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Profiling of compounds and degradation products from the postharvest treatment of pears and apples by ultra-high pressure liquid chromatography quadrupole-time-of-flight mass spectrometry

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ABSTRACT

This study deals with a simple strategy to pinpoint potential unknown compounds in full scan mass spectrometry (MS) experiments. Forty samples of apples and pears intended for human consumption were analyzed by ultra-high performance liquid chromatography quadrupole-time-of-flight (UPLC–QqTOF-MS), after extraction of the possible contaminants by rinsing the peel of the fruit with ethyl acetate. The peaks were visually recognized in the total ion chromatogram (TIC). Two major types of postharvest treatments were detected in this set of samples: imazalil (IMZ)/ethoxyquin (EQ) and thiabendazole (TBZ)/diphenylamine (DPA). The present work also describes the metabolites formed by degradation of EQ (to our knowledge not previously reported) and DPA (there was mass spectral evidence of some of them but full identification was not pursued). Hydroxy-DPA, n-phenyl-4-quinoneimine, methoxy-DPA, demethyl-EQ, demethyldehydro-EQ, EQ-dimer, methyl-EQ, EQ-N-oxyl and 2′,2,4,-trimethyl-6-quinolone were unequivocally identified and confirmed. Some relationships between the applied postharvest treatment and the metabolites formed were established. Remarkably, they may constitute a useful fingerprint in further investigations of postharvest treatments. Among other significant results, the study also reveals for the first time the presence of some EQ metabolites in fruits, which are different from those previously reported in animal tissues. There is not information on the occurrence of EQ metabolites in fruits and the DPA ones have not been studied extensively in pears and apples. The levels of the metabolites found exceeded several times those of the parent compounds.

1. Introduction

Recent advances in mass spectrometric instrumentation have provided the scientists with highly valuable tools to gain deeper insight into transformation processes of food contaminants and residues, which are extremely important to fully-understand toxicological effects on humans [1–3]. Studies about the identification, mainly of pesticides and their transformation products but also of other unknown food contaminants, have been multiplied in the last years [4–12]. This issue deserves further research efforts as clearly illustrated by the latest safety alarms activated within the field, such as the antibiotic metabolites in shrimps imported from Asia, the melamine in composite foods containing milk or milk products originating from China and the discovery of dioxin contamination in pork meat from Ireland distributed within the European Union [13,6,14,15].

Apples and pears are postharvest treated by dipping or spraying with fungicides to prevent rotting caused by *Phlyctaena*, *Penicillium*, *Botrytis*, *Rhizopus*, *Geosporium* and other pathogenic fungi. In addition, different physiological disorders (core flush, scald...) can also appear [16–19]. For the control of rot several fungicides, including conazoles, benzoimidazoles, bezamides, carbamates and dinitrophenols, are the major classes recommended [20,21]. Recent studies on the subject have pointed out the occurrence of postharvest fungicides, as well as some of their metabolites in different fruits [22–25]. In this field sophisticated liquid chromatography (LC) and mass spectrometry (MS) instrumentation provide a variety of platforms for sensitive detection of many types of molecules [8,12,25–28]. Research on metabolites is critical to understand the degradation processes of pesticides and other chemical compounds applied to food.

Likewise, susceptible apples and pear cultivars for medium or long-term storage are also treated with antioxidants, which are commonly restricted to ethoxyquin (EQ) and diphenylamine (DPA), to prevent superficial scald. Although both compounds have been used for more than 30 years, recent concerns on their innocuous-

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ness have raised because several studies indicated a possible risk of sporadic significant exposure levels [29,30]. Hence, these antioxidants are potentially harmful for medium and long-term exposure of consumers, and may posse severe implications in public health [31–33].

The objective of this study was to establish the profiling of compounds and degradation products (DPs) from the postharvest treatment of apples and pears by a non-target approach. Some pesticides and their DPs formed in fruits have been identified and confirmed. However, no other related compounds have been studied in the same way. There are only few reports for DPA (mass spectral evidence of some of its DP has been reported but full identification was not pursued) [33,34] and we are not aware of a similar study of EQ. These studies are critical to understand the processes involved in their degradation, and thus, in the assessment of human hazards associated therewith. The present work tackled this issue and, to our knowledge, constitutes the first report describing the degradation products of EQ in apples and pears. For the structure elucidation of the DPs formed, an ultra-high performance quadrupole-time-of-flight mass spectrometry (UPLC–QqToF-MS) was used. The high-resolution mass spectrometry, providing accurate mass measurements on MS/MS data, confirms to be very powerful in the identification of the unknown compounds and could lead to a characteristic fragmentation pattern of EQ metabolites. A case study is represented by the quantitative determination of some of the DPs identified.

2. Experimental

2.1. Reagents and samples

Ethyl acetate (organic trace analysis) was from Merck (Darmstadt, Germany). Ammonium formate was Analytical grade (Aldrich, Madrid, Spain). Water was purchased from Sigma–Aldrich (St. Louise, MO). Methanol of HPLC grade was from J.T. Backer (Stanford, UK). All the solvents and solutions were filtered through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain) before use.

Forty commercial fruit samples taken from local supermarkets were analyzed. Of them, twenty eight were pear samples of four varieties (Conference, Alexandrine Douillard, Abate Fetel and Blanquilla) and twelve were apple samples of five varieties (Golden, Granny, Red chief and Fuji). The samples of around 1 kg were in plastic trays and draped with plastic film. These samples were taken to the laboratory, stored at 4 °C until analysis, which was carried out within 48 h of the reception. Pears and apples from organic farming obtained in the same orchard prior to the postharvest treatment were used as sample blank. These organic samples were taken in paper-bags and stored in the laboratory in the same conditions as postharvest treated samples.

2.2. Sample preparation

A whole fruit of each sample was placed in a 500 mL glass beaker, covered with ethyl acetate and sonicated for 20 min. The ethyl acetate was filtered through a filter paper into a 250 mL rotator-evaporation flask. The sample was washed with 30 mL of ethyl acetate, which was filtered and collected with the first extraction fraction. The ethyl acetate was evaporated to less than 10 mL in a rotary evaporator set, at 40 °C and 250 mbar, and then, transferred to a cylindrical conical tube of 15 mL. The round bottom flask was rinse five times with 0.5 mL of methanol, and the rinses collected in the cylindrical conical tube. The solvent was evaporated almost to dryness using a multi-sample Turbovap LV Evaporator (Zymark, Hopkinton, USA) with a nitrogen stream and a water bath at 50 °C. The volume of the final extract was adjusted to 10 mL with methanol. In this study, three-fruits of each sample were analyzed.

2.3. UPLC–QqTOF-MS

Accurate MS and MS/MS measurements were performed using a Water/Micromass QTOF-Micro coupled to a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA). The chromatographic separation was carried on a 15 cm × 2.1 mm Waters Acquity C_{18} 1.7 μm column using a precolumn. The mobile phases were (A) water with 10 mM ammonium formate and (B) methanol with 10 mM of ammonium formate. The flow rate was 200 μL/min, with a gradient starting at 90% of A that was decreased linearly to 10% within 5 min and then, held from 10 min. The initial mobile phase composition was restored within 0.1 min and maintained another 3 min for column equilibration. The injection volume was 5 μL. The MS analysis was performed with an electrospray ionization (ESI) interface in positive ion mode with capillary and cone voltages 3000 and 20 V, respectively. The desolvation gas flow was 500 L/h at a temperature of 350 °C, the cone gas flow 50 L/h and the source temperature 120 °C. Nitrogen was used as nebulizer and drying gas. The instrument was operated at a resolution of 5000 (FWHM) and ESI mass spectra was acquired at 1 s intervals. Accurate mass measurements of the product ions were carried out in MS/MS mode using argon as collision gas at a pressure of ~20 psi. The system was tuned for optimum sensitivity and resolution using valine-tyrosine-valine (Val-Tyr-Val, m/z 380.2185, 5 ng/μL and syringe pump infused at 5 μL/min). TOF data were collected between m/z 50–500 with low collision energy of 4 eV. Data were centroided during acquisition using independent reference lock-mass ions (the Val-Tyr-Val solution) via the Lock Spray™ interface to ensure mass accuracy and reproducibility. The LockSpray frequency was set at 11 s, and data for the reference compound were averaged over 10 spectra/min. The MS/MS experiments were performed using variable collision energy (10–30 eV). The accurate mass and composition for the precursor ions and for the fragment ions were calculated using the MassLynx software incorporated in the instrument.

