SUPPORTING INFORMATION

Fluxapyroxad haptens and antibodies for highly sensitive immunoanalysis of food samples

Josep V. Mercader, Antonio Abad-Somovilla, Consuelo Agulló, Antonio Abad-Fuentes

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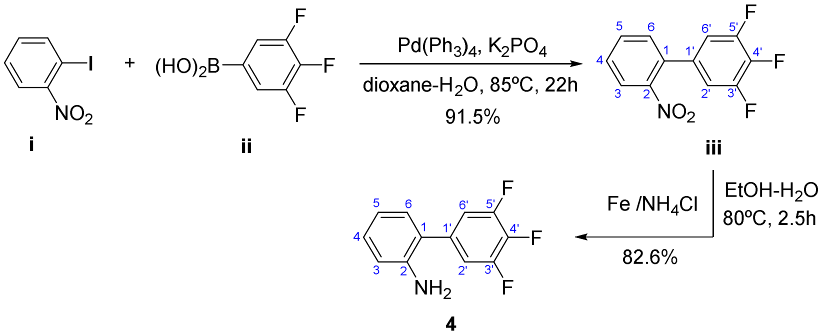
General experimental procedures and techniques

Solvents and reagents were purified by standard methods. All operations involving air-sensitive reagents were performed under an inert atmosphere of dry nitrogen or argon using syringe and cannula techniques, oven-dried glassware, and freshly distilled and dried solvents. The progress of reactions was monitored by thin layer chromatography (TLC) performed on F254 silica gel plates. The plates were visualized at 254 nm by immersion with aqueous ceric ammonium molybdate and heating. Column chromatography refers to flash chromatography and was performed on Merck silica gel 60, 230–400 mesh. All melting points were determined using a Kofler hot-stage apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on Bruker spectrometers (Billerica, MA, USA), in the solvent indicated, at 300 MHz and ^{13}C-NMR spectra at 75 MHz. ^{19}F-NMR spectra were acquired at 282 MHz with high power proton decoupling. All proton and carbon spectra were referenced to residual solvent (^{1}H-NMR: 7.26 ppm for $CDCl_3$); ^{13}C-NMR: 77.00 ppm for $CDCl_3$ and 49.00 ppm for CD_3OD). ^{19}F spectra were referenced to $CFCl_3$ as the internal reference which was set at δ 0.00 ppm. Carbon substitution degrees were established by DEPT pulse sequences. Abbreviations used for NMR signals are as follows: s =singlet, d = doublet, dd = double doublet, ddd = doublet of double doublet, t = triplet, dt = double triplet, ddt = double doublet of triplets, q = quadruplet, br = broad, quint = quintuplet, m = multiplet, BiPh = Biphenyl ring, Pz = Pyrazol ring. IR spectra were recorded using a Nicolet Avatar 320 FT-IR spectrophotometer using liquid films or ATR for solids. IR band intensities: w = weak, m = medium, s = strong. High-resolution mass spectra (HRMS) were run by the electrospray (ES) mode, which was obtained with a Q-TOF premier mass spectrometer with an electrospray source (Waters, Manchester, UK). The molar extinction coefficient of haptens was determined in 100 mM phosphate buffer (PB), pH 7.4.

Preparation of 3',4',5'-trifluoro-[1,1'-biphenyl]-2-amine (4)



Synthesis of 3',4',5'-trifluoro-2-nitro-1,1'-biphenyl (**iii**). $Pd(PPh_3)_4$ (92.8 mg, 0.080 mmol, 0.04 equiv) was added under nitrogen to a mixture of (3,4,5-trifluorophenyl)boronic acid (**ii**, 423.8 mg, 2.409 mmol, 1.2 equiv), 1-iodo-2-nitrobenzene (**i**, 500 mg, 2.008 mmol), and K_3PO_4 (1.278 g, 6.020 mmol, 3 equiv) in a mixture of degassed dioxane (10 mL) and water (2 mL). The resulting mixture was stirred at 85 ^{o}C for 22 h, cooled to room temperature, diluted with water and extracted with EtOAc. The organic extracts were washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure to yield yellowish oil that was purified by chromatography, using hexane–EtOAc 95:5) as eluent, to afford biaryl derivative **6** (465.2 mg, 91.5%) as an amorphous solid. ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 7.94 (1H, dd, *J* = 8.0, 1.2, Hz, H-3), 7.66 (1H, td, *J* = 7.6, 1.4 Hz, H-5), 7.56 (1H, td, *J* = 7.8, 1.5 Hz, H-4), 7.39 (1H, dd, *J* = 7.6, 1.4, Hz, H-6), 7.00–6.88 (2H, m, H-2' and H-6'). ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm): −133.9 (2F, d, *J* = 20.6 Hz, F-3' and F-5'), −161.1 (1F, t, *J* = 20.6 Hz, F-4'). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm): 151.1 (ddd, *J* = 251.2, 10.1, 4.1 Hz, C-3' and C-5'), 148.7 (C-1), 139.8 (dt, *J* = 253.5, 15.2 Hz, C-4'), 133.5 (C-2), 134.4 (td, *J* = 8.2, 5.1 Hz, C-1'), 132.4 (C-6), 131.6 (C-5), 129.4 (C-4), 124.5 (C-3), 112.6 (dd, *J* = 15.0, 7.3 Hz, C-2' and C-6').

