

Residues of 165 pesticides in citrus fruits using LC-MS/MS: a study of the pesticides distribution from the peel to the pulp

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Abstract

A sensitive LC–ESI-MS/MS method was developed for the determination of 165 pesticides in 50 citrus fruit samples collected in Sicily. Moreover, an evaluation of pesticides levels in the citrus layers (peel, albedo, and pulp) was carried out. The method presented acceptable trueness, precision, and linearity with LOQ of 5 µg/kg. The results obtained showed a high frequency of fungicides class pesticides in all the citrus samples examined (>95%) with the highest concentrations in the peel (4468 µg/Kg). A significant difference of concentrations was found between the layers of the citrus fruits analysed ($p < 0.05$). In particular, the peel and albedo present higher pesticides significantly higher than the pulp. Our findings confirming the widespread use of these substances in citrus cultivation and suggesting the importance of pesticides analysis in all the citrus fruit layers separately, considering the different interactions between the physicochemical characteristics of the matrices and the pesticides.

3. Experimental

Chemical and pesticides standards

Acetonitrile and Methanol were obtained from VWR (Radnor, Pennsylvania, USA). All organic solvents used in the multi-residue method were HPLC grade. Formic acid, sodium hydroxide, and ammonium formate were purchased from Fluka (Morris Plains, New Jersey, USA).

QuEChERS including solvent partitioning mixtures, extraction tube (4.00 g anhydrous magnesium sulfate, 1.00 g sodium chloride, 1.00 g sodium citrate and 0.50 g sodium hydrogen citrate sesquihydrate) and cleanup mixture (900 mg anhydrous magnesium sulfate and 150 mg PSA), were purchased from Sigma (Saint Louis, Missouri, USA).

A Millipore water purification system (Milli-Q) was used for deionized water production.

All the pesticide standards were purchased from Restek (Bellefonte, Pennsylvania, USA) at the concentration of 100 µg/mL. Working standard solution mixture of 1 mg/ml and 0.1 mg/ml was prepared by diluting a suitable aliquot of the stock solutions with acetonitrile and stored in a refrigerator at +4°C for the fortification of the blank samples. Standard Ethion was purchased from Sigma (Saint Louis, Missouri, USA).

3.1 Samples collection and preparation

A total of 50 samples of citrus fruits (oranges, lemons and mandarins) from 5 markets of Palermo and Trapani provinces (Sicily, Southern Italy) were analyzed for the evaluation of pesticides residues in peel, albedo, and pulp.

All the samples were stored in a freezer at -17 °C until the analysis. After homogenization, 10 g of the sample was weighed and put into a 50 ml centrifuge tube.

A 100 µL of Ethion 1 mg/L standard in acetonitrile was added as a quality control during the entire procedure. Then, 10 ml of acetonitrile, were added and vortexed for 1 min. The partition was obtained using the Extraction Tube, vortexing for 1 min and centrifuging at 3000 rpm for 5 min. An aliquot of 6 ml of the supernatant was transferred to a Cleanup Tube (900 mg anhydrous magnesium sulfate and 150 mg PSA) then mixed for 30 seconds and centrifuged at 3000 rpm for

5 min. 10 µl of formic acid 5% was added in 1 ml of the supernatant. Blank extracts were used for the matrix-matched standard calibration.

3.2 Instrumental analysis

The instrumental analysis was performed with a Quantum TSQ Vantage liquid chromatography coupled to a mass spectrometer equipped with a triple quadrupole mass analyzer (Thermo Scientific, Waltham, Massachusetts, USA). An electrospray ionization interface (ESI) was used for the determination of the studied pesticides. The separations were achieved using an Agilent Eclipse XDB-C18 (2,1x100mm, Agilent Technologies Santa Clara, California, USA). The injection volume was 10 µl. The analytes were separated with a mobile phase consisting of eluent A: H₂O, 2mM HCOONH₄, HCOOH 1% and eluent B: CH₃OH, 2mM HCOONH₄, HCOOH 1%. The gradient program followed a constant flow of 500 µl/min (table S3) ~~table 4~~. The mass spectrometer detector was operated using the electrospray (ESI) source in the positive mode and data were acquired in Selected Reaction Monitoring mode (SRM). ESI parameters were as follow: Spray voltage 5500, Vaporizer Temperature 350 °C, Capillary Temperature 220 °C, Sheath Gas Pressure 45 °C, Aux Gas Pressure 40°C. The data acquisition was performed using Trace Finder software (Thermo Scientific). The MS parameters were optimized to obtain the protonated molecule and selecting those transitions with higher molecular mass in order to avoid the disruptive effects of the matrix, as far as possible. The concentration of each analyte in the sample was calculated by interpolation with the matrix calibration curve and multiplying by the conversion factor determined by the following formula:

$$F_c = \frac{V_f}{P}$$

Where:

F_c = conversion factor from µg/l to µg/kg

V_f = final volume (ml) sample extract

P = weight (gr) of the sample.