2.4. Strategy for identification

The mass spectrum of all the visible peaks in the total ion chromatogram (TIC) were manually analyzed. No specialized software different of the MassLynx was used. Extracts of non-postharvest treated apples and pears were also injected in the chromatogram to rule out those peaks that can come from apples, pears, solvent and/or glass material. All those peaks different from that of these blanks were analyzed. The experimentally determined m/z values of these compounds were used to compute the possible calculated mass, empirical formula, mass accuracy (mDa and ppm), DBE (double bound equivalent) and i-FIT value (the likelihood that the isotopical pattern of the elemental composition matches a cluster of peaks in the spectrum). Tentative identification of parent compounds and/or known degradation products has been carried out searching those chemically coherent elemental compositions in the Google website or in the "Merck Index Data Base". Confirmation of the tentative identification was attained both, by studying the accurate product ion mass spectrum of the parent compounds and by further comparison with the analytical standard.

Unknown or unexpected metabolites were searched after the preliminary identification of the parent compounds, looking at those peaks that were not present in the blank extracts. A preliminary interesting knowledge are the data on degradation of these compounds already published. Some references concerning the degradation of DPA by hydroxylation, nitrosation or glycosylation in apple peel [33,34] and the EQ metabolism in fish [35–37] have been published. This information was useful in the assignation of the proposed structures. For identifying non-described DPs, it was taken into account that they have some common parts of their structure among them and to the parent compound that

can help to identified them looking for a basic empirical formula that can be modified by oxydation, hydroxylation, methylation, demethylation, dehydrogenation, etc. This is a tedious and dedicated task. Confirmation of the tentative identification was attained by studying the accurate product ion mass spectrum of the parent compounds and, if possible, by obtaining or to synthesizing the DP.

2.5. Standards

The different pesticides mention in the study, EQ (CAS No. 91-53-2) and DPA (CAS No. 122-39-4) were purchased from Riedel de Haën (Seelze, Germany). 4-Hydroxydiphenylamine (CAS No. 122-37-2) were from the Sigma–Aldrich (Madrid, Spain) library of rare chemicals. N-phenyl-4-quinoleimine and 4-methoxy diphenylamine were synthesized as previously outlined by Rudell et al. [34]. Briefly, the N-phenyl-4-quinoleimine was obtained by exposing DPA to light in the presence of the sensitizer-dye rose Bengal and the 4-methoxy diphenyl amine by methylation of 4-hydroxydiphenylamine.

The derivatives of the EQ were obtained from commercial EQ product (≥75% a.i. Fluka, Madrid, Spain), which was previously purified using column chromatography (SiO_2/hexane-ether). The pure standards of demethyldehydro-EQ, quinolone imine and the EQ-dimer were synthesized according to [35]. Basically, demethyldehydro-EQ was obtained by heating EQ at 280 °C for 13 min and separating them by column chromatography. Quinolone imine and the dimer were obtained by EQ oxidation with t-butylhydroperoxide in presence of ferrous sulfate and then, separated and purified by liquid–liquid partitioning followed by recrystallization.

Individual stock solutions were prepared dissolving 10 mg of each compound in 10 mL of methanol, and stored in stained glass-stopper bottles at 4 °C. Standard working solution, at appropriated concentrations of each antioxidant, was daily prepared by dilution of aliquots of the stock solutions in methanol or in matrix extract. Solutions were stable for at least 1 month preserving them protected from the light.

2.6. Quantitation

These standards were used to determine the concentration of compounds and their DPs in the samples using the external standard method. For each compound accurate mass chromatograms of the most abundant ion (200 mDa) were extracted from TIC and the peaks were integrated.

3. Results and discussion

3.1. Characteristics of the extraction procedure

The extraction procedure is important to recognize products from the postharvest treatment and their metabolites. Using a conventional multi-residue extraction procedure on chopped and homogenized samples, the extract is full of matrix components at high concentration, such as, carbohydrates, aromatic compounds, alkaloids, flavonoids, etc., in a way that the manual identification of trace-level contaminants can be impossible. It is necessary to obtain clean extracts that contain as much analytes and as less matrix compounds as possible. The extraction procedure used, which only implies to rinse the peel of the fruit with ethyl acetate, achieves clean extracts, slightly colored between yellowish and greenish without endogenous fruits components, in a way that the screening for postharvest treatment and DPs, even through time consuming and demanding task, is affordable. A number of chromatographic peaks, might not only originate from the substances applied in the postharvest treatment and their metabolites, but could be also attributed to the field treatment with pesticides or to the nutrient metabolism during the ripening process.

Other prerequisite for a successful extraction procedure is to obtain large pre-concentration factors that facilitate analyte enrichment to attain proper sensitivity. The weight of the individual pears oscillated from 124 to 370 g with a mean value of 210 g and that of the apples from 153 to 232 g with a mean of 202 g. At the worst pre-concentration conditions (sample weight: 124 g, final volume of the extract: 10 mL) a high pre-concentration factor (12) was attained.

The main disadvantage of the method is that the sample taken is not homogeneous because individual fruits were analyzed and the deposit of the postharvest compounds is not uniform among the fruits and on fruits. In this study, three different fruits of each sample were analyzed. The same compounds were always found but their concentrations showed high variability although not statistically differences were observed (data not shown). The lack of homogeneity is not crucial in this study because the aim is the identification of the compounds and DP instead of checking compliance with legislation.

3.2. Profiling of the postharvest-treatments

Pear and apple extracts were initially analyzed by UPLC–ESI-QqTOF-MS in positive ionization TOF-MS mode, with the first quadrupole operating in rf-only mode. Table 1 show the compounds from the postharvest treatment identified in the studied samples including the quantitation. Peaks were visually identified. The compound identity was established manually by searching the empirical formula in the Google website and confirmed injecting analytical standards since they are commercial. The reproducibility of the measurements (three injections of the same extract during a 1 month period) is supported by the RSD % values that are in all cases under 6.25%. The low RSDs values also indicated excellent stability of the extract at least during the studied period. Results showed that apples and pears were subjected to two main postharvest treatments. The most common applies imazalil (IMZ) and EQ (present in 22 samples, equivalent to 55%) and second one thiabendazole (TBZ) and DPA (3 samples, 8%). Making a short statistical of the other samples, of the 40 samples analyzed, 4 samples (10%) does not contain residues of the postharvest treatment, one has only EQ (4%), other only IMZ (4%), and the nine remaining ones, have a different mixtures of these four compounds (22.5%). These samples were from different geographical origins including not only different areas of Spain (Lleida, Alicante and Murcia) but also different European Countries, such as France and Italy. However, the postharvest treatments applied were almost equal.

The quantitative parameters of the method were validated when possible. Linearity was checked using standard prepared in methanol and in matrix extracts to assess matrix effect. It was only acceptable in a range of two orders of magnitude ($r^2 > 0.99$), requiring sometimes sample dilution but not matrix effect was observed (data not shown). An overall summary of the quantitative performance of the proposed method for the postharvest compounds found in the samples, highlighting its sensitivity, selectivity, accuracy and precision, is provided in Table S1 in the supplementary material. The accuracy value, close to 100%, in all fortified samples; the recovery test, higher than 72%; the detection and quantification limits ranged from 0.02 to 0.34 μg and from 0.05 to 0.60 μg, respectively, and the reproducibility (RSDs), always lower than 19%, checked on different days from independent extraction of the same analytes, highlight the uniqueness of the proposed method.

The levels of antioxidants used to prevent the scald are below the MRLs fixed by the EU and the USA for EQ and DPA in apples and pears. The concentrations of EQ ranged from 0.001 to 0.672 μg/kg and those of DPA from 0.024 to 0.369 μg/kg. However, the fungi-

Table 1
Concentration of identified chemicals from the postharvest treatment in the apples and pears[a].

