DETERMINATION OF PATULIN IN APPLE AND DERIVED PRODUCTS BY UHPLC-MS/MS. STUDY OF MATRIX EFFECTS WITH ATMOSPHERIC PRESSURE IONIZATION SOURCES

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ABSTRACT

Sensitive and reliable analytical methodology has been developed for patulin in regulated foodstuffs by using ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) with triple quadrupole analyzer. Solid samples were extracted with ethyl acetate, while liquid samples were directly injected into the chromatographic system after dilution and filtration without any clean-up step. Chromatographic separation was achieved in less than 4 min. Two atmospheric pressure ionization sources (electrospray (ESI) and atmospheric pressure chemical ionization (APCI)) were evaluated in order to assess matrix effects. The use of ESI source led strong signal suppression in samples; however, matrix effect was negligible using APCI, allowing to perform quantification with standards calibration prepared in solvent. The method was validated in four different apple matrices (juice, fruit, puree and compote) at two concentrations at the low $\mu g \cdot Kg^{-1}$ level. Average recoveries (n=5) ranged from 71 to 108%, with RSDs lower than 14 %. The optimized methodology has been applied to the analysis of apple and derived products of local markets. Confirmation of patulin in samples was made by intensity ion ratios and retention time agreements with reference standards.

Keywords: Mycotoxins, Patulin, ESI, APCI, UHPLC, tandem MS

1. INTRODUCTION

Patulin is a toxic secondary metabolite produced by a wide range of fungal species of the genera *Penicillium, Aspergillius* and *Byssochlamys*. Among the different genera, the most important patulin producer is *Penicillium expansum* (Moake, Padilla-Zakour & Worobo, 2005). Patulin has been found as a contaminant in many mouldy fruits, vegetables, cereals and other foods, however, the major sources of contamination are apples and apple products, which also are the most important source of patulin in the human diet (Reddy, Salleh, Saad, Abbas, Abel & Shier, 2010, Baert et al., 2007, Murillo-Arbizu, Amézqueta, González-Peñas & de Cerain, 2009).

The International Agency for Research on Cancer (IARC) has classified patulin as not carcinogenic (group 3), although it has been demonstrated it causes neurotoxic and mutagenic effects in animals (IARC, 2002). In 1995, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) recommended a provisional tolerable daily intake (pTDI) of 0.4 μg patulin/kg body weight-day based on long-term exposure (JECFA, 1995). As a result, the levels of patulin in fruits are subjected to legislative control. The Codex Alimentarius recommends levels of patulin in fruits and fruit juices to be lower than 0.05 $mg \cdot Kg^{-1}$. In 2006, European Commission established the following maximum levels of patulin in apple products: 0.05 $mg \cdot Kg^{-1}$ for fruit juices and other drinks derived from apple or apple juice; 0.025 $mg \cdot Kg^{-1}$ for solid apple products; and, 0.01 $mg \cdot Kg^{-1}$ for apple products intended for infants and young children and baby foods different from cereals-based products (Commission Regulation 1881/2006, 2006).

Considering the maximum levels allowed for patulin, it is necessary to apply sensitive, selective and reliable analytical methodology for its determination. Several strategies have been developed for patulin determination in a wide range of matrices, especially in apple products, based on thin layer chromatography (Martins, Gimeno, Martins & Bernardo, 2002), capillary

electrophoresis (Murillo-Arbizu, González-Peñas, Hansen, Amézqueta & Østergaard, 2008), gas chromatography (GC) with derivatization (Cunha, Faria & Fernandes, 2009) and liquid chromatography (LC), mainly using UV detection (Fernández-Cruz, Mansilla & Tadeo, 2010, González-Osnaya, Soriano, Moltó & Mañes, 2007); the latter has been adopted as AOAC official method for patulin in apple juice (AOAC International, 2000). The main disadvantage of this approach is the lack of selectivity, being 5-hydroxymethylfurfural and phenolic compounds typical interferences from apple-based products (Desmarchelier, Mujahid, Racault, Perring & Lancova, 2011). Therefore, additional sample treatments, such as SPE or matrix solid-phase dispersion are required to remove sample interferences (Li, Wu, Hu & Wang, 2007, Wu, Dang, Niu & Hu, 2008).

More recently, LC coupled to tandem mass spectrometry (MS/MS) has rapidly growth as one of the most advanced techniques in mycotoxins determination (Malik, Blasco, & Picó, 2010, Cappriotti et al., 2012,). Although a notable number of multi-methods have been reported, patulin has not been usually included in the analyte target list due to its high polarity and low molecular mass, which commonly lead to low recoveries and/or low sensitivity, hampering its determination at the regulatory levels (Beltrán, Ibáñez, Sancho & Hernández, 2009, Sulyok, Berthiller, Krska & Schuhmacher, 2006). For these reasons, specific LC-MS/MS methodologies need to be developed for its analysis, especially in regulated matrices.

Making use of LC-MS/MS methods, different atmospheric pressure ionization (API) sources have been applied, usually in negative ionization mode, for patulin determination: electrospray (ESI) (Desmarchelier, Mujahid, Racault, Perring & Lancova, 2011, Ito, Yamazaki, Inoue, Yoshimura, Kawaguchi & Nakazawa, 2004, Kataoka, Itano, Ishizaki & Saito, 2009), atmospheric pressure chemical ionization (APCI) (Sewram, Nair, Nieuwoudt, Leggott & Shephard, 2000) and atmospheric pressure photoionization (APPI) (Takino, Daishima & Nakahara, 2003, Zöllner & Mayer-Helm, 2006) . Although LC-MS/MS has clear advantages for

mycotoxin analysis derived from its excellent sensitivity and selectivity, API sources (especially ESI) are prone to suffer ionization problems due to the presence of matrix compounds that compete with the analyte in the ionization process. This matrix effect normally leads to signal suppression, although signal enhancement may also occur in some occasions. Matrix effects notably affect quantitative analysis being troublesome an accurate quantification unless they are reduced or corrected. The use of isotopically labeled reference standard or extensive sample treatment have been used to deal with matrix effects in patulin determination (Desmarchelier, Mujahid, Racault, Perring & Lancova, 2011, Ito, Yamazaki, Inoue, Yoshimura, Kawaguchi & Nakazawa, 2004, Kataoka, Itano, Ishizaki & Saito, 2009) .

