. Initial oven temperature optimization for "on column" injection. Each height represents the mean area of TIC (The standard deviation is represented by the error bar). A 2.5 ng amount of patulin was injected ($n = 3$) at different temperatures with an initial ramped pressure on injection, 200 kPa at 0 min, ramped at 650 kPa/min to 350 kPa and hold 0.3 min with a HP5-MS column.

When standards and spiked samples were analyzed, matrix interference was observed with respect to the target ion (*m*/*z* 110) for patulin (**Figure 3A**). As a result of that matrix interference, spiked samples did not meet the ratio limits stated on the Experimental Section, especially at low levels of patulin. **Figure 4A** evidences that the relative abundance of *m*/*z* 110 is higher than the expected when the other patulin ions are

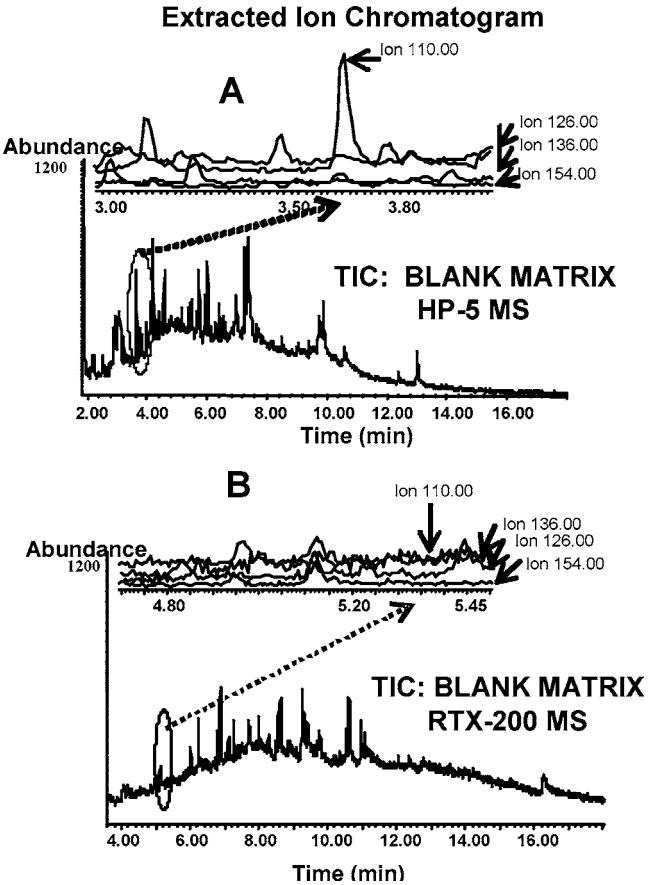

Figure 3. TICs and the extracted ions chromatograms of a blank sample matrix analyzed in a (**A**) HP-5 MS and a (**B**) RTX-200 MS chromatographic column. A zoom of where patulin is expected is presented. The arrow linked to ion *m*/*z* 110 indicates patulin retention time.

considered. The resulting calibration curves consequently exhibited considerable values for the intercept (data not shown). This problem was overcome by using the *m*/*z* 126 fragment as a target ion to quantify patulin. Linearities were acceptable ($r^2 > 0.99$) in both cases. By using the ion 126 to quantify patulin, a reduction in the slope value in the calibration line was observed. It was a consequence of the minor relative abundance of that ion in the patulin fragmentation pattern. The ratio calculated with respect to 110 was *m*/*z* 126/110 = 0.55 ± 0.05. The calibration range was established from 10 to 50 μg/L. Linearity was validated over the whole range. For each IS assayed, a correlation coefficient (r^2) greater than 0.99 was obtained (**Table 3**). Under such conditions, the best calibration parameters were obtained when using IS-4 as the IS. In such cases, the estimated standard deviations calculated for the slope, intercept, and regression were smaller than the respective values obtained for the other IS tested.

The precision of the replications determined at each spiked level did not exceed 15% of the relative standard deviation (RSD %). Results were shown in **Table 7**. The results obtained confirmed the low standard error (S_x) calculated for predicted values (**Table 4**). Of the four compounds tested, (5-oxo-2,5-dihydrofuran-3-yl)methyl chloroacetate (IS-4) showed the smallest standard error in the prediction of the concentration of patulin in spiked samples. This compound was therefore shown to be the best IS for determining patulin residues in samples with a 5% phenylmethylpolysiloxane stationary phase.