Sample		EQ		IMZ		DPA		TBZ	
Code	Type	mg/kg	RSD, %	mg/kg	RSD, %	mg/kg	RSD, %	mg/kg	RSD, %
1	Pear	0.075	2.45	2.990	1.05	ND		ND	
2	Pear	0.032	4.54	8.089	1.00	0.902	4.01	ND	
3	Pear	0.337	4.22	5.632	1.75	ND		ND	
4	Pear	0.672	3.70	10.012	1.25	ND		ND	
5	Pear	0.050	2.19	3.956	2.92	ND		ND	
6	Pear	0.028	3.22	4.078	1.82	ND		ND	
7	Pear	0.009	3.97	4.405	1.64	ND		ND	
8	Pear	0.001	5.99	5.820	1.35	ND		ND	
9	Pear	0.323	5.70	4.919	1.05	ND		ND	
10	Pear	0.005	6.25	5.659	1.02	0.120	2.29	ND	
11	Pear	ND		2.002	1.28	0.280	3.33	ND	
12	Pear	0.124	3.92	2.493	2.89	ND		ND	
13	Pear	0.112	2.63	4.188	2.55	ND		ND	
14	Apple	ND		ND		ND		ND	
15	Apple	ND		ND		0.228	4.77	0.839	3.21
16	Apple	ND		ND		0.369	5.09	1.234	1.66
17	Apple	ND		ND		ND		ND	
18	Apple	ND		ND		ND		ND	
19	Pear	0.024	5.26	2.216	2.03	ND		ND	
20	Pear	0.004	4.78	4.051	2.77	ND		ND	
21	Pear	0.452	3.08	4.052	2.83	ND		ND	
22	Pear	0.515	2.90	4.358	2.65	ND		ND	
23	Apple	0.006	6.03	ND		ND		ND	
24	Pear	0.007	6.15	3.788	1.24	ND		ND	
25	Pear	0.442	2.19	11.856	1.00	ND		ND	
26	Apple	0.035	2.25	ND		ND		0.245	3.22
27	Pear	0.183	5.45	8.580	2.88	ND		ND	
28	Apple	0.027	5.88	ND		ND		0.056	5.39
29	Pear	0.068	4.35	0.936	1.88	0.123	5.19	0.012	5.69
30	Pear	0.001	2.75	4.812	1.25	ND		ND	
31	Pear	ND		ND		ND		ND	
32	Pear	ND		7.931	1.52	0.239	4.78	ND	
33	Apple	ND		3.251	2.00	0.024	4.67	2.093	1.34
34	Apple	0.002	5.55	1.310	2.41	0.038	5.02	2.588	1.86
35	Apple	ND		0.590	2.66	0.262	5.98	1.029	2.76
36	Apple	ND		ND		0.057	4.68	0.028	5.99
37	Pear	0.006	6.00	2.989	3.01	ND		ND	
38	Pear	0.428	3.75	6.909	3.46	ND		ND	
39	Pear	0.216	2.99	9.466	0.97	ND		ND	
40	Pear	0.033	4.00	3.758	1.25	ND		ND	

[a] Concentration and RSDs were calculated from three different injections of the same extract performed in three nonconsecutive days with a difference between the first and the last of 1 month.

cides – IMZ and TBZ – were sometimes over the MRLs (applicable at that time) of 10 mg/kg for IMZ and 5 mg/kg for TBZ. Residues from 0.590 to 10.012 μg/kg of IMZ were found in the samples whereas residues of TBZ were significantly inferior (between 0.012 and 2.588 μg/kg). These results cannot be taken into account for enforcement purposes because samples are not homogeneous.

3.3. Identification and confirmation of the degradation products of the compounds used in the postharvest treatments

Figs. 1 and 2 illustrate the two main patterns of peaks observed in the chromatograms, in which, in addition to the compounds from the postharvest treatment, the possible DPs were also checked. DPs not previously described were visually recognized and identified searching resemblance among their empirical formula and that of the parent compound. Confirmation was performed by studying the product ion mass spectrum. Several peaks of the chromatogram could be potential contaminants. However, the attempt to assign their empirical formula was not successful. Screening of the forty samples showed three possible DPs from DPA and six from EQ. In contrast, IMZ and TBZ were stable against degradation throughout the period of the study. For IMZ, the occurrence of a DP, 1-(2,4-dichloro-phenyl)-2-imidazol-1-yl-ethanol, in orange extracts was reported by Thurman et al. [23]. A probably explanation of this divergence is that formation of DPs can be strongly influence by storage conditions and time. UPLC provides clear advantages in rapidity and efficiency against the conventional LC separations eluting all the possible compounds of the sample in less than 15 min. Although the low flow-rate appropriate for the MS detector (0.2 mL min^{-1} in the present study), is not the most suitable for real UPLC conditions, the saving in time in the chromatographic run is important.

The identity of the possible metabolites was established by the QqTOF using the experimentally determined *m*/*z* values to compute the possible calculated mass, empirical formula, mass accuracy (mDa and ppm), double bond equivalents (DBE) and i-FIT. The results are presented in Table 2. The mass difference between DPA and DPA-DP1 (16 Da) is indicative of monohydroxylated DPA (OHDPA) species. Considering the *m*/*z* increase of 14 and the empirical formula proposed in Table 2, DPA-DP2 correspond to N-phenyl-4-quinoneimine (n-PhQI) formed by the addition of oxygen and the concurrent loss of two hydrogens (see Fig. 1). Further, the *m*/*z* increase of DPA-DP3 is indicative of the monomethoxylated derivatives of DPA (MeOHDPA). Their structures were confirmed by MS/MS, the product ion mass spectra of the $[M+H]^+$ ion of DPA and its three DPs being in agreement with the proposed identity and characterized by neutral loss of benzene. The most intense fragment ions were *m*/*z* 93.0573 for DPA, *m*/*z* 108.0444 for OHDPA and n-PhQI and *m*/*z* 123.0684 for MeOHDPA. In the case of OHDPA and MeOHDPA, the precursor and product ion mass

Table 2
Accurate mass analysis of TOF-MS mass spectrum of samples 16 and 38 as representative of the postharvest treatment residues remaining in apples and pears.

	t_R (min)	Measured mass (m/z)	RA[a] (%)	Calculated mass (m/z)	Mass error (mDa)	Mass error (ppm)	DBE[b]	i-FIT[c]	Empirical formula	Tentative identification
Sample 16										
TBZ	5.04	202.0435	100	202.0439	0.44	−2	8.5	0.2	$C_{10}H_8N_3S$	
DPA-DP1	5.45	186.0922	100	186.0919	0.3	1.6	7.5	2.3	$C_{12}H_{12}NO$	Hydroxy-DPA
DPA-DP2	5.53	184.0751	100	184.0762	−1.1	−6	8.5	18.7	$C_{12}H_{10}NO$	N-phenyl-4-quinoneimine
DPA-DP3	6.28	200.1071	100	200.1075	−0.4	−2	7.5	3	$C_{13}H_{14}NO$	Methoxy-DPA
DPA	6.43	170.0966	100	170.0970	−0.4	−2.4	7.5	11.6	$C_{12}H_{12}N$	
Sample 38										
EQ-DP1	5.40	188.1066	100	188.1075	−0.9	−4.8	6.5	3	$C_{12}H_{14}NO$	2,2,4-Trimethyl-6-quinolone
		173.0849	56.09	173.0841	−0.8	4.6	7.0	5.0	$C_{11}H_{11}NO$	
EQ-DP2	6.02	232.1329	100	232.1338	−0.9	−3.9	6.5	4.5	$C_{14}H_{18}NO_2$	EQ-N-oxyl
		218.1552	42	218.1545	0.7	3.2	5.5	1.1	$C_{14}H_{20}NO$	
		188.1073	82	188.1075	−0.2	−1.1	6.5	4.5	$C_{12}H_{14}NO$	
EQ-DP3	6.19	204.1382	100	204.1388	−0.6	−2.9	5.5	3	$C_{13}H_{18}NO$	Demethyl-EQ
EQ-DP4	6.43	202.1231	100	202.1232	−0.1	−0.5	6.5	30.8	$C_{13}H_{16}NO$	Demethyldehydro-EQ
EQ	6.48	218.1540	100	218.1545	−0.5	−2.3	5.5	73.8	$C_{14}H_{20}NO$	
IMZ	6.65	297.0556	100	297.0561	−0.5	−1.7	7.5	1.3	$C_{14}H_{15}N_2O^{35}Cl_2$	
		299.055	63.48	299.0532	1.8	6	7.5	2.1	$C_{14}H_{15}N_2O^{35}Cl^{37}Cl$	
EQ-DP5	7.25	232.1696	100	232.1701	−0.5	−2.2	5.5	0.4	$C_{15}H_{22}NO$	Methyl-EQ
		216.1384	39	216.1388	−0.4	−1.9	6.5	15.6	$C_{14}H_{18}NO$	
EQ-DP6	10.93	433.2858	100	433.2855	0.3	0.7	11.5	0.5	$C_{28}H_{37}N_2O_2$	EQ-dimer

[a] RA, relative abundance.
[b] DBE, double equivalent.
[c] i-FIT, likelihood that the isotopical pattern of the elemental composition matches a cluster of peaks in the spectrum.

spectra did not allow for confident assignation of the position of the hydroxy and methoxy groups. Despite that, it was already established that metabolism of DPA in biological systems, including apples and pears, largely results in C-hydroxylation of one or both rings in the *para* position [34]. Furthermore, the formation of n-PhQI can only result from the oxidation of 4-OHDPA, the *meta* and *ortho* hydroxyl-DPA derivatives did not display this property.