The aim of this paper is to develop a rapid and sensitive analytical methodology for patulin determination in regulated foodstuffs by using ultra-high-performance liquid chromatography coupled to MS/MS. The signal suppression caused by co-eluted matrix interfences in ESI and APCI has been evaluated and sample treatment has been minimized avoiding any clean-up step. The method has been validated in four different apple matrices (juice, fruit, puree and compote) at concentration levels of few $\mu g \cdot Kg^{-1}$. Unambiguous identification has been assured by the acquisition of three selected reaction monitoring (SRM) transitions and evaluation of their Q/q ratios.

2.EXPERIMENTAL SECTION

2.1 Reagents and chemical

Patulin pure standard (>98%) was purchased from Sigma-Aldrich Spain (Madrid, Spain). HPLC-grade water was obtained from water passed through a Milli-Q water purification system (Millipore LTD, Bedford, MA, USA). Supragradient HPLC grade acetonitrile (ACN), supragradient HPLC grade methanol (MeOH), residue analysis grade acetone, ammonium acetate (NH_4Ac), triethyl amine (Et_3N), residue analysis methyl tert-butyl ether (MTBE), ultra trace analysis grade ethyl acetate (AcOEt), residue analysis grade anhydrous sodium sulfate anhydrous (Na_2SO_4), and LC-MS grade formic acid (HCOOH) were obtained from Scharlau (Barcelona, Spain). Anhydrous sodium sulphate was dried 18 h at 300ºC before its use.

2.2 Apparatus

A mechanical shaker (S.B.S. Instruments S.A, Barcelona, Spain) was used for extraction of the samples. Nylon filters (Iso-disc, Supelco, 0.22 µm) were used for filtering the final extract before injection.

2.3 UHPLC-MS/MS

An UPLC^{TM} system (Acquity, Waters, Milford, MA, USA) was interfaced to a triple quadrupole mass spectrometer (TQD, Waters Micromass, Manchester, UK). LC separation was performed with an Acquity UPLC BEH C18 analytical column (50x2.1mm, 1.7 µm) (Waters). The mobile phases employed consisted on water (A) and acetonitrile (B) without modifiers, at a flow rate of 0.3 $mL \cdot min^{-1}$. The gradient program started with 2% B for 1 min. Afterwards, the lineal gradient was programmed up to 90% of B for 2 min. and maintained during 1 min. Finally

the gradient was held to initial conditions (2% of B) in order to re-equilibrate the column. Temperature column was set to 40ºC. 100 µL were selected as injection volume.

APCI-MS and ESI-MS experiments were performed on a TQD triple quadrupole mass spectrometer (Waters Micromass, Manchester, UK). The MS parameters were optimized by direct infusion of 1 $mg \cdot L^{-1}$ in methanol:water (1:1) at a flow rate of 20 $\mu L \cdot min^{-1}$. Source temperature was set to 120 ºC. Drying as well as nebulising gas was nitrogen (Praxair, Valencia, Spain). The gas flow was set to 800 $L \cdot h^{-1}$ and the cone gas to 60 $L \cdot h^{-1}$. For operating in MS/MS mode, collision gas was argon (99.995%; Praxair, Valencia, Spain) with a pressure of approximately 4.10^{-3} mbar in the collision cell (0.15 mL min^{-1}). For APCI mode the corona discharge needle voltage, probe temperature and corona discharge current were set at 2.5 kV, 600ºC and 20 µA, respectively. For ESI mode, capillary voltage and desolvation gas temperature were set at 3.0 kV and 500ºC respectively. TargetLynx (MassLynx v. 4.1, Waters, Manchester, UK) software was used to process the quantitative data obtained from calibration standards and samples.

2.4 Sample preparation

2.4.1 Solid Samples

For solid samples (fruit, puree and compote), a 5 g portion was weighed into a 50 mL tube. After adding 20 mL of ethyl acetate samples were shaked during 30 minutes in a mechanical automated shaker. Samples were filtered through a paper filter with Na_2SO_4 anhydrous in order to remove water. The extract was collected in a volumetric flask and the final volume was adjusted to 25 mL with ethyl acetate. A 10 mL aliquot was evaporated to dryness at 40ºC under gentle N_2 stream and reconstituted with 1 mL of 0.01% HCOOH aqueous solution. Finally, the extract was filtered through a 0.2 µm nylon filter before being injected into the chromatographic system.

2.4.2 Liquid Samples

5 mL of liquid samples (juices) were 4-fold diluted with water. After being filtered through a 0.2 µm nylon syringe filter, the samples were directly injected into the LC-MS/MS system.

2.5 Validation study

Validation of the method was performed by evaluating the following parameters:

-Linearity: The calibration curves were obtained by injecting seven reference standard solutions in duplicate (2.5-250 $\mu g \cdot L^{-1}$). Linearity was assumed when the regression coefficient was greater than 0.995 with residuals lower than 20 %.

-Accuracy and precision: Accuracy was evaluated by means of recovery experiments, analyzing three different solid matrices (fruit, puree and compote) at two different concentration levels (0.01 and 0.05 $mg \cdot Kg^{-1}$). Regarding liquid matrices, apple juice was validated at three different concentration levels (0.05, 0.1 and 0.5 $mg \cdot L^{-1}$). In all cases, recovery experiments were performed by quintuplicate. Precision, expressed as the repeatability of the method, was determined in terms of relative standard deviation (RSD) from recovery experiments at each fortification level (n=5, each). Quantitation was performed by means of external calibration curves. Acceptable recoveries (between 70-120 %) and RSDs (below 20 %) were established according to Document SANCO/12495/2011 (Document SANCO, 2011).

