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Determination of Pesticide Residues in Fruit-Based Soft Drinks

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Here we report the first worldwide reconnaissance study of the presence and occurrence of pesticides in fruit-based soft drinks. While there are strict regulations and exhaustive controls for pesticides in fruits, vegetables, and drinking water, scarce attention has been paid to highly consumed derivate products, which may contain these commodities as ingredients. In the case of the fruit-based soft drinks industry, there are no clear regulations, relating to pesticides, which address them, even when there is significant consumption in vulnerable groups such as children. In this work, we have developed a screening method to search automatically for up to 100 pesticides in fruit-based soft drinks extracts based on the application of liquid chromatography−electrospray time-of-flight mass spectrometry (LC−TOF MS). The sample extracts injected were obtained by a preliminary sample treatment step based on solid-phase extraction using hydrophilic−lipophilic balanced polymer-based reverse phase cartridges and methanol as eluting solvent. Subsequent identification, confirmation, and quantitation were carried out by LC−TOF MS analysis: the confirmation of the target species was based on retention time matching and accurate mass measurements of protonated molecules ($[M + H]^+$) and fragment ions (obtaining accuracy errors typically lower than 2 ppm). With the proposed method, we measured over 100 fruit-based soft drink samples, purchased from 15 different countries from companies with brands distributed worldwide and found relatively large concentration levels of pesticides in most of the samples analyzed. The concentration levels detected were of the micrograms per liter level, low when considering the European maximum residue levels (MRLs) set for fruits but very high (i.e., 300 times) when considering the MRLs for drinking or bottled water. The detected pesticides (carbendazim, thiabendazole, imazalil and its main degradate, prochloraz and its main degradate, malathion, and iprodione) are mainly those applied to crops in the final stages of production (postharvest treatment), some of them contain chlorine atoms in their structures. Therefore, steps should be taken with the aim of removing any traces of pesticides in these products, in order to avoid this source of pesticide exposure on the consumer, particularly on vulnerable groups with higher exposure, such as children.

Pesticide residue research supports the establishment and control of safe levels of pesticides in food. It is important not only for trade purposes but also for ensuring human health. For this reason, maximum residue levels (MRLs) are set in order to ensure appropriate agricultural practices.[1] Surprisingly, while there are strict regulations and exhaustive controls for pesticides in fruits, vegetables, and drinking water, scarce attention has been paid to highly consumed derivate products, which may contain these commodities as ingredients. In the case of the fruit-based soft drinks industry, there are no clear regulations, relating to pesticides, which address them, even when there is significant consumption in vulnerable groups such as children. Despite the large quantity of these products consumed daily, no attention has been paid to enforce the safety of these products in terms of their chemical composition, even though we know they are considered representative and relevant in terms of consumption. For instance, the annual average soft drink consumption in the U.K. and the U.S. is 4 times higher that the bottled water consumption.[2]

There is more than one regulation which could be potentially applicable to fruit-based soft drinks. This would lead to very different figures in respect to permitted pesticide levels. The hypothetic application of the European Union Council Directive for drinking water, 98/83/EC of November 3, 1998 (80/778/EC)[3] dealing with the "quality of water intended for human consumption", involves a maximum admissible concentration for individual pesticides (and related products) of 0.1 $\mu g\ L^{-1}$ and 0.5 $\mu g\ L^{-1}$ for the total amount of pesticides (i.e., the sum of all individual pesticides detected and quantified in the monitoring procedure).

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(1) Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in food and feed of plant and animal origin and amending Council Directive 91/414/EEC, 16.3.2005, Official Journal of the European Union L 70/1.

(2) www.nationmaster.com.

(3) Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Official Journal of the European Communities, L330/32.

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The primary objective of the proposed work is to search for noncommon and unexpected sources of pesticides and other chemicals in a broad suite of foodstuffs, which are now neglected by regulations worldwide but which could be easily consumed daily, particularly by vulnerable groups such as children. In this sense, the presence of large amounts of postharvest fungicides in citrus samples, compounds applied to the peel of fruits to avoid rotting during storage and lengthen the market life of the crops, for the whole fruit but particularly for the peel of the fruit, triggered our interest in searching for these compounds in fruit-based soft drinks: products with varying amounts of juice/fruit extract percentages (~5−10%). We focused our study on fruit-based soft drinks of companies distributed worldwide. These beverages contain juice from concentrate in a percentage varying typically from 5 to 10%.

Since over 900 pesticides are used throughout the world, screening approaches are being developed to analyze as many pesticides as possible.[4] Classical, nonpolar pesticides are normally detected by GC/MS whereas modern polar pesticides are preferentially measured by the use of LC−MS/MS techniques.[5−7] A relatively new technique for the control of pesticides in food is liquid chromatography−time-of-flight mass spectrometry (LC−TOF MS).[8−13] Linearity of up to 3 orders of magnitude and LODs at low picogram levels injected are features of LC−TOF MS for quantitative target pesticide residue in crops, obtaining limits of quantitation in compliance with established MRLs.[14−17] The identification and confirmation is provided by retention time together with accurate mass measurements for each (de)protonated molecule ($[M + H]^+$) and characteristic fragment ions, allowing unambiguous identification even in complex matrixes (babyfood, fruits, vegetables, olive oil, etc).[14−17] The accuracies obtained in the measurements of the protonated molecules of the pesticides are better than 2 parts per million (ppm), typically below 0.5 mDa in small molecules. This ability to perform routine accurate mass measurements of ions with high mass accuracy and high full-scan sensitivity makes LC−TOF MS a unique tool for the development of screening methods based on accurate mass database searching. This approach has been implemented successfully for toxicology and forensics applications[18,19] and has been reported recently by our research group for pesticide residue screening in fruits and vegetables.[14,20]

Conventional methods based on triple quadrupole instruments offer high specificity allowing for identification and quantitation of pesticides in complex matrix samples.[7] However, because of limitations in the number of parallel MRM transitions in a single run for all LC−MS/MS, the chromatographic conditions, and the number of time segment windows hamper the development of comprehensive screening methods. Additionally, detection limits are sacrificed by increasing target numbers, and MRM method development require the availability of primary standards.

In contrast, state-of-the-art LC−TOF MS easily meets the required specificity for this application due to the high mass resolution and accuracy without limiting the number of targets.[10] Accurate mass measurements are specific and universal for every target analyte and do not depend on the instrumentation used. For these reasons, these advantageous LC−TOF MS features are extremely convenient for pesticide residue research field. In this work, we have exploited these features to develop a screening method for the multianalyte determination of 100 pesticides applied to fruit-based soft drinks, based on SPE followed by LC−TOF MS analysis using an automated screening method based on a database including information of retention time and accurate masses of characteristic ions for each individual compound. We measured over 100 samples, purchased from 15 different countries of brands from companies which distribute worldwide and have reported the presence of relatively large concentration levels of pesticides in fruit-based soft drinks. The detected pesticides are mainly those applied to crops during the final stages of production (postharvest treatment); some of them contain chlorine atoms in their structures and might have hazardous effects.