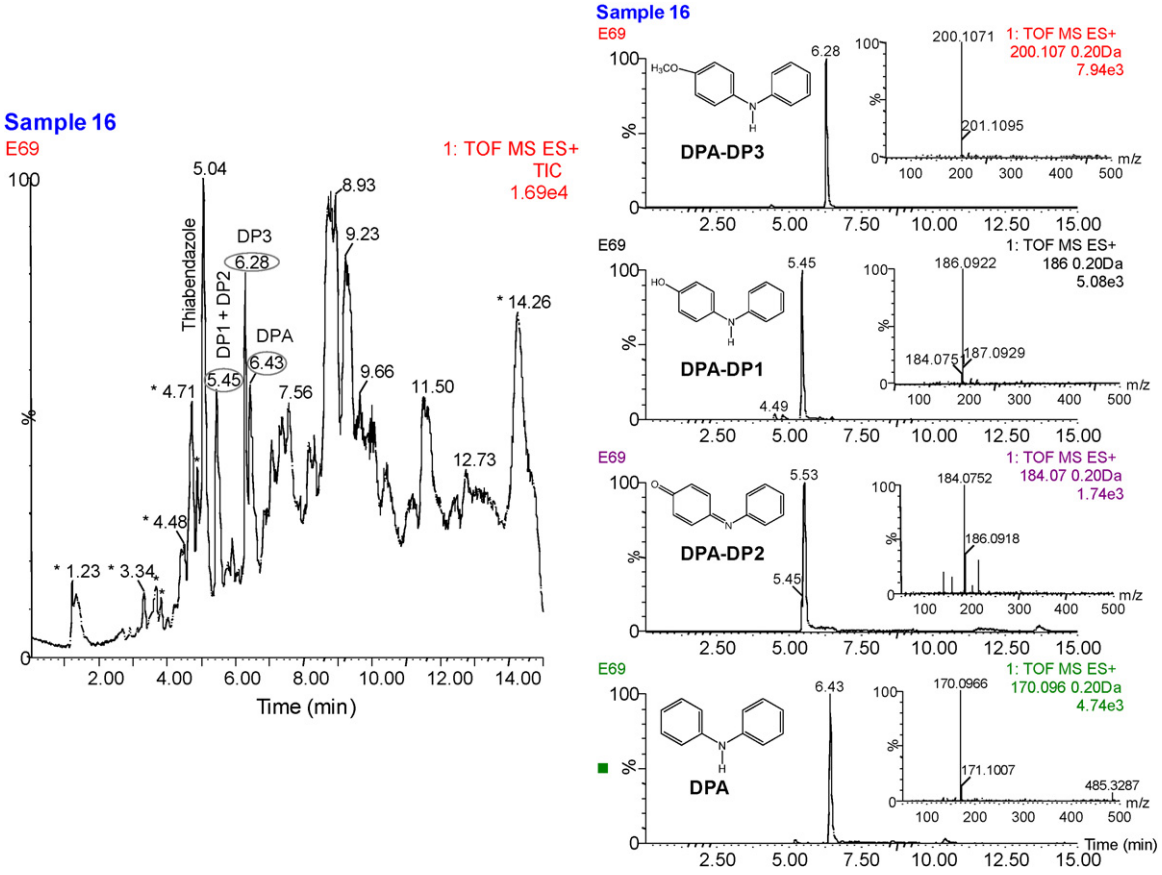


Fig. 1. UPLC–ESI-QqTOF-MS chromatograms corresponding to sample no. 16. Left: total ion chromatogram (TIC); right: extracted ion chromatograms (XIC) of DPA and its identified DPs, the mass spectrum and the proposed structure of which are shown as inserts. Peaks marked as (*) can be an unidentified contaminant. The other peaks of the chromatogram were also present in the extracts from non-postharvest treated apples and pears.

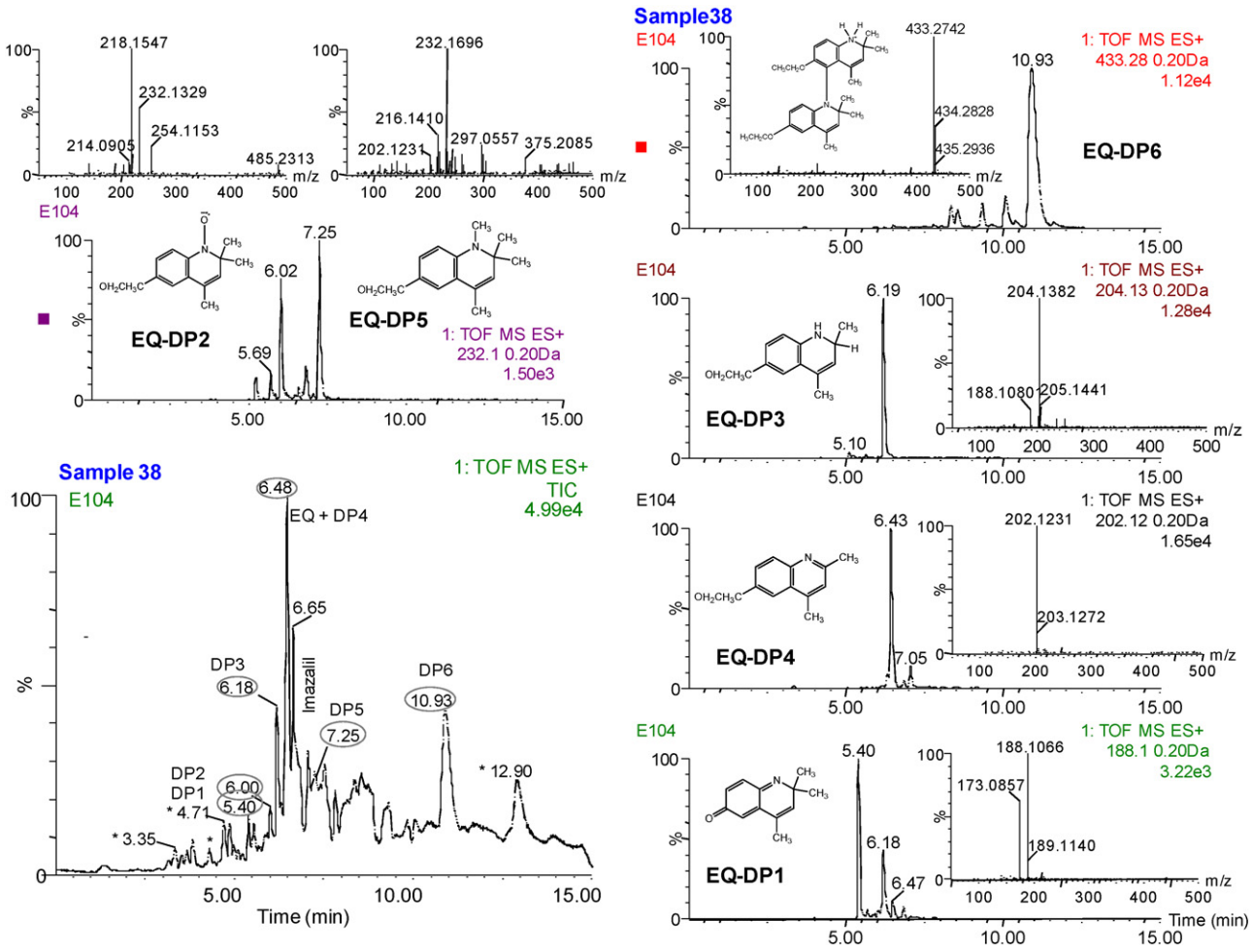


Fig. 2. UPLC–ESI-QqTOF-MS chromatograms corresponding to sample no. 38. Left bottom: total ion chromatogram (TIC); left upper and right: extracted ion chromatograms (XIC) of EQ identified degradation products, the mass spectrum and the proposed structure of which are shown as inserts. Peaks marked as (*) can be an unidentified contaminant. The other peaks of the chromatogram were also present in the extracts from non-postharvest treated apples and pears.