Synthesis of 3',4',5'-trifluoro-[1,1'-biphenyl]-2-amine (**4**). A solution of NH_4Cl (49.1 mg, 0.917 mmol, 0.5 equiv)) in water (1.8 mL) was added dropwise to a solution of nitro-biphenyl **iii** (464.4 mg, 1.835 mmol) in EtOH (5.7 mL). Iron powder (328.1 mg, 5.872 mmol, 3.2 equiv) was added in small portions to the resulting turbid solution and the reaction mixture was heated at 80 ^{o}C with stirring for 2.5 h, cooled to room temperature, and filtered. The filtrate was diluted with water and extracted with EtOAc, the combined extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give a residue that was purified by chromatography, using hexane–Et_2O 9:1 as eluent, to afford aminobiphenyl **4** (338.1 mg, 82.6%) whose spectroscopic properties were in accord with literature reports.[1] ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 7.18 (1H, ddd, *J* = 8.0, 7.4, 1.6, H-6), 7.15–7.02 (3H, m, H-2', H-6' and H-4), 6.82 (1H, td, *J* = 7.5, 1.2 Hz, H-5), 6.76 (1H, br dd, *J* = 8.0, 1.2 Hz, H-3), 3.73 (2H, br s, NH_2). ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm): −134.5 (2F, d, *J* = 20.6 Hz, F-3' and F-5'), −162.8 (1F, t, *J* = 20.6 Hz, F-4'). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm): 151.3 (ddd, *J* = 250.6, 10.0, 4.4 Hz, C-3' and C-5'), 143.25 (C-2), 138.9 (dt, *J* = 251.6, 15.3 Hz, C-4'), 135.5 (td, *J* = 7.7, 4.5 Hz, C-1'), 130.1 (C-4), 129.5 (C-6), 124.4 (C-1), 118.9 (C-5), 116.0 (C-3), 113.2 (dd, *J* = 14.4, 6.6 Hz, C-2' and C-6').

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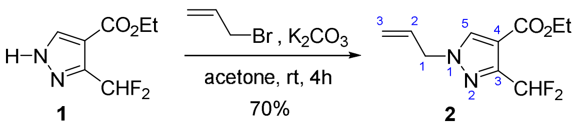
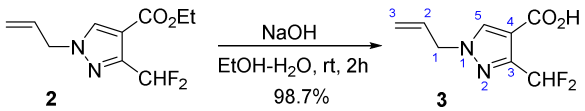
[1] a) Maleckis, A.; Kampf, J. W.; Sanford, M. S. A detailed study of acetate-assisted C-H activation at palladium(IV) centers. *J. Am. Chem. Soc*. *135*(17), 6618-6625, **2013**. b) Jasch, H.; Scheumann, J.; Heinrich, M. R. Regioselective radical arylation of anilines with arylhydrazines. *J. Org. Chem*. *77*(23), 10699-10706, **2012**.

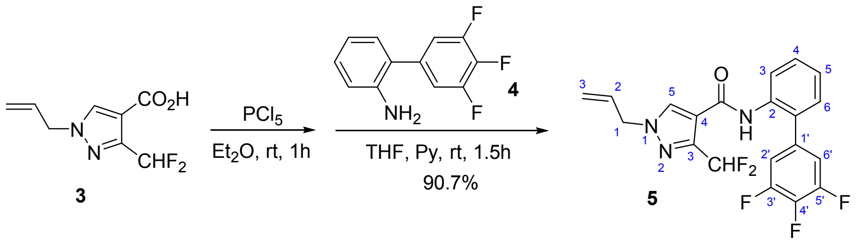
Characterization data of intermediates of the synthesis of hapten FX*n*



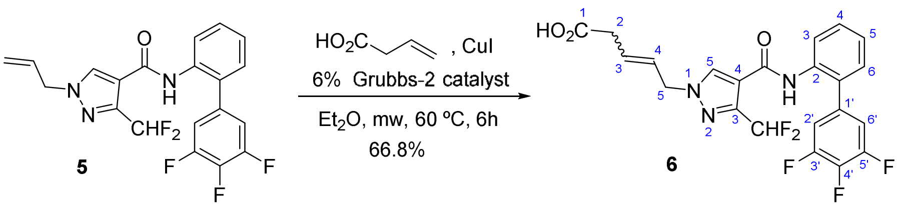
Characterization data of ethyl 1-allyl-3-(difluoromethyl)-1H-pyrazole-4-carboxylate (**2**). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 7.93 (1H, t, *J* = 1.2 Hz, H-5 Pz), 7.10 (1H, t, *J* = 54.0 Hz, CHF_2), 6.02 (1H, ddt, *J* =16.4, 10.2, 6.2 Hz, H-2 allyl), 5.40–5.27 (2H, m, H-3 allyl), 4.79 (2H, br d, *J* = 6.2 Hz, H-1 allyl), 4.31 (2H, q, *J* = 7.1 Hz, OCH_2CH_3), 1.35 (3H, t, *J* = 7.1 Hz, OCH_2CH_3), ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm): −116.6 (s). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm): 161.8 (CO_2), 146.2 (t, *J* = 24.7 Hz, C-3 Pz), 133.9 (C-5 Pz), 131.1 (C-2 allyl), 120.5 (C-3 allyl), 115.0 (C-4 Pz), 109.3 (t, *J* = 236.8 Hz, CHF_2), 60.7 (OCH_2CH_3), 55.5 (C-1 allyl), 14.2 (OCH_2CH_3).



Characterization data of 1-allyl-3-(difluoromethyl)-1H-pyrazole-4-carboxylic acid (**3**). Mp 102.5–103.8 °C (from hexane–EtOAc). IR (neat) ν_{max} (cm^{-1}): 3300–2350 (br, m), 2145 (w), 2989 (w), 1685 (s), 1681 (w), 1542 (s), 1278 (s), 1076 (s), 1049 (s), 863 (m). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 11.0 (1H, br s, CO_2H), 8.01 (1H, br t, *J* = 1.0 Hz, H-5 Pz), 7.10 (1H, t, *J* = 53.8 Hz, CHF_2), 6.03 (1H, ddt, *J* = 16.4, 10.2, 6.2 Hz, H-2 allyl), 5.42-5.28 (2H, m, H-3 allyl), 4.81 (2H, br d, *J* = 6.2 Hz, H-1 allyl). ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm): −116.7 (s). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm): 167.2 (CO_2H), 146.9 (t, *J* = 24.7 Hz, C-3 Pz), 134.9 (C-5 Pz), 130.8 (C-2 allyl), 120.9 (C-3 allyl), 112.3 (t, *J* = 3.2 Hz, C-4 Pz), 109.1 (t, *J* = 237.2 Hz, CHF_2), 55.6 (C-1 allyl). HRMS (TOF MS ES+): *m/z* calcd for $C_8H_9F_2N_2O_2$ $[M+H]^+$ 203.0627, found 203.0624.

