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A rapid gas chromatographic injection-port derivatization method for the tandem mass spectrometric determination of patulin and 5-hydroxymethylfurfural in fruit juices☆

Alexis Marsol-Vall, Mercè Balcells, Jordi Eras, Ramon Canela-Garayoa*

Departament de Química, Universitat de Lleida-Agrotecnio Center, Avda. Alcalde Rovira Roure, 191, E-25198, Lleida, Spain

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ABSTRACT

A novel method consisting of injection-port derivatization coupled to gas chromatography–tandem mass spectrometry is described. The method allows the rapid assessment of 5-hydroxymethylfurfural (HMF) and patulin content in apple and pear derivatives. The chromatographic separation of the compounds was achieved in a short chromatographic run (12.2 min) suitable for routine controls of these compounds in the fruit juice industry. The optimal conditions for the injection-port derivatization were at 270 °C, 0.5 min purge-off, and a 1:2 sample:derivatization reagent ratio (v/v). These conditions represent an important saving in terms of derivatization reagent consumption and sample preparation time. Quality parameters were assessed for the target compounds, giving LOD of 0.7 and 1.6 μg/kg and LOQ of 2 and 5 μg/kg for patulin and HMF, respectively. These values are below the maximum patulin concentration in food products intended for infants and young children. Repeatability (%RSD n = 5) was below 12% for both compounds. In addition, the method linearity ranged between 25 and 1000 μg/kg and between 5 and 192 μg/kg for HMF and patulin, respectively. Finally, the method was applied to study HMF and patulin content in various fruit juice samples.

1. Introduction

Patulin (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one) is a mycotoxin produced by approximately 60 species of micro-organisms, including *Aspergillus*, *Penicillium*, *Mucor*, and *Fusarium* [1–3]. Some of these micro-organisms are responsible for the rotting of fruits like apples, pears, and cherries. Consequently, the presence of patulin in fruit derivatives is an indicator of the quality of the feedstock used in the manufacturing process [4]. Although the toxicity of patulin has been widely reviewed [5], no general consensus has been reached about its true degree of toxicity. Nevertheless, government agencies in the European Union have regulated the following maximum patulin concentration in food products intended for infants and young children: 50 μg/kg in juices; 25 μg/kg in solid apple products; and 10 μg/kg in apple products [6].

5-Hydroxymethylfurfural (HMF) is one of the main products of the Maillard reaction, which may occur during food processing and storage, particularly at high temperatures in carbohydrate-rich products. Moreover, HMF can also be produced during the acid-catalyzed dehydration of hexoses via 1,2 enolisation [7] or by glucosamine hydrolysis [8]. It is present naturally in products in which water coexists with monosaccharides in acid medium, such as balsamic vinegar and fruit juice [9].

In this context, analyses of patulin and HMF are now routine procedures for some widely consumed agro-food products, especially apple-derived products. These two compounds can be considered markers of the quality of a fruit-derived product [10]. The presence of patulin and HMF has commonly been determined by HPLC, either using a diode array detector (DAD) [4,11–13] or a mass spectrum (MS) detector, the latter allowing a substantial increase in the selectivity of the analytical methods [14–17].

Patulin and HMF have also been determined independently by GC analysis of their trimethylsilyl ether, acetate or chloroacetate derivatives using either GC-FID [18,19] or GC–MS in SIM mode, the latter allowing improved selectivity in complex matrices [20–24]. The derivatization process improves the otherwise low volatility of these two compounds, which have also been analyzed without this derivatization step, although analytical performance diminished

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* Corresponding author.
E-mail address: canela@quimica.udl.cat (R. Canela-Garayoa).

