Supporting Information

Stereoselective Metabolism of the Sterol Biosynthesis Inhibitor Fungicides Fenpropidin, Fenpropimorph, and Spiroxamine in Grapes, Sugar Beets, and Wheat

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Separation of Stereoisomers by Chiral HPLC. The enantiomers of fenpropidin and fenpropimorph were separated by HPLC on a chiral Lux Cellulose 3 column (cellulose tris(4-methylbenzoate), 250×4.6 mm, 5 µm particle size, Phenomenex, Torrance, CA), using a Dionex P680 pump and a UVD170U UV/vis detector (Dionex, Sunnyvale, CA). HPLC conditions were as follows: isocratic elution with heptane/isopropanol (99/1 for fenpropidin and 98/2 for fenpropimorph) and 0.1% diethylamine at a flow rate of 1 mL/min and detection at 264 nm. These conditions were used to manually collect fractions of the pure enantiomers. Fraction 1 of fenpropidin was collected at a retention time of ≈3.15-3.25 min and fraction 2 at ≈3.40-3.50 min, fractions 1 and 2 of fenpropimorph at ≈3.2-3.5 min and ≈3.7-4.1 min, respectively (Figure S1). Fractions from 20 injections of ≈1 mg fenpropidin, or 10 injections of ≈1 mg fenpropimorph, initially dissolved in 10 µL of heptane, were combined. The pooled fractions contained ≈3-7 mg of each enantiomer with >99% enantiomeric purity as determined by enantioselective GC-MS/MS (see "Materials and Methods" in the manuscript). The pure enantiomers were then used to treat single sugar beet plants in the greenhouse. These HPLC methods were also used to determine the optical rotation of the two enantiomers of fenpropidin and fenpropimorph. For that, the exit of the UV-cell was connected to a Polar Monitor optical rotation detector (Büchi, Flawil, Switzerland).

The four stereoisomers of spiroxamine could not fully be separated on this chiral column (chromatogram not shown). Nevertheless, using heptane/isopropanol/diethylamine (99.8/0.1/0.1) as eluent at a flow rate of 1 mL/min, assignment of the optical rotation of the four stereoisomers was possible. Fractions of enriched stereoisomers were then collected (fraction 1 at a retention time of ≈3.3-3.4 min, fraction 2 at ≈3.5-3.6 min, fraction 3 at ≈4.3-4.7 min, and fraction 4 at ≈5.0-5.5 min) and analyzed by enantioselective GC-MS/MS.

Fractions of the two diastereomers of spiroxamine were collected after separation on an achiral Luna phenyl-hexyl column (250×3.0 mm, 5 µm, Phenomenex) using 10 mM ammonium acetate buffer (adjusted to pH 8.3 with ammonia) and methanol as eluents, with the following gradient: 60% methanol to 99% methanol within 1 min, followed by 24 min of isocratic elution, then back to initial composition within 1 min, and 4 min equilibration time (flow rate, 0.15 mL/min; detection at 235 nm). Fraction 1 (*trans*-spiroxamine, assigned by GC analysis, see manuscript) was collected at a retention time of ≈22.3-23.5 min and fraction 2 (*cis*-spiroxamine) at ≈23.8-26.0 min. Fractions from 6 injections of ≈8 µg spiroxamine, initially dissolved in 50 µL of water/methanol (86/14), were combined. The pooled fractions contained ≈20 µg of each diastereomer with >99% and 95% purity (*trans*- and *cis*-spiroxamine, respectively) as determined by LC-MS/MS (see below). The diastereomers were used for a hydrolysis experiment.

Hydrolysis of Spiroxamine Diastereomers in Acidic Water. The small amounts of pure *trans*- and *cis*-spiroxamine, collected by achiral HPLC, were used to study the selectivity of hydrolysis and possible interconversion of the diastereomers in acidic water. For that, three 20-mL glass vials were filled with 15 mL of 0.056 M citrate buffer (pH 4.0, Titrisol from Merck, Darmstadt, Germany), closed with screw caps with silicone/PTFE septa, sparged with nitrogen for 15 min, and autoclaved at ≈120°C during 30 min. A methanolic spiking solution of *trans*-, *cis*-, or of the diastereomer mixture of spiroxamine was then added with a gas tight glass syringe (15 µg *trans* or *cis*/30 µg diastereomer mixture in 150 µL) to obtain initial concentrations of ≈1 mg/L of each diastereomer. The vials were placed into an oven at 50°C. Aliquots of 50 µL were taken at defined sampling times (0, 0.17, 0.33, 1, 2, 3, 4.3, 7, 9, 11.3, and 15 d), diluted in 5 mL of water, and frozen until LC-MS/MS analysis.