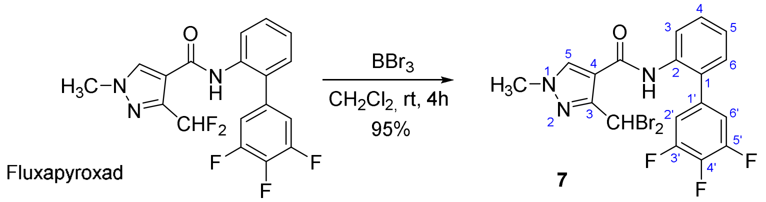


Characterization data of 1-allyl-3-(difluoromethyl)-N-(3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)-1H-pyrazole-4-carboxamide (**5**). IR (neat) ν_{max} (cm^{-1}): 3209 (s), 3126 (m), 2995 (m), 1632 (s), 1618 (s), 1534 (s), 1482 (s), 1065 (s), 1042 (s), 760 (s). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.18 (1H, d, *J* = 8.4 Hz, H-3 BiPh), 8.01 (1H ,s, H-5 Pz), 7.84 (1 H, br s, NH), 7.48–7.38 (1H, m, H-4 BiPh), 7.24–7.19 (2H, m, H-5 and H-6 BiPh), 7.06–6.94 (2H, m, H-2’ and H-6’ BiPh), 6.67 (1H, t, *J* = 54.2 Hz, CHF_2), 5.99 (1H, ddt, *J* = 16.9, 10.2, 6.2 Hz, H-2 allyl), 5.37 (1H, dq, *J* = 10.2, 1.2 Hz, H-1 allyl), 5.31 (1H, dtd, *J* = 17.0, 1.5, 1.0 Hz, H’-1 allyl), 4.73 (2H, d, *J* = 6.2 Hz, H-3 allyl). ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm): −109.1 (2F, s, CHF_2), −134.2 (2F, d, *J* = 20.6 Hz, F-3’ and F-5’ BiPh), −162.0 (1F, t, *J* = 20.6 Hz, F-4’ BiPh). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm): 159.4 (CONH), 151.3 (ddd, *J* = 251.2, 10.0, 4.1 Hz, C-3’ and C-5’ BiPh), 142.1 (t, *J* = 29.9 Hz, C-3 Pz), 139.5 (dt, *J* = 252.3, 15.2 Hz, C-4’ BiPh), 135.4 (C-5 Pz), 134.5 (C-2 BiPh), 134.1 (td, *J* = 7.6, 5.0 Hz, C-1’ BiPh), 131.1 (C-1 BiPh), 130.8 (C-2 allyl), 130.0 (C-6 BiPh), 129.2 (C-4 BiPh), 125.1 (C-5 BiPh), 123.3 (C-3 BiPh), 120.7 (C-3 allyl), 116.6 (C-4 Pz), 111.7 (t, *J* = 233.0 Hz, CHF_2), 113.7 (dd, *J* = 14.6, 7.0 Hz, C-2’ and C-6’ BiPh), 55.3 (C-1 allyl). HRMS (TOF MS ES+): *m/z* calcd for $C_{20}H_{15}F_5N_3O$ $[M+H]^+$ 408.1130, found 408.1130.

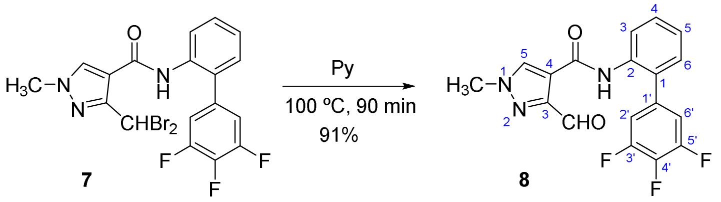


Characterization data of 6-(3-(difluoromethyl)-4-((3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)carbamoyl)-1H-pyrazol-1-yl)hex-4-enoic acid (**6**). IR (neat) ν_{max} (cm^{-1}): 3500–2500 (br, s), 3422 (m), 3058 (w), 2927 (w), 1707 (s), 1660 (s), 1617 (s), 1584 (s), 1541 (s), 1046 (s), 761 (s)738 (s). ^{1}H-NMR data of the major (*E*) isomer (300 MHz, $CDCl_3$) δ (ppm): 8.83 (1H, br s, CO_2H), 8.15 (1H, d, *J* = 8.1 Hz, H-3 BiPh), 8.06 (1H ,s, H-5 Pz), 7.87 (1 H, br s, NH), 7.48–7.37 (1H, m, H-4 BiPh), 7.24–7.21 (2H, m, H-5 and H-6 BiPh), 7.05–6.94 (2H, m, H-2’ and H-6’ BiPh), 6.67 (1H, t, *J* = 54.1 Hz, CHF_2), 6.03–5.68 (2H, m, H-3 and H-4), 4.73 (2H, d, *J* = 6.1 Hz, H-5), 3.20-3.10 (2H, m, H-2). ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm): −109.1 (2F, s, CHF_2), −134.2 (2F, d, *J* = 20.6 Hz, F-3’ and F-5’ BiPh), −162.0 (1F, t, *J* = 20.6 Hz, F-4’ BiPh). ^{13}C-NMR data of the major (*E*) isomer (75 MHz, CD_3OD) δ (ppm): 176.8 (CO_2H), 163.1 (CONH), 152.2 (ddd, *J* = 248.0, 10.1, 4.3 Hz, C-3’ and C-5’ BiPh), 147.1 (t, *J* = 24.1 Hz, C-3 Pz), 140.3 (dt, *J* = 248.8, 15.0 Hz, C-4’ BiPh), 137.3 (td, *J* = 8.1, 4.6 Hz, C-1’ BiPh), 137.1 (C-2 BiPh), 135.2 (C-1 BiPh), 133.0 (C-5 Pz), 131.4 (C-4 BiPh), 130.5 (C-3), 130.2 (C-5 BiPh), 128.9 (C-3 BiPh), 128.5 (C-6 BiPh), 128.2 (C-4), 117.1 (t, *J* = 2.8 Hz, C-4 Pz), 114.3 (dd, *J* = 15.0, 6.6 Hz, C-2’ and C-6’ BiPh), 111.1 (t, *J* = 235.1 Hz, CHF_2), 55.3 (C-5), 37.1 (C-2). HRMS (TOF MS ES+): *m/z* calcd for $C_{22}H_{17}F_5N_3O_3$ $[M+H]^+$ 466.1185, found 466.1194.