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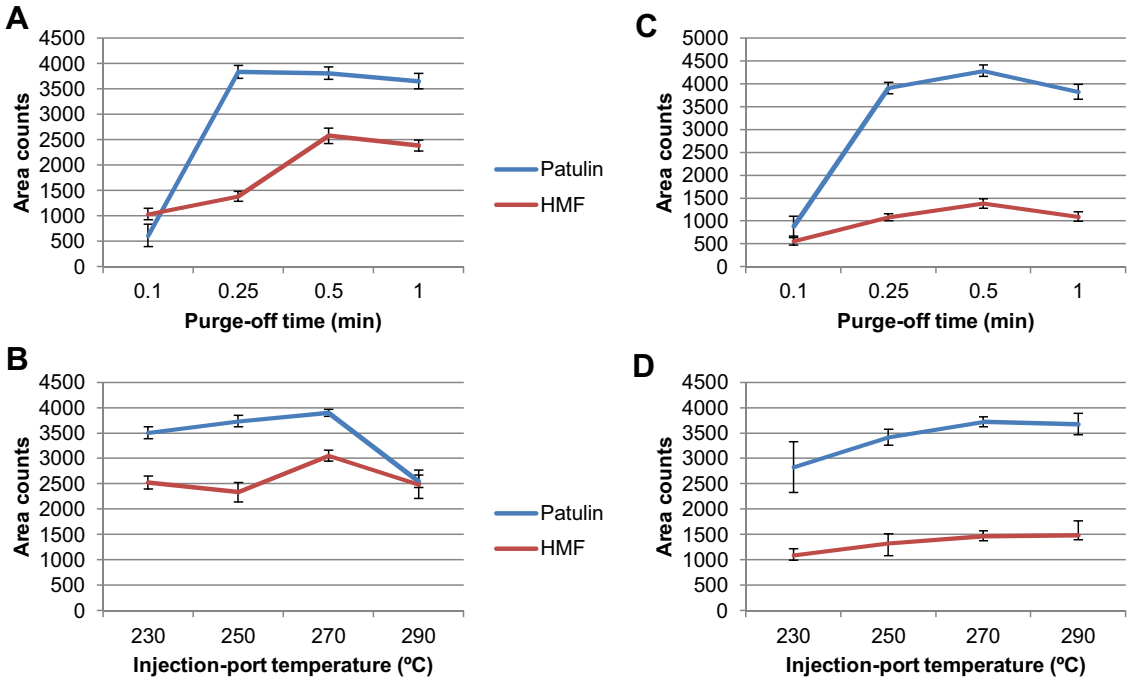

Fig. 1. Optimization of injection-port conditions in cloudy apple juice (A and B) and concentrate apple juice (C and D). Error bars mean standard deviation (n = 3).

[20,25]. To the best of our knowledge, there is only one publication reporting the simultaneous GC analysis of patulin and HMF in apple juice in a qualitative rather than quantitative manner [22].

Derivatization is often performed off-line after the extraction of the sample. Off-line silylation procedures suffer from experimental errors such as loss of analyte through evaporation and re-suspension steps, contamination of samples during work-up, and the interference of moisture in the reaction system, since silylating reagents and the resulting derivatives are extremely sensitive to the presence of water. However, the possibility to perform this derivatization on-line has emerged [26,27]. These approaches allow the reduction of time-consuming sample processing steps, a decrease in the amount of reagents required, and an increase in the speed and efficiency of the analysis. Inlet-based or in-port derivatization is one of these alternative approaches. This on-line process involves introducing the sample and the derivatization reagent directly into the hot GC inlet, where the derivatization reaction takes place in the gas-phase [28]. The sample and the derivatization reagent can be injected separately. This can be achieved by first injecting the sample or the derivatization reagent manually [29], thus making the presence of the analyst inevitable in order to start each analysis. Alternatively, injection of the sample and reagent can be attained simultaneously by using a software-controlled sandwich injection, which fills the syringe with both the sample and the derivatization reagent, allowing an air gap between them. The latter approach is expected to give better results in terms of repeatability and automation of the analytical sequence.

Here we sought to develop a new method for the simultaneous analysis of patulin and HMF in fruit juice. The sample was initially extracted with ethyl acetate because of its accepted efficiency [4]. Secondly, the ethyl acetate solution was analyzed using injection-port derivatization and GC–MS/MS. Derivatization was optimized in terms of purge-off time, temperature, sample: derivatization reagent volume ratio, and MS/MS transitions selected for both compounds. The method circumvents the evaporation of the extract prior to derivatization, thus reducing both analyte degradation and sample preparation time. Furthermore, a cleanup step was discarded because the enhanced sensitivity and selectivity achieved with the triple quadrupole were considered to prevent both the concentration and purification of the sample.