EXPERIMENTAL SECTION

Chemicals and Materials. HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Formic acid was obtained from Fluka (Buchs, Switzerland). A Milli-Q-Plus ultrapure water system from Millipore (Milford, MA) was used throughout the study to obtain the HPLC-grade water used during the analyses. All pesticide analytical standards were purchased from Dr. Ehrenstorfer (Ausburg, Germany) and from Riedel de Haën, Pestanal quality (Seelze, Germany). Individual pesticide stock solution (200−300 $\mu g\ mL^{-1}$) were prepared in methanol and stored at −20 °C. Oasis HLB SPE cartridges (200 mg, 6 mL) purchased from Waters (Milford, MA) and a Supelco (Bellefonte, PA) Visiprep SPE vacuum system were also used.

Samples. 102 samples, soft drink bottles and cans of different brands, were collected and purchased from 15 countries. The samples were collected from the following locations: (1) Porto (Portugal), (2) Madrid (Spain), (3) Alicante (Spain), (4) Jaca (Spain), (5) Jaén (Spain), (6) Almería (Spain), (7) Olouron St.

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Marie (France), (8) Lille (France), (9) London (U.K.), (10) Cambridge (U.K.), (11) Edinburgh (U.K.), (12) St. Andrews (U.K.), (13) London-Gatwick (U.K.), (14) Nador (Morroco), (15) La Massana (Andorre), (16) Bologne (Italy), (17) Vevey (Switzerland), (18) Frankfurt (Germany), (19) Berlin (Germany), (20) Krakow (Poland), (21) Hradek Kralove (Czech Republic), (22) Cesky Krumlov (Czech Republic), (23) Bratislava (Slovakia), (24) Vienna (Austria), (25) Budapest (Hungary), (26) Moscow (Russia), (27) Orlando (Florida, U.S.). For more details of the samples, see the Supporting Information. The list of the studied samples including origin and date of collection are included in Tables S1 and S2 of the Supporting Information. The compounds detected and concentrations are shown in Table S3 of the Supporting Information.

Sample Treatment. The extraction method was based on a sample preparation step involving solid-phase extraction (SPE), using HLB cartridges (200 mg) from Oasis (Waters, Milford, MA). The SPE step was carried out using a Visiprep SPE vacuum manifold (Supelco). The cartridges were preconditioned with 5 mL of MeOH and 5 mL of mQ water at a flow rate of 2 mL min^{-1}. After the conditioning step, aliquots of 15 mL of degassed sample (without pH adjustment) were loaded into the cartridge. Soft drink samples were passed through the cartridges at a flow rate of 3 mL min^{-1}. The retained analytes were eluted with 5 mL of MeOH at 1 mL min^{-1}, and this eluate was collected in a 15 mL graduated centrifuge tube. This eluate was then evaporated until near dryness by a gentle nitrogen stream and taken up with 500 μL of MeOH and 1000 μL of mQ water. Prior to LC−MS, this extract was filtered through a 0.45 μm PTFE filter (Millex FG, Millipore, Milford, MA). For validation purposes, matrix-matched standards were prepared by spiking the soft drinks extracts with appropriate amounts of the studied analytes. The matrixes used for recovery studies and calibration were analyzed to make sure they did not contain any of the studied pesticides before performing the validation studies. For recovery studies, the soft drinks samples were spiked with the studied pesticides before the SPE extraction. Recoveries close to 100% were obtained with the proposed SPE method for the pesticides under study.

Liquid Chromatography−Time-of-Flight Mass Spectrometry. The separation of the species from the soft drink SPE extracts was carried out using an HPLC system (consisting of a vacuum degasser, autosampler, and a binary pump) (Agilent Series 1100, Agilent Technologies, Santa Clara, CA) equipped with a reversed phase C_8 analytical column of 150 mm × 4.6 mm and 5 μm particle size (Zorbax Eclipse XDB-C8). A volume of 50 μL of extract was injected in each study. Mobile phases A and B were water with 0.1% formic acid and acetonitrile, respectively. The chromatographic method held the initial mobile phase composition (10% B) constant for 5 min, followed by a linear gradient to 100% B at 30 min. The flow-rate used was 0.6 mL min^{-1}. Then the mobile phase composition was kept constant for 5 min at 100% B, and finally a 12 min postrun time at initial mobile phase composition (10% B) (0.4 mL min^{-1}) was included in order to re-equilibrate the column.

The HPLC system was connected to a time-of-flight mass spectrometer Agilent MSD TOF (Agilent Technologies, Santa Clara, CA) equipped with an electrospray interface operating in the positive ion mode, using the following operation parameters: capillary voltage, 4000 V; drying gas, 9 L min^{-1}; gas temperature, 325 °C; nebulizer pressure, 40 psig; skimmer voltage, 60 V; octapole dc 1, 37.5 V; octapole rf, 250 V; fragmentor voltage, 190 V. LC−TOF MS accurate mass spectra were recorded across the range 50−1000 m/z. Accurate mass measurements of each peak from the total ion chromatograms were obtained by means of an automated calibrant delivery system using a dual-nebulizer electrospray source that introduces the flow from the outlet of the chromatograph together with a low flow of a calibrating solution (calibrant solution A, Agilent Techologies), which contains the internal reference masses (purine ($C_5H_4N_4$ at m/z 121.050 873 and HP-921 [hexakis-(1*H*,1*H*,3*H*-tetrafluoropentoxy)-phosphazene] ($C_{18}H_{18}O_6N_3P_3F_{24}$) at m/z 922.009 798). Besides, a software package is autocalibrating and continuously recording the results of the internal reference masses along with the raw data. The instrument worked providing a typical resolution of 9700 ± 500 (m/z 922). The full-scan data recorded was processed with Applied Biosystems/MDS-SCIEX Analyst QS software (Frankfurt, Germany) with accurate mass application-specific additions from Agilent MSD TOF software.