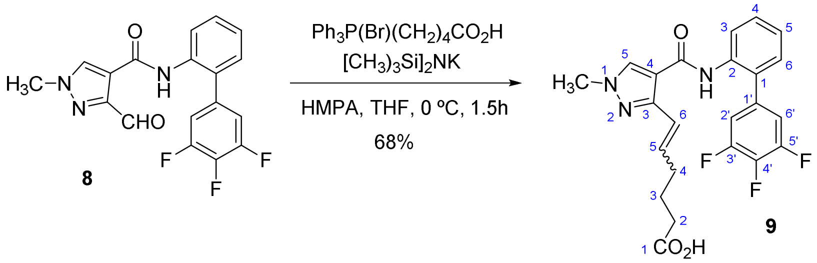
Characterization data of intermediates of the synthesis of hapten FX*c*



Characterization data of 3-(dibromomethyl)-1-methyl-N-(3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)-1H-pyrazole-4-carboxamide (**7**). Mp 198.6–199.4 °C (crystals obtained from slow evaporation from CH_2Cl_2 solution). IR (neat) ν_{max} (cm^{-1}): 3213 (s), 3117 (w), 3039 (w), 1640 (s), 1541 (s), 1532 (s), 1516 (s), 1492 (s), 1042 (s), 760 (s). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.09 (1H, br d, *J* = 7.8 Hz, H-3 BiPh), 7.54 (1H, br s, NH), 7.52 (1H, s, H-5 Pz), 7.43 (1H, ddd, *J* = 8.1, 6.3, 2.9 Hz, H-4 BiPh), 7.29–7.23 (2H, m, H-5 and H-6 BiPh), 7.19 (1H, s, $CHBr_2$), 7.10–6.99 (2H, m, H-2' and H-6' BiPh), 3.93 (3H, s, NMe). ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm): −133.2 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), −161.1 (1F, t, *J* = 20.6 Hz, F-4' BiPh).^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm): 160.3 (CONH), 151.4 (ddd, *J* = 251.7, 10.0, 4.1 Hz, C-3' and C-5' BiPh), 151.3 (C-3 Pz), 139.5 (dt, *J* = 253.5, 15.1 Hz, C-4' BiPh), 134.4 (td, *J* = 7.8, 5.0 Hz, C-1' BiPh), 133.8 (C-2 BiPh), 131.7 (C-5 Pz), 131.1 (C-1 BiPh), 130.1 (C-6 BiPh), 129.4 (C-4 BiPh), 125.7 (C-5 BiPh), 123.9 (C-3 BiPh), 113.6 (C-4 Pz), 113.6 (dd, *J* = 14.5, 7.0 Hz, C-2' and C-6' BiPh), 39.9 (NMe), 30.1 ($CHBr_2$). HRMS (TOF MS ES+): *m/z* calcd for $C_{18}H_{13}{}^{79}Br_2F_3N_3O$ $[M+H]^+$ 501.9372, found 501.9377.

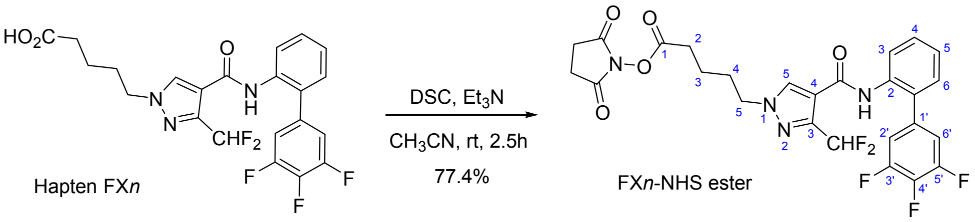


Charaterization data of 3-formyl-1-methyl-N-(3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)-1H-pyrazole-4-carboxamide (**8**). Mp 160.3–161.2 (crystals obtained from slow evaporation from $CHCl_3$ solution). IR (neat) ν_{max} (cm^{-1}): 3276 (w), 3251 (w), 3126 (m), 3074 (w), 2919 (w), 1656 (s), 1613 (s), 1586 (s), 1556 (m), 1421 (s), 1034 (s), 766 (m). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 10.6 (1H, br s, NH), 9.73 (1H, d, *J* = 0.8 Hz, CHO), 8.16 (1H, s, H-5 Pz), 8.04 (1H, d, *J* = 7.8 Hz, H-3 BiPh), 7.43 (1H, ddd, *J* = 8.5, 5.7, 3.3 Hz, H-4 BiPh), 7.30–7.21 (2H, m, H-5 and H-6 BiPh), 7.12–6.96 (2H, m, H-2' and H-6' BiPh), 4.04 (3H, s, NMe). ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm): −135.1 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), −162. 7 (1F, t, *J* = 20.6 Hz, F-4' BiPh). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm): 188.5 (CHO), 159.1 (CONH), 151.1 (ddd, *J* = 250.5, 10.2, 4.3 Hz, C-3' and C-5' BiPh), 145.7 (C-3 Pz), 138.1 (C-5 Pz), 139.3 (dt, *J* = 251.5, 14.5 Hz, C-4′ BiPh), 134.9 (td, *J* = 8.0, 4.9 Hz, C-1′ BiPh), 134.6 (C-2 BiPh), 132.5 (C-1 BiPh), 130.0 (C-6 BiPh), 128.9 (C-4 BiPh), 125.5 (C-5 BiPh), 125.0 (C-3 BiPh), 120.6 (C-4 Pz), 113.7 (dd, *J* = 14.6, 6.8 Hz, C-2′ and C-6′ BiPh), 40.2 (NMe). HRMS (TOF MS ES+): *m/z* calcd for $C_{18}H_{13}F_3N_3O_2$ $[M+H]^+$ 360.0954, found 360.0934.