2. Material and methods

2.1. Reagents, solvents, standard solutions and samples

N-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) was purchased from Sigma-Aldrich (Buchs, Switzerland), ethyl acetate (EtOAc) from J.T. Baker (Deventer, The Netherlands), anhydrous sodium sulfate from Acros (Pittsburgh, PA, USA), and sodium chloride from Panreac (Barcelona, Spain).

Stock standard solutions of patulin, 1000 μg/mL, and 5-(hydroxymethyl)-2-furaldehyde (HMF), 100 μg/mL, were prepared from the corresponding solid chemical reagents (Sigma Aldrich). Working solutions of 10 μg/mL and 5 μg/mL were prepared with EtOAc from consecutive dilutions of the stock solutions. All standard solutions were stored at −20 °C and warmed to room temperature before using. In addition, the exact concentration of the patulin standard solution was determined to correct the possible losses of this compound by interaction with the glassware container [4]. A volume of 1 mL of patulin solution was evaporated to dryness under a stream of N_2. The residue was immediately dissolved in 20 mL of ethanol. The absorption spectrum was recorded between λ = 250 nm and λ = 350 nm in a 1-cm quartz glass cell in a spectrophotometer with ethanol in the reference path. The concentration of patulin was calculated using the following equation [30]:

$$p_m = \lambda_{max} \times m \times 100/\varepsilon \times \delta$$

where p_m is the mass concentration of patulin in μg/mL; λ_{max} is the absorbance of patulin solution determined at λ = 276 nm; m is the molecular mass of patulin (m = 154 g/mol); ε (1460 m^2/mol in ethanol) is the molar absorptivity of a patulin solution at λ = 276 nm; and δ is the path length of the quartz cell in cm (δ = 1 cm).

Apple and pear juice concentrate samples from different production units were supplied by Zucasa (Fraga, Spain). Additional

Table 1
Retention time, ions monitored in SIM mode, and selected transitions in the MS/MS method for the target compounds.

Compound		GC–MS(SIM)	GC–MS/MS		
	R.T. (min)	Monitored ions (m/z)[a]	Parent ions (m/z)[a]	Daughter ions (m/z)[a]	C.E. (eV)[a]
HMF	5.7	183, 128[b], 109, 97[b]	183/109	111/81	20/10
Patulin	6.9	226, 183, 154[b], 136[b]	183/226	152/170	20/10

[a] Underlined values were used for quantification.
[b] Ions corresponding to the non-derivatized species.