RESULTS AND DISCUSSION

Automated Screening of Target Pesticides by LC−TOF MS Accurate Mass Measurements and Retention Time Information: Method Development and Screening Results. The automated screening procedure described elsewhere by our research group[10,14,20] enables the analysis of a large number of pesticides and degradates (i.e., 100−300) in any complex food extract using LC−TOF MS in the positive ion mode with full-scan accurate mass spectra. The main strength of the proposed approach is the theoretically unlimited number of compounds that can be screened simultaneously at low concentration levels. In fact, no overlapping or interference was observed in the 100 compounds list (see Table 1). The screening criteria consisted of ±10 mDa accurate mass window, ±0.25 min retention time window, and a minimum area count of 5000 (approximately the typical area corresponding to the approximate LOD of a large number of the studied compounds).

The screening method comprised exact monoisotopic masses for 100 multiclass pesticides. Once the screening method was established, fruit-based soft drink SPE extracts were injected. Each ion of interest in the database was searched and extracted (at the t_R window of interest) from the sample file in an automated fashion. An analysis of 100 pesticides takes from 2 to 5 min to be processed in a laptop, depending on the length of the file (a 30 min run analysis is ~80 MB).[14] From the LC−TOF MS acquisition data, the automated target database search reported hits within a selected retention time window (t_R ± 0.25 min), area counts and mass tolerance ($[M + H]^+$ ± 10 mDa). Subsequent confirmation of the findings is accomplished by accurate mass analysis, using a 5 ppm-mass accuracy threshold for final confirmation.

Interestingly, the results obtained using the automated screening method of the pesticides showed the presence of the following pesticides in a significant percentage of samples tested (over 100): carbendazim, thiabendazol, imazalil and its main degradation product, prochloraz and its main degradation product, and malathion. In these positive samples, accurate mass analysis of characteristic fragment ions and isotopic signatures if available

Table 1. Accurate Mass Measurements of Ions of Interest and Retention Time (t_R) of the Compounds Included in the Method for the Screening of Pesticides in Fruit-Based Soft Drinks

pesticide	selected ion ($[M + H]^+$)	$m/z_{calculated}$	t_R (min)	pesticide	selected ion ($[M + H]^+$)	$m/z_{calculated}$	t_R (min)
cyromazine	$C_6H_{11}N_6$	167.103 97	3.6	thiofanox[a]	$C_9H_{18}N_2O_2NaS$	241.098 12	21.1
butoxicarboxin	$C_7H_{15}N_2O_4S$	223.074 70	3.9	metalaxyl	$C_{15}H_{22}NO_4$	280.154 33	21.2
carbendazim	$C_9H_{10}N_3O_2$	192.076 75	7.0	isoproturon	$C_{12}H_{19}N_2O$	207.149 18	21.2
thiabendazole	$C_{10}H_8N_3S$	202.043 34	8.6	diazoxon	$C_{12}H_{21}N_2O_4P$	289.131 17	21.2
oxamyl[a]	$C_7H_{13}N_3O_3SNa$	242.056 98	11.2	difenoxuron	$C_{16}H_{19}N_2O_3$	287.139 01	21.2
aldicarb-sulfone	$C_7H_{15}N_2O_4S$	223.074 7	11.4	diuron	$C_9H_{11}N_2OCl_2$	233.024 29	21.3
nitenpyram	$C_{11}H_{16}N_4O_2Cl$	271.095 63	12.1	monolinuron	$C_9H_{12}N_2O_2Cl$	215.058 18	21.5
methomyl[a]	$C_5H_{10}N_2O_2SNa$	185.035 52	12.2	ethiofencarb	$C_{11}H_{16}NO_2S$	226.089 44	21.6
chloridazon	$C_{10}H_9N_3OCl$	222.042 86	14.7	spinosyn D	$C_{42}H_{68}NO_{10}$	746.483 77	21.6
ethiofencarb sulfoxide	$C_{11}H_{16}NO_3S$	242.084 54	13.6	metobromuron	$C_9H_{12}N_2O_2Br$	259.007 66	22.0
thiofanox-sulfoxide	$C_9H_{19}N_2O_3S$	235.111 09	13.7	dimethomorph	$C_{21}H_{23}NO_4Cl$	388.131 01	22.1/22.5
thiamethoxam	$C_8H_{11}N_5O_3ClS$	292.026 56	13.7	flazasulfuron	$C_{13}H_{13}N_5O_5F_3S$	408.058 4	22.3
methiocarb sulfoxide	$C_{11}H_{16}NO_3S$	242.084 54	14.4	ioxynil	$C_7H_4NOI_2$	371.837 69	22.6
metamitron	$C_{10}H_{11}N_4O$	203.092 73	14.5	triadimenol	$C_{14}H_{19}N_3O_2Cl$	296.116 03	22.7/23.1
imazalil-Met	$C_{11}H_{11}N_2OCl_2$	257.024 29	14.6	prochloraz	$C_{15}H_{17}N_3O_2Cl_3$	376.038 08	22.8
cambendazole	$C_{14}H_{15}N_4O_2S$	303.091 02	14.9	propazine	$C_9H_{17}N_5Cl$	230.116 69	23.0
ethiofencarb sulfone	$C_{11}H_{16}NO_4S$	258.079 45	15.1	cyproconazole	$C_{15}H_{19}N_3OCl$	292.121 11	23.3
imidacloprid	$C_9H_{11}N_5O_2Cl$	256.059 57	15.5	methiocarb	$C_{11}H_{16}NO_2S$	226.089 62	23.4
oxfendazole	$C_{15}H_{14}N_3O_3S$	316.075 03	15.5	terbuthylazine	$C_9H_{17}N_5Cl$	230.116 69	23.4
dimethoate	$C_5H_{12}NO_3PS_2$	230.006 9	16.1	chloroxuron	$C_{15}H_{16}N_2O_2Cl$	291.089 48	23.5
acetamiprid	$C_{10}H_{12}N_4Cl$	223.074 5	16.3	bromuconazole	$C_{13}H_{13}N_3OBrCl_2$	375.961 35	23.6/24.5
thiofanox-sulfone	$C_9H_{19}N_2O_4S$	251.106 00	16.4	linuron	$C_9H_{10}N_2O_2Cl_2$	249.019 2	23.7
prochloraz-Met	$C_{11}H_{15}NOCl_3$	282.021 37	16.8	methidathion[a]	$NaC_6H_{11}N_2O_4PS_3$	324.951 08	23.7
cymoxanil	$C_7H_{11}N_4O_3$	199.082 56	17.4	fenamiphos	$C_{13}H_{23}NO_3PS$	304.113 08	23.8
albendazole	$C_{12}H_{16}N_3O_2S$	266.095 77	17.8	chlorbromuron	$C_9H_{11}N_2O_2BrCl$	292.968 69	24.0
butocarboxin[a]	$C_7H_{14}N_2O_2SNa$	213.066 82	17.1/17.6	azoxystrobin	$C_{22}H_{17}N_3O_5$	404.124 09	24.1
methiocarb sulfone	$C_{11}H_{16}NO_4S$	258.079 45	17.3	promecarb	$C_{12}H_{18}NO_2$	208.133 2	24.1
thiacloprid	$C_{10}H_{10}N_4ClS$	253.030 92	17.8	tebuconazole	$C_{16}H_{23}N_3OCl$	308.152 41	24.5
imazalil	$C_{14}H_{15}N_2OCl_2$	297.055 9	17.9	triadimefon	$C_{14}H_{17}N_3O_2Cl$	294.100 38	24.5
mebendazole	$C_{16}H_{14}N_3O_3$	296.102 96	18.0	tetraconazole	$C_{13}H_{12}N_3OF_4Cl_2$	372.028 8	24.6
aldicarb	$C_7H_{15}N_2O_2S$	191.084 87	18.4	diflubenzuron	$C_{14}H_{10}N_2O_2ClF_2$	311.039 33	24.9
oxadixyl	$C_{14}H_{19}N_2O_4$	279.133 93	18.7	iprodione	$C_{13}H_{14}N_3O_3Cl_2$	330.040 67	25.3
simazine	$C_{10}H_8N_3OCl$	202.085 39	18.7	triflumizol	$C_{15}H_{16}N_3OF_3Cl$	346.092 85	25.5
fluroxypyr	$C_7H_6N_2O_3FCl_2$	254.973 4	18.8	malathion	$C_{10}H_{20}O_6PS_2$	331.043 34	25.6
monuron	$C_9H_{11}N_2OCl$	199.063 06	18.8	procymidone	$C_{13}H_{12}NO_2Cl_2$	284.023 96	25.6
flubendazole	$C_{16}H_{13}N_3O_3F$	314.093 54	18.8	neburon	$C_{12}H_{17}N_2OCl_2$	275.071 24	25.8
lenacil	$C_{13}H_{19}N_2O_2$	235.144 1	19.2	vinclozolin	$C_{12}H_{10}NO_3Cl_2$	286.003 22	26.3
methyl-thiophanate	$C_{12}H_{15}N_4O_4S_2$	343.052 92	19.4	mecarbam	$C_{10}H_{21}NO_5PS_2$	330.059 33	26.4
pyrimethanil	$C_{12}H_{14}N_3$	200.118 22	19.6	triflumuron	$C_{15}H_{11}N_2O_3F_3Cl$	359.040 48	26.5
spiroxamine	$C_{18}H_{36}NO_2$	298.274 05	19.8	dichlofluanid	$C_9H_{12}N_2O_2Cl_2F_2S_2$	332.969 58	26.6
ethoxyquin	$C_{14}H_{20}NO$	218.153 94	19.9	hexaflumuron	$C_{16}H_9N_2O_3F_6Cl_2$	460.988 89	27.2
prometryn	$C_{10}H_{20}N_5S$	242.143 39	20.1	buprofezin	$C_{16}H_{23}N_3OS$	306.163 46	27.2
fenbendazole	$C_{15}H_{14}N_3O_2S$	300.080 12	20.2	diazinon	$C_{12}H_{21}N_2O_3PS$	305.108 32	27.5
carbofuran	$C_{12}H_{16}NO_3$	222.112 46	20.4	teflubenzuron	$C_{14}H_7N_2O_2F_4Cl_2$	380.981 52	27.6
chlorotoluron	$C_{10}H_{14}N_2OCl$	213.078 91	20.4	thiobencarb	$C_{12}H_{16}NOClS$	258.071 39	27.6
bendiocarb	$C_{11}H_{14}NO_4$	224.091 73	20.6	lufenuron	$C_{17}H_8N_2O_3F_8Cl_2$	510.985 70	28.6
spinosyn A	$C_{41}H_{66}NO_{10}$	732.468 12	20.9	pyriproxyfen	$C_{20}H_{20}NO_3$	322.143 77	29.2
fluometuron	$C_{10}H_{12}N_2OF_3$	233.089 62	21.0	flufenoxuron	$C_{21}H_{12}N_2O_3F_6Cl$	489.043 51	29.3
atrazine	$C_8H_{15}N_5Cl$	216.101 04	21.1	chlorfluazuron	$C_{20}H_{10}N_3O_3F_5Cl_3$	539.970 24	29.7
miconazole	$C_{18}H_{15}N_2OCl_4$	414.993 3	21.1	hexythiazox	$C_{17}H_{22}N_2O_2ClS$	353.108 5	30.0