The present study confirms the presence of various previously reported DPA derivatives in apple peel whereas other derivatives where not detected. Using this extraction method, only the detection of the glycosidic conjugates of the hydroxylated derivatives would be unfeasible because it would require additional hydrolysis. Rudell et al. [34] reported large quantities of DPA, 4-OHDPA and smaller quantities of 2-OHDPA, 3-OHDPA, 2,4-dihydroxydiphenylamine (2,4-diOHDPA) and their glycosidic conjugates. Small amounts of N-nitrosoDPA (NODPA) and 2-nitroDPA (NO_2DPA) have also been reported in the peel of DPA treated apples [33]. However, the formation of DPA-DPs was affected by harvest maturity and storage conditions, specially, NODPA and NO_2DPA contents were lower in fruits treated with 1-methylcyclopropene and/or stored in controlled atmospheres [33,34]. That theory could explain the nitroderivates absence in the selected samples. However, there was no appropriate explanation for the non-occurrence of 2,4-diOHDPA.

Some metabolites of the EQ were also identified after analyzed the exact masses (Table 2, Fig. 2). The *m*/*z* decreases of 14, 16 and 30 Da observed for EQ-DP3, EQ-DP4 and EQ-DP1, respectively, are indicative of loss of methyl, methyl and hydrogen, and ethyl and hydrogen (2,2,4-trimethyl-6-quinolone or quinoline imine, QI) from the corresponding protonated molecule. The *m*/*z* increase of 14 is indicative of two situations: the methyl EQ (EQ-DP5) and the N-oxyl EQ (EQ-DP2). Finally, the *m*/*z* increase of 216 is symptomatic of a dimer formation (EQ-DP6). The proposed metabolic pathway according to the obtained information is shown in the Scheme S1 in the supplementary. Two of these DPs, the EQ-dimer and the QI were already described as degradation products formed in fish, with EQ-dimer as a main metabolite [36,37]. Possible toxicological effects of EQ-dimer are not known.

This tentative identification was supported by MS/MS fragmentation. As illustrates Figure S1 in the supplementary, the product ion mass spectrum presents lower or higher degree of fragmentation and different abundance of product ions depending on the collision energy (CE) applied. A CE of 20 V was chosen because it provides the most distinctive product ions for all DPs with adequate sensitivity. Figs. 3 and 4 display the MS/MS mass spectra of EQ and its DPs (see supplementary Table S2 that provides the summary of the accurate mass analysis of the product ion mass spectra of possible EQ metabolites). Schemes 1 and 2 show the fragmentation pattern of EQ and its DPs. EQ, demethyl-EQ and methyl-EQ (Scheme 1) showed very similar product mass spectra characterized by groups of products ions with mass differences of ca. 1 Da, which have higher abundance that that would be expected of the isotopical pattern. This is a sign of the capacity of EQ, demethyl-EQ and methyl-EQ to act as hydrogen atom donors towards radicals or ions and an indicative of extensive delocalization of unpaired electrons. This shows the DPs of EQ also posse antioxidant properties by the same mechanisms as the parent EQ and should be considered as an alert of the possibility of synergic negative effects due to the presence of EQ DPs. The principal products ions of EQ,

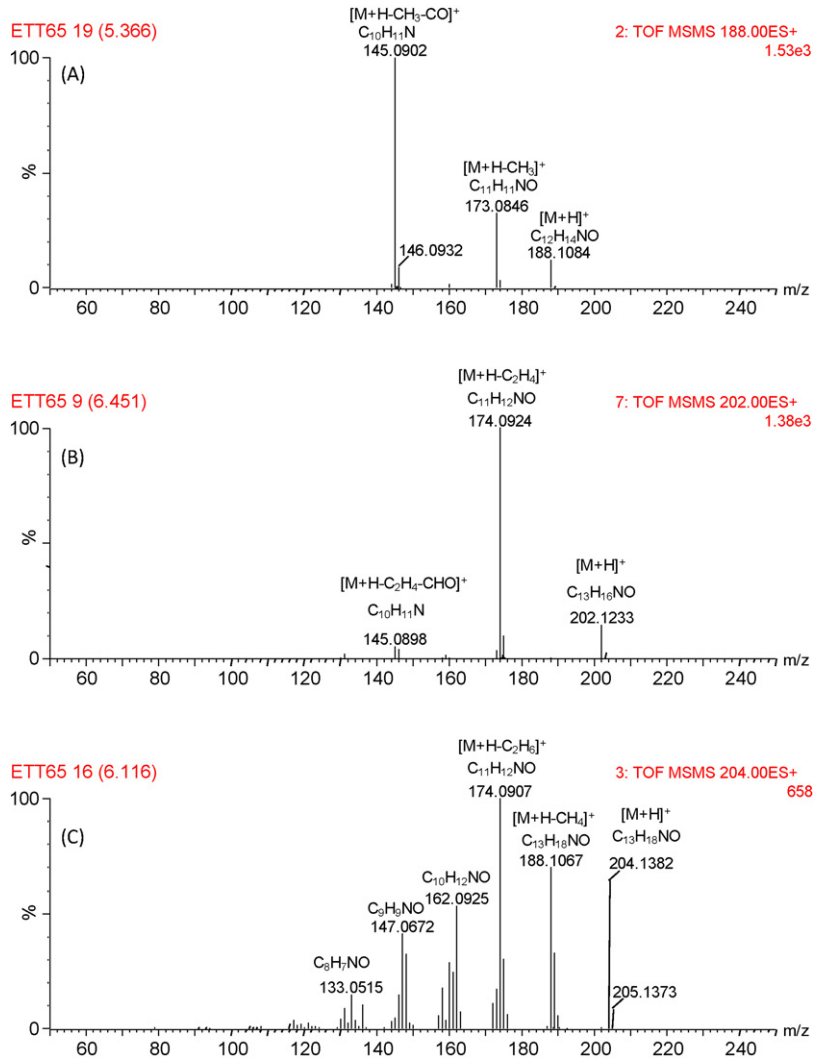


Fig. 3. UPLC–QqTOF product ion mass spectra of (A) EQ-DP1 (m/z 188) possible 2,2′,4-trimethyl-6-quinolone; (B) EQ-DP4 (m/z 202) possible demethyldehydro-EQ; (C) EQ-DP3 (m/z 202) possible demethyl-EQ. All spectra were acquired at collision energy of 20 eV.

methyl-EQ and demethyl-EQ were at m/z 202, 188, 178, 162, 148 and 138. The product ions at m/z 202 (for EQ) and of m/z 216 (for methyl-EQ) were produced by losing of a methyl radical and a hydrogen. The product ion at m/z 188 represents the loss of the second methyl radical from position 2. The product ion at m/z 178 had the structure of a quinine. It is probably produced by losing a neutral ethene molecule from the molecular ion due to the Mac Lafferty re-arrangement and then by further loss of a methyl group. This fragmentation pattern can be explained by the conjugation of the lone electron pairs of N and O atoms in the *para*-position of the aromatic cycle and by the electronic-structure stabilization of the aminyl radical formed in radical reactions,

The structure of the other product is also reported in the Schemes 1 and 2. The 1,2-dihydro and N-oxyl DPs have loss capacity as hydrogen atom donors and have much more localized the unpaired electrons by the extra double bound in between atoms 1 and 2 or by the electronegative oxygen atom linked to the nitrogen. The product ion mass spectrum of these DPs was also characterized by the loss of methyl radicals and carbon monoxide (Scheme 2). Finally, the MS/MS of EQ-dimer shows product ions at m/z 416, 377, 231 and 216, the two first product ions correspond to the loss of ammoniac and the loss the two ethane groups that form an ether with the oxygen, the structure of the two last is shown in Scheme 2. The results demonstrated clearly that, both within the collision cell of the mass spectrometer, the EQ and its DPs follow a universal fragmentation pattern, producing a series of product ions that corresponded precisely to the theoretical structure of these molecules (Schemes 1 and 2 and see supplementary Table S2). The reliability and robustness of the approach is illustrated by the comparability and reproducibility of the product ion mass spectra of each EQ-DP in different samples is shown in Figure S2 in the supplementary. The EQ has been described as an unexpected residue found in different non-target surveys performed in fruits [22]. However, its DPs have not been previously identified nor even mentioned. The universal fragmentation pattern described in this study can be used to easily predict the fragmentation of new compounds that have the same general structural backbone. In addition, single or multiple ion monitor reactions can be performed for any quantitative studies of fruits treated with these antioxidants.

