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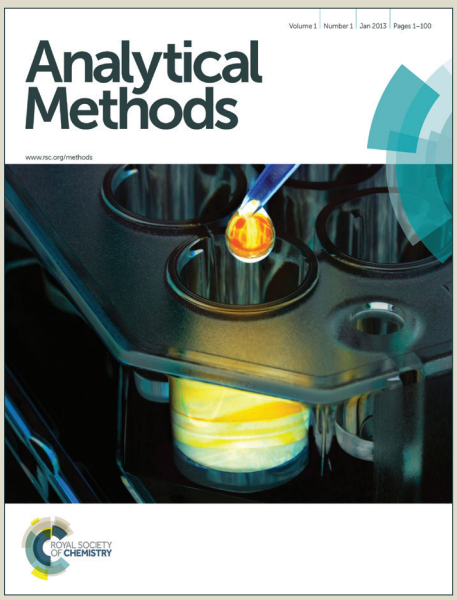

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Simultaneous analysis of kasugamycin and streptomycin in vegetables by liquid chromatography-tandem mass spectrometry

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Abstract

In this work an LC-MS/MS method has been developed for the simultaneous analysis of kasugamycin (KAS) and streptomycin (STR) in vegetable samples. The use of a mixed-mode HPLC column and a ternary mobile phase acetonitrile:water:0.5% aqueous formic acid in gradient elution mode are proposed providing enough retention and resolution for these highly polar compounds. Heated-electrospray (H-ESI) has been used to ionize KAS and STR when coupling liquid chromatography to mass spectrometry. In contrast to what happens with most aminoglycosides, KAS only showed single charged ions in the full-scan mass spectrum. The $[M+H]^+$ of both KAS and STR are proposed as precursor ions for tandem mass spectrometry since more stable product ions and better ion ratio precisions were obtained. Fragmentation of KAS has been studied, showing that the cleavage of the glycosidic bonds provided the main product ions and the most abundant and selective ones are proposed for quantitation and confirmation purposes in MRM mode. The LC-MS/MS method developed has been applied to the analysis of vegetables. A simple clean-up procedure using hydrophilic-lipophilic-balanced cartridges was applied to several vegetable extracts from tomato, zucchini, chard and lettuce, obtaining recoveries >80% for both compounds. The high matrix suppression observed for KAS in all matrices was reduced by a 1:5 sample dilution with acetonitrile, providing a 100-fold improvement in sensitivity for this compound. Method quality parameters have been established for tomato matrices, obtaining method limits of quantitation in the low µg kg^{-1} level (5-10 µg kg^{-1}) for both compounds, precisions expressed in %RSD better than 7% and accuracies expressed as relative error better than 8% at 100 µg kg^{-1} level, making this method suitable for routine analysis.

1. Introduction

Kasugamycin (KAS) and streptomycin (STR) are two aminoglycoside (AGs) antibiotics active against certain types of gram-negative bacteria[1]. Their general structure includes several aminosugars linked by glycosidic bonds to a streptidine (STR) or a 2-deoxystreptamine (KAS) unit. STR, produced by *Streptomyces griseus*, was the first antibiotic applied against plant diseases[2] to control fire blight of apple and pear, wild fire of tobacco and bacterial leaf blight of the rice plant, among others and its use is being reassessed due to the spread of resistant bacterial strains.[3] KAS is produced by *Streptomyces kasugaensis*, it has a strong preventive effect against rice blast caused by *Piricularia oryzae*, and it has been also used for the treatment of bacterial diseases in bell peppers, tomatoes, citrus, cucumbers and many other fruiting crops. Moreover, the use of KAS as a substitute of STR for the treatment and prevention of fire blight in pears and apples has been suggested[4] due to the low toxicity of KAS to crops and mammals and its activity against resistant strains. Thus, residues of these aminoglycosides might be present in fruits and vegetables.

Since public concern over pesticide residues in foods has become an important issue, recommended maximum residue levels (MRL) for these compounds in agricultural products have been established. Nevertheless, different levels have been set depending on the food commodity and the country. For instance, in the United States MRLs for KAS have been set at 0.04 mg kg^{-1} in fruiting crops and at 0.05 mg kg^{-1} for apples[5] whereas for STR the MRLs range from 0.25 to 0.5 mg kg^{-1}, depending on the matrix.[6] In the European Community their use is not authorised since 2005[7] and thus a default MRL of 0.01 mg kg^{-1} is recommended.[8]