3.3 LC-MS/MS method validation

Certified homogenised orange, lemon and mandarin samples with no pesticides detected were used for recovery studies, and for the preparation of matrix-matched standards for calibration.

Calibration standards in a blank matrix were prepared by diluting the standard mixture solution (1 mg/mL and 0.1 mg/mL) and adding the respective spiking solution and internal standard solution to blank extract, to produce a final concentration of 2.5, 5.0, 10.0, 20.0 and 40.0 µg/L and 10 µg/L for internal standards, respectively.

The accuracy and precision, as well as selectivity e specificity, linearity, and limit of quantification (LOQ) analytical parameters were assessed according to the SANTE/11813/2017. Five replicates were used to check the recovery and precision at the targeted LOQ or MRL of the method, and at least two levels 2 and 5x higher than the targeted LOQ or the MRL. A range of 70–120% was considered acceptable for the recovery study for all the analytes studied, with an associated repeatability $RSD_r \leq 20\%$. The within laboratory reproducibility (RSD_w) was considered at $\leq 20\%$, excluding any contribution due to sample heterogeneity. The linearity was evaluated by spiking blank lemon, orange and mandarin extracts at the concentrations 2.5, 5, 10, 20 and 40 µg/L. The first calibration level was always equal to, or lower than, the MRLs established by the European Union, EU, legislations (Commission Directive 2000/42/EC) The correlation coefficient (r^2) for each pesticide was calculated by plotting signal responses against concentrations of each pesticide. The r^2 values for each pesticide analysed, was ≥ 0.99 , indicating excellent linearity for the analysed pesticides using the method developed. The selectivity of the method was evaluated by injecting blank sample (lemon, orange and mandarin) extracts. The absence of signal above a signal-to-noise ratio of 3 at the retention times of the target compounds showed that the method is free of interferences. The trueness was obtained by recovery measurements of five spiking samples of 5, 10, and 25 µg/kg. All the pesticides studied showed recoveries at range 70–100% with $RSDs \leq 20\%$ at the three concentration levels considered, in agreement with SANTE/11813/2017 document. The precision was considered as relative standard deviation (RSD %). Intra-day precision was

calculated for the recoveries of five repeated spiked lemon, orange and mandarin samples (at spiking levels of 5, 10, and 25 µg/kg) in the same day. The LOQ was considered as the lowest spiked level that achieved the method performance criteria for trueness and precision. The Estimations of LOQ was calculated taking into account the Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food. The LOQs were much lower than the MRLs established by the EU legislations. The measurement uncertainty study was carried out on the basis of a combination of methodologies described by the EURACHEM guide (2014). The main sources of uncertainty for the method were: (1) the mass measurements of the standards for the preparation of solutions; (2) dilution of the standard solutions; (3) the measurements of the volume of the extraction solution; (4) the MMC curves and (5) intermediate precision. The uncertainties related to the measurements of volume and mass are negligible compared to the other sources raised. The main contribution to the total uncertainty arises from the MMC curves, considering that the MMC curves encompass all the steps, from the weighing of standards until the final quantification steps (including the whole extraction process, the instrumental analysis and statistical processing of the data). The expanded uncertainty, expressed as percentage (MU%), for each pesticide was determined at each fortification level considered for repeatability and reproducibility. The MU% calculated for each pesticide showed values below 50%. The uncertainty values at all levels studied were in the range 11.2-48.4%. These results were in agreement with the acceptable criteria established by the SANCO 12495/2011 document.

3.3 Statistical analysis

The assumptions of normality distribution have been met for orange and mandarin (Shapiro-Wilk test $p > 0.05$) therefore, ANOVA and t test were carried out in order to verify significant differences between the orange and mandarin layers, respectively. For lemon samples, a non-parametric Kruskal-Wallis test was carried out. The statistical analysis were carried out using the statistical software R@3.4.1.

5. References

Commission Directive 2000/42/EC of 22 June 2000 on the fixing of maximum levels for pesticide residues in and on cereals, foodstuffs of animal origin and certain products of plant origin, including fruit and vegetables respectively.

