分析质控与制剂

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Determination of streptomycin and dihydrostreptomycin in honey by hydrophilic interaction chromatography combined with tandem-mass spectrometry

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ABSTRACT **Objective** A specific and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed to determine streptomycin and dihydrostreptomycin residues in honey. **Methods** Streptomycin and dihydrostreptomycin were extracted from honey samples using a liquid extraction with a phosphate buffer followed by a solid-phase extraction based on weak cation-exchange (WCX) columns. After sample preparation, the hydrophilic interaction chromatography (HILIC) was used to retain streptomycin and dihydrostreptomycin on the column for the separation of the analytes from endogenous compounds. The aminoglycosides were detected and quantified with electrospray ionization tandem mass spectrometry (ESI-MS/MS). To decrease or avoid effectively the spectra overlap of streptomycin and dihydrostreptomycin, isotopologue ion of the latter was used as parent ion, which differed 3 mass units from the former. **Results** The lower limits of quantification (LLOQ) of streptomycin and dihydrostreptomycin in honey were 0.5 μg/kg and 1.0 μg/kg, respectively. **Conclusion** This HILIC-MS/MS method is sensitive and specific to monitor and analyze of streptomycin and dihydrostreptomycin residues in honey.

KEY WORDS Liquid chromatography-tandem mass spectrometry; Hydrophilic interaction chromatography; Streptomycin; Dihydrostreptomycin; Honey

亲水作用色谱-串联质谱法测定蜂蜜中的链霉素和双氢链霉素

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摘要: 目的 建立一种专属、灵敏的液相色谱-串联质谱法(LC-MS/MS)测定蜂蜜中链霉素和双氢链霉素的残留量。**方法** 用磷酸盐缓冲溶液提取蜂蜜中的链霉素和双氢链霉素后,经弱阳离子交换柱(WCX)进行固相萃取,样品制备后采用亲水作用色谱(HILIC),使链霉素和双氢链霉素实现保留,从而与内源性物质达到分离,采用电喷雾离子化串联质谱法(ESI-MS/MS)检测和定量。双氢链霉素的同位素离子较链霉素大 3 个质量单位,以此为母离子可有效减少或避免二者之间的光谱重叠现象。**结果** 蜂蜜中链霉素和双氢链霉素的最低定量限(LLOQ)分别为 0.5 μg/kg 和 1.0 μg/kg。**结论** 该 HILIC-MS/MS 法灵敏、专属,适用于监测和分析蜂蜜中链霉素和双氢链霉素的残留量。

关键词: 液相色谱-串联质谱法; 亲水作用色谱; 链霉素; 双氢链霉素; 蜂蜜
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Streptomycin (STR) and its derivative dihydrostreptomycin (DHS) (Fig. 1) belong to the group of aminoglycosides, which are characterized as a large class of antibiotics containing two or more aminosugars linked by glycosidic bonds to an aminocyclitol component[1]. They are widely used for the treatment of serious infections caused by Gram-negative and some Gram-positive bacteria with a good clinical effectiveness,

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a low rate of resistance and low cost. STR and DHS are utilized in apiculture to protect honeybees against a variety of diseases, such as American or European foulbrood[2, 3]. Besides, streptomycin has also been used to control the highly contagious fruit-tree disease 'fire blight' by spraying infected trees with solutions of STR[2, 4]. The above actions all can lead to the occurrence of STR and DHS in honey. It is well known that aminoglycoside antibiotics may cause serious toxic effects, such as ototoxicity, nephrotoxicity and allergic reactions[3]. To avoid these adverse effects, a residue control in edible animal or animal derived food is necessary. Many countries have fixed maximum residue limits (MRLs) for STR and DHS in food. In the EU, it is more rigorous for honey, in which, these drugs are not permitted to exist. It is desired to develop a sensitive and reliable analytical method to monitor STR and DHS in honey.