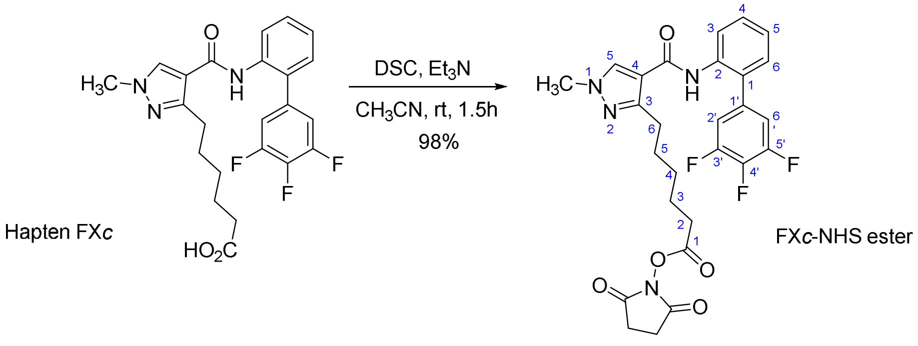


Characterization data of (E/Z)-6-(1-methyl-4-((3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)carbamoyl)-1H-pyrazol-3-yl)hex-5-enoic acid (**9**). IR (neat) ν_{max} (cm^{-1}): 3330–2500 (s), 3120 (w), 2003 (w), 2929 (s), 1720 (s), 1670 (m), 1617 (m), 1534 (s), 1487 (m), 1298 (s), 1195 (s), 986 (s), 725 (s). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.31 (0.3H, br d, *J* = 8.3 Hz, H-3 BiPh of *Z*-isomer), 8.16 (0.7H, br d, *J* = 8.1 Hz, H-3 BiPh of *E*-isomer), 7.83 (0.3H, s, H-5 Pz of *Z*-isomer), 7.62 (0.7H, s, H-5 Pz of *E*-isomer), 7.54 (0.3H, br s, NH of *Z*-isomer), 7.44–7.36 (1H, m, H-4 BiPh of *Z*- and *E*-isomers), 7.38 (0.7, br s, NH of *E*-isomer), 7.23–7.16 (2H, m, H-5 and H-6 BiPh of *Z*- and *E*-isomers), 7.08–6.96 (2H, m, H-2′ and H-6′ BiPh of *Z*- and *E*-isomers), 6.43 (1.4H, m, H-5 and H-6 of *E*-isomer), 6.10 (0.3H, dt, *J* = 11.1, 1.5 Hz, H-6 of *Z*-isomer), 5.67 (0.3H, dt, *J* = 11.1, 7.5 Hz, H-5 of *Z*-isomer), 3.88 (0.9H, s, NMe of *Z*-isomer), 3.87 (2.1H, s, NMe of *E*-isomer), 2.32–2.04 (4H, m, H-3 and H-4 of *Z*- and *E*-isomers), 1.65 (2H, m, H-3 of *Z*- and *E*-isomers). ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm): −131.2 and -133.2 (2F, each d, *J* = 20.6 Hz, F-3′ and F-5′ BiPh of *Z*- and *E*-isomers), −161.2 and −161.3 (1F, each t, *J* = 20.6 Hz, F-4′ BiPh of *Z*- and *E*-isomers). HRMS (TOF MS ES+): *m/z* calcd for $C_{23}H_{21}F_3N_3O_2$ $[M+H]^+$ 444.1530, found 444.1528.

Hapten activation and characterization of active esters



Synthesis of 2,5-dioxopyrrolidin-1-yl 6-(3-(difluoromethyl)-4-((3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)carbamoyl)-1H-pyrazol-1-yl)hexanoate (FX*n*-NHS ester). Et_3N (17.5 µL, 0.125 mmol, 3.8 equiv) was added to *a* solution of hapten FX*n* (15.3 mg, 0.033 mmol) and *N,N'*-disuccinimidyl carbonate (10.9 mg, 0.042 mmol, 1.3 equiv) in anhydrous CH_3CN (300 µL) at 0 ^oC under nitrogen. The mixture was stirred at room temperature for 2 h, then diluted with EtOAc and washed with 5% aqueous $NaHCO_3$ and brine and dried over anhydrous $MgSO_4$. The residue left after evaporation of the solvent was filtered through a short pad of silica gel, using $CHCl_3$ as eluent, to afford FX*n*-NHS (14.4 mg, 77.4%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.17 (1H, d, *J* = 8.1 Hz, H-3 BiPh), 8.01 (1H ,s, H-5 Pz), 7.82 (1 H, br s, NH), 7.49–7.35 (1H, m, H-4 BiPh), 7.24–7.20 (2H, m, H-5 and H-6 BiPh), 7.05–6.95 (2H, m, H-2' and H-6' BiPh), 6.69 (1H, t, *J* = 54.3 Hz, CHF_2), 4.16 (2H, d, *J* = 6.9 Hz, H-5), 2.83 (4H, $COCH_2CH_2CO$), 2.65 (2H, t, *J* = 6.9 Hz, H-2), 2.02 (2H, m, H-4), 1.75 (2H, m, H-3). ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm): −109.2 (2F, s, CHF_2), −134.2 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), −162.0 (1F, t, *J* = 20.6 Hz, F-4' BiPh).