After thawing, the samples were fortified with fenpropimorph that was used here as an internal standard (50 ng in 50 µL methanol). The compounds were then analyzed with liquid chromatography - tandem mass spectrometry. The instrument was configured with an autosampler (HTS PAL, CTC Analytics, Zwingen, Switzerland), a binary HPLC pump (Agilent 1100 Series, with micro-vacuum degasser, Agilent, Santa Clara, CA), an auxiliary HPLC pump (Jasco PU980, Gross-Umstadt, Germany), and a triple quadrupole mass spectrometer (API 4000, with turbo ion spray source, AB Sciex, Framingham, MA).

Enrichment of the analytes was achieved by online coupling of a SPE cartridge (cartridge pre-column packed with Gemini C_{18}, 8×3 mm, 5 µm, Phenomenex) to the achiral HPLC column (Luna phenyl-hexyl, see above) using a column switching technique with a dual 6-port valve system (instrumental scheme, see Figure S1 in supporting information of ref[1]). First, sample volumes of 1 mL were injected into a PEEK loop. The valves were then switched so that the sample was transferred to the SPE cartridge during 90 s with H_2O as mobile phase (flow rate, 1 mL/min, delivered by the auxiliary HPLC pump). After transfer was complete, the valves were switched to allow elution of the enriched compounds to the analytical column.

The HPLC method described above for preparation of the pure diastereomers of spiroxamine was slightly adapted for LC-MS/MS analysis. The gradient was as follows: initial composition 10 mM ammonium acetate buffer/methanol (40/60), gradient to 100% methanol within 1 min, followed by 26 min of isocratic elution, then back to initial composition within 3 min, and 5 min equilibration time (flow rate, 0.2 mL/min). The mass spectrometer was operated in positive mode (ion spray voltage, 4 kV; 400°C) and the following ion transitions were monitored: spiroxamine, *m/z* 298→144 with a collision energy of 27 eV (and for confirmatory purposes, *m/z* 298→100, 42 eV) and fenpropimorph, *m/z* 304→147, 41 eV (*m/z* 304→98, 42 eV). Quantification was based on peak area ratios relative to the internal standard and in reference to suitable standard solutions.

Field Trials with Grapes. Two field trials were conducted in a vineyard at the research institute of Agroscope in Wädenswil, Switzerland (coordinates, 47°13’30’’N, 8°40’37’’E). The grapevines (variety Riesling-Silvaner) had been planted in 1998 in rows and were Guyot cane pruned (≈1.9 m distance between rows and ≈0.9 m between the individual plants). On July 15, 2014, the fungicides Astor and Prosper were separately applied to 19 grapevines each, at BBCH growth stage 77 (bunch closure). One row was left untreated for blank samples. Application rates of Astor and Prosper were 0.4 and 0.8 L/ha, respectively, corresponding to 300 g fenpropidin/ha and 400 g spiroxamine/ha. During application, care was taken not to spray plants of the other trial, since fenpropidin was used as internal standard for spiroxamine, and vice versa. Moreover, the amount of internal standard added to the homogenized plants (10 mg/kg, see chapter “Extraction” in the manuscript) was clearly higher than potential residues resulting from spray drift of the adjacent field trial. The grapevines were also treated with a number of further pesticides (Table S1). Some basic weather data during the field trials (daily mean temperatures and precipitation) are available in Table S2. Leaf samples were taken three hours, 8 d, 14 d, 30 d, 50 d, and 76 d after application, and were stored in a freezer at -20°C before they were homogenized and extracted. Young leaves, which developed after fungicide application, were not sampled. Mature grapes were harvested on September 29 (day 76).