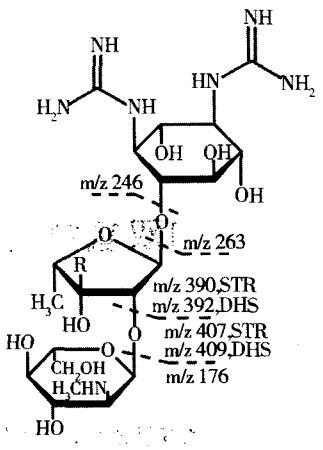


Fig. 1　Chemical structures of streptomycin and dihydrostreptomycin, where R is CHO for streptomycin and R is CH_2OH for dihydrostreptomycin

Trace level detection of aminoglycoside antibiotics in food matrices has been reported using both biological and chromatographic methods. The former were generally used for screening purpose, but results obtained with these assays must always be confirmed by a more selective method such as chromatography, because they were susceptible to generating false-positives for the high cross-reactions with other aminoglycosides and food matrices[3, 5]. The latter involved a variety of analytical methods. HPLC with fluorescence or UV detection methods were possible only after sample preparation followed by pre- or post-column derivation, since aminoglycosides do not possess strong chromophores or fluorophores[3, 6~11]. For this reason, some other detection techniques, such as electrochemical and ELSD detection were described[12, 13]. Because aminoglycosides are not volatile, derivation is also required prior to analysis by gas chromatography (GC)[14, 15]. In recent years, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has gradually become a powerful technique for the detection of such components. It is more specific, sensitive and robust. In addition, no derivation is required and the risk of false-positives is reduced. Until now, several LC-MS/MS methods have been reported for the analysis of aminoglycosides in biological matrices[2, 4, 16~20]. Among these, ion-pair chromatography was always used by adding the volatile ion-pairing reagents in the mobile phase because aminoglycosides are very hydrophilic and always show no retention on routine reversed-phase chromatographic systems. However, ion-pair chromatography is not ideal for electrospray mass spectrometric detection because the signal can be suppressed and the sensitivity is reduced. Recently, hydrophilic interaction chromatography (HILIC) was used for the analysis of neomycin[21] and six aminoglycosides[22] in human serum after solid phase extraction with Oasis MCX cartridges. STR and DHS have the higher alkalinity and polarity than other aminoglycosides. The chromatographic system and SPE condition described in the above literatures were not suitable for STR and DHS. In Waters application note[23], a hydrophilic interaction chromatography (HILIC) column and a weak cation exchange SPE procedure were used to determine STR using DHS as the internal standard, where STR and DHS had the same retention time.

The molecular ion ($[M+H]^+$) of STR (m/z 582) is only 2 u lower than that of DHS (m/z 584). The isotopologue ion ($[M+H+2]^+$) of STR might affect the accuracy of DHS when the two compounds were simultaneously determined with the same retention time. In this study, the interaction of STR and DHS due to the spectra overlap was evaluated when they were determined

simultaneously by LC-MS/MS method. The isotopologue ion ($[M+H+1]^+$) of DHS (m/z 585) was used as parent ion to decrease the spectra overlap of STR and DHS. By optimizing mass spectral conditions, a sensitive and specific LC-MS/MS method was described to monitor and analysis of STR and DHS residues simultaneously in honey using hydrophilic interaction chromatography (HILIC) technique.

1 Experimental

1.1 Reagents and chemicals

Streptomycin sulfate (STR), and dihydrostreptomycin sesquisulfate (DHS), were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA, purity ≥ 98.0%). Acetonitrile (for HPLC) and methanol (for HPLC) were purchased from Merck (Darmstadt, Germany). Formic acid (for HPLC), acetic acid (for HPLC) and ammonium formate (for HPLC) were obtained from Tedia Company Inc. (Fairfield, OH, USA). Ultrapure water was produced by a Milli-Q Gradient A10 system (Millipore, France). Stock solutions and working standards of STR and DHS were prepared in water and stored at 4 ℃. These solutions were not stored in glass materials due to their binding tightly to the glass. Polypropylene volumetric flasks, centrifuge tubes and conical autosampler vials were used.

