Identification, isolation, characterization, and synthesis of impurities of pesticide spirotetramat

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**ABSTRACT**
Research work featured in this article describes the impurity profile of spirotetramat, a widely used broad-spectrum pesticide targeting acetyl-CoA carboxylase. Technical grade spirotetramat from four different sources were analyzed and compared with commercial Movento using UPLC-MS. Seven potential impurities were detected and six of them except for the trans-isomer of spirotetramat were subsequently isolated using preparative HPLC. All impurities were characterized mainly by MS and NMR spectroscopy and their structures were further confirmed by chemical synthesis. The formation of the impurities was described in this report as well.

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Spirotetramat; Movento; impurities; characterization; synthesis; UPLC-MS

**Introduction**
The assessment of drug impurities is one of the essential parts of drug development and is rigorously monitored. Impurities in pesticides, however, receive less attention comparatively. [3] Considering the vast amounts of pesticides that are used globally, even small percentages of impurities may accumulate in the environment and significantly harm the surrounding ecosystem, affecting wildlife and humans. [4,5]

We believe it is worth the effort to identify the impurities found in pesticides so that their bioactivities and toxicities can be studied.

Spirotetramat (1, Fig. 1), the cyclic ketoenol insecticide developed by Bayer CropScience, targets a broad spectrum of sucking insects on crops such as fruit and potatoes. [6–8] Two stereo isomers of spirotetramat, cis- and trans-isomer, are possible, but the cis-isomer is the active constituent. Spirotetramat is a proinsecticide, the hydrolysis of ethyl carbonate provides the corresponding 4-hydroxyprorol-2-one, "spirotetramat-enol" (2, Fig. 1), which functions as an inhibitor of acetyl-CoA carboxylase, a key enzyme in fatty acid biosynthesis. [9] Due to its mechanism of action, it affects primarily the juvenile stages of insects.

To the best of our knowledge, the impurity profile of spirotetramat and the characterization of the impurities have not ever been published yet. In this paper, we report the impurity analyses of technical grade spirotetramat from four different sources and the comparison with Movento from Bayer CropScience. [10] A total of seven impurities were identified on UPLC-MS, six of which were subsequently isolated using preparative HPLC and characterized using NMR spectroscopy. The chemical structures of all seven impurities, including the one that could not be separated in our hands, were further confirmed by chemical synthesis.

**Materials and methods**

**Chemicals and reagents**
Technical grade spirotetramat samples were either purchased commercially or provided from pesticide manufacturers and laboratories. Movento, a 22.4% (w/w%) suspension concentrate of spirotetramat, was purchased commercially. All identified impurities (purity >95%) were either separated using preparative HPLC or synthesized in our laboratory. HPLC grade acetonitrile and methanol were supplied by Honeywell (Shanghai, China). Water was purified using a Milli Q Plus purification system (Millipore, Bedford, MA, USA). Chemicals used in the synthesis of impurities were purchased from Energy Chemical and used without purification unless otherwise stated.

**UPLC-MS and method**
Assay studies were performed on a Waters Acquity UPLC-MS system (Waters PDA Detector, QDa Detector, Sample Manager—FTN, Quaternary Solvent Manager). The analyses of all samples were carried out on an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm) with a mobile phase consisting of 0.1% (v/v) formic acid in water and methanol (38:62, v/v) with a flow rate of 0.3 mL/min for 12 min. The injection volume and detection wavelength were fixed at 2.0 μL and 245 nm, respectively. Column and sample
temperatures were 35 and 25°C, respectively. Mass spectrometry data was acquired in positive ESI mode over an m/z range from 100 to 800 Da. The optimized operating parameters included: capillary voltage of 0.8 kV, cone voltage of 30 V, and source temperature of 120°C. All data were acquired and processed by Waters Empower v. 3 software.

Technical grade spirotetramat samples for UPLC-MS analysis were prepared at a concentration of 1.0 mg/mL in methanol. Movento (22.4%, w/w) was used directly without removing other possible matrix components and was prepared at a concentration of 1.0 mg/mL in methanol for UPLC-MS analysis.