Some studies dealing with the same and other strategies on this subject have already been published [4,8–11,22–24]. Thurman et al. [23,24] applied LC–TOF-MS accurate mass measurements to generate elemental compositions of ions. One disadvantage of the LC–TOF-MS in front of LC–QqTOF-MS is that it does not provide information on the identity of the compounds detected because cannot perform ion accurate MS^2 experiments. This drawback has been palliated in several ways, for instance, supplementing the

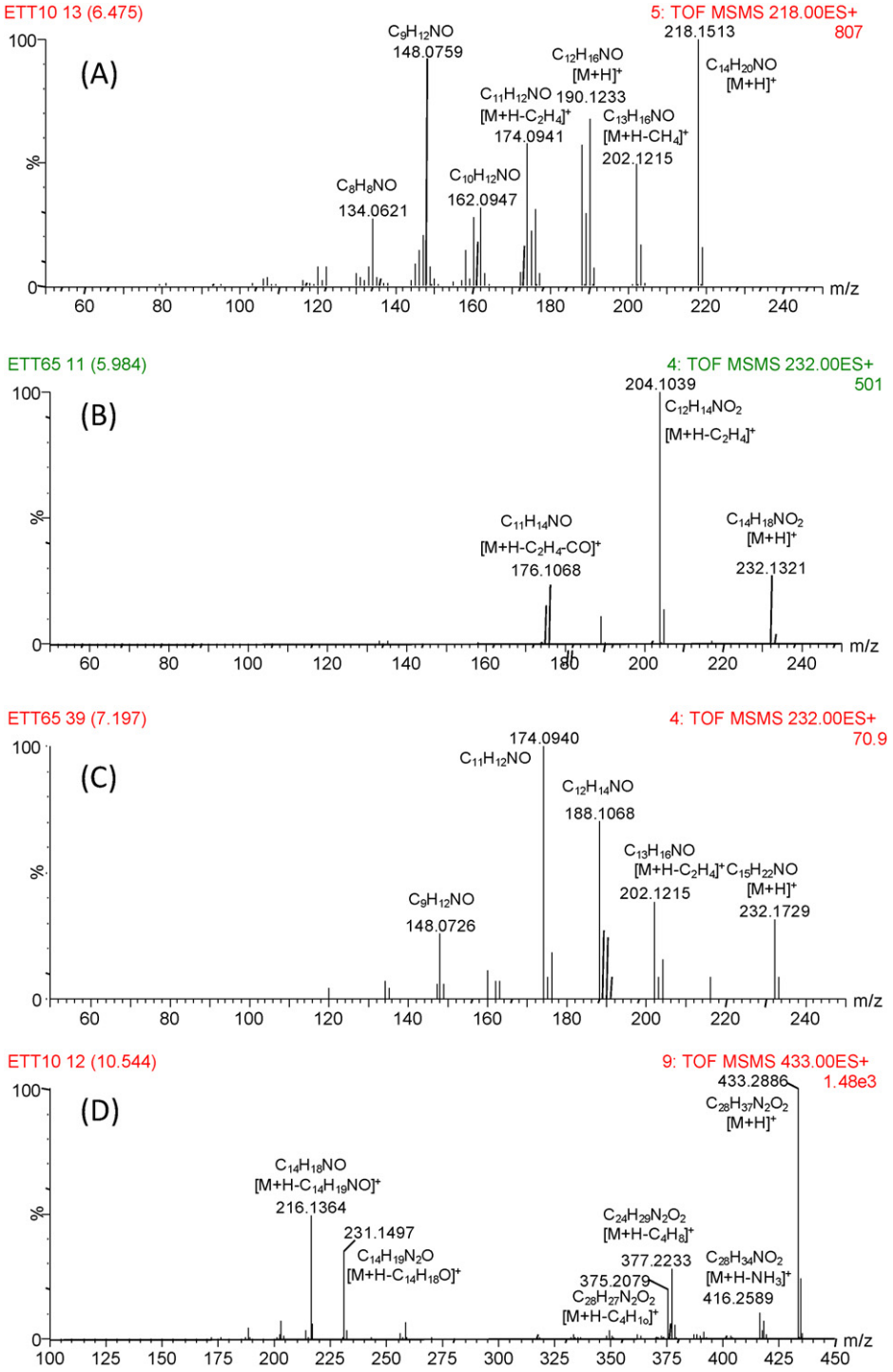
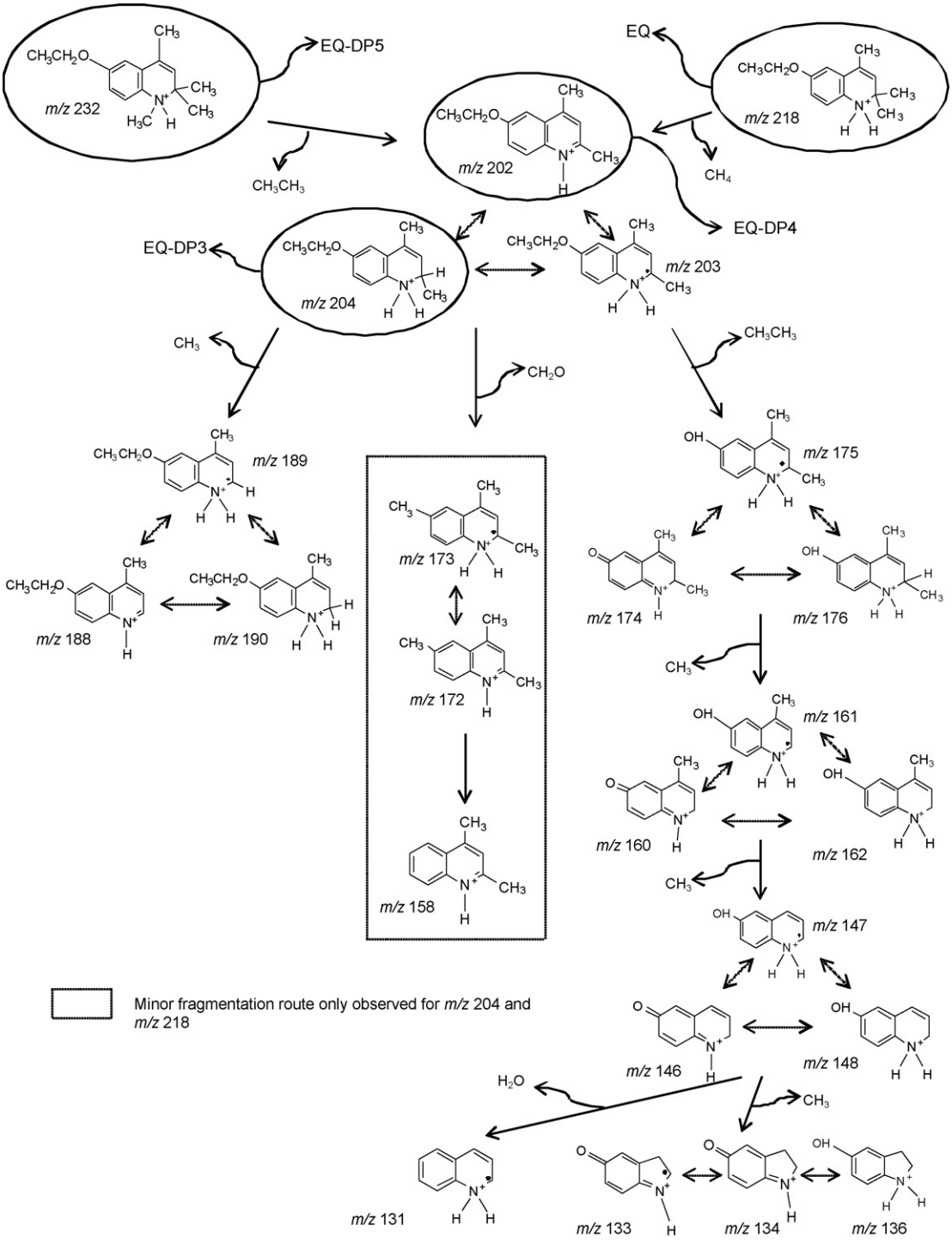


Fig. 4. UPLC–QqTOF product ion mass spectra of (A) EQ (*m*/*z* 218); (B) EQ-DP2 (*m*/*z* 232) possible EQ-N-oxyl; (C) EQ-DP5 (*m*/*z* 232) possible methyl-EQ, and (D) EQ-DP6 possible EQ-dimer. All spectra were acquired at collision energy of 20 eV.

LC–TOF-MS data with those of LC/ion trap multiple MS (MS^n), which provides useful complementary structural information [23] or studying resolved isotopic clusters to obtain a reduced number of possible elemental compositions (typically 1-2) [25]. García-Reyes et al. [11] proposed a elegant strategy based on the use of LC–TOF-MS: accurate mass measurements of (molecule and fragment) ions of interest are used in order to establish relationships between fragmentation of the parent pesticides in the instrument (in-source CID fragmentation) and possible degradation products of these pesticides in food. This strategy has shown an interesting potential for identification of malathion and amitraz metabolites.