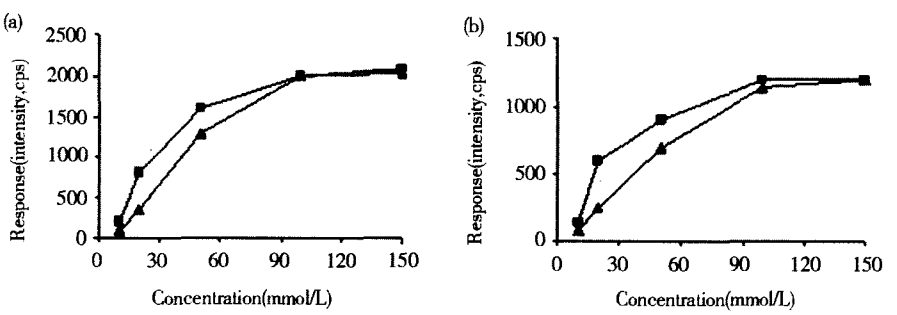
Waters Oasis WCX cartridges (60 mg, 3 ml, 30 μm, Waters Corporation, Milford, MA, USA) were used for solid phase extraction.

1.2 Instruments and LC-MS/MS conditions

(1) HPLC system An Agilent 1100 system consisted of a quaternary gradient pump (G1311 A), a Thermo Well Plate autosampler (G1367 A), a thermostatted column compartment (G1316 A) and a degasser (G1379A). The chromatographic separation was performed on an Atlantis™ HILIC Silica column (50 mm × 2.1 mm, 3 μm) in combination with a guard column of the same material (10 mm × 2.1 mm, 3 μm) from Waters (Milford, MA, USA), which were thermostatted at 30 ℃. The concentrations of the volatile buffer salt and formic acid in the mobile phase were investigated separately (See Fig. 2). Finally, the mobile phase was applied with solvent A (100 mmol/L ammonium formate and 100 mmol/L formic acid in water) and solvent B (100 mmol/L formic acid in acetonitrile) as the following gradient procedure:

The flow rate was 0.3 ml/min and the retention times of STR and DHS were both about 6.7 min.

	Time (min)				
	0	4	8	9	12
A (%)	10	60	60	10	10
B (%)	90	40	40	90	90



■: Effect of the concentrations of ammonium formate when formic acid was fixed at 100 mmol/L;

▲: Effect of the concentrations of formic acid when ammonium formate was fixed at 100 mmol/L

Fig. 2 Effect of the concentrations of ammonium formate and formic acid on the response of STR (a) and DHS (b)

(2) Mass spectrometer The LC effluent was interfaced with a MDS Sciex API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo IonSpray source in the positive ion mode. The instrument was tuned by direct infusion of 0.5 μg/ml STR and DHS at both 5 μl/min, respectively, first without and thereafter in combination with the LC mobile phase. Tuning parameters were optimized manually to give the values shown in Tab. 1. Source parameters were optimized as follows: CUR (curtain gas) 14 psi, ion spray voltage 4500 V, Gas 1 (nebulizer gas) 60 psi, Gas 2 (heater

gas) 50 psi, Ihe (interface heater) on, CAD (collision gas pressure) 5 psi, TEM (turbo gas temperature) 500 ℃. Under these conditions, the analytes were detected in the multiple reaction monitoring (MRM) mode. Data were collected and processed using Analyst 1. 4. 1 software (Applied Biosystems).

Tab. 1　API 4000 tuning parameters

Tune parameters	STR		DHS	
	m/z 582.2→263.4	*m/z* 582.2→246.3	*m/z* 585.4→263.3	*m/z* 585.4→246.3
DP (V)	110	110	77	77
EP (V)	10	10	10	10
CE (eV)	45	53	45	55
CXP (V)	13	10	12	11
Dwell time (ms)	200	200	200	200