-Limit of quantification. LOQ was estimated for a signal-to-noise ratio of 10 from the chromatogram of samples spiked at the lowest fortification level tested.

-Limit of detection. The LOD was estimated, from the quantification transition, as the analyte concentration that produced a peak signal 3 times the background noise from the chromatogram at the lowest fortification tested.

3.RESULTS AND DISCUSSION

3.1 MS/MS optimization

Full scan and MS/MS spectra of patulin were obtained from infusion of 1 $mg \cdot L^{-1}$ methanol:water (50:50) solution at a flow rate of 20 $\mu L \cdot min^{-1}$. Experiments were carried out in both positive and negative ionisation modes. In this work two different atmospheric pressure ionization (API) interfaces have been used to compare its suitability for the determination of patulin in different matrices.

When checking electrospay ionization (ESI) in full scan mode, no signal was obtained for positive ionization mode. However, negative ionization mode presented an abundant $[M-H]^-$ for patulin at *m/z* 153 **(Figure 1a)**. MS/MS spectra of the precursor ion $[M-H]^-$ were acquired in product ion scan mode at different collision energies to obtain optimum product ions. As it can be seen in **Figure 1 (b)**, three different product ions were selected for patulin determination. The most abundant ion at *m/z* 109, optimized at 10 eV collision energy and corresponding to $[M-CO_2]^-$ was chosen for quantification purposes. Two less abundant ions, at m/z 81.0 and 53.0 respectively corresponding to $[C_5H_5O]^-$ and $[C_4H_5]^-$, were selected for confirmation purposes (q_i) **(Table 1)**.

In the same way, when atmospheric pressure chemical ionization (APCI) source was tested, the scan spectrum only showed signal in negative ionization mode. Under this ionization mode, apart from the deprotonated molecule $[M-H]^-$ (*m/z* 153), it was also observed the molecule radical ion $[M^{\cdot}]^-$ at *m/z* 154 and its in-source fragment corresponding to the loss of water at m/z 136 **(Figure 1c)**. This is in accordance with literature (Takino, Daishima & Nakahara, 2003), which reports that ionization of patulin by APCI in the negative ion mode simultaneously

proceeds via electron capture and/or charge exchange to produce $[M^{\cdot}]^{-}$ and proton transfer to produce $[M-H]^{-}$.

Fragmentation of the molecule radical ion $[M^{\cdot}]^{-\cdot}$(*m/z* 154) and of the deprotonated molecule $[M-H]^{-}$ was studied under product ion scan mode at different collision energies. As it can be seen in **Figure 1d**, the same fragmentation than obtained in ESI ionization was observed for $[M-H]^{-}$. However, the molecule radical ion (*m/z* 154) followed a different fragmentation pathway **(Figure 1e)**. The most abundant product ions observed were at *m/z* 124 (10eV collision energy) corresponding to $[M^{\cdot}-H_2CO]^{-}$, and at *m/z* 123 (20 eV collision energy) corresponding to $[M^{\cdot}-H_3CO]^{-}$. An additional product ion at *m/z* 64 $[C_5H_4^{\cdot}]^{-}$ was obtained product of the fragmentation of the in-source fragment (*m/z* 136) **(Figure 1f)**.

3.2 LC optimization

An Acquity UPLC BEH C18 analytical column (50x2.1mm, 1.7 µm) was selected in this work to perform the analysis of patulin. ESI and APCI experiments were performed at 0.3 $mL \cdot min^{-1}$. When working in APCI source it is common the selection of higher flow rates due to ionization in APCI ion source is mass dependent. For this reason, additional experiments were carried out at higher flow rates using an Atlantis C18 (4.6x100 mm, 5 µm particle size) column (Waters) which allows working at higher flows due to its higher particle size. Furthermore, its stationary phase is designed to enhance the retention of polar compounds when compared with traditional reversed phase columns. Results obtained with the Atlantis column (5 µm particle size) working at 0.8 $mL \cdot min^{-1}$ showed a similar behavior in terms of sensitivity than experiments carried out with the Acquity column (1.7 µm particle size) working at 0.3 $mL \cdot min^{-1}$. Finally, it was selected the Acquity column due to the lower time and solvents used per chromatographic run compared with the analysis performed with higher particle size column.

220 The selection of the mobile phase can be relevant to enhance the detector response. In the
221 present work, water-methanol and water-acetonitrile with and without modifiers (ammonium
222 acetate and triethyl amine) were tested in order to select the most suitable mobile phase, i.e., that
223 leads to the highest sensitivity maintaining a good chromatographic peak shape. The use of
224 ammonium acetate was dismissed as its use deteriorated the peak shape. Triethyl amine was tested
225 in order to maximize the deprotonation of the molecule, although not differences were observed
226 when it was compared with no additives in the mobile phase (Grimalt, Pozo, Marín, Sancho &
227 Hernández, 2005). As it was not observed any improvement in sensitivity when modifiers were
228 added to the mobile phase, experiments were performed with solvents without modifiers. It was
229 preferred the use of acetonitrile in front of methanol as narrower peaks were obtained.

230 Finally, although different SRM transitions could be acquired for patulin, depending on
231 ionization via electron capture or proton transfer, it was preferred the selection of SRM transitions
232 based on ionization via electron capture as they showed higher sensitivity. The most sensitive
233 transition (154>124) was selected for quantification and the next two more sensitive transitions
234 (154>123, and 136>64) were selected for confirmation purposes **(Table 1)**.