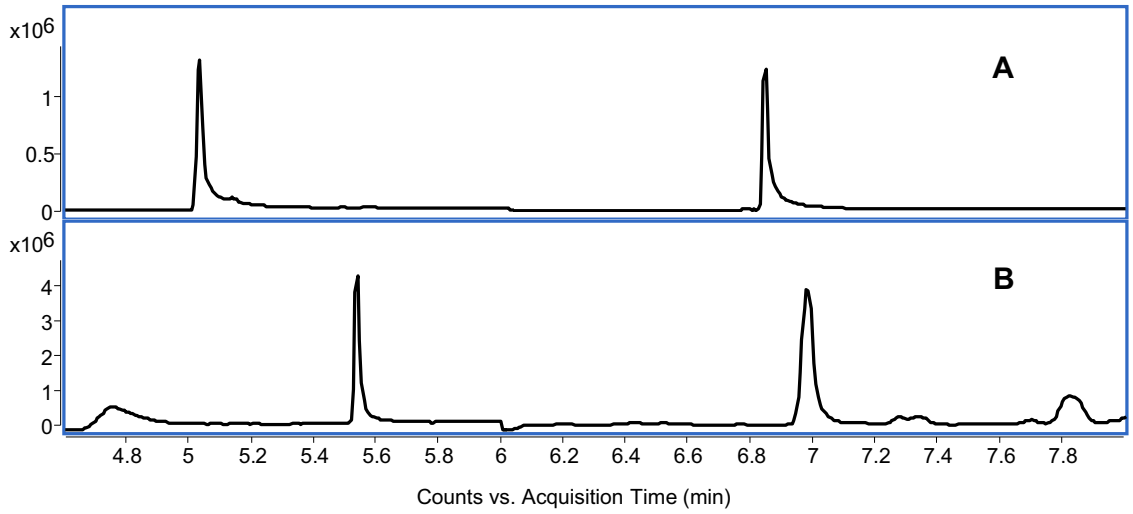

Fig. 2. SIM chromatograms of ethyl acetate extracts spiked at 25 μg/mL for the non-derivatized (A) and 2.5 μg/mL for the derivatized sample (B) (retention times for HMF: 5.05 and 5.55 for the non-derivatized and derivatized compound, respectively. Retention times for patulin: 6.90 and 6.98 for the non-derivatized and derivatized compound, respectively).

samples of commercial cloudy apple and pear juice were purchased from a local supermarket.

2.2. Instrumentation

The GC–MS and GC–MS/MS analyses were performed with an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with a multimode injector and a splitless liner containing a piece of glass wool. A fused silica high-temperature capillary column J&W DB–5MS (30 m × 0.25 mm i.d.; 0.25 μm film thickness) from Agilent was used at constant flow. The detector was an Agilent 7000B triple quadrupole mass spectrometer with inert EI ion source. The mass spectrometer worked in SIM or MRM mode with EI ionization source at 70 eV. Helium with a purity of 99.9999% was used as carrier gas and quenching gas, and nitrogen with a purity of 99.999% as collision gas, both supplied by Air Liquide (Madrid, Spain).

For control purposes and data analysis, the Agilent Mass Hunter B.06.00 software was used.

2.3. Analytical procedure

We placed 5 g of the homogenized sample into a 50-mL centrifugation tube. Subsequently, 10 mL of EtOAc was added. The mixture was vigorously shaken for 1 min by hand. Next, the tube was centrifuged for 5 min at 5000 rpm (Multi Reax; Heidolph, Schwabach, Germany). A volume of 1.5 mL of the upper layer was transferred into a 2-mL Eppendorf vial containing 100 mg of anhydrous sodium sulfate. The vial was manually shaken for 1 min and centrifuged for 3 min at 12,000 rpm (Hettich Eppendorf Centrifuge MIKRO 22 R; Germany). Finally, the organic phase was transferred to a crimp-cap vial for injection into the gas chromatograph.

Sandwich injections of the sample and the derivatization reagent (MSTFA) in various volume ratios were carried out in splitless mode between 230–290 °C using a silanized glass insert containing a piece of glass wool with various purge-off time values. The gas chromatograph temperature was programmed as follows: 70 °C (held for 1 min) to 320 °C at 25 °C/min (held for 2 min) at a constant flow regime of 1 mL/min. In addition, the cap of the vial containing the derivatization reagent was PTFE/Silicone/PTFE which allows repeating injections and replaced every 20 injections to prevent contamination from the septum.

The temperatures of the ion source and the transfer line were 250 °C and 300 °C, respectively. The mass spectra detector operated in selected ion monitoring (SIM) mode, monitoring three ions per compound (Table 1). An MRM method was developed with the same instrument, keeping the temperature of the two quadrupoles at 150 °C. Two transitions were monitored for each analyte, the first for quantification purposes and the second for confirmation. Table 1 shows the mass spectrometer conditions selected. Resolution was adjusted to 1.0 Da for quadrupoles 1 and 3. The solvent delay was 5 min.

3. Results and discussion

3.1. Gas chromatography and MS/MS optimization

The separation of the two analytes of interest was achieved rapidly with a non-polar capillary column. Derivatized HMF appeared earlier in the chromatogram due to its lower boiling point. The molecular ion (m/z 198) was very small, as previously reported for cyclic alcohols [31], and thus its use as parent ion was unfeasible. On the basis of the fragmentations selected, 183 → 111 was used for quantification in HMF, resulting in the parent ion from the loss of methyl radical from the molecular ion $[M-CH_3]^+$. The daughter corresponded to the loss of the ODMS moiety followed by an opening of the furan ring. The transition selected for con-

Table 2
Performance parameters of the GC–MS/MS method in terms of LOD, LOQ, intra- and inter-day repeatability (expressed as relative standard deviation), coefficient of determination (r^2), and recoveries.