(3) Mass spectrometry STR and DHS have very similar structures. There is only 2 u difference between their molecular weights. In the Q1 full scan mass spectra, STR and DHS both formed predominately the protonated molecules $[M+H]^+$ at *m/z* 582 and 584, respectively, and the corresponding isotopologue ions $[M+H+1]^+$ and $[M+H+2]^+$. Their abundant ratios were about 100:27:6. In addition, adduct ions with H_2O were also observed at *m/z* 600. 2 and *m/z* 602. 1 for STR and DHS, respectively. The product ion spectra of $[M+H]^+$, $[M+H+1]^+$ and $[M+H+2]^+$ from STR and DHS showed the same base fragment ion at *m/z* 263 (See Fig. 3 and Fig. 4). When STR was detected using transition of protonated molecular (*m/z* 582. 2) to *m/z* 263, the response of its isotopologue channel with transition of $[M+H+2]^+$ at *m/z* 584 to *m/z* 263 was about 4% (see Fig. 5). In other words, when the same concentrations of STR and DHS were determined simultaneously both using the transitions of the respective protonated moleculars to *m/z* 263, the isotopologue ion $[M+H+2]^+$ of STR resulted in an error of about 4% for DHS. Since STR and DHS could not be separated by chromatography, it was necessary to decrease or avoid the above mass spectra overlap. In our study, the isotopologue ion transition of DHS (*m/z* 585. 4 → *m/z* 263. 3) was chosen for quantification, instead of the transition of the protonated molecular (*m/z* 584. 2) to *m/z* 263. Fig. 5 showed the respective typical chromatogram channels of STR for a sample which only contained 100 μg/kg of STR. As can be seen, the spectral overlap was only about 0.3%.

1.3 Sample preparation

Sample preparation was carried out as follows: 2.0 g of honey samples were weighed in a capped 15-ml polypropylene centrifuge tube and dissolved in 5 ml of the extraction solvent, consisting of a 20 mmol/L Na_2HPO_4 buffer solution (pH 7. 4, adjusted with HCl of 6 mol/L). After vortex-mixing for 30 s, the samples were centrifuged at 4000 r/min for 10 min. The clear supernatants were decanted into next fresh polypropylene centrifuge tubes and set aside for further solid-phase clean-up. The Oasis WCX SPE columns were installed on a vacuum manifold and preconditioned with 3 ml methanol and 3 ml water. All of the previously centrifuged honey extracts were loaded on the SPE cartridges at an average speed of one drop per second. The cartridges were washed with 3 ml water, dried for 10s (under pressure ca. 20 kPa). The analytes were eluted with 3 ml elution solution (2% acetic acid solution in water: acetonitrile 80: 20, *V/V*). A 10-μl aliquot of the eluate was injected onto the LC column.

2 Results

A series of blank matrices, blank matrix-spiked calibration standards and quality control (QC) samples were analyzed to determine method linearity, accuracy, precision, reproducibility, recovery and matrix effect.

2.1 Assay selectivity and matrix effect

Selectivity was assessed by comparing the chromatograms of 12 different types of blank honey with the corresponding spiked honey. Fig. 6 and Fig. 7 showed the typical chromatograms of a blank honey sample, blank honey sample spiked with STR and DHS at the LLOQ and 20 μg/kg concentration levels, and incurred