Eurachem 2014. The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics.

SANCO/12495/2011. Method validation and quality control procedures for pesticide residues analysis in food and feed.

SANTE/11813/2017. Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed.

Table S1. Retention time windows and MS/MS conditions of each compound analysed.

Name	Ret Time [min]	Parent	Product 1	Product 2	Collision Energy 1	Collision Energy 2
Pymetrozine	0.57	218.0	78.0	105.0	61	27
Methamidophos	0.58	142.0	94.0	125.0	19	19
Formetanate HCl	0.59	222.1	120.0	165.0	37	23
Aminocarb	0.59	209.1	137.1	152.0	33	19
Propamocarb	0.60	189.2	102.0	144.0	25	19
Omethoate	0.60	214.0	124.9	182.8	31	17
Butoxycarboxim	0.63	223.1	106.0	166.0	13	11
Carbendazim	0.63	192.1	132.1	160.1	25	20
Acephate	1.37	184.1	49.0	143.0	35	11
Dinotefuran	1.38	203.1	129.2	157.2	17	11
Aldicarb sulfone	1.38	240.1	86.2	148.2	28	19
Nitenpyram	1.46	271.0	126.0	225.2	35	17
Mexacarbate	1.87	223.2	151.0	166.1	31	21
Clothianidin	3.93	250.0	132.0	169.0	21	19
Fenuron	3.99	165.1	46.0	72.1	29	45
Imidacloprid	4.02	256.0	175.1	209.1	25	21
Dioxacarb	4.11	224.1	123.0	167.0	21	11
3-Hydroxycarbofuran	4.13	238.1	163.0	181.0	19	15
Dimethoate	4.17	230.0	125.0	199.0	29	13
Acetamiprid	4.2	223.0	99.0	126.0	53	29
Pirimicarb	4.28	239.2	72.1	182.1	33	21
Mevinphos	4.30	225.1	127.1	193.2	21	11
Cymoxanil	4.39	198.9	111.2	128.2	23	12
Ethirimol	4.5	210.2	98.1	140.1	39	31
Trichlorfon	4.53	256.9	109.1	127.0	25	23
Thiacloprid	4.54	253	99.0	126.0	59	29
Tricyclazole	4.66	190.0	136.0	163.0	39	33
Oxadixyl	4.85	279.1	132.1	219.1	43	15
Simetryn	4.95	214.0	124.0	144.0	29	29
Carbetamide	4.96	237.1	118.1	192.0	17	13

Thiophanate-methyl	5.1	343	151.1	311.0	31	17
Propoxur	5.11	210.1	111.0	168.1	19	11
Prometon	5.12	226.1	86.0	142.0	39	33
Metribuzin	5.14	215.1	84.1	187.1	31	25
Bendiocarb	5.14	224.1	109.0	167.1	33	15
Terbumeton.Secbumeton	5.14	226.1	100.0	170.1	41	23
Carbofuran	5.15	222.1	123.0	165.1	29	17
Thidiazuron	5.22	221.1	102.1	127.9	23	23
Pyracarbolid	5.23	218.1	97.0	125.0	37	25
Tebuthiuron	5.26	229.1	116.1	172.4	37	25
Carboxin	5.31	236.1	87.0	143.0	33	21
Carbaryl	5.33	202.1	127.0	145.0	39	13
Imazalil	5.35	297.0	159.0	201	29	25
Monolinuron	5.44	215.1	99.0	126.1	47	23
Imidacloprid	5.44	256.0	175.1	209.1	25	21
Ethiofencarb	5.46	226.1	107.2	164.0	21	21
Ametryn	5.48	228.1	96.0	186.1	35	25
Fluometuron	5.53	233.1	46.0	235.1/72.1	37	35
Diuron	5.53	233.1	72.0	235.1/72.1	37	33
Methoprotryne	5.61	272.2	198	240.2	31	27
Chlorotoluron	5.63	213.1	46.2	72.2	35	31
Metobromuron	5.65	259.0	148.2	170.2	21	25
Isoprocab	5.69	194.1	95.0	137.0	21	13
Methabenzthiazuron	5.7	222.1	150.3	165.2	45	21
Flutriafol	5.7	302.1	70.1	123.0	59	37
Metalaxyl	5.8	280.1	192.2	220.2	25	19
Pyrimethanil	5.81	200.0	82.0	107.0	37	33
Isoproturon	5.84	207.2	46.1	72.1	35	29
Cycluron	5.92	199.1	89.0	89.1	21	21
Forchlorfenuron	6.00	248.0	93.1	129.1	49	25
Fenpropimorph	6.00	304.0	117.0	147.0	73	39
Desmedipham	6.08	318.1	154.0	182.0	35	19
Chlorantraniliprole	6.14	484.0	285.9	452.9	17	21