			Repeatability				Recoveries		
Compound	LOD (μg/kg)	LOQ (μg/kg)	intra-day (%RSD, n = 5)	inter-day (%RSD, n = 10)	r^2	linear range (μg/kg)	lower level	higher level	Ref.[a]
HMF	1.6	5	2.3–4.6	6.2–8.4	0.991	25–1000	74 ± 7	82 ± 6	[b]
	12	39	2.5	5.0	0.999	25–700	n.e.		[9]
	0.06	n.e.	6	15	0.98	50–10000	91–94		[10]
	6.52	19.75	5.0	8.0	0.992	5–110[c]	77–101	[25]	
					0.998	114–2210[c]			
Patulin	0.7	2	4.6–5.7	8.3–11.7	0.999	5–192	87 ± 7	97 ± 4	[b]
	0.4	2.0	0.6–7.5	n.e.	0.998	5–250	83 ± 8		[12]
	0.5	10	1.9–4.8	5.7–8.1	n.e.	12.5–100	97–102		[17]
	0.4	1.6	10–16	n.e.	0.992	1.6–100	80 ± 13		[24]
	2.1	n.e.	7.5–16.4	n.e.	0.999	2–100	72.7–94.6		[33]
	0.1[c]	0.3[c]	3.3–8.1	n.e.	0.999	10–150[c]	70–82		[34]

n.e.: Parameter not specified in the manuscript.
[a] References of other methods that analyze the same compounds in similar samples.
[b] The performance parameters of the developed method are indicated in this row.
[c] Data are provided in mg/L and linear range was determined in the two intervals showed.

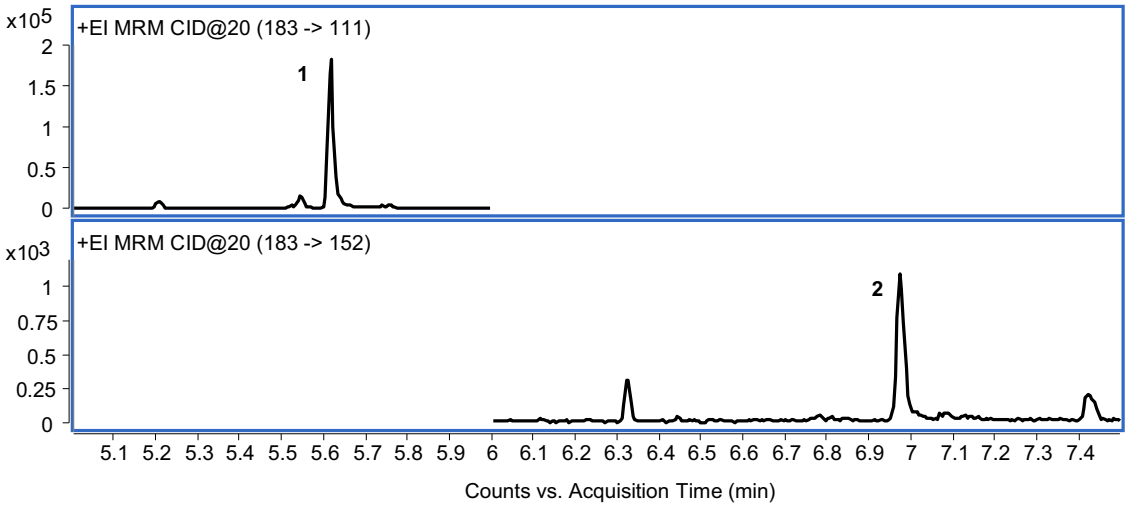

Fig. 3. MRM chromatogram obtained for a derivatized apple juice sample with the quantification transition for: (1) HMF and (2) patulin.

firmation was 109 → 81, corresponding to a loss of OTMS to give the parent ion and a loss of COH to furnish the daughter ion of *m*/*z* 81 $[M-(OTMS+COH)]^+$. Concerning patulin, quantification transition 183 → 152, corresponding to the base peak $[M-(CH_3+CO)]^+$ followed by a loss of OCH_3, was selected. The transition used for confirmation was 226 → 170 from to consecutive losses of CO from the molecular ion (*m*/*z* 226). A reaction scheme of the fragmentations obtained can be found in Supplementary material.