Field Trials with Wheat. Spring wheat (variety Fiorina) was sown on March 17, 2014 with a density of ≈520 seeds/m^2 on a 6.6×30 m plot at Agroscope in Wädenswil (47°13'19''N, 8°40'12''E). On May 05, the fungicides Capalo, Input Xpro, and Orius Top were separately applied to two subplots of 3×7 m each, at BBCH growth stage 31 (first node at least 1 cm above tillering node). Two further subplots were left untreated. Since fenpropidin and spiroxamine were used as internal standards for each other, an untreated subplot or a subplot treated with fenpropimorph was cultivated between these subplots, to minimize possible cross-contamination from spray drift (note that already in directly adjacent, fenpropimorph-treated wheat from the day of application, only traces of spiroxamine and fenpropidin were found, <0.1 and <0.01 mg/kg, respectively). Application rates of Capalo, Input Xpro, and Orius Top were 2.0, 1.5, and 1.5 L/ha, respectively, corresponding to 400 g fenpropimorph/ha, 375 g spiroxamine/ha, and 225 g fenpropidin/ha. For further pesticide applications and weather data, see Tables S1 and S2. Samples of wheat forage were taken two hours, 14 d, 29 d, and 57 d after application, and were stored at -20°C before they were homogenized and extracted. Mature wheat was harvested on July 23, 79 d after application, but was not analyzed since already in the previous sampling, very low residues were observed.

Field Trials with Sugar Beets. Sugar beets were sown in rows on March 19, 2014 with a density of 11 seeds/m^2 (≈50 cm distance between rows and ≈18 cm between seeds) on a 44×100 m plot at Agroscope in Zürich, Switzerland (47°25'45''N, 8°30'52''E). Two varieties of treated seeds were sown in alternating rows, Ribera (Syngenta) with 4 g thiram, 18 g hymexazol, 60 g thiamethoxam, and 8 g tefluthrin per 100'000 seeds, and Amalia (KWS) with 4 g thiram, 18 g hymexazol, and 90 g imidacloprid per 100'000 seeds. On July 17, the fungicide Opus Top was applied at BBCH growth stage 45 (crop cover complete). The application rate of 1.2 L/ha corresponded to 300 g fenpropimorph/ha. For further pesticide applications and weather data, see Tables S1 and S2. In this field trial, possible leaching of pesticides to a drainage system was also investigated, and for that, potassium bromide was applied as a conservative tracer (on March 14, 29 kg/ha). Samples of sugar beet leaves were taken 20 hours, 6 d, 11 d, 14 d, 20 d, 32 d, 48 d, and 77 d after application, and were stored at -20°C before they were homogenized and extracted. Roots were not extracted since residues were expected to be very low.[2]

An additional small field trial with fenpropidin was performed in a private garden in Zürich (47°23'54''N, 8°35'15''E). Twelve seeds of the sugar beet variety Amalia (see above) were sown on March 19, 2014 (≈25 cm distance between seeds). On July 17, the fungicide Spyrale was applied with a target application rate of 0.1 mL/m^2, corresponding to 375 g fenpropidin/ha. Other pesticides were not used, except for the molluscicide metaldehyde that was applied weekly in March and April, and every 3-7 d in May and June. Samples of sugar beet leaves were taken two hours, 6 d, 14 d, 26 d, 42 d, and 62 d after application.

Application of Pure Enantiomers of Fenpropidin and Fenpropimorph to Sugar Beets in the Greenhouse. The small amounts of pure enantiomers of fenpropidin and fenpropimorph, collected by HPLC (see above), were sufficient to study the possible interconversion of enantiomers in single sugar beet plants grown in a greenhouse in Wädenswil. For that, 1-L pots were filled with soil from the trial site in Zürich (47°25'45''N, 8°30'52''E). On November 27, 2013, one seed grain was sawn per pot (variety Amalia, see above) and then kept in the greenhouse (mean temperature, 16°C; relative moisture, 60%; additional artificial light until March).

Fungicide applications were performed in April 2014, when the sugar beets had developed 10-15 leaves (height of plants, ≈30 cm). The pure enantiomers were initially dissolved in the HPLC eluent heptane/isopropanol, solvents that may cause phytotoxic injuries when sprayed on plants. Therefore, the solvents were carefully evaporated to a small volume and replaced by ethanol. A volume of 0.5-1.0 mL of these ethanolic solutions was then applied with a mini perfume sprayer to one sugar beet plant each, corresponding to amounts of ≈1-2 mg of the pure enantiomers. Leaf samples were taken 1 hour, 14 or 17 d, 31 or 35 d, and 49 or 51 d after application. These samples were extracted immediately.

Figure S1

Enantioselective HPLC separation of fenpropidin and fenpropimorph using the chiral Lux Cellulose 3 column with UV detection (arbitrary scales). Injected amounts were ≈0.01 mg (lower traces) or ≈1 mg (upper traces, used to collect fractions).