Liquid chromatography coupled to mass spectrometry is the analytical technique of choice for the analysis of KAS and STR, which are highly hydrophilic compounds that lack chromophore groups. Due to the amino groups present in their structures, both STR and KAS are poorly retained in reversed-phase columns such as C18[9-11] and for this reason, alternative chromatographic separation modes such as ion pair liquid chromatography (IPLC), hydrophilic interaction liquid chromatography (HILIC) and mixed-mode chromatography have been used. The analysis of KAS presents a high analytical challenge and this compound is not currently included in the analysis of other AGs. In fact, only two studies have been published dealing with the determination of KAS in foods. The first one proposes a method for the analysis of both KAS and STR,

and other AGs in animal tissues, milk and eggs using IPLC with trifluoroacetic acid[12] and in the second one, KAS is determined in soils and chili peppers[13] using HILIC. This last study is the only one published regarding the analysis of KAS in vegetables. Moreover, most of the developed methods for STR have been established for its determination in foods from animal origin. Some of them use IPLC with ion pair reagents like heptafluorobutyric acid,[14-20] perfluoropentanoic acid,[21] and trifluoroacetic acid,[12] while others use HILIC, often with high buffer concentrations in the mobile phase, such as ammonium acetate or formate at concentrations between 150 and 200 mM, and with percentages of formic acid ranging from 0.1 to 1%.[22-26] Additionally, mixed-mode chromatography with low ionic strength mobile phases has also been proposed for the analysis of STR and other AGs in meat.[27] However, only two methods dealing with the analysis of STR in fruits and vegetables can be found in the literature. One of these studies proposes the use of IPLC with heptafluorobutyric acid as ion pair reagent for the determination of STR and tetracycline in pomegranate[28] and the other one applies HILIC to determine STR in apples.[29]

All these methods use electrospray (ESI) in positive mode as the ionization technique because protonation is favored by the presence of amine groups in their chemical structure. Low resolution analyzers such as triple quadrupole[12-17,19-21,24-27,29] and quadrupole-ion trap[22, 28] combined with multiple reaction monitoring (MRM) acquisition have been used to determine KAS and STR at low concentration levels. The fragmentation pathways of STR has been studied,[30, 31] but to the best of our knowledge, the collision-induced dissociation (CID) mass fragmentation and the product ions assignment for KAS have not been proposed. Moreover, when coupling HILIC and IPLC methods to mass spectrometry, the presence of ion pair reagents and the high concentration of salts needed for ion exchange chromatography or HILIC could affect the performance of the mass spectrometric systems in both sensitivity and maintenance. So there is a need for LC-MS methods to analyze aminoglycosides that avoid the use of ion pair reagents or high concentration buffers in the mobile phase.

The aim of this work is to develop a liquid chromatography-tandem mass spectrometry method for the simultaneous analysis of KAS and STR, which are the only aminoglycoside antibiotics used as pesticides, in vegetables. For this purpose, the use of a mixed-mode chromatographic column that combines different retention mechanisms is evaluated in order to retain and separate both compounds without using ion pair reagents or high buffer concentrations as required for HILIC. The ionization of these

compounds under electrospray conditions is studied and their tandem mass spectrometry fragmentation is discussed, especially for KAS which has not been previously reported. Finally, the LC-MS/MS method proposed is applied to the simultaneous determination of KAS and STR in vegetables at low μg kg^{-1} level.

2. Experimental

2.1. Reagents and materials

Streptomycin sulfate (STR, 98%, CAS No. 3810-74-0), and kasugamycin hydrochloride hydrate (KAS, 79%, CAS No. 19408-46-9) were purchased from Dr. Ehrenstorfer Gmbh (Augsburg, Germany). Figure 1 shows the structures of the studied compounds. The glycosidic rings of the structures have been labeled with capital letters to help with the discussion of the fragmentation patterns. Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate (98.5%) was purchased from Sigma-Aldrich (Steinheim, Germany). LC/MS grade methanol, acetonitrile (ACN) and water and trichloroacetic acid (TCA) (99.5%) were obtained from Fluka (Steinheim, Germany) and formic acid (98-100%) was provided by Merck (Darmstadt, Germany). Stock standard solutions of KAS and STR (2.0 g kg^{-1}) were individually prepared by weight in water and stored at -20ºC. Intermediate solutions were prepared monthly from stock standard solution by appropriate dilution. As aminoglycosides present high sorption affinity to polar surfaces all contacts with glass were avoided and only polypropylene laboratory material was used. All samples and mobile phases were filtered through a 0.22 μm nylon membrane filters purchased from Whatman (Clifton, NJ, USA) to avoid clogging. Nitrogen (99.8% pure) supplied by gas line from Air Liquide (Madrid, Spain) was used for the API source. The collision-induced dissociation gas used for tandem mass spectrometry experiments was high-purity Argon (ALPHAGAZ 1 Ar) also from Air Liquide (Madrid, Spain). Sample clean-up was performed using Oasis HLB cartridges (150 mg, 6 cm^3) and extraction manifold from Waters (Milford, MA, USA). For the control of the pH of buffered solvents a pHmeter Basic 20 from Crison Instruments (Alella, Spain) was used.