235

236 **3.3 Extraction/sample treatment**

237 Liquid samples were injected into the chromatographic system after a 4-fold dilution step
238 with water avoiding tedious sample treatments, such as liquid-liquid extraction or SPE processes.
239 Desired sensitivity was achieved increasing the sample volume injection up to 100 µL. Regarding
240 solid samples, it was necessary to apply an extraction step. Different solvents (ethyl acetate,
241 methyl tert-butyl ether, acetone and acetonitrile) were tested in apple matrix to determine the
242 extraction efficiency. The best recoveries in apple spiked at 0.1 $mg \cdot Kg^{-1}$ for the overall process
243 were obtained with ethyl acetate (94%). Acceptable recoveries were also obtained for methyl tert-

butyl ether (89%), whereas acetone and acetonitrile showed lower recoveries (62% and 69%, respectively) probably due to the high content of co-extracted interferences that could affect the ionization process. On the basis of these results, ethyl acetate was selected as extractant solvent due to the favourable recoveries obtained in apple matrix and its supported use by official methods (AOAC International, 2000). Na_2SO_4 anhydrous was added in the filtration step in order to remove water traces present in the sample that could difficult the evaporation process. Dried extracts were reconstituted with water acidified with 0.01 % HCOOH in order to preserve patulin, as it is known its instability under alkaline conditions (Desmarchelier, Mujahid, Racault, Perring & Lancova, 2011).

3.4 Matrix effect

It is well known that co-eluted matrix components are susceptible to compete in the ionization process, typically producing signal suppression. Matrix effect was investigated in both ESI and APCI interfaces, by comparison of spiked extracts and reference standards in solvent in apple juice (as an example of liquid sample) and fruit (as solid sample). The ratio was expressed in terms of percentage, corresponding to signal decrease values below 100%.

When matrix effects were studied in the ESI ionization source, a strong signal suppression was observed for apple and juice, showing only 16% and 5%, respectively, of the expected signal when compared with that of reference standard . These results showed the need for reducing or compensating matrix effect for an accurate quantification of patulin. Although different approaches could be applied to correct matrix effect, as matrix matched calibration or the use of isotope internal standard, these strategies were not evaluated as the method would not show enough sensitivity to reach required levels in studied matrices.

On the contrary, when APCI ionisation was used, matrix effects were tolerable in this source, showing low signal suppression in apple and juice (92% and 85%, respectively, when

compared with the reference standard). This fact made feasible the quantification of patulin using an external calibration curve, without the need of applying any correction as it would be occur in the ESI source. **Figure 2** shows the LC-MS/MS chromatograms obtained for a reference standard and a spiked apple juice under both ionisation sources. As it can be seen, the sensitivity for the standard in solvent was similar, or even though slightly higher in the case of ESI. However, the strong matrix suppression did not allow to achieve the desired sensitivity in ESI; therefore, APCI was selected for subsequent experiments.

3.5 Method validation

For validation purposes three different matrices (apple fruit, compote and fruit puree) were used as representative solid matrices whilst apple juice was selected as liquid sample. Samples were analyzed before its use as blanks in the validation in order to determine the absence of patulin. The method was found highly specific as no relevant signals were observed in the blanks at the patulin retention time.

Linearity of the method was studied in the range 2.5-250 $\mu g \cdot L^{-1}$, obtaining satisfactory results, with linear correlation coefficients higher than 0.99 and residuals below 20 %.

Accuracy and precision (repeatability, expressed as relative standard deviation (RSD) in %) of the overall analytical procedure were evaluated by spiking blank samples, at least, at two different concentration levels and analyzing them in quintuplicate. For solid samples, validation was carried out at 0.01 and 0.05 $mg \cdot Kg^{-1}$, and for liquid samples at 0.05, 0.1 and 0.5 $mg \cdot L^{-1}$.

The method was found to have satisfactory precision and accuracy, with recoveries between 70-108% and RSD<14% in solid samples. The results obtained in liquid samples were also satisfactory, showing recoveries in the range of 71-75% with RSDs lower than 9%. (Table 2).

292 The lower recoveries (around 75%) in liquid matrices were attributed to slight signal suppression
293 in the ionization process.

294 As it can be seen in **Table 2**, LOQs were fixed in the range of the lowest level validated,
295 whereas LODs were established into the range 0.002-0.003 $mg \cdot Kg^{-1}$ in solid samples and 0.015
296 $mg \cdot L^{-1}$ in liquid samples.

297 **Figure 3** shows the SRM chromatograms for the quantification (Q) and confirmation (q1
298 and q2) transitions for the four matrices studied at the lowest level validated (0.01 $mg \cdot kg^{-1}$ for
299 solid samples and 0.05 $mg \cdot L^{-1}$ in liquid samples)

300

301 **3.6 Confirmation and application to real samples**

302 Following EU guidelines recommendation, in order to assure analyte identification at least
303 two transitions should be acquired (Document SANCO, 2011). In this work, up to three SRM
304 transitions have been used for confirmation purposes at concentration levels as low as the LOQ.
305 The Q/q ratio, defined as the ratio between the signal obtained from the quantification transition
306 (Q) and the signal obtained from the confirmation transitions (q_i), was used to confirm the identity
307 of the peak in the samples.