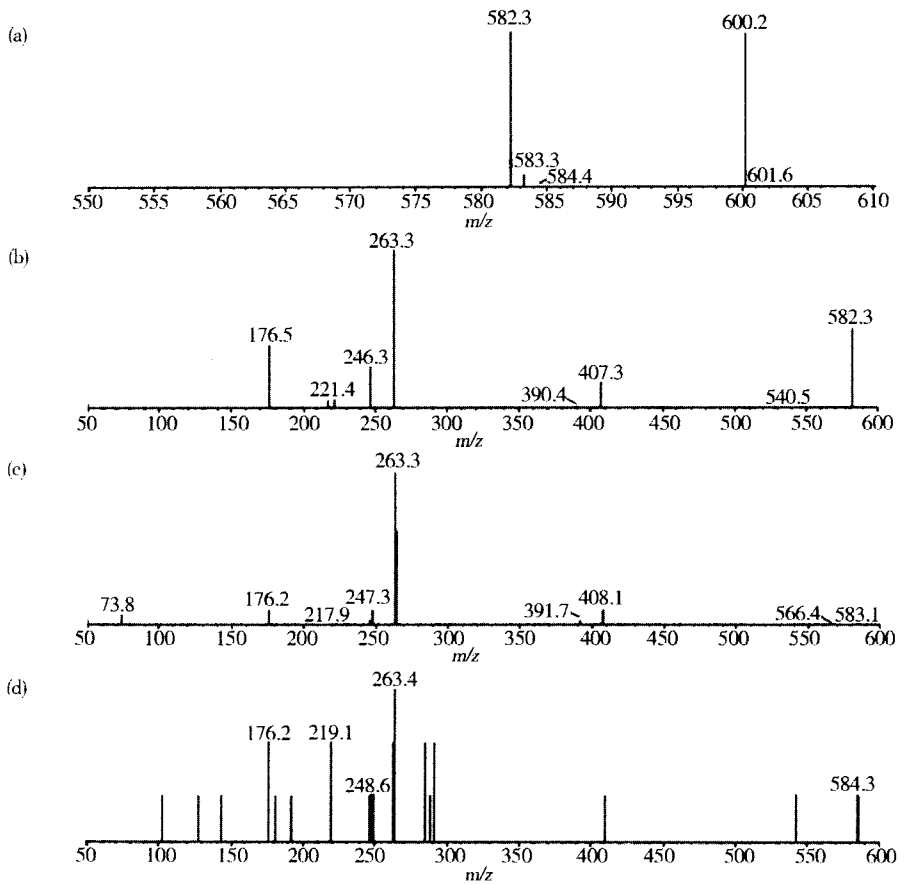


Fig. 3 Q1 full scan mass spectrum (a) and product ion spectra of $[M+H]^+$ (b), $[M+H+1]^+$ (c) and $[M+H+2]^+$ (d) from STR.

honey samples. No interference from endogenous substances was observed at the retention times of STR and DHS.

Matrix effect was tested by comparing signals of blank honey extracts spiked with both STR and DHS prior to injection with the signals of standard solution in eluation solvent at the same levels of LLOQ, 20 μg/kg and 100 μg/kg, respectively, in sets of 5 replicates each. As a result, distinct matrix effects were shown. The responses of STR were enhanced to 127%, 121% and 125%, respectively, at the above concentration levels. For DHS, the signals were increased to 135%, 137% and 130%, respectively. Twelve different types of blank honeys were tested for the matrix effect. It was found that the influence of the type of honey on the matrix effect were negligible, which was consistent with the results reported previously[2].

2.2 Linearity and lower limit of quantification (LLOQ)

The linearity was performed using spiked blank honey samples with the regression of peak area (Y) versus concentrations (X). The calibration curves were plotted at 0.5, 1.0, 2.0, 5.0, 20 and 100 μg/kg for STR, 1.0, 2.0, 5.0, 20 and 100 μg/kg for DHS, respectively. Each solution was run in duplicate on three consecutive days ($n=6$). The calibration curves were $Y=(4640\pm66)X+(832\pm207)$ for STR and $Y=(2374\pm35)X(94\pm58)$ for DHS, respectively, with $1/X$ as the weighting factor. Excellent linearities of STR and DHS were obtained with correlation coefficients (r) of both above 0.99.

The lower limits of quantification (LLOQ) were 0.5 and 1.0 μg/kg for STR and DHS in honey. The accuracy and precision at the concentration of LLOQ were shown in Tab. 2 and Tab. 3. Under the present LLOQ, the concentrations of STR and DHS could be sensitively determined.