Preparative HPLC and method
Preparative chromatography was performed on a Waters preparative HPLC system (2545 quaternary gradient module, 2998 photodiode array detector, 2707 autosampler, Waters fraction collector III). Separations were carried out on a Waters XBridge C18 OBD column (150 × 30 mm, 5 μm) with a mobile phase consisting of 0.1% (v/v) formic acid in water and acetonitrile (50:50, v/v) at 35 mL/min flow rate for 15 min. The injection volume was 0.4 mL. UV detection was set at 245 nm. Fractions were collected based on time mode. Waters ChromScope v. 2.0 software was used for instrument operation as well as data acquisition and processing.

Technical grade spirotetramat and Movento samples were prepared at a concentration of 100 mg/mL in methanol for preparative HPLC isolation.

NMR spectroscopy
The 1H NMR and 13C NMR spectra were recorded on a Bruker Avance 500 MHz NMR instrument at 25°C using CDCl3 or DMSO-d6 as a solvent and the residual signal of deuterated solvent as the internal standard.

Results and discussion
Detection of impurities by UPLC-MS
Technical grade spirotetramat samples from four different sources, together with Movento, were subject to UPLC-MS analysis using the afore-described UPLC-MS method. The representative UPLC chromatograms are shown in Figure 2 and the retention time of each impurity and spirotetramat in these five samples are summarized in Table 1 (Please see Supplementary Data for impurity profiles of each sample). The targeted impurities (>0.06% content) are marked as
IMP-1 to IMP-6 according to their ascending retention time in sample A. The impurity in Movento at RT 1.973 min is marked as IMP-7. As can be seen, a total of seven impurities were detected even though the identity and concentrations of these impurities varied between the samples. For example, IMP-1 and IMP-4 were present in all four spirotetramat samples and in Movento, whereas IMP-5 exists only in sample A, IMP-7 was only found in Movento. The mass spectra of spirotetramat and each individual impurity that were tested through UPLC-MS are displayed in Figure 3.

**Isolation of impurities by preparative HPLC**

With the exception of IMP-4, which could not be isolated from spirotetramat either on silica gel column or preparative HPLC, the other six impurities were successfully isolated by preparative HPLC using the method discussed in the method section. The isolated solids were characterized by 1H NMR as summarized in Table 2, and 13C NMR which can be found in Supplementary Data.

**Structure elucidation of impurities**

**Impurity 1**

IMP-1 was commonly found in all the samples that were tested, ranging from 0.14 to 0.24%. The ESI mass spectrum exhibited protonated molecular ions at m/z 302 [(M + H)+] and Na+ adduct ion at m/z 324 [(M + Na)+], indicating a molecular weight of 301, which was 72 amu less than that of spirotetramat. The 1H NMR spectrum of IMP-1 indicated the absence of an ethyl group suggesting the hydrolysis of ethyl carbonate. Based on this spectral data, the molecular structure was characterized as cis-3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate, also known as “spirotetramat-enol”.

**Impurity 2**

The ESI mass spectrum of IMP-2 exhibited protonated molecular ions at m/z 360 [(M + H)+] and Na+ adduct ion at m/z 382 [(M + Na)+], indicating a molecular weight of 359, which was 14 amu less than that of spirotetramat. This difference in molecular weight possibly corresponds to a methyl group. Compared with the 1H NMR of spirotetramat, no methoxy signal was found for IMP-2. Furthermore, the fragment ions of the mass spectrum showed m/z 288 and 316, matching the hydrolysis product of IMP-2 (IIb, Fig. 4) and the corresponding formate (IIIb, Fig. 4), respectively. Based on the spectral data, the molecular structure was identified as cis-3-(2,5-dimethylphenyl)-8-hydroxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate.