Prometryne	6.17	242.2	158.1	200.1	33	25
Phenmedipham	6.19	301.2	107.9	168.0	44	12
Terbutryn	6.23	242.1	68.1	186.1	61	25
Spiroxamine	6.33	298.2	100.1	144.2	43	29
Fenobucarb	6.39	208.2	95.3	152.1	19	13
Furalaxyl	6.4	302.1	95.0	242.1	39	21
Azoxystrobin	6.42	404.1	344.1	372.1	33	19
Diethofencarb	6.44	268.1	124.0	226.1	43	13
Ethofumesate	6.49	287.1	121.1	259.1	23	15
Acibenzolar-S-methyl	6.5	211.0	91.2	136.2	29	41
Linuron	6.5	249.1	160.0	182.1	25	21
Nuarimol	6.5	315.0	81.0	252.1	49	37
Fenamidone	6.58	312.1	92.0	236.1	35	21
Methiocarb	6.6	226.1	121.1	169.1	27	13
Siduron	6.62	233.3	94	137.2	31	23
Ethiprole	6.7	397.3	255.2	350.9	47	27
Dimethomorph	6.75	388.1	165.1	301.0	45	29
Boscalid	6.75	343.0	140.0	307.0	25	27
Promecarb	6.8	208.1	109.0	151.0	21	13
Mandipropamid	6.82	412.1	328.1	356.1	19	15
Triadimenol	6.87	296.1	70.0	227.1	33	17
Paclobutrazol	6.87	294.0	70.0	125.0	49	41
Flutolanil	6.95	324.1	242.1	262.1	35	31
Bupirimate	6.96	317.0	166.1	108.0	33	35
Mepronil	7.05	270.1	119.1	228.0	31	21
Triadimefon	7.08	294.0	197.1	225.0	21	19
Methoxyfenozide	7.08	369.1	149.1	313.2	21	11
Cyprodinil	7.15	226.0	77.0	93.0	61	47
Myclobutanil	7.17	289.0	70.0	125.0	41	39
Cyproconazole	7.20	292.0	70.0	125.0	31	29
Clethodim Isomer 1	7.21	360.1	164.0	268.1	29	17
Mefenacet	7.22	299.0	120.1	148.1	35	21
Bifenazate	7.33	301.1	170.1	198.1	27	13

Mepanipyrim	7.34	224.0	77.0	106.0	55	35
Chloroxuron	7.34	291.1	72.4	218.1	47	33
Iprovalicarb	7.43	321.2	119.0	203.1	47	13
Fluoxastrobin	7.5	459.2	188.0	427.2	47	23
Fenhexamid	7.54	302.0	55.0	97.0	61	33
Butafenacil	7.54	492.1	331.0	349.0	27	19
Fenarimol	7.57	331.0	81.0	268.0	55	35
Spirotetramat	7.59	374.2	302.2	330.2	27	23
Etaconazole	7.62	328.1	159.0	205.0	31	23
Triticonazole	7.64	318.1	70.0	125.0	35	49
Flufenacet	7.64	364.1	152.1	194.2	27	17
Bromucanazole	7.70	378.0	70.0	159.0	47	37
Tetraconazole	7.73	372.1	70.0	159.0	47	35
Epoxiconazole	7.85	330.0	101.1	121.1	69	49
Cyazofamid	7.91	325.2	108.0	261.2	18	14
Fenbuconazole	8.09	337.0	70.0	124.9	39	55
Rotenone	8.13	395.1	192.1	213.1	33	31
Diflubenzuron	8.18	311.0	141.1	158.2	43	19
Flusilazole	8.18	316.1	165.1	247.1	37	21
Fenoxycarb	8.26	302.1	88.0	116.1	29	17
Picoxystrobin	8.29	368.0	145.0	205.0	29	13
Tebufenozide	8.31	353.2	133.0	297.2	23	11
Dimoxystrobin	8.40	327.1	116.0	205.0	29	23
Neburon	8.43	275.0	88.0	114.0	23	21
Diclobutrazol	8.50	328.2	59.1	70.2	48	48
Prochloraz	8.60	376.0	70.0	308.0	43	15
Tebuconazole	8.74	308.2	70.0	125.0	51	55
Flubendiamide	8.75	683.1	274.1	408.0	41	9
Propiconazole	8.88	342.1	69.0	159.0	39	31
Amitraz	8.89	294.2	91.2	148.3	57	22
Benalaxyl	8.89	326.2	148.1	294.1	29	15
Prothioconazole	8.91	344.1	125.1	188.9	33	31
Zoxamide	9.02	336.1	159.0	187.0	55	29