[a] Sodium adducts.

were also used in order to provide unambiguous confirmation of positive samples containing the pesticides detected during the screening step. Table 2 shows the results from accurate mass analysis of a fruit-based soft drink sample spiked at 5 μg L^{-1} of the pesticides which were usually found in the tested samples. Mass accuracies obtained for both protonated molecules and fragment ions were within the 3 ppm threshold. On the other hand, electrospray ionization conditions were studied to achieve the best possible sensitivity and selectivity for the selected pesticides. We selected default values from previous experience on multiresidue methods.[14,15,21] Fragmentor voltage, which affects in-source fragmentation and thus sensitivity, was set at 190 V, as a compromise value between sensitivity for quantitation and additional mass spectral information for confirmation purposes.

(21) Gómez, M. J.; Malato, O.; Ferrer, I.; Agüera, A.; Fernández-Alba, A. R. *J. Environ. Monit.* **2007**, *9*, 718–729.

Validation of the Sample Treatment Procedure for the Studied Pesticides. To perform the untargeted search for pesticide residues in the studied fruit-based soft drink samples, a generic, broad sample treatment based on SPE was used. With this method, multiclass compounds are extracted with recovery rates between 70−110% for most of the analytes.[20] Besides, blanks of solvents and cartridges were examined throughout the study to secure the absence of both carryover effects and cross-contamination phenomena. The occurrence and concentration found on the preliminary studies prompted us to conduct a detailed validation of the extraction method on the compounds usually found in the tested samples, in order to assess the recovery of the studied compounds, beyond the conventional sample treatment applied initially for the screening method.