2.2. Sample treatment

Samples of tomato, chard, lettuce, zucchini and red pepper bought at local supermarkets in Barcelona (Spain), were cut, ground and homogenized with an Ultraturrax T25 basic (IKA-Werke, Staufen, Germany) and kept frozen at -20ºC until analysis. Subsamples of 2 g were weighed in a 15 mL polypropylene centrifuge tube (Serviquimia, Barcelona, Spain) and extracted in a Sonorex RK100 ultrasonic bath (Bandelin Electronic GmbH & Co., Berlin, Germany) for 10 min with 2 mL of acetonitrile:aqeuous 5% TCA, 2 mM EDTA (v/v 1:1) and then centrifuged at 4,000 rpm (Selecta-Macrotronic, J.P. SELECTA S.A., Abrera, Spain) for 10 min. The supernatant was then loaded onto an Oasis HLB SPE cartridge previously conditioned with 3 mL of methanol and 3 mL of water for clean-up. After discarding the first 0.75 mL of the eluate the rest was collected and filtered through a 0.22 µm nylon membrane. Finally, 200 µL of the filtered extract were diluted with 800 µL of acetonitrile and injected into the LC-MS/MS system. Matrix-matched calibration standards were prepared with blank matrix samples spiked at 6 concentration levels ranging from 10 to 500 µg kg^{-1} and submitting them to the same sample treatment described above.

2.3. HPLC-MS/MS

HPLC separation was performed on an Open Accela liquid chromatography system (Thermo Fisher Scientific, San José, CA, USA), equipped with a quaternary UHPLC pump, and a CTC PAL autosampler (CTC Analytics, Zwingen, Switzerland). An Obelisc R (150 x 2.1 mm, 5 µm) column (SIELC Technologies, Prospect Heights, IL, USA) was used for the proposed method. Mobile phases consisted of a mixture of acetonitrile (A), aqueous 0.5% formic acid (B) and water (C). The chromatographic initial conditions were 85% A, 3.8% B and 11.2% C, and a linear ramp was performed in 1.5 min to 85% A, 7.5% B and 7.5% C. A second linear ramp was carried out in 4.5 min to 5% A, 50% B and 45% C and these conditions were maintained for 4 min, moving back to initial conditions in 2 min and equilibrating the column for 8 min. The flow rate was 300 µL min^{-1} and the column was kept at ambient temperature. The injection mode was full loop with a 5 µL loop.

Mass spectrometry was performed in a TSQ Quantum Ultra AM (Thermo Fisher Scientific, San José, CA) triple quadrupole mass spectrometer equipped with heated-electrospray (H-ESI) as ionization source. Electrospray voltage was set at 3.0 kV and the temperatures of the ion transfer tube and the vaporizer were both held at 300ºC.

Sheath gas, auxiliary gas and ion sweep gas flow rate were 35, 10 and 0 a.u. (arbitrary units), respectively. In tandem mass spectrometry experiments, both quadrupoles worked in low resolution mode (0.7 full width half maximum, FWHM), the collision gas pressure was 1.5 mTorr and the collision energies (CE) ranged from 11 eV to 18 eV. Two transitions were monitored for each compound using a dwell time of 250 ms (1 scan/cycle time). Transitions for quantitation and confirmation purposes and the corresponding collision energies and ion ratios are listed in Table 1. Other instrument settings were optimized by infusion of standard solutions (10 mg L^{-1}) at a flow rate of 5 µL min^{-1}, using the built-in syringe pump and a zero dead volume T-piece to mix them with the mobile phase.

3. Results and discussion

3.1. HPLC-MS/MS

Due to the high polarity of KAS and STR their analysis by LC is usually performed by IPLC or HILIC. However, the use of these chromatographic methods requires the addition of ion pair reagents or buffers at high concentration to the mobile phase, thus making difficult the compatibility with mass spectrometry. In this work a mixed-mode Obelisc R column has been used to explore the simultaneous chromatographic separation of KAS and STR, as this column has been proposed for the separation of STR and other AGs using gradient elution with a mobile phase composed of a mixture of acetonitrile:1% aqueous formic acid:water.[27] These conditions worked well for STR but KAS eluted too early in the chromatogram due to the high elution strength of the mobile phase, so alternative chromatographic conditions were optimized. To this end, different aqueous formic acid gradients were tested. When the initial formic acid concentration was lowered, the retention of KAS increased because at low pHs hydrophilic interaction is the prevailing retention mechanism, enhancing the retention with the decrease of the ionic strength. However, the retention and peak width of STR also increased for the same reason, resulting in poor chromatographic efficiency (peak width > 2 min) and long analysis times. To achieve good retention and peak efficiency, a ternary gradient simultaneously decreasing acetonitrile and increasing the formic acid concentration was used, providing the optimal conditions. The optimized gradient has been indicated in the experimental section and Figure 2 shows the chromatogram

obtained under those chromatographic conditions. As can be seen, the compounds were baseline separated in less than 6 minutes with good peak shape and far enough from the elution front.