Synthesis of 2,5-dioxopyrrolidin-1-yl 6-(1-methyl-4-((3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)carbamoyl)-1H-pyrazol-3-yl)hexanoate (FX*c*-NHS ester). Et_3N (20 µL, 0.133 mmol, 3.8 equiv) was added to a stirred solution of hapten FX*c* (15.4 mg, 0.035 mmol) and *N,N'*-disuccinimidyl carbonate (11.7 mg, 0.038 mmol, 1.1 equiv) in anhydrous CH_3CN (0.4 mL) at 0 ^oC under nitrogen. The mixture was stirred at this temperature for 30 minutes and then at room temperature for an additional 1.5 hours. The reaction mixture was diluted with EtOAc, washed with a 10% aqueous solution of $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Chromatography of the residue through a short pad of silica gel, using $CHCl_3$ as eluent, afforded FX*c*-NHS *e*ster (18.4 mg, 98%).^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.21 (1H, d, *J* = 8.2 Hz, H-3 BiPh), 7.48 (1H, s, H-5 Pz), 7.42 (1H, ddd, *J* = 8.5, 4.4, 4.4 Hz, H-4 BiPh), 7.23-7.15 (3H, m, H-5 and H-6 BiPh and NH), 7.15-6.96 (2H, m, H-2' and H-6' BiPh), 3.83 (3H, s, NMe), 2.80 (4H, br s, $COCH_2CH_2CO$), 2.71 (2H, t, *J* = 7.5, H-6), 2.59 (2H, t, *J* = 7.5, H-2), 1.71 (4H, m, H-3 and H-5), 1.43 (2H, m, H-4); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -133.1 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), -161.0 (1F, t, *J* = 20.6 Hz, F-4' BiPh).

Preparation of bioconjugates

The purified active ester of the hapten was dissolved in *N*,*N*-dimethylformamide (DMF) and drop wise added to a protein solution in 50 mM carbonate buffer, pH 9.6. The reaction was performed with a 10% (v/v) DMF maximum content. After 2 h at room temperature under gentle stirring, the protein conjugate was purified by size-exclusion chromatography using 100 mM phosphate buffer, pH 7.4 as eluent. BSA conjugates were filter sterilized and stored frozen at −20 °C. OVA conjugates were diluted in elution buffer and kept at −20 °C. HRP conjugates were diluted with PBS (10 mM phosphate buffer, pH 7.4, with 140 mM NaCl) containing 1% (w/v) BSA and stored at 4 °C.

MALDI mass spectrometry analysis of immunizing and assay bioconjugates

Sample preparation. 100 µL of each of the protein conjugate solutions (0.5–1 mg/mL) were dialyzed against milliQ water and then freeze-dried and lyophilized. The samples were dissolved in MilliQ H_2O to theoretical final concentration 1 µg/µL. Then, 1 µL of every sample solution was spotted onto the MALDI plate. After the droplets were air dried at room temperature, 1 µL of matrix (10 mg/mL sinapinic acid (Bruker) in 0.1% trifluoroacetic acid–CH_3CN/H_2O (7:3 v/v) was added and allowed to air-dry at room temperature.

Mass spectrometry analysis. The resulting mixtures were analyzed in a 5800 MALDI TOF/TOF (ABSciex) in positive linear mode (1500 shots every position) in a mass range of 10000–100000 m/z. Previously, the plate was calibrated with 1 µL of the TOF/TOF calibration mixture (ABSciex), in 13 positions. Every sample was calibrated by ‘close external calibration’ method with a BSA, OVA, or HRP spectrum acquired in a close position.

Competitive ELISA procedures

Antibody-coated direct format

Polystyrene 96-well microplate wells were coated with 100 µL per well of antibody dilution in 50 mM carbonate buffer, pH 9.6, by overnight incubation at 4 °C. Then, plates were rinsed four times with 150 mM NaCl containing 0.05% (v/v) Tween 20 (washing solution). The competitive reaction was carried out by mixing 50 µL per well of fluxapyroxad solution in PBS and 50 µL per well of peroxidase tracer in PBST (PBS containing 0.05% Tween 20, v/v). Plates were incubated at room temperature during 1 h and rinsed four times with washing solution. Then, the immunochemical reaction was revealed with 100 µL per well of enzyme substrate solution (2 mg/mL of *o*-phenylenediamine and 0.012% (v/v) H_2O_2 in 25 mM citrate and 62 mM phosphate buffer, pH 5.4). After 10 min at room temperature, the peroxidase activity was stopped with 100 µL per well of 1 M H_2SO_4 and the absorbance was immediately read.

Conjugate-coated indirect format

Coating was accomplished with 100 µL per well of OVA conjugate solution in 50 mM carbonate buffer, pH 9.6, overnight at room temperature. After each incubation step, plates were washed as indicated for the direct format. The competitive step was performed by mixing 50 µL per well of fluxapyroxad standard solution in PBS and 50 µL per well of antibody dilution in PBST, and incubating during 1 h at room temperature. After washing, 100 µL per well of GAR–HRP solution (diluted 1/10000) in PBST, supplemented with 10% (v/v) ABS, was applied and plates were incubated again 1 h at room temperature. Next, plates were washed as before. Finally, the presence of peroxidase activity was revealed and the absorbance was read as described for the previous ELISA format.

Table S1. Antibody specificity evaluated as cross-reactivity (%).