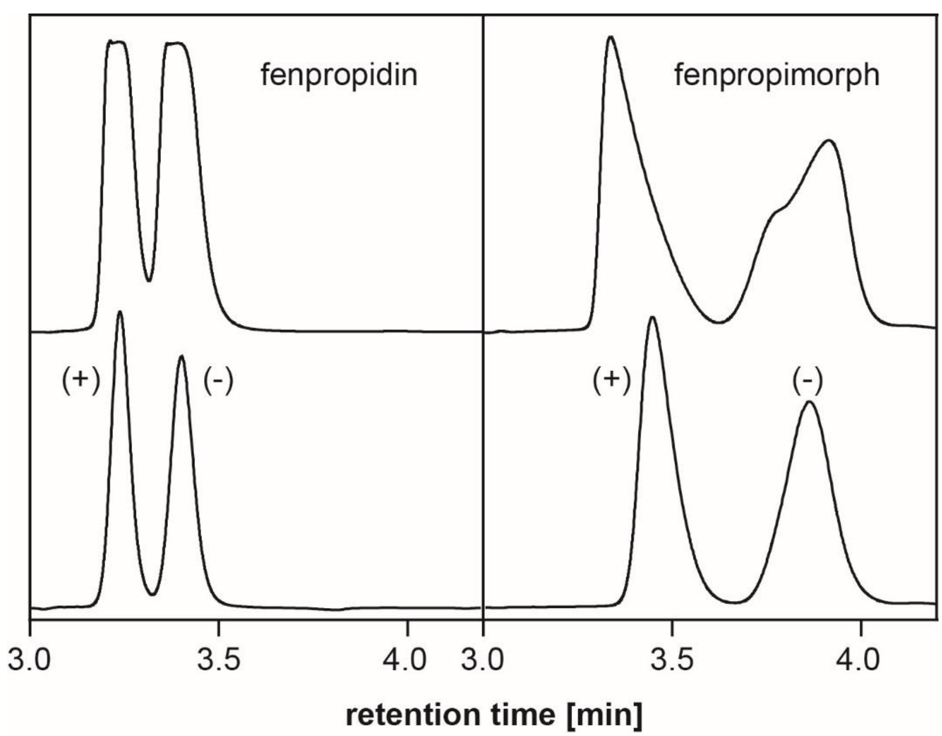

Table S1
Pesticide Applications in Field Trials with Grapes, Spring Wheat, and Sugar Beets, in 2014.

date	grapes	spring wheat	sugar beets
March 21			0.5 L/ha Dual Gold
April 07	4.0 L/ha Oleodan		
April 15			1.5 L/ha Sugaro Gamma and 1.5 L/ha Sugaro Pro
April 16	5.0 L/ha Roundup		
April 17	1.2 kg/ha Folpet 80 WDG		
May 05		**2.0 L/ha Capalo or 1.5 L/ha Input Xpro or 1.5 L/ha Orius Top**	
May 20 and 28	1.25 kg/ha Folpet 80 WDG and 2.0 kg/ha Tiovit Jet		
June 03			1.5 L/ha Sugaro Gamma and 1.5 L/ha Sugaro Pro
June 05	1.5 kg/ha Folpet 80 WDG and 2.4 kg/ha Tiovit Jet	3 L/ha Banvel M	
June 13	1.5 kg/ha Folpet 80 WDG and 2.4 kg/ha Tiovit Jet		
June 25	2.0 kg/ha Folpet 80 WDG and 3.2 kg/ha Tiovit Jet		
July 02	5.0 L/ha Roundup		
July 07	2.0 kg/ha Folpet 80 WDG and 3.2 kg/ha Tiovit Jet		
July 15	0**.4 L/ha Astor or 0.8 L/ha Prosper**		
July 17	2.0 kg/ha Folpet 80 WDG		**1.2 L/ha Opus Top** in tank mix with 1.0 L/ha Allegro
August 08	4.0 kg/ha Cuprosan U-DG		

Allegro: 125 g/L epoxiconazole, 125 g/L kresoxim-methyl
Astor: 750 g/L fenpropidin
Banvel M: 360 g/L MCPA, 30 g/L dicamba
Capalo: 200 g/L fenpropimorph, 75 g/L metrafenone, 62.5 g/L epoxiconazole
Cuprosan U-DG: 36% folpet, 18% copper
Dual Gold: 960 g/L *S*-metolachlor
Folpet 80 WDG: 80% folpet
Input Xpro: 250 g/L spiroxamine, 100 g/L prothioconazole, 50 g/L bixafen
Oleodan: 96 g/L chlorpyriphos-methyl, 701 g/L rapeseed oil
Opus Top: 250 g/L fenpropimorph, 84 g/L epoxiconazole
Orius Top: 150 g/L fenpropidin, 200 g/L prochloraz, 100 g/L tebuconazole
Prosper: 500 g/L spiroxamine
Roundup: 480 g/L glyphosate
Sugaro Gamma: 700 g/L metamitron
Sugaro Pro: 15 g/L desmedipham, 115 g/L ethofumesate, 75 g/L phenmedipham
Tiovit Jet: 80% sulfur