3.2. Extraction

EtOAc is a suitable extraction solvent for patulin and HMF in juice matrices [32]. Although other solvents such as acetonitrile [12,17] and long-chain alcohols [13] could be used, acetonitrile requires a QuEChERS procedure to achieve phase separation. This procedure is followed by a cleanup step due to the presence in the extract of many sugars present in the sample. In addition, acetonitrile is not highly suited for GC because of its high expansion volume in the gas phase, which can increase the risk of exceeding the injection liner capacity since sample is injected together with the derivatization reagent in the injection-port derivatization approach. On the other hand, long-chain alcohols contain hydroxyl groups that would interfere in the injection-port derivatization, which is conducted without solvent evaporation, as previously stated.

3.3. Optimization of injection-port derivatization

Initially, injection mode was studied by using splitless, split, and pulsed pressure split/splitless mode to optimize the derivatization process performed in the injection port. Finally, the splitless mode was selected to perform further optimization of the methodology. Moreover, the injection-port silylation conditions were optimized in terms of time (purge-off), temperature, and sample volume/MSTFA ratio [26,29] for both compounds. Two apples derivatives were used, namely a sample of cloudy juice (Fig. 1a and b) and a sample of concentrate (Fig. 1c and d). Samples had an original HMF concentration and were spiked to obtain a concentration of 50 ng/mL of patulin. Both matrices showed a similar behavior. With respect to purge-off time, a high increase in the area counts was observed from 0.1 to 0.25 min. The area counts values were very similar from 0.25 to 0.5 min and started to decrease at 1 min. On the other hand, peak areas decreased at an injection-port temperature of 290 °C in cloudy juices, although this decrease was found to be less significant for concentrate. The highest area for the two compounds was achieved using a 0.5 min purge-off time

Table 3
Slopes (area counts × mL/ng) achieved with the standard addition calibration curve.

	Standard	Concentrate	Cloudy
HMF	35.7 ± 2.2	37.4 ± 1.3	42.9 ± 3.9
Patulin	55.6 ± 2.6	56.2 ± 4.3	55.6 ± 3.2

and 270 °C injection-port temperature. The sample:derivatization reagent ratio was also optimized by studying ratios of 1:1, 1:2, 2:1 and 1:3 (v/v). The highest area was achieved using a sample: derivatization reagent ratio of 1:2. Fig. 2 shows the chromatograms obtained in SIM mode monitoring the characteristic ions of the non-derivatized (Fig. 2a) and derivatized (Fig. 2b) compounds under study. Results showed complete derivatization of HMF and patulin as no peaks of the non-derivatized species were observed in the chromatogram of the derivatized compounds. Moreover, these figures show the increase on the chromatographic response of both compounds after derivatization.

3.4. Method performance

The performance parameters of the GC–MS/MS method for the optimized conditions described in Section 3.1 were assessed in terms of LOD, LOQ, and intra- and inter-day repeatability (expressed as relative standard deviation), coefficient of determination (r^2), and linear range, as summarized in Table 2. A freshly prepared spiked apple juice not initially containing HMF or patulin was used to determine the LOD and LOQ. These two parameters were calculated as the analyte concentration, giving S/N = 3 and S/N = 10, respectively. The results showed adequate performance in terms of LOD and LOQ, which are particularly critical for patulin in order to assure compliance with the strict regulations of the European Commission (10 μg/kg for food products destined for infants). The performance of the developed method for patulin was in the same range as other GC methods reported for the analysis of fruit juices (LOD = 0.4 and LOQ = 1.6 μg/kg) [24] and HPLC with DAD (LOD = 0.4 and LOQ = 2 μg/kg) [12] or MS (LOD = 0.5 and LOQ = 10 μg/kg) [17]. The analysis of HMF also offered a similar performance when compared with existing methods with LOD = 6 and LOQ = 20 μg/kg [9] and LOD = 6.52 and LOQ = 19.75 μg/kg when using SPME for liquid samples [25]. Regarding repeatability, the values were within those commonly accepted, whereas linearity proved excellent, with $r^2 > 0.991$.