Pico et al. [22,8,9] applied a similar strategy to that reported here to identify several fenthion and amitraz metabolites as well as for non-target screening of pesticides. The clear advantage of these strategies based on the employ of QqTOF-MS/MS offers more possibilities for further investigating the identity of the compounds detected due to the valuable information obtained from MS^2 experiments on product-ion accurate-mass spectra. This information has also been complemented by that obtained using alternative LC–MS techniques, such as LC/ion trap multiple MS (MS^n) [8]. The main disadvantages of this strategy is the difficulty and the time spend identifying manually all the peaks, and that sometimes the



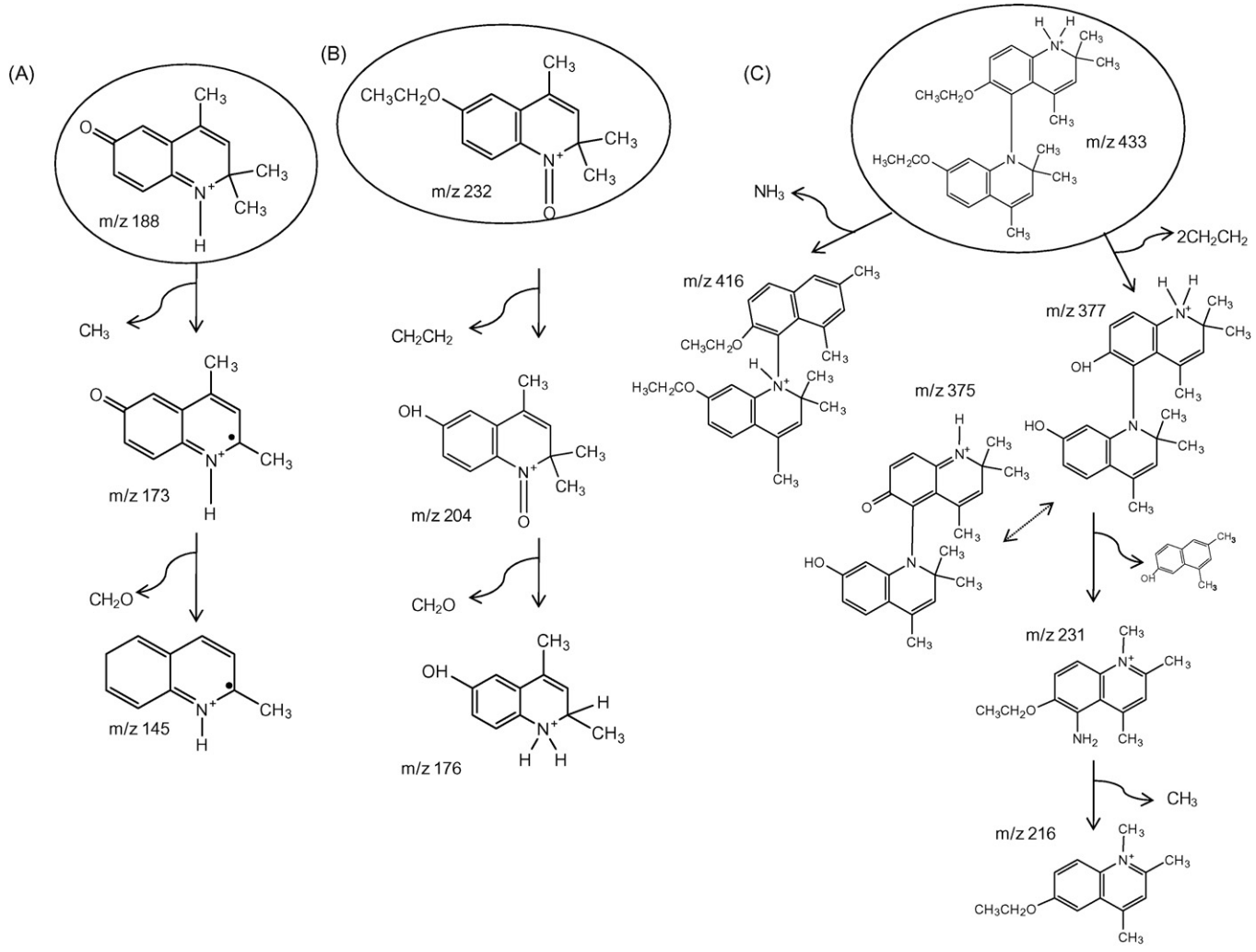
Scheme 1.

assignation of chemical structure to an empirical formulation is complicated [22]. The contribution of the present study relays on the identification of several new EQ DPs.

Hernandez et al. [4] and Grimalt et al. [12] proposed the use of large libraries (theoretical and/or empirical) and specific deconvolution software to facilitate identification and discovery of unknowns in this food. The measurements of the accurate masses of several representative ions using powerful software, has proved to be an efficient approach for non-target screening in water and food samples saving time and improving the identification capabilities as well as the assignment of the chemical structure to the empirical formula. Some limitations have been observed in this challenging task, as the deconvolution software has failed trying to discriminate ions from background when the ions were present in samples at low levels of concentration. Future improvements are needed in deconvolution software in order to increase the success in detecting components, especially at low levels of concentration.

3.4. Identification of other compounds

These apples and pears were also found to contain other products derived from the plastic films (polyolefin), in which the sample



Scheme 2.

was draped as well as several pesticides from the pre-harvest treatment in the orchard. The identification was done as for identifying the parent compound of postharvest treatment. According to UPLC–ESI-QqTOF-MS analysis, the most frequent (six samples) was erucamide. This compound is used as a slip agent, anti-fogging or lubricant. Isopthalamide (two samples) and amidinobenzoic acid (one sample) were detected in small amounts. Of the pesticides detected, the high occurrence was for chlorpyriphos (ten samples) followed by boscalid (three samples), bitertanol (two samples) and tolclophos methyl (two samples). Chlorpyriphos, likely used in apples and pears to control of coddling moth in spring, corresponds to the labeled small peak showed in Fig. 5 at an elution time of 7.52 min. Although its mass spectrum (showed as an insert) already has some fragment ions that unequivocally identify the compounds, higher confidence level can still be attained by obtaining the product ion mass spectrum of the protonated molecule. Finally, in three samples carbendazim was detected at very low concentrations. Carbendazim is also a postharvest residue from the treatment of the sample with benomyl or with the carbendazim itself. However, the low concentrations indicated that it could be a contamination from the residues remaining in the treatment lines or in the materials of any previous treatment performed in the same installations.

3.5. Occurrence of degradation products in apples and pears

This study goes one step beyond on the evaluation of EQ and DPA-DPs by establishing the concentration of some of them in apples and pears and its implications for food safety. EQ, DPA, IMZ and TBZ residues in fruits has been widely described in the monitoring surveys and control programs of many different countries. However, up to now, no data have been published on the levels of their principal DPs. One reason that justifies this ignorance, once a new DP was detected and its structure elucidated, is the lack of commercially available analytical standards or the difficulty of synthesizing them in sufficient quantity to validate the analytical method. There are few methods described for the production of pure n-PhQI, MeOH DPA [33,34], QI, EQ-dimer [35,36] and even demethyldehydro-EQ [35] as standards (>99% purity) for calibration of quantitative determination methods (Table S3 in the supplementary outlines the performance of the analytical method to quantify these DPs).