Regarding mass spectrometry, thermally assisted electrospray (H-ESI) was used as ionization source in LC/MS, as it improved desolvation, especially with mobile phases with a high percentage of water. Full-scan mass spectra were studied to select the most adequate precursor ions for tandem mass spectrometry. The mass spectrum of KAS (Figure 2) is very simple showing only the protonated molecule $[M+H]^+$ (*m/z* 380) and the loss of CO_2 from the protonated molecule due to in-source collision-induced dissociation (*m/z* 336). In contrast, the mass spectrum of STR (Figure 2) is more complex, because STR has two guanidine groups with high pKa values (13.40 ± 0.70)[32] that favored the generation of multiple charged ions. So, in the mass spectrum of STR the single and double charged ions, $[M+H]^+$ and $[M+2H]^{2+}$ (*m/z* 582 and 292, respectively) due to protonation and also the single and double charged ions due to the hydrated form of the aldehyde group, $[M+H_2O+H]^+$ and $[M+H_2O+2H]^{2+}$ (*m/z* 600 and 301 respectively), can be observed. Moreover, some in-source fragmentation from the cleavage of the glycosidic bonds is also present (Figure 2). The $[M+H]^+$ was selected as precursor ion for tandem mass spectrometry since it showed the best fragmentation performance, leading to more stable product ions and better precisions than the double charged ions. As regards the hydrated form of the single charged ion, it showed the unspecific loss of water as the most intense product ion, which is more susceptible to interferences than the fragmentation from the protonated molecule. The chosen SRM transitions for STR are listed and assigned in Table 1. For KAS, the $[M+H]^+$ was also selected as precursor ion and the triple quadrupole product ion scan spectrum is shown in Figure 3. The fragmentation pattern of KAS has been discussed using the nomenclature defined by Goolsby and Brodbelt,[30] which proposes the use of the subscript "1" for the cleavage of the bond between the ring designated with the uppercase letter and the glycosidic oxygen, and the subscript "2" for that between the glycosidic oxygen and the next ring. As happens with most AGs, the main product ions of the $[M+H]^+$ of KAS involved the cleavage of the glycosidic bond (Figure 3), whether arising from the loss of the glucose ring ($[M+H - B_2]^+$, *m/z* 200) or from the combined loss of the glucose ring and the amino(imino)acetic side chain ($[M+H - B_2 - C_2H_4O_2N_2]^+$, *m/z* 112). The presence of the carboxylic group in the side chain favored also the loss of CO_2 from the protonated molecule (*m/z* 336) or from the $[M+H - B_2]^+$

(*m/z* 156). Additionally, further cross-ring cleavage and rearrangement of the *m/z* 112 would produce the product ion at *m/z* 70. Fragmentation of KAS is shown in Figure 3 and the elemental compositions of all product ions have been confirmed by MS/HRMS experiments in a Q-Exactive (Thermo Fisher Scientific) at a mass resolution of 35,000 FWHM (full with half maximum) at *m/z* 200, obtaining mass errors below 2 ppm. The most abundant product ions, at *m/z* 112 and *m/z* 200, were selected for quantitation and confirmation, respectively, of KAS when working in MRM mode (Table 1).

Instrumental quality parameters including limits of detection (LOD), linearity and run-to-run and day-to-day precisions were calculated (Table 2). LODs, based on a signal-to-noise ratio of 3:1 for the confirmation transition, were estimated by injecting standard solutions at concentrations down to 0.1 µg kg^{-1}, obtaining results lower than 10 pg injected for both compounds. KAS provided slightly better instrumental sensitivity, probably because for this compound only one precursor ion was obtained in ESI in contrast with what happened to STR, where the total ion signal was split into different species. Standard calibration curves with 6 concentration levels between 5 and 300 µg kg^{-1} showed good linearity ($R^2 > 0.996$). Run-to run (n=5) and day-to-day (n=5, 3 days) precisions were calculated at two concentration levels, 15 µg kg^{-1} and 150 µg kg^{-1}, and were always lower than 7% for both compounds. Retention time precision was also evaluated in the same way as the concentration precision, and low values, even for the day-to-day precision (below 1%), were obtained. Regarding the ion ratio (relative areas for quantifier vs qualifier transitions) precisions, the %RSD values were below 10% for both compounds.