308 The method was applied to ensure that patulin levels were under the legislation level in
309 nine samples (three apple juice, three apple fruit samples, two compote and one puree) randomly
310 selected from big supermarkets and organic produce retail outlets in Castellon (Spain). Quality
311 control (QC) samples prepared from blank samples spiked at the highest and lowest level
312 validated were included in each sample batch. Satisfactory recoveries (between 70 and 120 %)
313 were obtained for all QCs, ensuring the reliability of the method. In the absence of positive
314 samples, Q/q ratios were calculated in the spiked samples and compared with the Q/q ratios
315 obtained with reference standards in solvent in order to demonstrate the applicability of Q/q ratios

316 criteria to ensure a correct confirmation of the peak identity in matrix. Deviations in the Q/q ratio
317 did not exceed 15% in any of the studied matrices. **Figure 4** shows the SRM chromatograms for
318 all SRM transitions corresponding to a spiked blank apple and spiked blank apple juice samples
319 used as quality control.
320

4. CONCLUSIONS

A sensitive and reliable method has been optimized for the determination of patulin in apple and derived products by using UHPLC-MS/MS system with a QqQ analyzer. Two different atmospheric ionisation sources have been compared in order to choose the optimal source. Although ESI and APCI showed similar behaviour when patulin standards in solvent were tested, differences were magnified in presence of matrix. ESI showed a strong signal suppression that hampered the determination of patulin at the 0.05 $mg \cdot Kg^{-1}$ required by the European Commission (Commission Regulation 1881/2006, 2006). However, ionization in the APCI source was not affected by the presence of matrix interferences, allowing an accurate patulin determination using calibration in solvent. The sensitivity achieved with APCI source allowed the determination of patulin in juices by direct injection. A main advantage of this methodology is the minimisation of sample treatment, avoiding tedious tasks as SPE.

The method applicability to different solid matrices as well as liquid matrices was confirmed by the analysis of different spiked samples in the method validation, with satisfactory results in terms of accuracy and precision. The use of three SRM transitions, one of them for quantification purposes and the other two for confirmation purposes, is an excellent approach that would make feasible a reliable confirmation of patulin in positive samples by means the accomplishment of the Q/q ratios.

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FIGURE CAPTIONS

Figure 1. MS and MS/MS spectra for patulin. (a) ESI MS scan, (b) product ion scan for *m/z* 153. (c) APCI MS scan, (d) product ion scan for *m/z* 153, (e) product ion scan for *m/z* 154, and (f) product ion scan for *m/z* 136.

Figure 2. UHPLC-MS/MS chromatograms for patulin in: (a1) reference standard at 0.1 $mg \cdot L^{-1}$ under ESI ionization and (a2) apple juice spiked at 0.5 $mg \cdot L^{-1}$ (0.1 $mg \cdot L^{-1}$ in extract) under ESI ionization. (b1) reference standard 0.1 $mg \cdot L^{-1}$ under APCI ionization and (b2) apple juice spiked at 0.5 $mg \cdot L^{-1}$ (0.1 $mg \cdot L^{-1}$ in extract) under APCI mode. (Q: Quantification transition; q_i: confirmation transitions)

Figure 3. UHPLC-MS/MS chromatograms for patulin at the lowest level validated in: (a) fruit , (b) puree , (c), compote and (d) juice. (Q: Quantification transition; q_i: confirmation transitions)

Figure 4. UHPLC-MS/MS chromatograms for patulin in: (a) reference standard 0.1 $mg \cdot L^{-1}$, (b) apple juice sample spiked at 0.5 $mg \cdot L^{-1}$ (0.1 $mg \cdot L^{-1}$ in extract), and , (c) apple sample spiked at 0.05 $mg \cdot Kg^{-1}$ (0.1 $mg \cdot L^{-1}$ in extract). (Q: Quantification transition; q_i: confirmation transitions)

449 **TABLES**

450 **Table 1.** Optimized MS/MS parameters for patulin in ESI and APCI ionization modes. (Q:
451 Quantification transition; qi: confirmation transitions)
452
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454
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Ionisation source	Precursor Ion	Cone (V)	Product ion	Collision energy (eV)	Ion ratio
ESI	153.1 $[M-H]^-$	20	109.0 (Q)	10	
			81.0 (q_1)	10	1.3
			53.0 (q_2)	15	4.4
APCI	154.0 $[M^{\cdot}]^-$	20	124.0 (Q)	10	
			123.0 (q_1)	20	5.7
	136.0 $[M-H_2O^{\cdot}]^-$	20	64.0 (q_2)	10	5.3