Table S2
Daily Mean Temperatures [T, °C] and Precipitation [P, mm] in the Field Trials with Grapes, Spring Wheat, and Sugar Beets, in 2014.

Wädenswil, vineyard			**Wädenswil, spring wheat**			**Zürich, sugar beets**		
Date	T	P	Date	T	P	Date	T	P
15-Jul	19.8	0.0	5-May	11.4	0.0	17-Jul	21.6	0.0
16-Jul	21.0	0.0	6-May	15.1	0.0	18-Jul	22.2	0.0
17-Jul	22.3	0.0	7-May	11.8	34.8	19-Jul	23.0	0.0
18-Jul	23.0	0.0	8-May	14.6	0.0	20-Jul	20.7	11.8
19-Jul	24.2	0.0	9-May	15.2	4.6	21-Jul	17.1	12.7
20-Jul	22.3	7.8	10-May	17.0	0.2	22-Jul	17.6	27.9
21-Jul	17.0	19.2	11-May	11.5	18.4	23-Jul	20.7	0.1
22-Jul	17.5	38.0	12-May	9.9	1.2	24-Jul	19.3	0.0
23-Jul	20.9	0.0	13-May	8.2	8.2	25-Jul	20.6	0.4
24-Jul	20.4	0.0	14-May	7.8	11.6	26-Jul	17.2	10.3
25-Jul	21.1	0.0	15-May	7.8	7.8	27-Jul	17.7	0.7
26-Jul	17.3	11.8	16-May	10.6	0.2	28-Jul	18.7	4.5
27-Jul	17.3	0.8	17-May	12.6	0.0	29-Jul	16.7	6.1
28-Jul	18.9	42.8	18-May	13.9	0.0	30-Jul	17.1	5.0
29-Jul	17.2	4.2	19-May	17.8	0.0	31-Jul	19.7	0.0
30-Jul	17.5	4.8	20-May	18.4	0.0	1-Aug	18.9	0.0
31-Jul	20.4	0.2	21-May	20.4	0.0	2-Aug	19.8	1.1
1-Aug	19.8	0.0	22-May	19.8	4.0	3-Aug	18.0	5.4
2-Aug	20.0	2.8	23-May	14.1	16.4	4-Aug	18.6	0.5
3-Aug	18.0	3.6	24-May	14.9	0.0	5-Aug	19.1	0.0
4-Aug	18.5	5.4	25-May	17.0	0.0	6-Aug	18.1	0.0
5-Aug	18.6	1.8	26-May	16.1	0.0	7-Aug	19.3	0.0
6-Aug	19.0	0.0	27-May	12.5	9.4	8-Aug	21.0	0.0
7-Aug	20.0	0.0	28-May	13.9	0.0	9-Aug	20.7	5.5
8-Aug	21.1	2.8	29-May	14.9	1.8	10-Aug	21.0	15.0
9-Aug	21.4	1.8	30-May	12.4	4.2	11-Aug	16.7	19.1
10-Aug	22.1	26.2	31-May	12.4	0.0	12-Aug	17.1	0.0
11-Aug	16.7	36.4	1-Jun	15.3	0.0	13-Aug	15.5	2.1
12-Aug	18.1	0.0	2-Jun	15.2	1.4	14-Aug	14.9	2.3
13-Aug	16.1	3.8	3-Jun	14.9	2.4	15-Aug	13.2	11.5
14-Aug	16.3	0.0	4-Jun	13.9	5.6	16-Aug	13.4	2.0
15-Aug	13.1	21.4	5-Jun	15.3	0.2	17-Aug	14.2	0.0
16-Aug	13.2	3.8	6-Jun	18.9	0.0	18-Aug	16.1	0.0
17-Aug	15.2	0.0	7-Jun	22.3	0.0	19-Aug	16.0	3.5
18-Aug	17.3	0.0	8-Jun	24.1	0.0	20-Aug	15.6	0.0
19-Aug	16.6	3.4	9-Jun	25.7	1.8	21-Aug	15.2	0.0
20-Aug	16.0	2.4	10-Jun	25.3	14.6	22-Aug	15.2	0.0
21-Aug	15.8	0.2	11-Jun	22.8	0.0	23-Aug	13.6	1.2
22-Aug	16.4	0.0	12-Jun	22.0	11.4	24-Aug	13.2	3.5
23-Aug	14.7	0.4	13-Jun	19.7	10.4	25-Aug	13.2	0.6
24-Aug	13.9	5.6	14-Jun	18.2	0.0	26-Aug	16.0	15.4
25-Aug	14.2	0.6	15-Jun	17.7	0.0	27-Aug	16.7	4.3
26-Aug	15.5	11.4	16-Jun	18.4	0.0	28-Aug	18.0	0.0