A chromatographic column with trifluoropropylmethylpolysiloxane as stationary phase was studied in order to assess whether matrix interference at *m*/*z* 110 could be prevented by using a more selective stationary phase. The uses of this phase for patulin GC-MS analysis had previously been proposed by Roach et al. (*11*). When this column was used, no matrix interference was observed in the *m*/*z* 110 quantification ion for

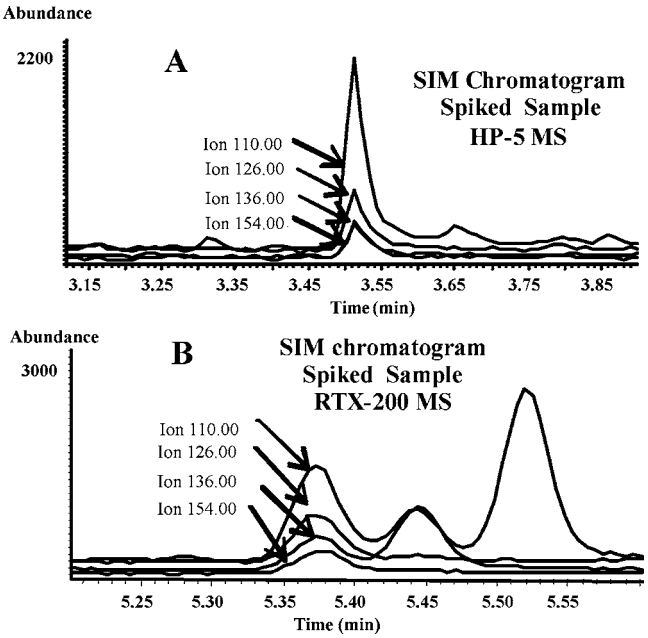

Figure 4. SIM chromatograms corresponding to a spiked sample with 6 μg/L of patulin analyzed in (**A**) a HP-5 MS and (**B**) a RTX-200 MS chromatographic columns, respectively. The abundance of each single ion chromatogram is showed. A zoom of where patulin is expected is presented. The arrow linked to ion *m*/*z* 110 indicates patulin retention time.

Table 3. Regression Parameters for the Different Curves Obtained Using *m*/*z* 126 to Quantify Patulin and the Four ISs Tested in a 5% Diphenylmethylpolysiloxane Stationary Phase[a]

	regression parameters/HP-5 MS Y = [area *m*/*z* 126/area IS]			
	IS-1	IS-2	IS-3	IS-4
slope (b)	0.02	0.03	0.02	0.01
standard deviation of the slope (S_b)	0.0005	0.001	0.001	0.0003
intercept (a)	–0.03	–0.03	–0.04	–0.02
standard deviation of the intercept (S_a)	0.01	0.02	0.02	0.01
residual standard deviation ($S_{y,x}$)	0.02	0.03	0.03	0.01
correlation coefficient (r^2)	0.998	0.996	0.996	0.998
calibration range (μg/L)	10.0–50.0	10.0–50.0	10.0–50.0	10.0–50.0
LOD (μg/L)	3.7	3.8	4.1	2.5

[a] N = 5; each calibration point is the average of two series of standards.

Table 4. Predicted Concentration in Spiked Samples Deduced from Calibration Curves Described in **Table 3**[a]

	HP-5 MS	*m*/*z* 126		
spiked concn (μg/L)	predicted concn (μg/L)			
	IS-1	IS-2	IS-3	IS-4
9.9	12.9 ± 0.7	10.3 ± 0.9	11.4 ± 0.9	10.7 ± 0.5
29.8	38.2 ± 0.7	37.3 ± 0.9	31.2 ± 0.9	29.8 ± 0.5
49.8	60.8 ± 0.7	60.0 ± 0.9	53.1 ± 0.9	50.4 ± 0.5

[a] Each value is the mean of $n = 3 \pm S_x$, the standard error for predicted concentration.

Table 5. Regression Parameters for the Different Curves Obtained Using *m*/*z* 110 to Quantify Patulin and the Four ISs Tested in a Trifluoropropylmethylpolisiloxane Stationary Phase[a]

	regression parameters Rtx-200 MS Y = [area *m*/*z* 110/area IS]			
	IS-1	IS-2	IS-3	IS-4
slope (*b*)	0.07	0.06	0.05	0.04
standard deviation of the slope (S_b)	0.001	0.002	0.002	0.003
intercept (*a*)	0.01	0.05	0.01	-0.13
standard deviation of the intercept (S_a)	0.02	0.05	0.03	0.05
residual standard deviation ($S_{y,x}$)	0.02	0.05	0.03	0.05
correlation coefficient (r^2)	0.999	0.996	0.997	0.985
calibration range (μg/L)	6.0–30.0	6.0–30.0	6.0–30.0	6.0–30.0
LOD (μg/L)	1.0	2.2	1.9	4.5

[a] $N = 5$; each calibration point is the average of two series of standards.