**Impurity 3**

The ESI mass spectrum of IMP-3 exhibited protonated molecular ions at m/z 360 [(M + H)+] and Na+ adduct ion at m/z 382 [(M + Na)+], indicating a molecular weight of 359, which was identical to that of IMP-2. However, the 1H NMR of IMP-3 showed a singlet signal at δ 3.61 ppm rather than the ethoxy signal in the 1H NMR of spirotetramat. The spectral data confirmed the molecular structure to be cis-3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl methyl carbonate.
Impurity 4

IMP-4, which shared the same protonated molecular ion and similar fragmentation pattern with spirotetramat (Figs. 3 and 4), is the second impurity that was commonly found in all five samples. The composition of IMP-4 ranged from 0.54 to 0.94%. The retention time of IMP-4 and spirotetramat were too close to be effectively separated. Considering the synthetic route of spirotetramat,\(^{\text{[11]}}\) it was speculated that IMP-4 should be the trans isomer of spirotetramat. However, the structure could not be confirmed until the

Figure 3. Mass spectra of spirotetramat and its impurities. spirotetramat (m/z 396.25); IMP-1 (m/z 324.18); IMP-2 (m/z 382.23); IMP-3 (m/z 382.23); IMP-4 (m/z 396.23); IMP-5 (m/z 366.22); IMP-6 (m/z 651.32); IMP-7 (m/z 340.19).
compound was synthesized and analyzed by NMR. The synthesized trans isomer was co-injected with sample A to confirm the identity of IMP-4 based on matching retention times (Fig. 5). This confirmed the identity of IMP-4 as trans-3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate.

**Impurity 5**

The ESI mass spectrum of IMP-5 exhibited protonated molecular ions at m/z 344 [(M + H)⁺] and Na⁺ adduct ion at m/z 366 [(M + Na)⁺], indicating a molecular weight of 343, which was 30 amu less than that of spirotetramat. Therefore, it was likely a methoxyl group fragment. Unlike the ¹H NMR of spirotetramat, no methoxyl signal was found in the ¹H NMR spectrum of IMP-5. Furthermore, the fragment ions of the mass spectrum displayed m/z 272 and 300 (Fig. 4), which matched the hydrolysis product of IMP-5 and the corresponding formate, respectively. Therefore, the molecular structure was determined to be 3-(2,5-dimethylphenyl)-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate.

**Impurity 6**

The ESI mass spectrum of IMP-6 exhibited protonated molecular ions at m/z 629 [(M + H)⁺], Na⁺ adduct ion at m/z 651 [(M + Na)⁺] and K⁺ adduct ion at m/z 667 [(M + K)⁺], indicating a molecular weight of 628. Interestingly, the ¹H NMR of IMP-6 was quite similar to that of IMP-1, and neither spectrum exhibited an ethoxy group signal. Comparatively, the ¹³C NMR of IMP-6 showed a carbon signal at 146.32 ppm, which did not exist in the ¹³C NMR of IMP-1. All the spectral data supported a symmetrical carbonate and therefore, the molecular structure was characterized as bis(cis-3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl) carbonate.

**Impurity 7**

The ESI mass spectrum of IMP-7 exhibited protonated molecular ions at m/z 318 [(M + H)⁺] and Na⁺ adduct ion at m/z 340 [(M + Na)⁺], indicating a molecular weight of 317, which was 16 amu more than that of IMP-1. It was speculated that IMP-7 could be the oxidation product of IMP-1, and the ¹H NMR of IMP-7 supported the speculation very well. The structure of IMP-7 was finally confirmed after the compound was synthesized using well-established oxidation chemistry. It showed that the ¹H NMR of the synthesized compound was the same as the ¹H NMR of IMP-7 isolated from Movento using UPLC. Furthermore, the synthesized IMP-7 was co-injected with Movento to confirm the identity based on matching retention times (Fig. 6). This confirmed the molecular structure of IMP-7 as cis-3-(2,5-dimethylphenyl)-3-hydroxy-8-methoxy-1-azaspiro[4.5]decane-2,4-dione.
Synthesis of impurities

To further confirm the chemical structures of the seven impurities, especially the structure of IMP-4, the synthesis of each impurity was conducted. The spectral data for the isolated impurities from preparative HPLC were found to be identical to those which were prepared.