Table 3 summarizes the concentrations of all DPA-DPs and three EQ-DPs in the forty analyzed samples. The MeOHDPA was the major DP of DPA and almost the only one when DPA and EQ coexist in the sample. The concentration of this compound in the samples was higher than that of DPA. The OHDPA was also one of the major DPs

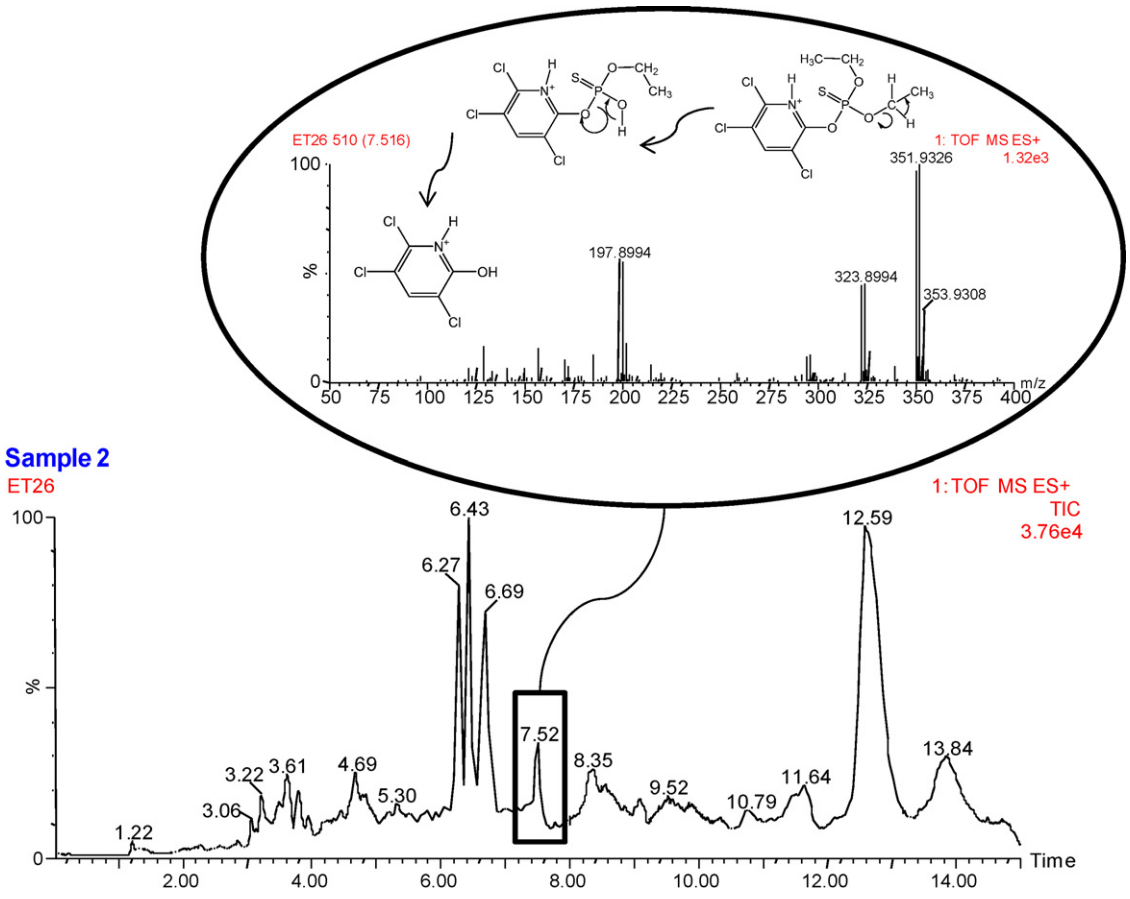


Fig. 5. UPLC–ESI-QqTOF-MS chromatogram corresponding to the sample no. 2. The insert correspond to the precursor ion mass spectrum of the peak at retention time of 7.52 min.

Table 3
Concentrations (μg/kg) of some DPA-DPs and EQ-DPs in apples and pears.

Code	Type	4-OHDPA	4-MeODPA	n-PhQI	∑DPA[a]	QI	DMDH-EQ	EQ-dimer	∑EQ[a]
1	Pear					0.035	0.209	0.152	**0.343**
2	Pear	0.005	0.898	0.001	**0.835**		0.025	0.022	**0.038**
3	Pear					0.035	0.409	0.399	**0.683**
4	Pear					0.101	0.425	4.025	**2.602**
5	Pear					0.007	0.335	0.225	**0.483**
6	Pear						0.045	0.020	**0.059**
7	Pear					0.001	0.022	0.026	**0.038**
8	Pear					0.001	0.395	0.624	**0.742**
9	Pear					0.002	0.215	0.223	**0.347**
10	Pear		0.229		**0.212**		0.020	0.006	**0.025**
11	Pear		0.523		**0.483**		0.225	0.252	**0.370**
12	Pear					0.017	0.458	0.305	**0.668**
13	Pear					0.023	0.291	0.346	**0.515**
14	Apple								
15	Apple	0.531	0.636	0.175	**1.222**				
16	Apple	0.601	0.724	0.237	**1.420**				
17	Apple								
18	Apple								
19	Pear					0.003	0.054	0.010	**0.067**
20	Pear					0.001	0.010	0.017	**0.021**
21	Pear					0.032	0.284	0.182	**0.435**
22	Pear					0.056	0.415	0.407	**0.718**
23	Apple						0.004	0.003	**0.006**
24	Pear					0.001	0.038	0.025	**0.055**
25	Pear					0.085	0.202	0.484	**0.560**
26	Apple					0.004	0.037	0.015	**0.052**
27	Pear					0.009	0.178	0.395	**0.401**
28	Apple					0.001	0.019	0.031	**0.037**
29	Pear		0.469		**0.433**	0.004	0.303	0.238	**0.451**
30	Pear					0.001	0.032	0.035	**0.053**
31	Pear								

Table 3 (*Continued*)

Code	Type	4-OHDPA	4-MeODPA	n-PhQI	∑DPA[a]	QI	DMDH-EQ	EQ-dimer	∑EQ[a]
32	Pear	0.004	0.342	0.002	**0.321**		0.058	0.011	**0.068**
33	Apple		0.034		**0.031**	0.001	0.033	0.354	**0.215**
34	Apple		0.052		**0.048**	0.004	0.021	0.048	**0.051**
35	Apple	0.323	0.538	0.202	**0.964**				
36	Apple	0.021	0.035	0.021	**0.069**				
37	Pear					0.004	0.060	0.237	**0.189**
38	Pear					0.047	0.326	0.823	**0.821**
39	Pear					0.045	0.454	0.724	**0.907**
40	Pear					0.004	0.096	0.280	**0.249**

4-OHDPA: Hydroxy-DPA; 4-MeOHDPA: Methoxy-DPA; n-PhQI: n-phenyl-4-quinoleimine; DMDH-EQ: demethyldehydroEQ.
[a] Sum of the DPs expressed as the parent compound.

in the samples treated only with DPA. Its concentration is slightly lower than that of MeOHDPA but also higher than the remaining DPA. n-PhQI concentration in pears and apples ranged from 0.001 to 0.237 μg/kg and were thus lower than the remaining DPA. On the quantitative determination of EQ-DPs, the levels of QI in pears and apples did not exceed 0.101 μg/kg and therefore, its residues were always much lower than the remaining EQ indicating that QI is a minor EQ-DP in fruits, as, according to the peak areas showed in Fig. 2, are EQ-N-oxyl and methyl-EQ. In contrast, demethyldehydro-EQ and EQ-dimer were both major DP and were present at higher concentrations that EQ, taking a look again to Fig. 2 they were major metabolites together with demethyl-EQ. According to the present study, the sum of DPA and EQ DPs concentrations expressed as the parent compounds (columns in black letters in Table 3) showed that amount of DPs can exceeded several times that of the parent compound.

4. Conclusions

The application of chemicals in the postharvest treatment, mainly fungicides and antioxidants as well as the emerging concern on the DPs of unknown identity, has prompted the evaluation of an analytical procedure suitable for the identification and confirmation of these compounds, even in difficult matrixes, such as food. The non-target analysis of different samples is, until the moment, the best source of information for the evaluation of any non-specific analyte present in food. UPLC–ESI-QqTOF-MS or MS/MS in positive ion mode has been used to assess the profiling of these substances in pears and apples.

Degradation of DPA in apples and pears largely results in OHDPA and MeOHDPA, and in lesser amounts of n-PhQI. The samples that evidence to be simultaneously treated with both antioxidants, DPA and EQ, showed a greater increase on MeOHDPA in front of OHDPA. This may constitute a useful fingerprint in further investigations of postharvest treatments. Six DPs of the EQ -demethyl-EQ, demethyldehydro-EQ, EQ-dimer, methyl-EQ, EQ-N-oxyl and QI- were identified and unambiguously confirmed in apples and pears. As virtually no information on metabolites of EQ transformation in fruits is currently available, the proposed DPs bring an important contribution to recognize the fate of this compound.

The data thus obtained allow, for the first time, a proper evaluation of the occurrence in fruits of the antioxidants used in the postharvest treatment and their metabolites. The new findings, of keen interest worldwide to researchers seeking insights into the processes involved in antioxidants degradation, also translate into a disturbing suggestion that the magnitude of the antioxidants threat in fruits have been significantly underestimated. Furthermore, the approach applied in this study for profiling of compounds and DPs from the postharvest treatment of pears and apples could be further extended in many other fields of research, which require the non-target analysis and the identification and confirmation of unexpected or unknown compounds, demonstrating the perspectives and adaptability of UPLC–QqTOF-MS.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2009.11.070.

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