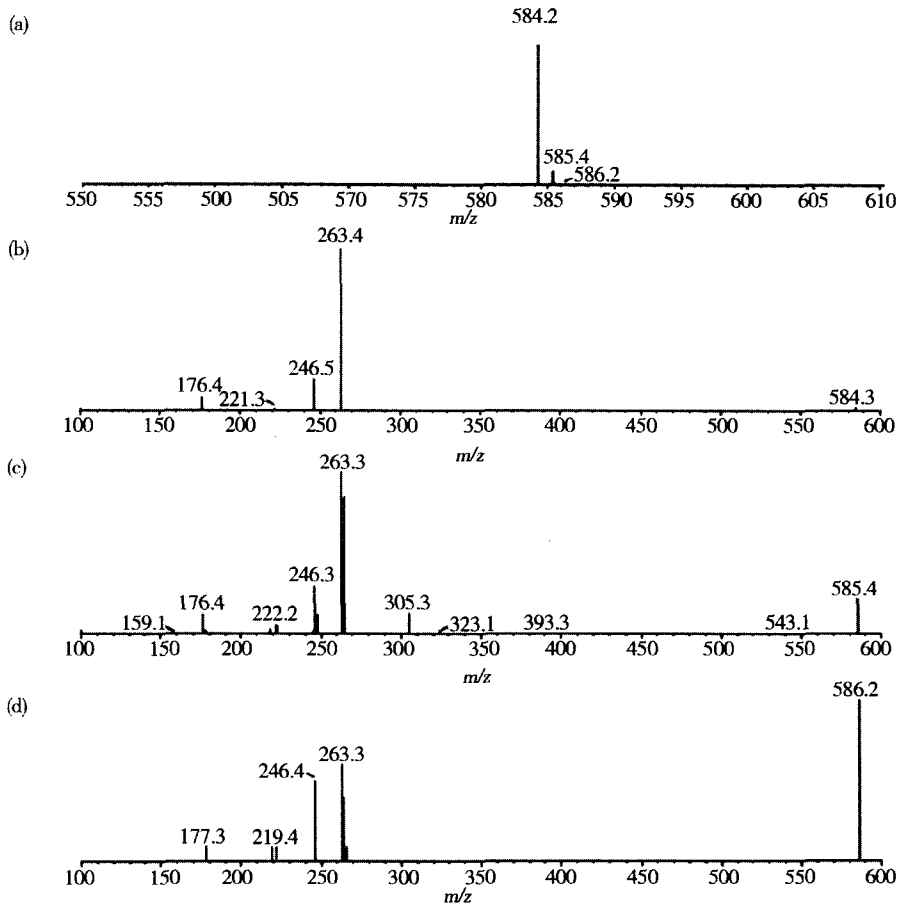


Fig. 4　Q1 full scan mass spectrum (a) and product ion spectra of $[M+H]^+$ (b), $[M+H+1]^+$ (c) and $[M+H+2]^+$ (d) from DHS

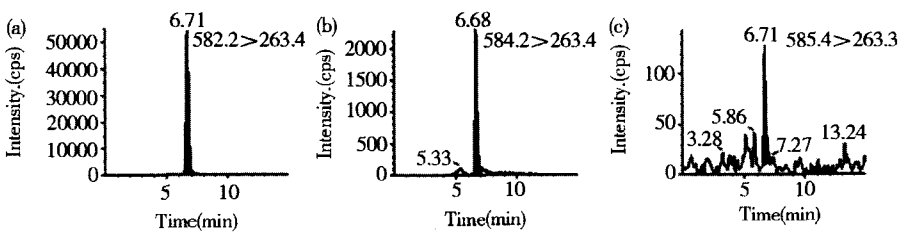


Fig. 5　MRM chromatograms of STR for a sample containing 100 μg/kg STR and 0 μg/kg DHS with the following ion transitions: (a) $[M+H]^+ \rightarrow 263$, (b) $[M+H+2]^+ \rightarrow 263$, (c) $[M+H+3]^+ \rightarrow 263$

2.3 Accuracy and precision

The accuracy and precision of the method were evaluated by determining spiked blank honey samples at three spiked levels (LOQ, 20 μg/kg and 100 μg/kg) in sets of six replicates on three consecutive days. Accuracy was expressed by relative error and precision by relative standard deviation (RSD). Tab. 2 and Tab. 3 summarized the intra- and inter-assay precision and accuracy for STR and DHS. All the results were satisfied.

2.4 Extraction recovery

Extraction recovery was performed by comparing the analytical results for spiked honey samples at three concentration levels (LOQ, 20 μg/kg and 100 μg/kg) subjected to the complete extraction procedure with that for blank honey extracts spiked with both STR and DHS standard solution at the same concentration levels. Average recoveries were 76%, 71% and 78% for STR, and 83%, 87% and 89% for DHS, respectively, at the above concentration levels.