3.2. Sample treatment

To analyse KAS and STR in vegetables, a sample treatment previously proposed for the analysis of STR and other AGs in meat[27] was evaluated, consisting of an extraction with an aqueous solvent containing a strong acid (TCA) and a chelating agent (EDTA) followed by a clean-up with hydrophilic-lipophilic-balanced cartridges (HLB). The clean-up with HLB cartridges served to retain the matrix coextractives, that gave a cloudy and slightly coloured aspect to the extract. The highly polar AGs were not retained in this sorbent and eluted directly. Recoveries were evaluated by means of the analysis of blank tomato samples (2 g) spiked at 250 µg kg^{-1}, and good recoveries (>80%) were obtained for STR, which were similar to those obtained in the previous

work. However, KAS, which had not been previously studied, was not recovered at all. Recoveries with solvent based standards were close to 100%, and when blank tomato extracts spiked after sample treatment were analysed poor results for KAS were also obtained. Thus, the lack of signal for KAS was due to high matrix suppression for this compound and not to poor recoveries. Variations of the sample treatment method such as changes in the extraction solvent, or the use of other SPE strategies like ion-exchange cartridges not only did not provide successful results for KAS but also worsened the recoveries for STR as well, so alternative method were sought. As a first step, the use of an ionization source less susceptible to matrix effects such as Atmospheric Pressure Chemical Ionization (APCI) was tested. Although the high polarity and low volatility of AGs would produce lower ionization efficiency than ESI, the decrease in the ion suppression might produce an overall improvement in sensitivity. In fact, the use of APCI has been previously proposed to overcome ion suppression due to ion pair reagents for the LC-MS/MS analysis of gentamicin impurities.[33] As expected, STR provided lower signal intensities in APCI than in ESI, but no signal was observed for KAS in APCI in both, positive and negative modes. The lack of response of KAS might be explained by the presence of a carboxylic group in a side chain in KAS (Figure 1) which might favor the formation of an internal hydrogen bond with the primary amino substituent in ring A that would make KAS difficult to ionize in APCI in both positive and negative modes. Anyhow, as the ionization of KAS was not possible, the use of the APCI source was discarded.

As a second step, the sample extract was diluted with ACN. Blank vegetable samples (lettuce, zucchini, red pepper, chard and tomato) spiked at 500 µg kg^{-1} were submitted to the sample treatment and the obtained extracts were analysed without dilution and diluting 1:1, 1:5, and 1:10 with acetonitrile. For all the samples, an important increase on the response of KAS was obtained when diluting with acetonitrile, which allowed its detection. As an example, Figure 4 shows the LC-MS/MS chromatograms of two aliquots of a blank tomato extract spiked with KAS and STR at 500 µg kg^{-1} without dilution (A) and diluted 1:5 with ACN (B). As it can be seen, KAS response increased considerably after a 1:5 dilution, showing up to a 100-fold enhancement in signal intensity, which provided a significant decrease of the LODs allowing the detection of KAS at the European MRL value (0.01 mg kg^{-1}) which is the most restrictive one. Further dilution of the extracts did not provide an improvement in signal intensity, probably because the decrease in signal due to dilution was higher than

the improvement due to the reduction of matrix suppression. As a result, a 1:5 dilution of the extract is proposed for the analysis of KAS and STR in vegetable samples. As can be seen in Figure 4, signal intensity of STR significantly decreased when diluting the sample, as apparently a less matrix effect was affecting this compound. Nevertheless, the response of STR after the dilution is similar to that of KAS.

3.3. Method performance

Method quality parameters such as method limit of quantitation (MLOQ), run-to-run and day-to-day precisions, accuracy and ion ratio precision were evaluated using tomato samples free of the analytes as blank samples, and the obtained values are listed in Table 2. As KAS was affected from ion suppression, matrix-matched calibration was used for the quantitation of the samples. Matrix-matched calibration curves were prepared in the 10-500 μg kg^{-1} range by spiking blank tomato samples and submitting them to the sample treatment detailed in the experimental section. The MLOQs (signal-to-noise ratio of 10:1) were estimated by injecting samples spiked at low concentration values (below 10 μg kg^{-1}). As can be seen in Table 2, in contrast to what happened with the instrumental LOD, the MLOQ obtained for STR was better than that of KAS, probably as a result of the higher matrix suppression that affected the latter. MLOQs were at or below the default MRL values established for these compounds in the European Union (10 μg kg^{-1}), and far below the MRL allowed in other countries such as USA (250 μg kg^{-1} for STR), [6] Japan (300 μg kg^{-1} for KAS)[34] or Canada (100 μg kg^{-1} for both compounds). [35,36] MLOQs in the other vegetable matrices also were studied, and in all cases the results were below 15 μg kg^{-1} for both compounds, only being above the MRL in the European Union in red pepper matrices. To evaluate run-to-run precision, five replicate analyses of blank samples spiked at two concentration levels, 10 μg kg^{-1} and 100 μg kg^{-1}, were analysed. Day-to-day precision was calculated by analysing 5 replicates each day during three days at the same two concentration levels. %RSD values based on concentrations (Table 2) ranged from 13 to 24% for the low concentration level, and were below 8% for the high concentration level. Accuracy expressed as relative error was also evaluated at the two concentration levels and as can be seen in Table 2, KAS provided worse accuracies than STR for the low level (23% versus 15%), as it corresponded to the MLOQ for this compound (10 μg kg^{-1}). Ion ratio relative errors with respect to those of standards were lower than 10% and ion ratio