	Antibody			
	FX*n*#1	FX*n*#2	FX*c*#1	FX*c*#2
Fluxapyroxad	100	100	100	100
Penthiopyrad	0.05	0.05	<0.01	<0.01
Fluopyram	0.05	0.05	<0.01	<0.01
Boscalid	0.2	0.2	2.6	2.9
Azoxystrobin	<0.01	<0.01	<0.01	<0.01
Picoxystrobin	<0.01	<0.01	<0.01	<0.01
Pyraclostrobin	<0.01	<0.01	<0.01	<0.01
Kresoxim-methyl	<0.01	<0.01	<0.01	<0.01
Fenhexamid	<0.01	<0.01	<0.01	<0.01
Fluopicolide	<0.01	<0.01	<0.01	<0.01
Pyrimethanil	<0.01	<0.01	<0.01	<0.01
Cyprodinil	<0.01	<0.01	<0.01	<0.01

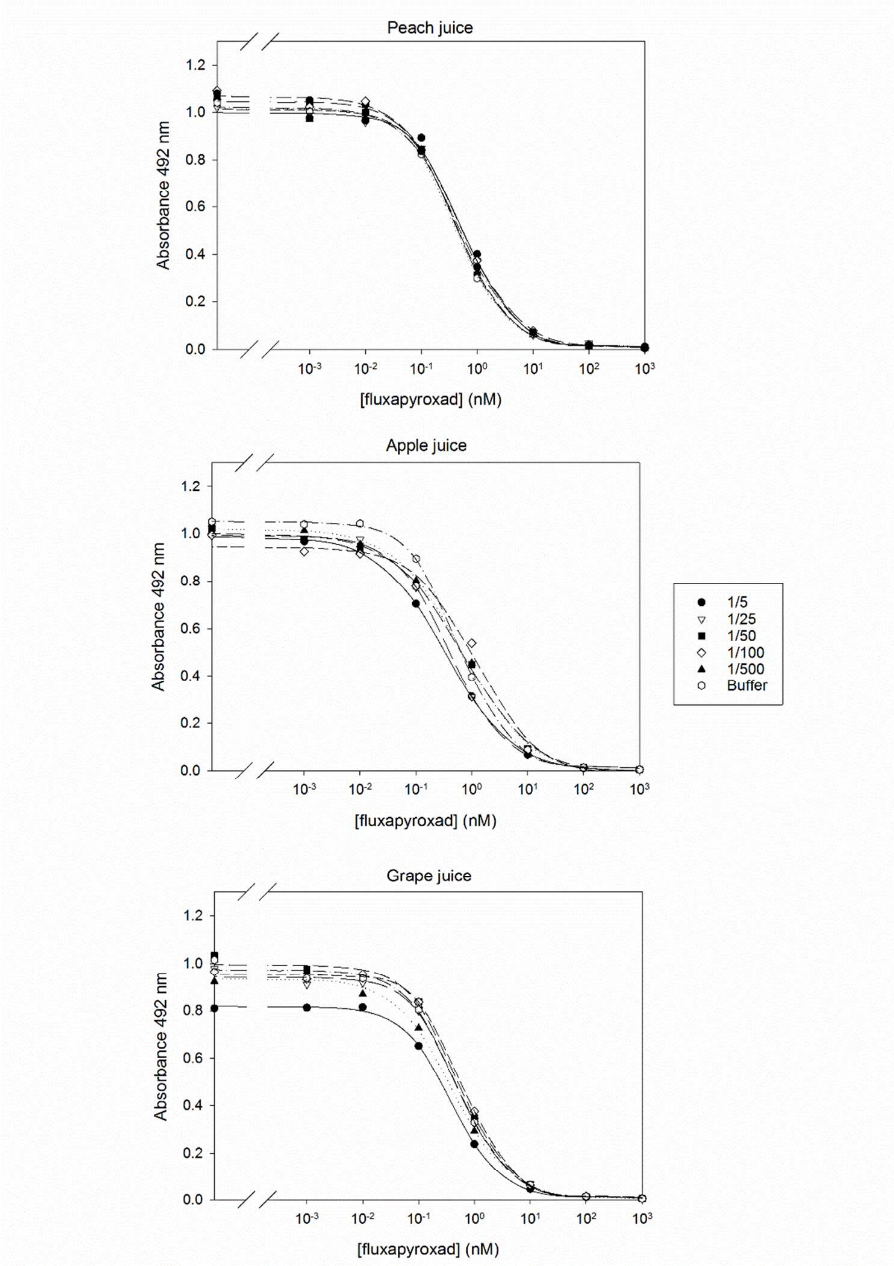


Figure S1. Matrix effects of different fruit juices over the direct immunoassay.