During the analytical procedures, retention times were stable (RSD < 0. 5%) and no deviation of more than 10% from the average ion ratio between quantification transition and the confirmation transition for STR and DHS was observed.

3 Discussion

3.1 Chromatography

The high specificity of tandem mass spectrometry (MS/MS) makes complete chromatographic separation of analytes and matrix unnecessary. However, sufficient chromatographic separation is desired for the analytes with similar molecular weights (≤2 u) to avoid mass spectra overlap. STR and DHS have similar structures, in which, only substituted groups in the middle saccharide ring are different. One is aldehyde group, the other is alcohol hydroxyl group. The very similar structures made the chromatographic separation of STR and DHS difficult. At the present time, only Cherlet et al[16] reported an ion-pair chromatographic system, which allowed a separation of STR and DHS. But a gradual decrease in retention time of DHS was found with increasing life-time of the column due to the low pH (<2) of the mobile phase. The robustness of the method was poor. On the other hand, ion-paring mobile phase could reduce the MS signal[16] and be not well compatible with the electrospray MS system.

Tab. 2 Intra-day accuracy and precision at three concentration levels for STR and DHS spiked in honey ($n=6$)

Analyte	Conc. spiked (μg/kg)	$\bar{x} \pm s$ (μg/kg)	Accuracy (%)	Precision (RSD,%)
STR	0.5	0.534 ±0.047	6.8	8.8
	20	18.86 ±1.13	-5.7	6
	100	95.87 ±5.52	-4.1	5.8
DHS	1	0.903 ±0.063	-9.7	7
	20	20.57 ±1.91	2.8	9.3
	100	93.55 ±5.03	-6.4	5.4

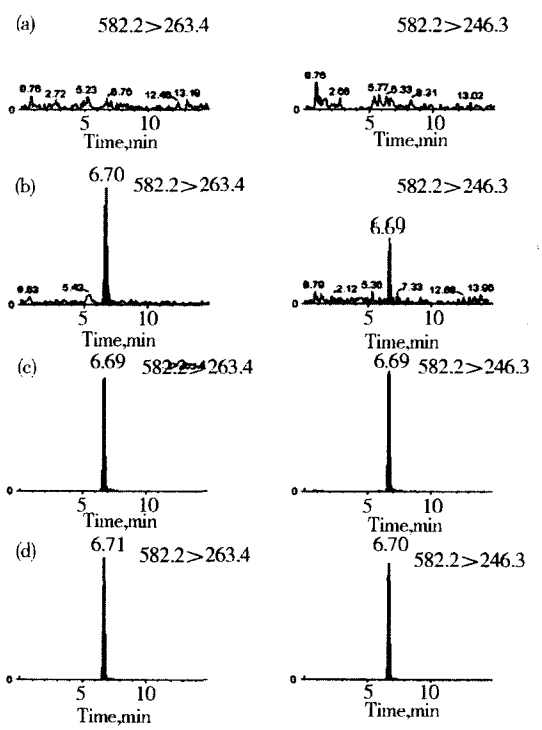


Fig. 6 Typical MRM chromatograms for the quantification (m/z 582. 2 > 263. 4, left) and confirmation (m/z 582. 2 > 246. 3, right) of STR extracted from honey: (a) Blank honey; (b) Blank honey spiked with STR at the LLOQ level (0. 5 μg/kg); (c) Blank honey spiked with 20 μg/kg STR; (d) An incurred honey sample (47 μg/kg)