For the SPE step, 15 mL of soft drink sample was selected as loaded volume. The preconcentration factor was set at 10:1, due

Table 2. Identification and Confirmation of Pesticide Residues in Fruit-Based Soft-Drinks By LC−TOF MS[a]

compound	t_R	ion	Elemental Compositions	m/z theoretical	m/z experimental	error mDa	error ppm
carbendazim	7.7	$[M + H]^+$	$C_9H_{10}N_3O_2$	192.0767	192.0765	−0.25	1.3
		fragment	$C_8H_6N_3O$	160.0505	160.0502	−0.33	2.1
thiabendazole	9.8	$[M + H]^+$	$C_{10}H_8N_3S$	202.0433	202.0432	−0.14	0.7
imazalil	14.7	$[M + H]^+$	$C_{11}H_{11}N_2OCl_2$	257.0242	257.0245	0.20	0.8
metabolite		^{37}Cl ion	$C_{11}H_{11}N_2OCl\ ^{37}Cl$	259.0213	259.0216	−0.27	1.0
prochloraz	16.7	$[M + H]^+$	$C_{11}H_{15}NOCl_3$	282.0213	282.0211	−0.27	1.0
metabolite		^{37}Cl ion	$C_{11}H_{15}NOCl_2{}^{37}Cl$	284.0184	284.0180	−0.42	1.5
		$^{37}Cl_2$ ion	$C_{11}H_{15}NOCl\ ^{37}Cl_2$	286.0154	286.0150	−0.47	1.6
imazalil	17.9	$[M + H]^+$	$C_{14}H_{15}N_2OCl_2$	297.0555	297.0549	−0.69	2.3
		^{37}Cl ion	$C_{14}H_{15}N_2OCl\ ^{37}Cl$	299.0526	299.0526	−0.25	0.8
prochloraz	22.9	$[M + H]^+$	$C_{15}H_{17}N_3O_2Cl_3$	376.0380	376.0384	0.31	0.8
		^{37}Cl ion	$C_{15}H_{17}N_3O_2Cl_2{}^{37}Cl$	378.0351	378.0356	0.46	1.2
		$^{37}Cl_2$ ion	$C_{15}H_{17}N_3O_2Cl\ ^{37}Cl_2$	380.0321	380.0326	0.41	1.1
		fragment 1	$C_{12}H_{13}NO_2Cl_3$	308.0006	308.0012	0.56	1.8
		^{37}Cl ion	$C_{12}H_{13}NO_2Cl_2{}^{37}Cl$	309.9976	309.9983	0.61	2.0
		$^{37}Cl_2$ ion	$C_{12}H_{13}NO_2Cl\ ^{37}Cl_2$	311.9947	311.9953	0.66	2.1
malathion	25.3	$[M + H]^+$	$C_{10}H_{20}O_6PS_2$	331.04334	331.0433	−0.05	0.2
		$[M + Na]^+$	$C_{10}H_{19}O_6PS_2Na$	353.0252	353.0254	0.11	0.3
		fragment 1	$C_8H_{14}O_5PS_2$	285.0015	285.0019	0.41	1.5
		fragment 2	$C_2H_8O_2PS_2$	158.9698	158.9697	−0.09	0.6
		fragment 3	$C_6H_7O_3$	127.0389	127.0388	0.17	1.3
		fragment 4	$C_2H_6O_2PS$	124.9821	124.9820	0.006	0.5
		fragment 5	$C_4H_3O_3$	99.00767	99.0078	0.13	1.3
iprodione	25.5	$[M + H]^+$	$C_{13}H_{14}N_3O_3Cl_2$	330.0406	330.0412	−0.52	1.6
		^{37}Cl ion	$C_{13}H_{14}N_3O_3Cl\ ^{37}Cl$	332.0377	332.0382	0.47	1.4
		fragment 2	$C_9H_7N_2O_2Cl_2$	244.9879	244.9880	0.09	0.4
		^{37}Cl ion	$C_9H_7N_2O_2Cl\ ^{37}Cl$	246.9849	246.9853	0.34	1.4

[a] Accurate mass analysis of a fruit-based soft drink extract spiked at 5 $\mu g\ L^{-1}$ of the pesticides found throughout the study.

to the complexity of the matrix. It should be noted that the proposed method is based on a direct SPE procedure without further cleanup stages. Therefore, the obtained extracts are relatively dirty to be injected in the LC−MS instrument, so that the use of relatively small preconcentration factors was mandatory. Preconcentration factors tested of 20:1 or higher involved complex extracts that yielded signal/sensitivity losses, making daily cleaning and maintenance of the electrospray source necessary. In addition, under these conditions, matrix effects were significant (over 35% suppression in all the studied analytes). In contrast, the use of preconcentration factors of 10:1 did not affect strongly the sensitivity and signal stability of the MS source over large periods of operation. Furthermore, matrix effects were negligible for all the analytes (suppression percentages below 5% in most cases), which secures the accurate quantitation of the samples.

To evaluate the effectiveness of the extraction method, different recovery studies were carried out using an orange-flavored soft drink sample. Several aliquots were spiked at three different concentration levels (5, 10, and 20 $\mu g\ L^{-1}$) with the working standard solution. Then the spiked samples were extracted with the proposed SPE method described. The obtained extracts were analyzed with the developed LC−TOF MS method, obtaining recoveries between 74 and 106%, as can be seen in Table 3. The precision of the whole method including SPE and LC−MS analysis was remarkable, with RSD values below 12% in most cases. These results show the feasibility of the studied extraction method for the determination of the selected pesticides in fruit-based soft drinks.

Table 3. Recovery Studies on Fruit-Based Soft Drinks, Spiked with Selected Pesticides at Three Different Fortification Levels: 5, 10 and 20 $\mu g\ L^{-1}$

pesticide	spiking level ($\mu g\ L^{-1}$)	recovery (%)	RSD (%) ($n = 5$)
carbendazim	5	79.5	10.2
	10	96.7	9.1
	20	91.0	8.7
thiabendazole	5	95.3	10.1
	10	104.6	9.2
	20	102.1	6.9
imazalil metabolite	5	90.8	11.1
	10	85.7	9.0
	20	94.1	6.7
prochloraz metabolite	5	78.3	11.4
	10	82.0	9.8
	20	91.0	8.7
imazalil	5	96.3	8.4
	10	105.8	7.5
	20	96.1	8.9
prochloraz	5	75.8	12.1
	10	81.7	7.3
	20	89.1	8.7
malathion	5	79.1	12.0
	10	81.7	9.9
	20	83.0	10.1
iprodione	5	74.1	13.0
	10	76.7	11.2
	20	73.7	12.3

Analytical Performance. For identification and quantitation purposes, we used extracted ion chromatograms (XICs) using a mass-window width of 20 mDa ($[M + H]^+ \pm 10$ mDa). The protonated molecule ($[M + H]^+$) was used for both confirmation and quantitation purposes in most cases, except for prochloraz, where the relative abundance of its characteristic fragment ion (with m/z 308) was higher than that of the protonated molecule in the selected conditions. In addition, some studied fungicides present chlorine atoms (e.g., imazalil, imazalil metabolite, prochlo-