27-Aug	17.2	3.8	17-Jun	18.2	0.0	29-Aug	17.6	0.7
28-Aug	18.6	0.0	18-Jun	18.0	0.0	30-Aug	18.0	0.0
29-Aug	18.2	14.6	19-Jun	18.5	0.0	31-Aug	14.3	8.0
30-Aug	18.3	1.2	20-Jun	18.2	0.0	1-Sep	13.8	2.0
31-Aug	15.0	19.8	21-Jun	18.7	0.0	2-Sep	14.4	0.0
1-Sep	13.9	1.4	22-Jun	20.1	0.0	3-Sep	15.5	0.0
2-Sep	14.5	0.0	23-Jun	18.3	13.4	4-Sep	17.3	0.0
3-Sep	16.2	0.0	24-Jun	16.5	24.6	5-Sep	18.0	0.0
4-Sep	17.8	0.0	25-Jun	16.5	8.4	6-Sep	18.6	0.0
5-Sep	18.1	0.8	26-Jun	17.5	0.2	7-Sep	18.8	0.0
6-Sep	18.8	0.0	27-Jun	19.9	2.0	8-Sep	19.6	0.0
7-Sep	19.1	0.0	28-Jun	19.9	6.0	9-Sep	18.5	0.8
8-Sep	19.9	0.0	29-Jun	14.7	26.0	10-Sep	17.5	0.0
9-Sep	18.8	1.2	30-Jun	14.0	2.0	11-Sep	14.4	0.4
10-Sep	18.1	0.0	1-Jul	16.6	0.0	12-Sep	11.9	3.2
11-Sep	14.9	1.4	2-Jul	14.7	12.2	13-Sep	13.8	0.4
12-Sep	12.9	2.2	3-Jul	18.3	0.0	14-Sep	15.2	0.0
13-Sep	14.5	0.0	4-Jul	20.2	1.8	15-Sep	16.1	0.0
14-Sep	15.7	0.0	5-Jul	18.3	2.6	16-Sep	16.6	0.0
15-Sep	16.7	0.0	6-Jul	22.0	0.4	17-Sep	17.0	0.0
16-Sep	17.7	0.0	7-Jul	19.3	3.0	18-Sep	17.2	0.0
17-Sep	17.9	0.0	8-Jul	14.0	20.8	19-Sep	18.4	1.7
18-Sep	17.9	0.0	9-Jul	12.1	22.0	20-Sep	18.3	0.9
19-Sep	19.2	0.0	10-Jul	12.2	8.4	21-Sep	15.9	53.5
20-Sep	19.2	0.0	11-Jul	16.1	6.8	22-Sep	12.4	2.5
21-Sep	15.9	36.8	12-Jul	17.2	8.6	23-Sep	10.1	0.0
22-Sep	12.9	3.4	13-Jul	17.2	6.2	24-Sep	11.8	1.7
23-Sep	11.6	0.0	14-Jul	18.1	3.8	25-Sep	12.1	1.2
24-Sep	13.4	9.8	15-Jul	19.4	0.0	26-Sep	11.1	0.0
25-Sep	11.8	2.0	16-Jul	20.7	0.0	27-Sep	13.8	0.0
26-Sep	12.2	0.0	17-Jul	22.2	0.0	28-Sep	13.7	0.0
27-Sep	14.3	0.0	18-Jul	23.0	0.0	29-Sep	15.8	0.1
28-Sep	15.3	0.0	19-Jul	24.2	0.0	30-Sep	15.8	1.0
			20-Jul	22.0	8.0	1-Oct	16.2	8.7
			21-Jul	16.7	17.4	2-Oct	15.5	0.0
			22-Jul	17.1	38.2			

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