The synthesis of IMP-2, IMP-4, and IMP-5, was achieved using the synthetic route shown in Scheme 1. Following a modified literature procedure,[12] Strecker reaction of cyclohexanone derivative 3 provided α-aminonitrile 4. The reaction of 4 with 2-(2,5-dimethylphenyl)acetyl chloride gave amide 10, which was transformed to ester 12 via Pinac reaction. The ethylene ketal protecting group was deprotected under the reaction conditions spontaneously. Cyclization of 12 using t-BuOK as the base provided the spiro compound 14. After its reaction with ethyl chloroformate, the product ketone 17 was ready for reduction. It turned out that this transformation was highly substrate-dependent.

With several reducing agents that we tried, such as NaBH4, NaBH3CN, and L-Selectride, IMP-2 was obtained in 10:1 stereoselectivity.[13] α-Cyanoamination reaction of ketone 5 led to cis and trans products in a 1:1 ratio. The two isomers (6 and 7) could be roughly separated via column chromatography. After the reaction of 7 with 2-(2,5-dimethylphenyl)acetyl chloride, the amide was further purified by recrystallization to provide 11 with over 95% purity. Following a similar route mentioned above, IMP-4, the trans isomer of spirotetramat, was obtained. On the other hand, starting from cis-isomer 6, the same procedure provided spirotetramat.
The preparation of IMP-5 was rather straightforward with the experience of synthesizing IMP-2 and IMP-4. Starting from commercially available methyl 1-aminocyclohexanecarboxylate, a three-step transformation provided IMP-5 ultimately.

IMP-1, also named spirotetramat-enol, one of the major metabolites of spirotetramat, could be obtained either by the hydrolysis of spirotetramat or through a synthetic scheme similar to that used to prepare IMP-4, beginning with cis α-aminonitrile. The “spirotetramat-enol” could then be converted to IMP-3 or IMP-6 via its reaction with methyl chloroformate or triphosgene, respectively (Scheme 2). A typical analytical UPLC chromatogram of sample A spiked with the synthesized IMP-1 to IMP-6 is shown in Figure 5.

On the other hand, the oxidation of “spirotetramat-enol” with 2-Iodoxybenzoic acid provided IMP-7 (Scheme 2). An analytical UPLC chromatogram of Movento spiked with the synthesized IMP-7 is shown in Figure 6.

**Formation of impurities**

IMP-1 and IMP-4 are the two impurities that were found to be present in both technical grade spirotetramat samples and commercial Movento. As the active ingredient, IMP-1 may either result from the degradation of spirotetramat or be a residual reactant in the case of an incomplete reaction. IMP-4 is a process-related impurity, its presence depends heavily on the stereo-purity of the starting cis α-aminocyclohexane derivative, such as compound in Scheme 1. IMP-2 could be the metabolite of spirotetramat, otherwise it may also be an unmethylated derivative resulting from the starting material. A possible pathway for the formation of IMP-3 and IMP-6 could be the reaction of spirotetramat-enol with in situ prepared ethyl chloroformate. The presence of methanol would lead to IMP-3 while unreacted triphosgene would provide IMP-6. Since IMP-5 exists only in sample A, it could possibly derive from the corresponding
impurity in the raw starting material. IMP-7 is a known metabolite of spirotetramat[14] that formed over long-time storage, which might explain why it was not found in technical grade spirotetramat.

Conclusions

In conclusion, we have identified and characterized seven impurities that were found among four different technical grade spirotetramat sources and commercial Movento. IMP-1 and IMP-4 were detected in all four sources as well as commercial Movento. Structural elucidation of these impurities is accomplished by UPLC-MS and NMR spectral data followed by chemical synthesis. Moreover, we proposed possible pathways for the formation of the impurities. The present work has made a significant contribution to the impurity analysis of spirotetramat and will be of immense help to the quality control of spirotetramat and its related product.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary Materials.

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