Hexaconazole	9.17	314.1	70.0	159.0	55	33
Clofentezine	9.27	303.0	102.0	138.0	51	19
Metconazole	9.29	320.1	70.0	125.0	43	53
Spinosad (Spinosyn A)	9.3	732.5	98.1	142.2	95	39
Pyraclostrobin	9.34	388.0	163.0	194.0	31	17
Thiobencarb	9.39	258.0	89.0	125.0	65	23
Benzoximate	9.46	364.0	105.0	199.0	31	11
Bitertanol	9.48	338.2	70.0	269.2	29	13
Triflumuron	9.5	359.1	139.0	156.2	45	23
Diniconazole	9.64	326.2	70.2	159.0	50	43
(Monceren) Pencycuron	9.64	329.1	125.0	218.1	31	23
Difenoconazole	9.77	406.1	251.1	253.1	41	31
Triflumizole	9.82	346.1	73.0	278.1	27	17
Spinosad (Spinosyn D)	9.83	746.8	98.0	142.1	55	35
Hydramethylnon	9.95	495.2	151.1	323.2	77	41
Spinetoram	9.97	748.5	98.1	142.2	95	43
Ipconazole	10.00	334.2	70.0	125.0	37	47
Trifloxystrobin	10.00	409.0	186	206.0	21	19
Indoxacarb	10.04	528.0	203.0	218.0	47	
Clethodim Isomer 2	10.2	360.1	164.0	268.1	29	17
Buprofezin	10.23	306.2	116.2	201.1	23	17
Novaluron	10.32	493.0	141.1	158.1	65	29
Furathiocarb	10.45	383.1	195.1	252.1	25	17
Tebufenpyrad	10.47	334.0	117.0	145.0	47	37
Quinoxifen	10.64	308.1	162.1	197.1	63	45
Metaflumizone	10.65	507.1	178.1	287.1	33	33
Temephos	10.67	467.0	405.0	419.1	21	27
Ethion	10.70	385.2	171.0	199.2	17	12
Lufenuron	10.75	511.1	141.2	158.1	61	27
Hexythiazox	10.81	353.1	168.0	228.0	37	19
Spiromesifen	11.00	371.2	255.2	273.2	31	11
Etoxazole	11.01	360.1	57.2	141.0	51	59
Propargite	11.01	368.2	175.1	231.1	23	15

Flufenoxuron	11.05	489.0	158.0	141.1	27	63
Fenpyroximate	11.21	422.0	135.1	366.1	53	23
Spirodiclofen	11.23	411.3	71.3	313.3	31	17
Chlorfluazuron	11.26	540.0	158.0	383.0	29	27
Fenazaquin	11.40	307.1	147.0	161.1	25	27

Table S2. Frequencies and concentrations of the pesticides residues found in the citrus samples examined, sorted by fruit layer. MRL = Maximum residue levels regulated by the EU Plant Protection Products Directive (2005/396/EEC).

Sample type	N. samples examined	Pesticides	N. positive Samples	Range of concentration (µg/Kg)	MRL µg/Kg
Orange Peel	20	Imazalil	11	8-4468	5000
		Boscalid	1	18	2000
		Spirotetramat	1	110	1000
Orange Albedo	20	Imazalil	10	498-2750	5000
		Boscalid	1	5	2000
		Spirotetramat	1	41	1000
Orange Pulp	20	Imazalil	10	13-392	5000
		Spirotetramat	1	5	1000
Lemon Peel	20	Fenhexamide	12	15-66	10
Lemon Albedo	20	Fenhexamide	12	5-50	10
Lemon Pulp	20	Fenhexamide	6	5-21	10
Mandarine Peel	10	Imazalil	4	3233-4456	5000
		Carbendazim	1	73	700
Mandarine Pulp	10	Imazalil	4	34-87	5000
		Carbendazim	1	12	700

Table S3. Linear gradient program used for the separation of the compounds analysed.