precisions were consistent with those obtained with standard solutions (below 13%). In comparison with previous literature works that analyze either KAS or STR in vegetables,[13,28,29] the proposed method allows the simultaneous analysis of both compounds without losing sensitivity. Also, ion pair reagents are not needed for the chromatographic separation, that favours MS performance and maintenance, and provides slightly better LODs than those reported in the analysis of KAS and STR in animal tissues.[12]

3.4. Method application

Over 24 vegetable samples, including tomato (2 samples), lettuce (2 samples), chard (2 samples), red pepper (2 samples), zucchini (2 samples) and pear (4 samples), purchased at local supermarkets were analyzed. No residues of KAS or STR were detected at levels above the MLOQ in any of the analyzed samples. This fact shows the compliance of the European Community regulations concerning the use of pesticides and the control of the presence of residues of pesticides in foods. Negative results were also found in the analysis of 10 tomato samples acquired from a vegetable garden that had been treated with both compounds. Although it was expected that some residues should have been found in this samples, the short extinction time of these compounds, which is lower than 6 days for KAS in chili peppers,[13] might be a possible reason for the negative results. However, the method provided satisfactory results with spiked blank samples at levels below the MRLs established in most countries (Table 2), making it suitable for routine analysis in laboratories dealing with the control in countries where the use of this compounds as pesticides is allowed (e.g. USA and Canada) and also in imported and exported goods.

4. Conclusions

In this work a method is proposed for the simultaneous analysis of KAS and STR in vegetable samples. A base line chromatographic separation, good peak shapes and short analysis time (less than 6 minutes) are achieved using a mixed-mode column and an ternary mobile phase composed of acetonitrile:water:0.5% aqueous formic acid in reversed gradient elution. Single charged ions are proposed as precursors for tandem mass spectrometry since they showed better fragmentation performance than the

multiple charged ions. The CID MS/MS fragmentation of KAS was studied for first time concluding that the main product ions arose from the cleavage of the glycosidic bonds. The simultaneous loss of glucose and the amino(imino)acetic acid side chain (*m/z* 112) was used for quantitation, whereas the loss of glucose (*m/z* 200) was chosen for confirmation purposes. The high matrix suppression that affected the electrospray KAS response when analyzing vegetables can be greatly decreased by diluting the obtained extract 1:5 with ACN, achieving a 100-fold improvement in signal intensity. Under the optimal working conditions the LC-MS/MS developed method provided MLODs lower enough to determine KAS and STR at the lower MRL established for them in all countries (10 µg kg^{-1}). The applicability of the method has been assessed by analyzing spiked samples, and good method quality parameters have been obtained.

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Figure captions

Figure 1. Structures and acronyms of the studied compounds

Figure 2. Chromatogram of a standard mixture (100 ng g^{-1}) using the conditions given in the experimental section and full-scan mass spectra of KAS and STR

Figure 3. Triple quadrupole product ion scan and fragmentation of KAS

Figure 4. LC-MS/MS chromatogram of a blank tomato extract spiked at 500 µg kg^{-1} level A) without dilution and B) diluting 1:5 with acetonitrile

Tables

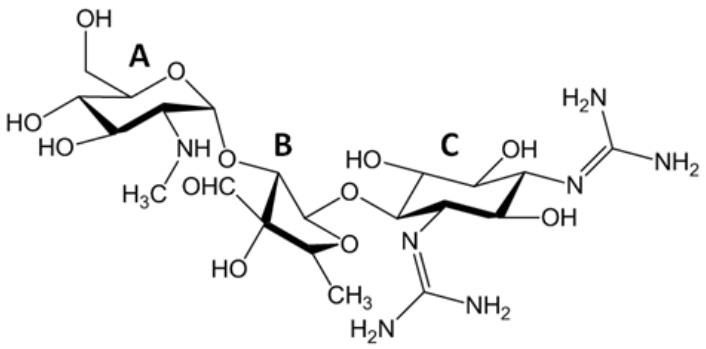
Table 1. Tandem mass spectrometry working conditions. Selected SRM transitions, collision energies (CE), ion assignments and ion ratios