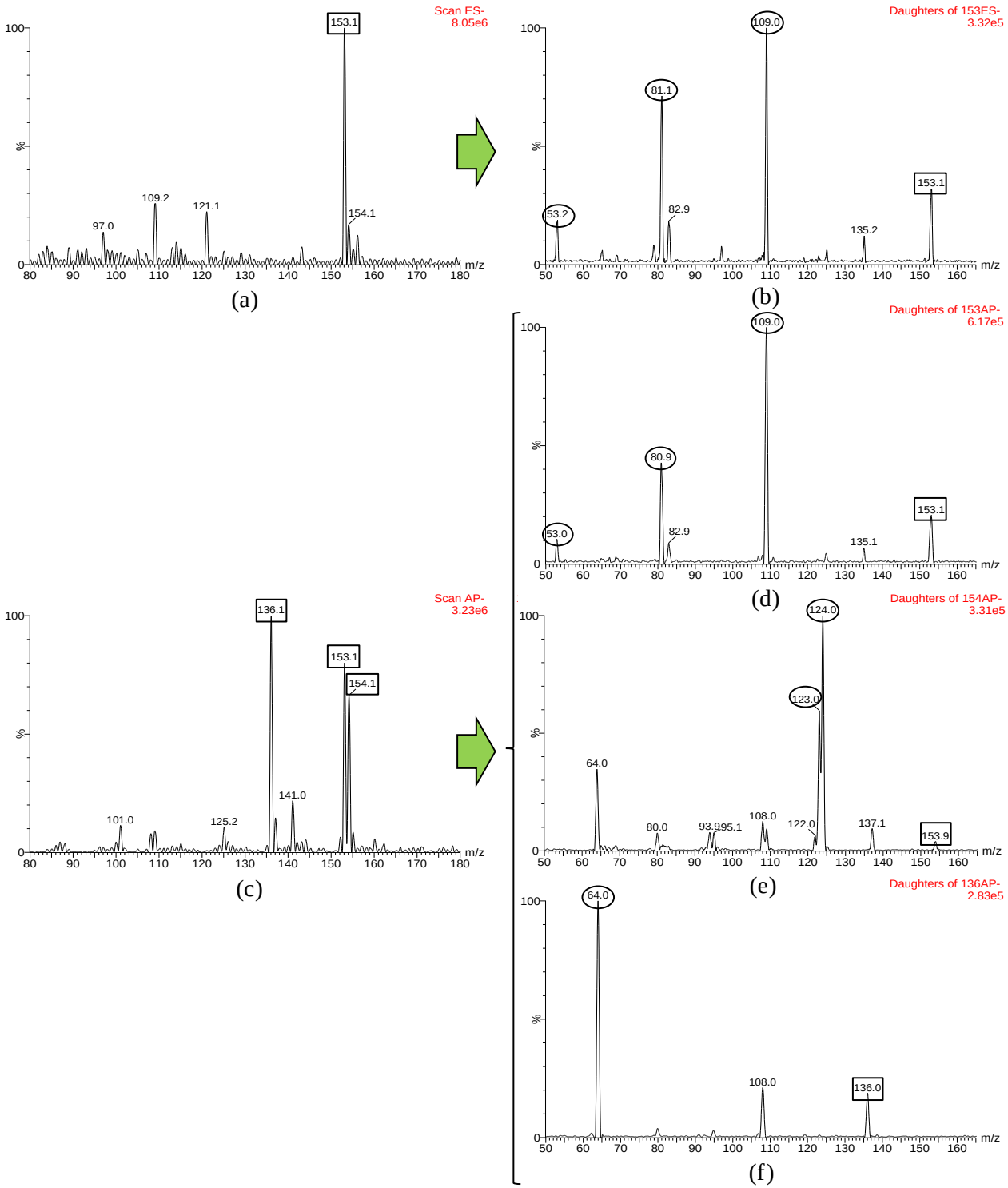
456
457
458

Table 2. Validation of the UHPLC-MS/MS method for patulin determination. Mean recoveries (%) and relative standard deviation (%, in brackets) of the overall procedure (n=5). Estimated limits of detection (LOD).

	Spiked Level		LOQ	LOD
Matrix	0.01 mg·Kg^{-1}	0.05 mg·Kg^{-1}	(mg·Kg^{-1})	(mg·Kg^{-1})
Fruit	70 (11)	89 (6)	0.007	0.002
Compote	98 (13)	93 (14)	0.008	0.003
Puree	108 (10)	79 (14)	0.006	0.002

	Spiked Level			LOQ	LOD
Matrix	0.05·mg·L^{-1}	0.1 mg·L^{-1}	0.5 mg·L^{-1}	(mg·L^{-1})	(mg·L^{-1})
Apple juice	75 (7)	71 (9)	71 (3)	0.047	0.015