Table 6. Predicted Concentration in Spiked Samples Deduced from Calibration Curves Described in **Table 5**[a]

	Rtx-200 MS *m*/*z*110 predicted concn (μg/L)			
spiked concn (μg/L)	IS-1	IS-2	IS-3	IS-4
6.0	6.9 ± 0.2	5.6 ± 0.5	5.3 ± 0.4	8.2 ± 1.0
10.0	9.8 ± 0.2	9.1 ± 0.5	7.2 ± 0.4	9.8 ± 1.0
30.0	28.1 ± 0.2	26.4 ± 0.5	21.8 ± 0.4	21.3 ± 1.0

[a] Each value is the mean of $n = 3 \pm S_x$, the standard error for predicted concentration.

Table 7. Precision of Replications Determined at Each Spiked Level in a HP-5 MS Using the Ion 126 to Quantify Patulin, Indicated by the RSD %

	HP-5 MS *m*/*z* 126 RSD % ($n = 3$)			
spiked concn (μg/L)	IS-1	IS-2	IS-3	IS-4
9.9	8.76	4.30	10.5	5.90
29.8	7.66	2.34	4.50	3.72
49.8	1.62	1.78	7.06	3.61

patulin (**Figure 3B**). This apparently indicates that the specificity of the method could be improved by changing the stationary phase. In that case, all spiked samples meet the criteria stated on the Experimental Section for patulin identification. **Figure 4B** represents a visual confirmation of the ratio agreement.

Table 5 shows the regression parameters for each of the putative ISs assayed when a trifluoropropylmethylpolysiloxane stationary phase was used. IS-1 displayed the best parameters in this case. A correlation coefficient of 0.999 was obtained, and this IS was also associated with the lowest standard errors when comparing slope, intercept, and regression calculations with other ISs. The highest level of accuracy was also achieved in the prediction of the patulin content of spiked samples, with a prediction error of $S_x = \pm 0.2$ (**Table 6**). **Table 8** summarizes the results of the precision of the replications determined at each spiked level calculated when the Rtx-200 MS chromatographic column was used. In all cases, the RSD % did not exceed 15%.

The lower calibration point achieved 6 μg/L was slightly better than the limit of quantification of 10 μg/L described by Sheu et al. (*7*). The authors proposed a GC-MS method that involves one derivatization step and a diphasic dialysis extraction prior to mass spectrometric determination and suggested using nitrobenzene as the IS. A derivatization step was also proposed by Rychlik et al. (*21*) that reported a very low LOD, 12 ng/L, using an isotopomer of patulin as the IS. Although this detection limit is much lower than our own—1 μg/L using a trifluoropropylmethylpolysiloxane column—it was achieved using a HRGC/HRMS system, involving 100 mL of sample and a more extensive clean-up procedure. Consequently, their procedure would be difficult to use for a routine quantification analysis of patulin in apple juice. Finally, although Roach et al. (*11*) used on-column injection mode of underivatized patulin by GC-MS-SIM, this was only done for the purpose of confirmation, as quantification was carried out by LC-UV.

Table 8. Precision of Replications Determined at Each Spiked Level in a RTX-200 MS Using the Ion 110 to Quantify Patulin, Indicated by the RSD %

	Rtx-200 MS *m*/*z* 110 RSD % ($n = 3$)			
spiked concn (μg/L)	IS-1	IS-2	IS-3	IS-4
6.0	9.50	13.4	9.49	5.01
10.0	8.88	5.51	13.1	10.4
30.0	1.07	7.72	7.98	10.3

In conclusion, the proposed analytical strategy combines the extractive step of an AOAC official method and a GC-MS determination. No extra cleanup is applied, and no derivatization step is required prior to GC determination, so errors due to incomplete reactions can be prevented. It shows a good level of calibration agreement; slope and intercept values calculated with data obtained from the matrix matched calibration standards and spiked samples. Following this approach, two ISs, IS-4 and IS-1, can be proposed for quantifying the presence of patulin in apple juice by GC-MS-SIM. (5-Oxo-2,5-dihydrofuran-3-yl)-methyl acetate (IS-1) is preferable when trifluoropropylphenyl polysiloxane, a more selective GC stationary phase, is used. The use of IS-1 as an IS represents a new alternative for the quantification of trace levels of patulin in apple juice by GC-MS-SIM at low levels (6—30 μg/L).

Polymer-assisted reaction can be regarded as a quick and simple strategy to obtain sufficiently pure, structurally closely related compounds. Some of these compounds can be useful ISs and valid alternatives to ILAs. By using structurally closed related compounds as ISs, with extraction and fragmentation properties that are very similar to those of patulin, it is possible to overcome some of the drawbacks associated with the use of IS with properties whose differences are chemically significant.

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Supporting Information Available: Analytical and spectroscopic data for synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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