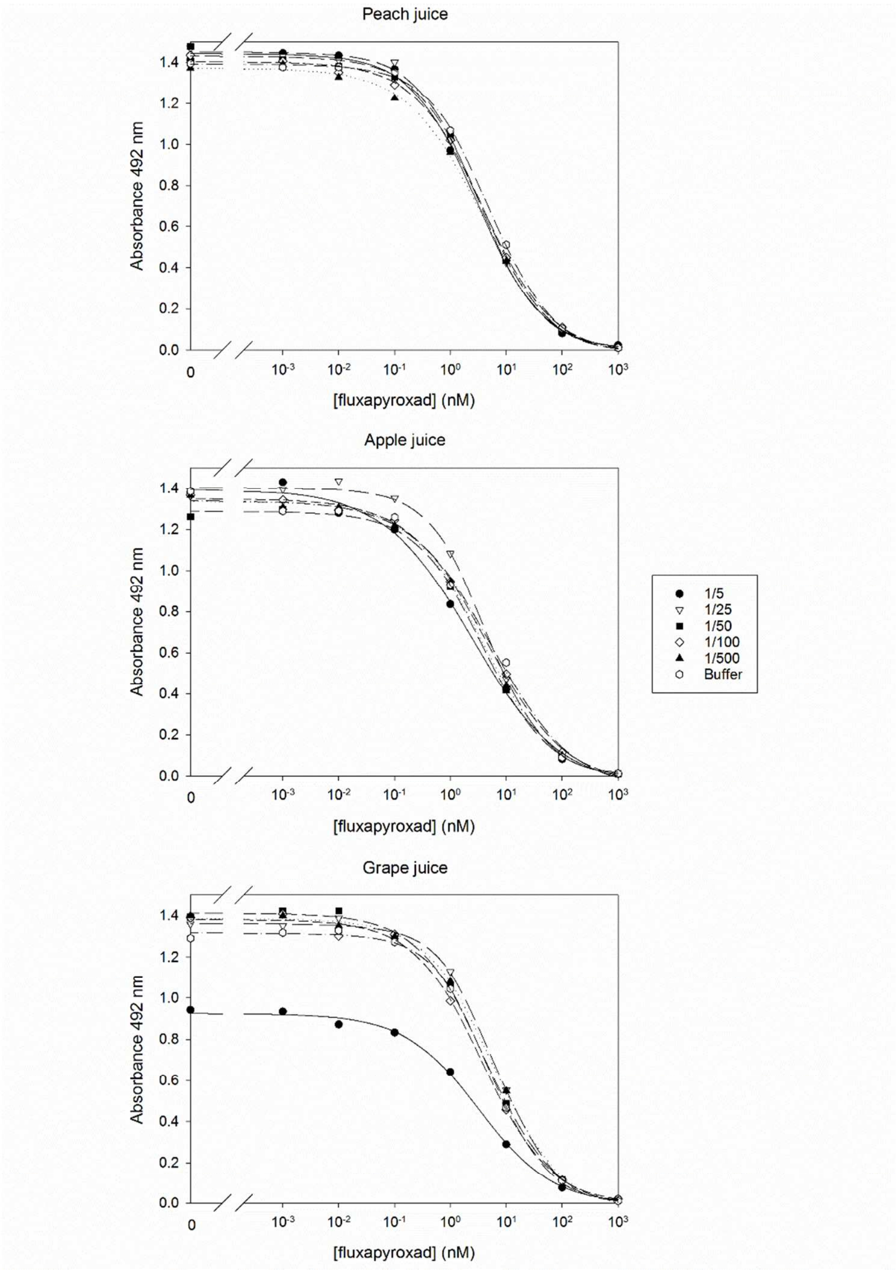
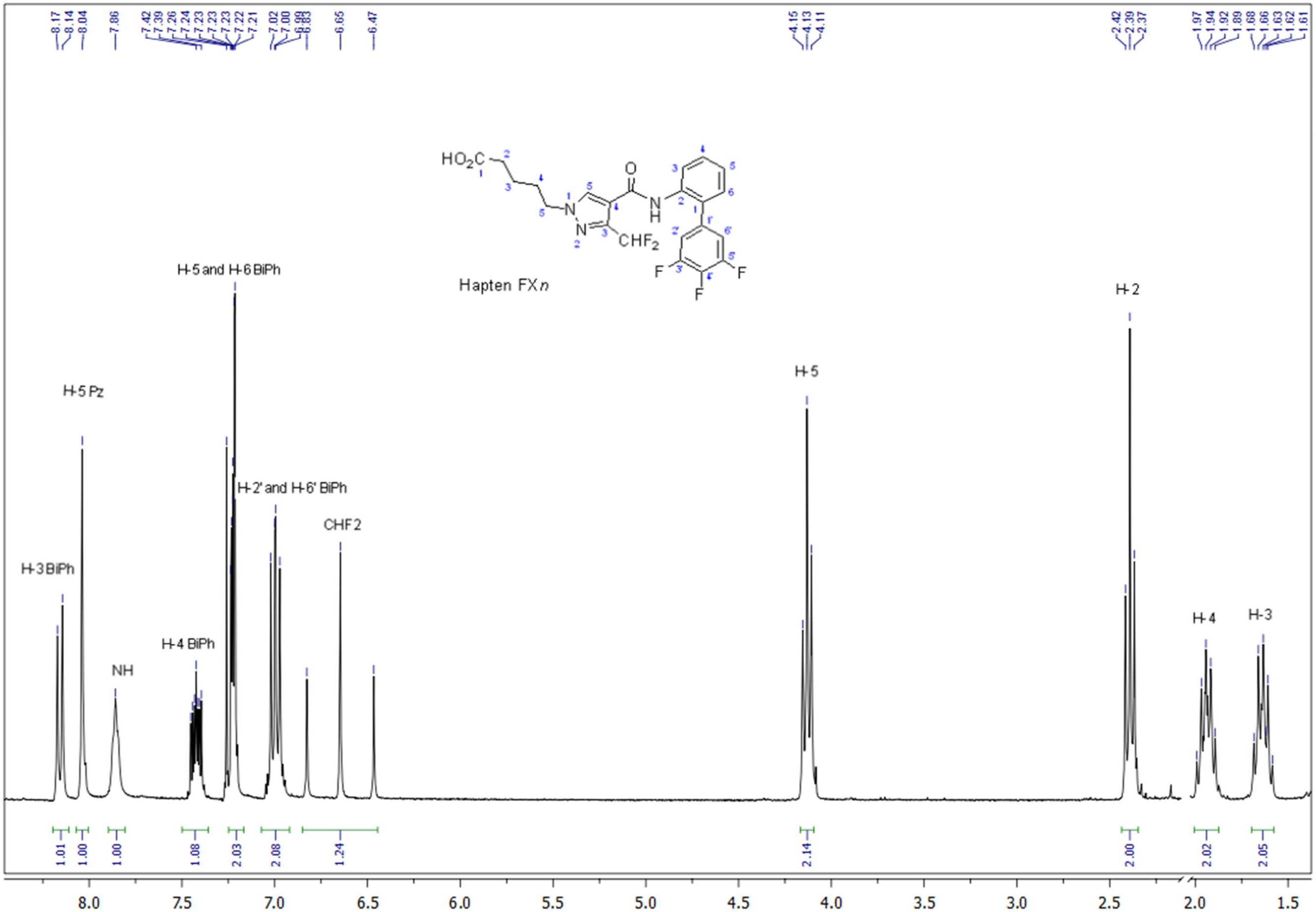


Figure S2 Matrix effects of different fruit juices over the indirect immunoassay.

^{1}H NMR spectrum of hapten FX*n* ($CDCl_3$, 300 MHz)



^{1}H NMR spectrum of hapten FX*c* ($CDCl_3$, 300 MHz)