Table 4. Analytical Parameters for the Analysis of Selected Pesticides in Fruit-Based Soft Drinks by LC−TOF MS

compound	concn range (μg L^{-1})	linearity (r)	LOD (μg L^{-1})	LOQ (μg L^{-1})	RSD (%) ($n = 6$)	
					intraday	interday
carbendazim	0.1−50	0.9990	0.03	0.1	2.9	7.8
thiabendazole	0.1−50	0.9996	0.009	0.03	3.7	8.2
imazalil metabolite	0.1−50	0.9997	0.006	0.02	1.9	6.1
prochloraz metabolite	0.1−50	0.9999	0.006	0.02	3.1	4.5
imazalil	0.1−50	0.9991	0.006	0.02	1.2	3.4
prochloraz	0.1−50	0.9998	0.006	0.02	2.4	4.9
malathion	0.1−50	0.9997	0.006	0.02	4.4	9.0
iprodione	1−50	0.9991	0.09	0.3	6.3	9.9

raz), which offer an isotopic pattern that yield further information for the unambiguous identification of the target compounds.[22]

Calibration curves of the analyzed compounds were constructed at different concentrations, in the range 0.1−50 μg L^{-1} using fruit-based soft drink extracts to prepare matrix-matched standards. The linearity of the analytical response across the studied range is excellent, taking into account that all the calibration curves of the analyzed compounds showed correlation coefficients higher than 0.999 as shown in Table 4, where these values are summarized together with the limits of detection and intra- and interday RSD (%). The relative standard deviation (RSD) ($n = 6$) values for the run-to-run study were in the range 1.2−6.3% and interday RSD ($n = 6$) values were between 3.4 and 9.9%. These results demonstrate the precision of the developed method and the potential of the proposed approach for quantitative purposes. The limits of detection (LODs) obtained were estimated from the injection of matrix-matched standard solutions at concentration levels corresponding to a signal-to-noise ratio (S/N) = 3. Similarly, limits of quantification (LOQs) were estimated on the basis of the 10:1 signal-to-noise ratio criterion. The results obtained for the target pesticides are shown in Table 4. The limits of detection obtained were as low as 6 ng L^{-1} for prochloraz or imazalil and below 0.03 μg L^{-1} for all the chemicals studied, enabling the appropriate monitoring of the soft drink samples at ultratrace levels.

Monitoring Results and Discussion. We purchased different brands which together make up most of the global market for fruit-based soft drinks. We measured 102 samples collected from around the world and investigated the presence of 100 compounds (see Table 1). Samples were collected from Spain (41), The United Kingdom (19), The United States (11), France (8), Italy (5), Russia (4) Germany (3), Austria (2), The Czech Republic (2), Morocco (2), Hungary (1), Poland (1), Portugal (1), Slovakia (1), and Switzerland (1). (see Tables S1 and S2 in the Supporting Information).

From the 102 samples analyzed, only 17 (16.7%) were found to be free of the studied pesticides. The rest of the samples were positive. Out of these, 14.6% contained at least 1 pesticide, 4% contained 2 pesticides, 65% contained at least 3 pesticides, and 58% of the studied samples contained 4 or more pesticide residues. The concentration found for the studied and detected compounds in each individual sample is included in the Supporting Information. For instance, there were cases of seven different classes of pesticides found in the same sample at relevant concentrations. It should be stated that the presence of more than one chemical can enhance the toxic effect of the others. The combined effect of a cocktail comprised of various pesticides can be more harmful than the sum of the individual effects from each of them alone.[4]

Interestingly, most of the samples collected from the United States (11 samples purchased in Orlando, FL) did not contain pesticides. In this country, as claimed on the label, the product is artificially flavored, and therefore no fruit extract is used. This explains the absence of pesticides. In Morocco and Russia, no significant concentrations were detected either, although the products contained a certain percentage of juice. In these cases, either the way the product is manufactured is different or it is possible that the raw material does not contain any pesticide. A more in-depth study, including a detailed analysis of other less used classes of pesticides (i.e., organochlorine and organophosphorus) or pesticides banned in the EU, should be performed to confirm these results. The rest of the samples collected in the EU contained relatively large concentrations of carbendazim, imazalil, imazalil metabolite, prochloraz, prochloraz metabolite, and thiabendazole.

The most frequently detected pesticides, mainly postharvest fungicides, were carbendazim (73%), imazalil (68%), imazalil metabolite, (60%), prochloraz (50%), prochloraz metabolite (40%), and thiabendazole (56%), although insecticides were also detected (malathion traces were detected in 22% of the samples). The concentration levels of thiabendazole and imazalil found in selected samples are shown in Figure 1. It should be noted that the MRL of each individual pesticide in drinking water, according to the EU, is 0.1 μg L^{-1}. As shown, the values are much higher than the EU MRL (by factors of up to 320 times).

Table 5 shows data on the concentration of the detected pesticides in the studied samples. For instance, the range of the imazalil concentration in the positive samples varied between 0.05 and 32.0 μg L^{-1} (from 0.5 to 320 times the tolerated EU MRL). Besides, the range of the thiabendazole concentration in the positive samples varied from 0.18 to 9.8 μg L^{-1} (from 1.8 to 98 times the tolerated EU MRL).

The average concentration of the detected compounds in the studied fruit-based soft drink samples classified per country is shown in Figure 2. The samples from Spain and the U.K. are those with a higher level of pesticides. See for instance, Figure 3, showing the LC−TOF MS analysis of a sample from the United Kingdom. The average concentration of the U.K. samples tested was 17.4 μg L^{-1}, which is 34.6 times the EU MRL for the sum of pesticides permitted. The average concentration of Spanish samples tested was 12.3 μg L^{-1}, 25-fold the EU standard. Except for the U.S., Russia, and Morocco, the MRL value is exceeded in all the studied samples/countries. In Germany, the average

(22) Garcia-Reyes, J. F.; Ferrer, I.; Thurman, E. M.; Molina-Díaz, A.; Fernández-Alba, A. R. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2780–2788.