Table 2 also shows the recoveries assessed by spiking a blank sample of freshly prepared apple juice at 5 and 50 μg/kg for patulin and 50 and 500 μg/kg for HMF (%RSD, n = 5 is expressed in brackets). Recoveries of 82 (±7)% and 97 (±4)% for patulin and 76 (±7)% and 83 (±5)% for HMF were achieved. These results are in agreement with others reported in the literature for the liquid-liquid extraction of patulin [33,34] and HMF [10] in fruit juices and prove the suitability of the methodology developed for the simultaneous analysis of patulin and HMF in these matrices.

3.5. Matrix effects

In an in-port derivatization method, matrix effects may occur in the injection liner, where the reaction takes place, due to the presence of co-extractives. For this reason, matrix effects were assessed by comparing the slopes attained with the standard additions method applied to two juice samples (concentrate and cloudy apple juice) and to standard dilutions. Six calibration concentrations were analyzed using the optimized experimental conditions (Table 3). Slopes were not found to be significantly different by a Students *t*-test ($\alpha < 0.05$). Hence, an external calibration curve was further applied to carry out the sample analyses.

Table 4
HMF and patulin concentration nm in fruit juice products.

Sample	HMF (μg/g) Mean ± RSD	Patulin (μg/kg) Mean ± RSD
Apple concentrate 1	2.86 ± 0.24	4.54 ± 0.24
Apple concentrate 2	1.99 ± 0.07	18.91 ± 0.07
Apple concentrate 3	0.57 ± 0.15	<LOQ
Apple concentrate 4	3.05 ± 0.23	n.d.
Pear concentrate1	0.09 ± 0.001	<LOQ
Pear concentrate 2	0.86 ± 0.02	<LOQ
Pear concentrate 3	1.04 ± 0.28	n.d.
Pear concentrate 4	0.64 ± 0.04	n.d
Pear concentrate 5	0.61 ± 0.01	n.d.
Cloudy apple juice 1	0.48 ± 0.05	n.d
Cloudy apple juice 2	0.36 ± 0.02	n.d.
Cloudy pear juice 1	0.14 ± 0.01	n.d.
Cloudy pear juice 2	0.20 ± 0.01	n.d.

n.d.: not detected;<LOQ: detected but with a S/N < 10.

3.6. Application to the analysis of real samples

The method described herein was applied to the analysis of samples of commercial fruit juice, including several samples of juice concentrate. These concentrate initially ranged from 66 to 73 Brix and were diluted to 12 Brix prior to the analysis. HMF was present in all samples, with a concentration ranging from 0.14 to 3.05 μg/g (Table 4). In contrast, only two samples of concentrate quantifiable values of patulin, registering 4.54 and 18.91 μg/kg, respectively. The concentration of patulin in concentrate 2 was above the maximum content allowed for infant consumption (10 μg/kg). In addition, patulin was detected in two pear samples but in these cases it was below the LOQ. Finally, Fig. 3 shows an MRM chromatogram resulting from the application of the described methodology to a juice sample.

4. Conclusions

The proposed analytical method, consisting of an injection-port derivatization coupled to gas chromatography–tandem mass spectrometry, offers a novel approach for the simultaneous analysis of two key compounds, HMF and patulin, in the fruit juice industry. Samples can be analyzed avoiding concentration and purification processes. The chromatographic separation of the two compounds was achieved in a short chromatographic run (12.2 min) suitable for routine controls of these compounds in the fruit juice industry. The optimal conditions for the injection-port derivatization represent an important saving in terms of derivatization reagent consumption and sample preparation time. With the proposed method LOD and LOQ are below the maximum patulin concentration permitted for food products for infants and young children, thus this methodology is compliant with current legal standards. Finally to test the usefulness of the method, it was applied to various apple and pear juice samples from different origin. All of them contained HMF. In contrast, patulin was detected only in a few samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.05.043.

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