485 **FIGURES**



486

487 **Figure 1**

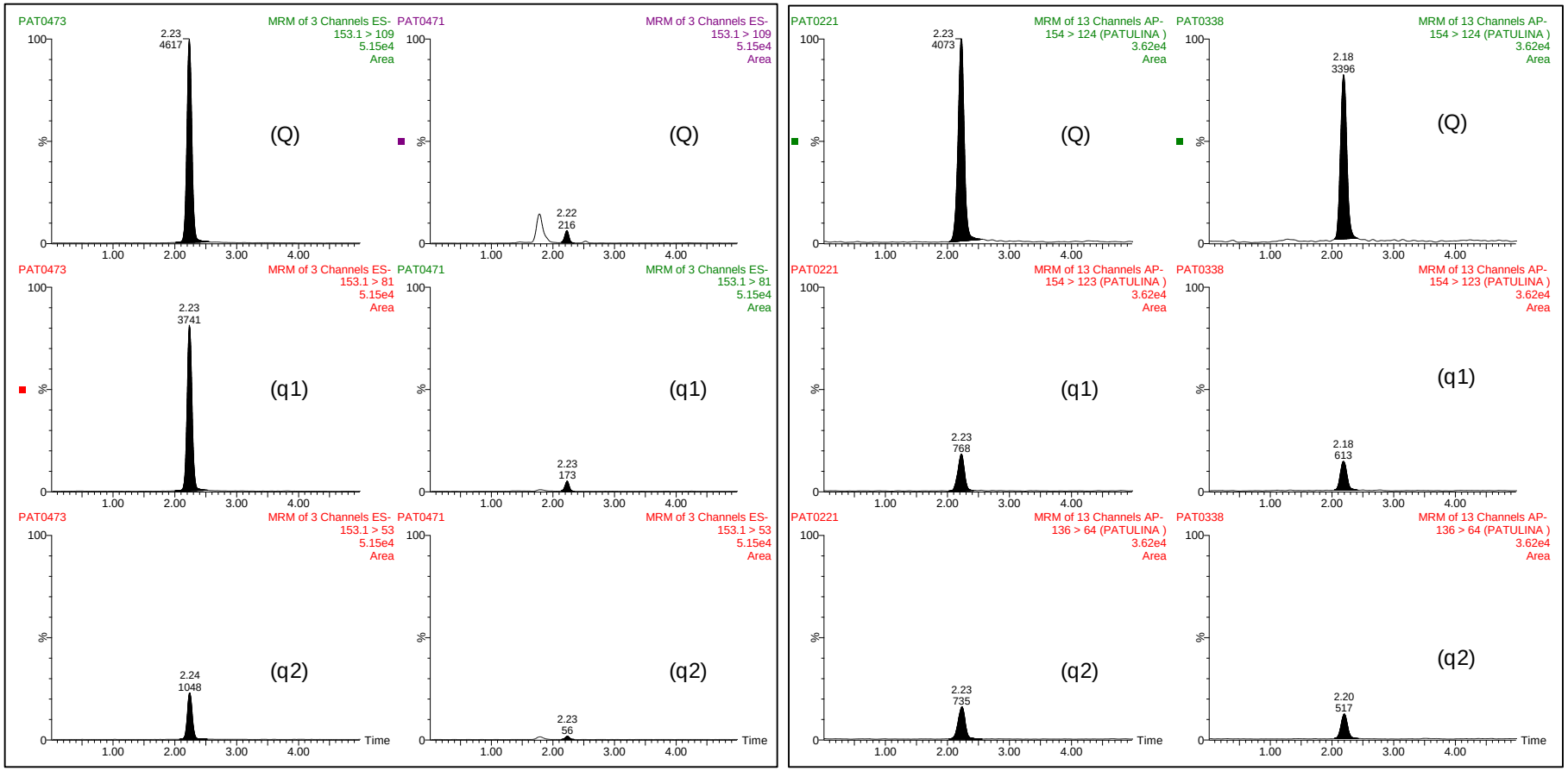
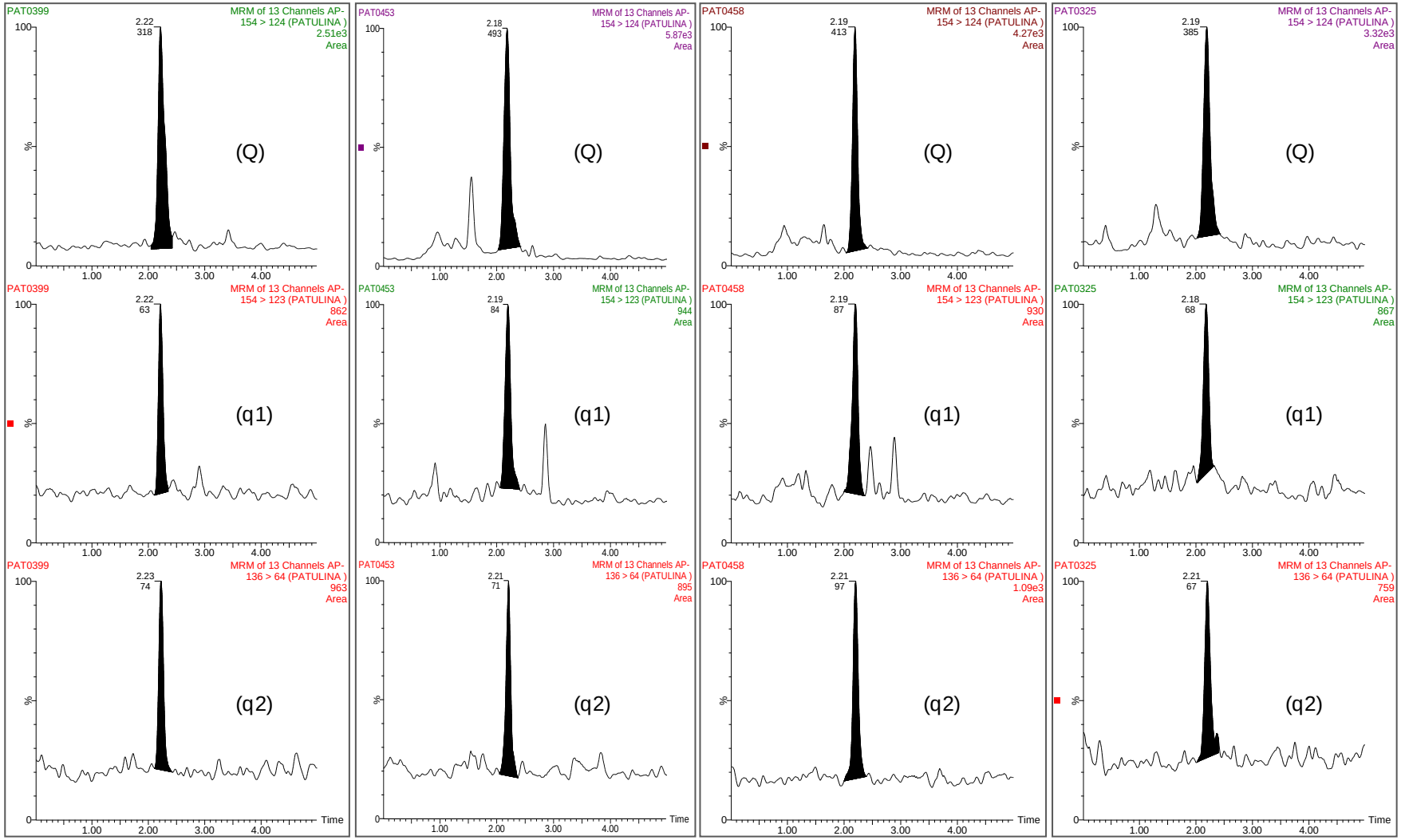

PAT0473
MRM of 3 Channels ES-
153.1 > 109
5.15e4
Area
2.23
4617
(Q)
PAT0471
MRM of 3 Channels ES-
153.1 > 109
5.15e4
Area
2.22
216
(Q)
PAT0473
MRM of 3 Channels ES-
153.1 > 81
5.15e4
Area
2.23
3741
(q1)
PAT0471
MRM of 3 Channels ES-
153.1 > 81
5.15e4
Area
2.23
173
(q1)
PAT0473
MRM of 3 Channels ES-
153.1 > 53
5.15e4
Area
2.24
1048
(q2)
PAT0471
MRM of 3 Channels ES-
153.1 > 53
5.15e4
Area
2.23
56
(q2)
Time
PAT0221
MRM of 13 Channels AP-
154 > 124 (PATULINA)
3.62e4
Area
2.23
4073
(Q)
PAT0338
MRM of 13 Channels AP-
154 > 124 (PATULINA)
3.62e4
Area
2.18
3396
(Q)
PAT0221
MRM of 13 Channels AP-
154 > 123 (PATULINA)
3.62e4
Area
2.23
768
(q1)
PAT0338
MRM of 13 Channels AP-
154 > 123 (PATULINA)
3.62e4
Area
2.18
613
(q1)
PAT0221
MRM of 13 Channels AP-
136 > 64 (PATULINA)
3.62e4
Area
2.23
735
(q2)
PAT0338
MRM of 13 Channels AP-
136 > 64 (PATULINA)
3.62e4
Area
2.20
517
(q2)
Time
(a1)
(a2)
(b1)
(b2)