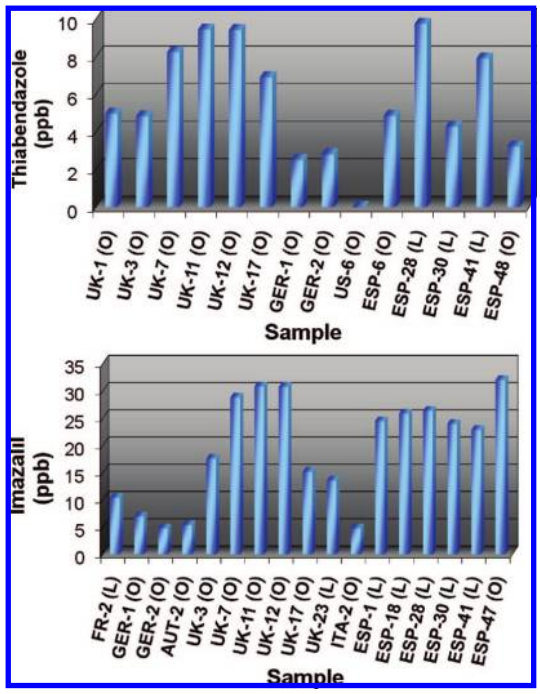

Figure 1. Individual concentration of pesticides thiabendazole and imazalil in selected samples expressed in parts ber billion (micrograms per liter) from different EU countries. The maximum residue level (MRL) tolerated for an individual pesticide in drinking water according to the EU standard is 0.1 μg L^{-1}. (O) Orange flavor; (L) lemon flavor. The concentration values detected for thiabendazole and imazalil are up to 320 times the EU MRL level.

Table 5. Concentration Levels of the Detected Pesticides in the Studied Fruit-Based Soft Drink Samples

pesticide	positive samples (%)	concentration range (μg L^{-1})	x MRL standard
carbendazim	73	0.11−4.8	1.1−48
imazalil	68	0.05−32	0.5−320
imazalil metabolite	60	0.025−0.74	0.25−7.4
thiabendazole	56	0.18−9.8	1.8−98
prochloraz	50	0.036−3.7	0.36−37
prochloraz metabolite	40	0.18−1.4	1.8−14
malathion	22	0.02−0.16	0.2−1.6
iprodione	1	0.71	7.1

concentration was 8.4 μg L^{-1}, 17 times the MRL. In France it was 4.9 μg L^{-1}, 9.8 times the MRL, and in Austria, 7.0 μg L^{-1}, 14 times the MRL.

In the study of the detected concentration levels in the tested soft drink samples, it becomes apparent that the total concentration of pesticides present in the raw extract of fruit used to flavor the soft drink, which represents 5−8% of the total product, is really high (i.e., 300−800 μg L^{-1} levels). This confirms that the peel is also used to prepare the extract that flavors the soft drinks. The source of contamination could be attributed to bad practices when manufacturing the products: the peels of the fruits (mainly citrus) are not removed or appropriately washed before being squeezed, probably to reduce costs. The peels contain large amounts of pesticides, and these compounds are then transferred to the final product. Therefore, it would not be difficult to remove this source of pesticide contamination. It is simply a matter of changing the way the raw (juice) extract is prepared from the fruits.

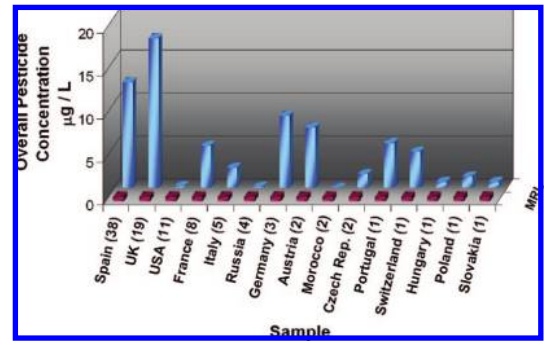

Figure 2. Overall average concentration of pesticides found in the studied fruit-based soft drink samples according to the country (average pesticide concentration per country, in blue), expressed in micrograms per liter and maximum residue level (MRL) tolerated for the sum of pesticides (0.5 μg L^{-1}) according to the EU standard, in purple. The number of samples is given in parentheses after the country. The overall concentration values detected in most of the countries exceed the EU MRL level in drinking water, especially in the U.K. and Spain, where the concentrations found are about 35 and 25 times the EU standard, respectively.

In a recent Indian survey report of pesticides in soft drinks, organophosphorus and organochlorine pesticide residues[23] were found in carbonated soft drinks. In this case, the pesticides detected were already present in the raw water used to prepare the soft drinks. The Centre of Science and Environment in India reported that the origin of pesticides in soft drink came from the raw water used, which represented 90% of its composition. They found that the samples of groundwater taken from inside the factory were contaminated with the same pesticides as were found in the finished product. Therefore, the origin of this source was the contamination of raw water used to prepare the products, which might be contaminated due to decades of pesticide use in agriculture.

Unlike the Indian scenario (where the presence of pesticides caused by environmental contamination is practically unavoidable unless a dedicated treatment is applied to raw water), the source of contamination in the case we are dealing with might be related to the way these products are manufactured, since pesticides might be transferred from the peels to the product, probably during squeezing.

The main obstacle is the absence of regulations and standards, which are necessary in deciding whether the presence of these concentration levels is tolerable or not. In the case of Indian soft drinks, the Drinks and Carbonated Beverages Sectional Committee, FAD14, of The Bureau of Indian Standards (BIS) deliberated on the issue of pesticide residue standards for soft drinks. In the end, they proposed the adoption of the same EU standard as used for drinking water. But this standard is voluntary and not mandatory in nature so far. In the following discussion we have used the EU drinking water standard as the reference value to evaluate the concentration levels found in the tested samples.

The toxicity effects of consuming these products are difficult to predict and evaluate. Equally, the effects of cocktails of pesticides included in the same sample and at relevant concentrations, for example, there were samples with six to seven pesticides that are not usually evaluated in toxicological tests. In these types

(23) India, C. CSE Report: Analysis of Pesticide Residues in Soft Drinks, August 2006 (available at www.cseindia.org).