Compound	Retention time (min)	Precursor ion		Product ion				Ion ratio
		m/z	Assignment	*m/z*	Assignment	CE (eV)		
KAS	4.44	380.2	$[M+H]^+$	112.1	$[M+H-B_2-C_2H_4O_2N_2]^+$	18	Quantitation	1.3
				200.1	$[M+H-B_2]^+$	11	Confirmation	
STR	5.07	582.3	$[M+H]^+$	263.1	$[C_2]^+$	31	Quantitation	1.7
				246.1	$[C_2-NH_3]^+$	35	Confirmation	

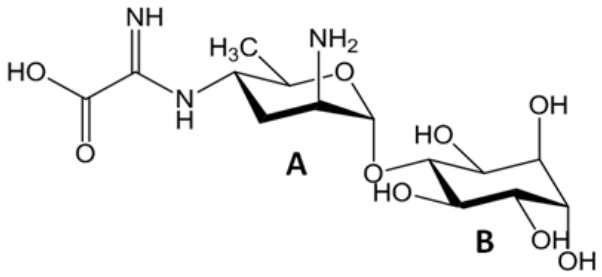
Table 2. Instrumental and method quality parameters.

Compound	Instrumental quality parameters								
	LOD	Run-to-run precision (%RSD, n=5)				Day-to-day precision (%RSD, n=5, 3 days)			
	(pg)	Concentration		Retention	Ion	Concentration		Retention	Ion
		5 μg kg^{-1}	150 μg kg^{-1}	time precision	ratio precision	5 μg kg^{-1}	150 μg kg^{-1}	time precision	ratio precision
KAS	3	4.1	2.5	0.2	4.8	6.0	4.6	0.7	7.1
STR	8	3.6	1.5	0.3	6.7	6.7	3.2	0.4	8.9
	Method quality parameters (tomato matrix)								
	MLOQ	Precision (%RSD)				Accuracy		Ion ratio	
	(μg kg^{-1})	Run-to-run (n=5)		Day-to-day (n=5, 3 days)		(%Rel. Error)		Rel. Error	Precision
		10 μg kg^{-1}	100 μg kg^{-1}	10 μg kg^{-1}	100 μg kg^{-1}	10 μg kg^{-1}	100 μg kg^{-1}	(%)	(%RSD)
KAS	10	13	5.2	24	6.7	23	8.0	10	13
STR	4	15	5.0	21	5.2	19	5.2	9.2	11

Figure 1. Structures and acronyms of the studied compounds



Streptomycin (STR)



Kasugamycin (KAS)

Figure 2. Chromatogram of a standard mixture (100 ng g^{-1}) using the conditions given in the experimental section and full-scan mass spectra of KAS and STR