Time (min)	Eluent A (%)	Eluent B (%)
0,0	90	10
1,50	90	10
4,00	40	60
8,00	30	70
11,00	0	100
12,00	0	100
12,01	90	10
15,00	90	10

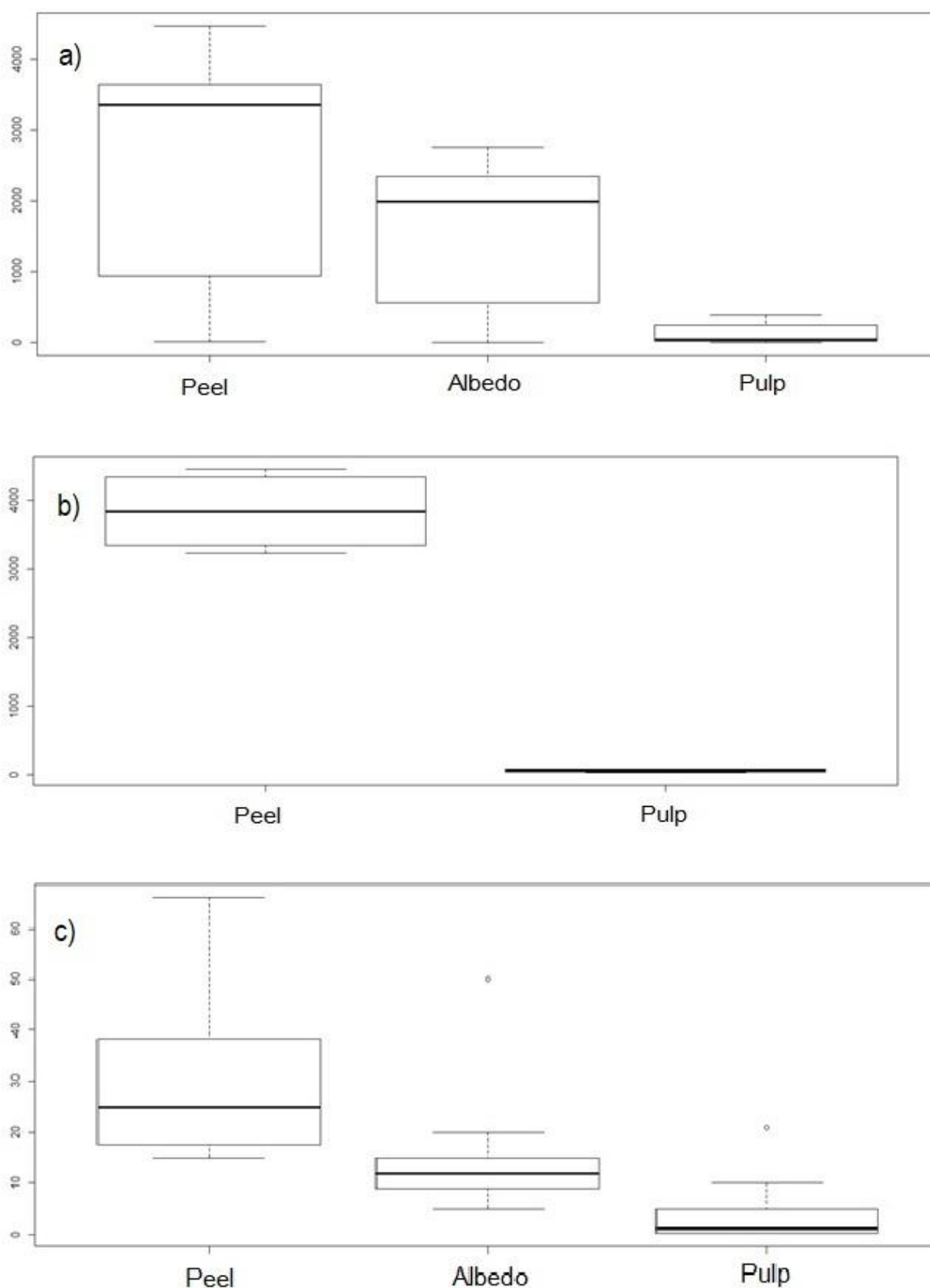


Figure S1. Box-plot of pesticides residues ($\mu\text{g/Kg}$) in orange (a), mandarin (b) and lemon (c) samples layers. The area of each box-plot represents the upper and lower 25% quartiles of the data distribution on either side of the median (black bar) and the whiskers represent the range of the data excluding outliers, represented as open circles.