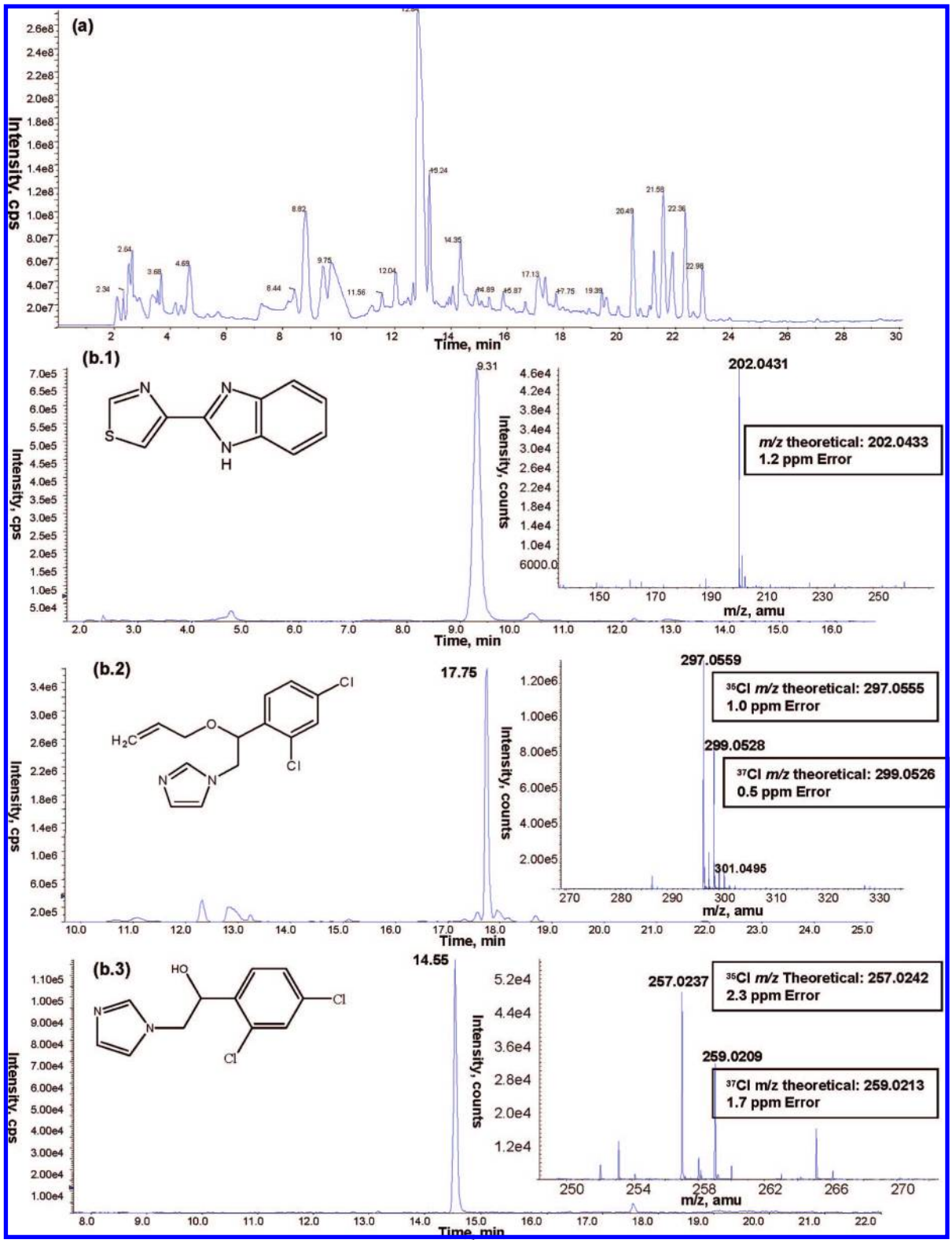

Figure 3. Liquid chromatography−time-of-flight mass spectrometry (LC−TOF MS) analysis of pesticides residues in a fruit based soft drink from Gatwick Airport (London), U.K. (a) Total ion chromatogram; (b) extracted ion chromatograms of the detected compounds: (b.1) thiabendazole (9.44 μg L^{-1}); (b.2) imazalil metabolite (0.689 μg L^{-1}); and (b.3) imazalil (30.8 μg L^{-1}). The total concentration of pesticides detected was 41.5 μg L^{-1} (83-fold the EU standard). Structures and accurate mass spectra of the detected compounds are included as insets.

of tests, only data on individual compounds is given, mainly acute toxicity tests, obtained from assays with animals and then a safety factor is applied. However, the chronic toxicity of these pesticides on humans is a difficult task to assess in depth, particularly when more than one chemical is present. Therefore, the exposure of children to these kinds of products should be limited.

Concern has been raised that exposure to pesticides might modulate or disrupt the endocrine system in humans. Many

pesticides are able to block or activate the steroid hormone receptors and/or to affect the levels of sex hormones, thereby potentially affecting the development or the expression of the male and female reproductive system, or both. According to recent studies, azole compounds (i.e., prochloraz and imazalil) may cause inhibition of aromatase activity, an unwanted side effect that might cause endocrine disruption (putative effects on steroid biosynthesis and sex hormone balance).[24−26] In these assays, both prochloraz and imazalil gave rise to a statistically significant inhibition of CYP19 aromatase activity in human placental microsomes.

It is clear that the MRLs are dramatically exceeded in most of the studied samples (according to EU regulations for drinking water). Taking into account (1) the high concentration levels found in most of the samples tested, particularly in the EU, in view of the 0.1 $\mu g\ L^{-1}$ threshold established by EU guidelines for drinking water and (2) given that cocktails of pesticides are usually found (up to six to seven compounds) with toxic effects difficult to predict and evaluate, action should be taken to ensure the food safety of these products, especially considering that children are the main target and consumers. It would seem apparent that steps should be taken toward the removal of pesticides in these beverages by changing the way these compounds are manufactured. In addition, new safety standards should be set urgently by official bodies to appropriately regulate the market for soft drinks (carbonated and fruit-based soft drinks and derivates). Unless safe limits are defined, we cannot refer to this situation as unsafe, despite these products containing unnecessarily large amounts of pesticides.

CONCLUDING REMARKS

In this work, we have exploited the advantaging features of LC−TOF MS (viz. high sensitivity full scan acquisition with accurate mass measurement capabilities) to develop a screening method for the multianalyte determination of 100 pesticides in fruit-based soft drinks. The proposed approach was based on SPE extraction followed by LC−TOF MS analysis using an automated screening method based on a database including information of retention time and accurate masses of characteristic ions for each individual compound. The proposed automatic screening method has been applied to the identification of pesticides residues in a new matrix in pesticide residue research: fruit-based soft drinks. To the best of our knowledge, this is the first method developed for the determination of these chemicals in this matrix and also the first time that monitoring results on the presence of these compounds are reported. The presence of these pesticides in fruit-based soft drinks could be attributed to the use of the peels in the extracts prepared which flavor the soft drink. As these compounds are concentrated in the fruit peel, they could be almost completely removed by the application of good manufacturing practices. Therefore steps should be taken in order to avoid pesticide contamination in these products by changing the way they are manufactured and establishing appropriate quality standards to regulate fruit-based soft drinks, in order to avoid this source of pesticide exposure, particularly on vulnerable groups with higher exposure such as children.

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SUPPORTING INFORMATION AVAILABLE

Tables with detailed information on the samples and results obtained including data concerning country of origin, date, and concentration of the detected pesticides. This material is available free of charge via the Internet at http://pubs.acs.org.

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