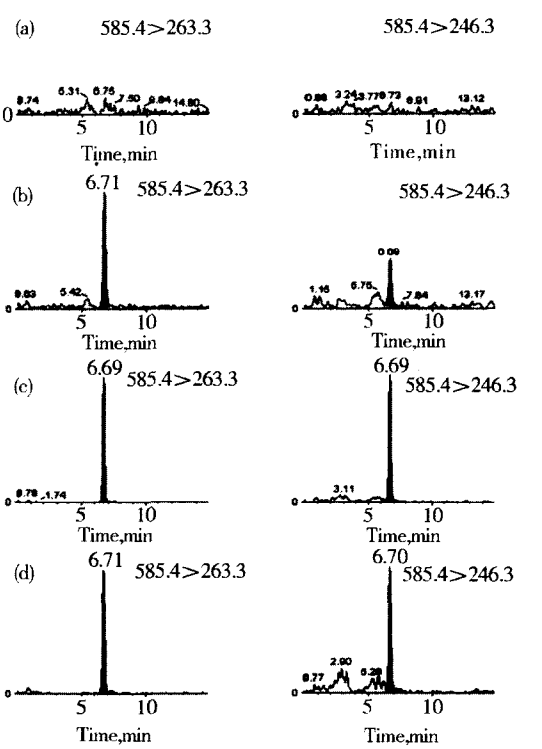


Fig. 7 Typical MRM chromatograms for the quantification (m/z 585. 4 > 263. 3, left) and confirmation (m/z 585. 4 > 246. 3, right) of DHS extracted from honey: (a) Blank honey; (b) Blank honey spiked with DHS at the LLOQ level (1. 0 μg/kg); (c) Blank honey spiked with 20 μg/kg DHS; (d) An incurred honey sample (6. 0 μg/kg)

Tab. 3　Inter-day accuracy and precision at three concentration levels for STR and DHS spiked in honey ($n = 18$)

Analyte	Conc. spiked (μg/kg)	$\bar{x} \pm s$ (μg/kg)	Accuracy (%)	Precision (RSD,%)
STR	0.5	0.481 ±0.056	-3.8	11.6
	20	18.37 ±0.82	-8.2	4.5
	100	103.5 ±8.1	3.5	7.8
DHS	1	0.957 ±0.098	-4.3	10.2
	20	21.37 ±0.64	6.8	3
	100	96.33 ±6.21	-3.7	6.4

In our study, the reversed-phase chromatographic system without ion-pairing reagent was investigated. Referring to Waters application note[23], hydrophilic interaction chromatography (HILIC) coupled with the reported mobile phase was adopted during the early stage of the experiment. Under that condition, these two analytes could be retained on the column; however, a gradual decrease of the signal was observed when 200 mmol/L ammonium formate in the mobile phase was used. It might be derived from the fact that the formed crystal blocked the orifice of the MS detector. Moreover, it was taken into consideration that the high concentration of ammonium formate and formic acid might produce signal suppression effect. As Fig. 2 showed, when the concentration of ammonium formate was fixed at 100 mmol/L, the responses of the STR and DHS reduced gradually with the decrease of formic acid from 100 mmol/L to 10 mmol/L. When the concentration of formic acid was fixed at 100 mmol/L, the same phenomenon was observed to ammonium formate. A sensitive and steady signal was observed in use of 100 mmol/L ammonium formate and formic acid. At this condition, STR and DHS could be eluted with good peak shape and sensitive response at the retention time of about 6.7 min.

3.2 Mass spectrometry

To exclude interferences from the biological matrix and improve specificity, two MRM transitions per compound were monitored. As Fig 6 and Fig 7 showed, the reaction transitions of m/z 582.2 to m/z 263.4 for STR and m/z 585.4 to m/z 263.3 for DHS were used for quantification, while the transitions of m/z 582.2 to m/z 246.3 for STR and m/z 585.4 to m/z 246.3 for DHS were used for confirmation of the identity. Chromatograms corresponding to the two transitions per compound were controlled separately. The ion ratios between the quantification transition and the confirmation transition for STR and DHS were both steady. No interferences were found. A good sensitivity and enough points for the peaks were achieved.

4 Conclusion

In this study, an HILIC-MS/MS method was developed and validated for the determination of highly hydrophilic STR and DHS in honey samples. A simple extraction step followed by a further solid-phase clean-up on WCX SPE columns was described. To decrease or avoid effectively the spectra overlap of STR and DHS, isotopologue ion of DHS (m/z 585) was used as parent ion, which differed 3 mass units from STR. The ion ratios between the quantification and confirmation transitions for STR and DHS enhanced the identity confirmation of the analytes and reduced the risk of false positive results. The method is sensitive, specific and can be used for the routine analysis of STR and DHS residues in honey.

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