Figure 2



(a) (b) (c) (d)

Figure 3

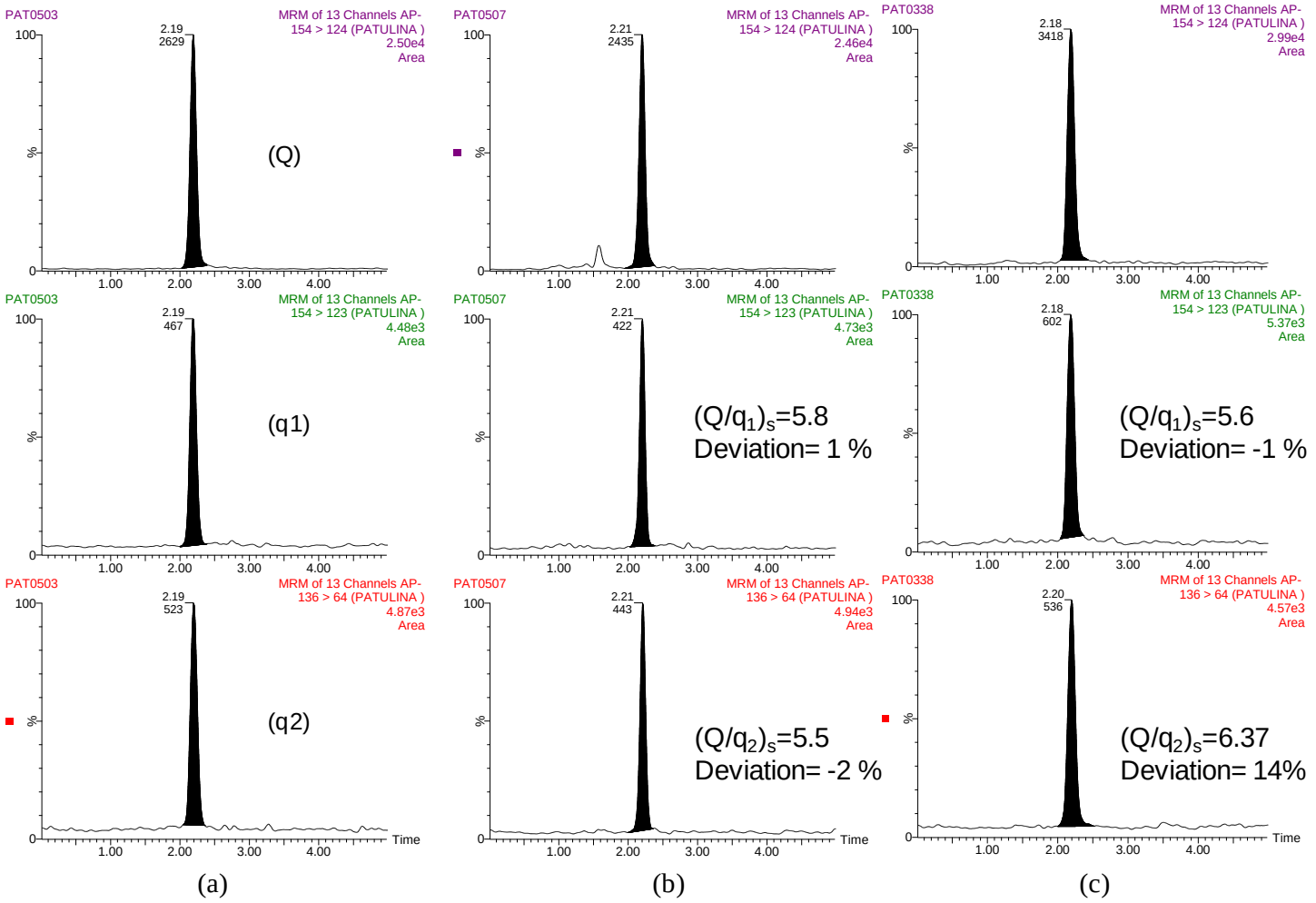

PAT0503
MRM of 13 Channels AP-
154 > 124 (PATULINA)
2.50e4
Area
2.19
2629
(Q)
PAT0507
MRM of 13 Channels AP-
154 > 124 (PATULINA)
2.46e4
Area
2.21
2435
PAT0338
MRM of 13 Channels AP-
154 > 124 (PATULINA)
2.99e4
Area
2.18
3418
PAT0503
MRM of 13 Channels AP-
154 > 123 (PATULINA)
4.48e3
Area
2.19
467
(q1)
PAT0507
MRM of 13 Channels AP-
154 > 123 (PATULINA)
4.73e3
Area
2.21
422
(Q/q1)s=5.8
Deviation= 1 %
PAT0338
MRM of 13 Channels AP-
154 > 123 (PATULINA)
5.37e3
Area
2.18
602
(Q/q1)s=5.6
Deviation= -1 %
PAT0503
MRM of 13 Channels AP-
136 > 64 (PATULINA)
4.87e3
Area
2.19
523
(q2)
PAT0507
MRM of 13 Channels AP-
136 > 64 (PATULINA)
4.94e3
Area
2.21
443
(Q/q2)s=5.5
Deviation= -2 %
PAT0338
MRM of 13 Channels AP-
136 > 64 (PATULINA)
4.57e3
Area
2.20
536
(Q/q2)s=6.37
Deviation= 14%
%
Time
1.00
2.00
3.00
4.00
0
100
(a)
(b)
(c)

Figure 4