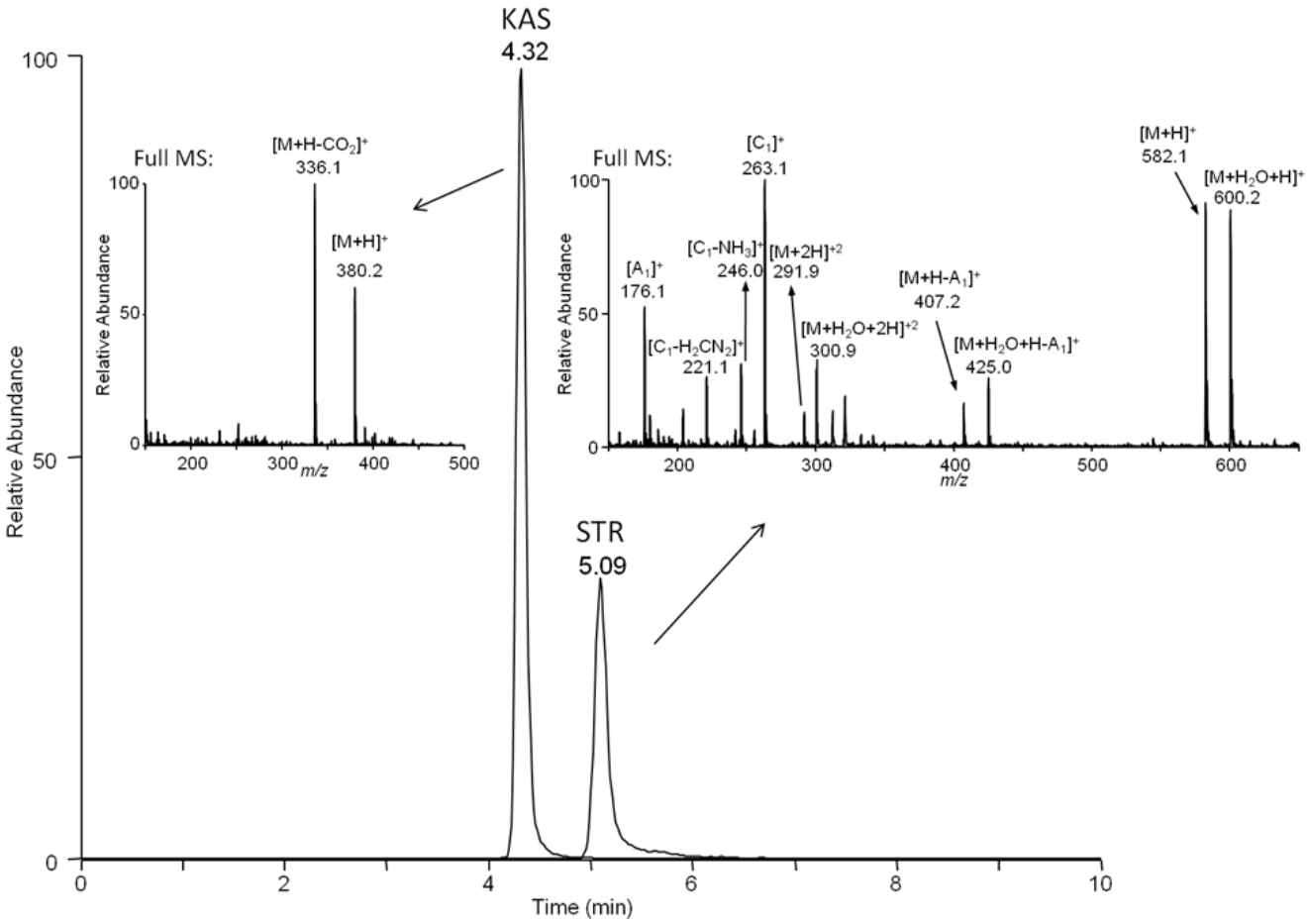


Figure 3. Triple quadrupole product ion scan and fragmentation of KAS

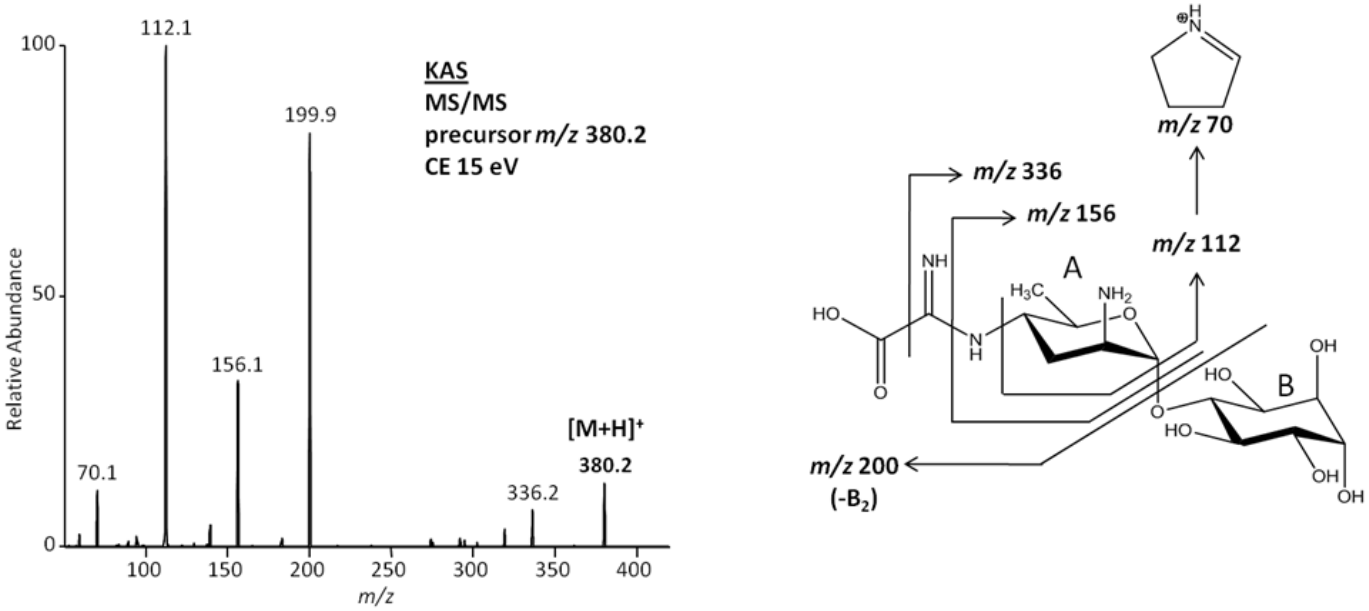


Figure 4. LC-MS/MS chromatogram of a blank tomato extract spiked at 500 µg kg^{-1} level A) without dilution and B) diluting 1:5 with acetonitrile