Evaluation and prevention of the negative matrix effect of terpenoids on pesticides in apples quantification by gas chromatography–tandem mass spectrometry

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ABSTRACT

The sample matrix can enhance the gas chromatography signal of pesticide residues relative to that obtained with the same concentration of pesticide in solvent. This paper is related to negative matrix effects observed in coupled gas chromatography-mass spectrometry ion trap (GC/MS^2) quantification of pesticides in concentrated extracts of apple peel prepared by the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) method. It is focused on the pesticides most frequently used on the apple varieties studied, throughout the crop cycle, right up to harvest, to combat pests and diseases and to improve fruit storage properties. Extracts from the fleshy receptacle (flesh), the epiderm (peel) and fruit of three apple varieties were studied by high-performance thin-layer chromatography hyphenated with UV–vis light detection (HPTLC/UV visible). The peel extracts had high concentrations of triterpenic acids (oleanolic and ursolic acids), reaching 25 mg kg^{-1}, whereas these compounds were not detected in the flesh extracts (<0.05 mg kg^{-1}). A significant relationship has been found between the levels of these molecules and negative matrix effects in GC/MS^2. The differences in the behavior of pesticides with respect to matrix effects can be accounted for by the physicochemical characteristics of the molecules (lone pairs, labile hydrogen, conjugation). The HPTLC/UV visible method developed here for the characterization of QuEChERS extracts acts as a complementary clean-up method, aimed to decrease the negative matrix effects of such extracts.

1. Introduction

Gas or liquid chromatography techniques coupled with mass spectrometry (MS or MS^n) are among the most powerful analytical tools currently available for monitoring pesticide residues in food. The use of mass spectrometry, particularly MS^n, has considerably improved the selectivity and sensitivity of the analysis. However, such methods may underestimate or overestimate pesticide levels in complex samples, such as food products, due to matrix effects. Such effects may result in significant differences in the signals obtained for chromatographic standards prepared in solvent and standards prepared in the matrix [1–8]. The chromatographic signal is increased by positive matrix effects and decreased by negative matrix effects. These effects may result from the adsorption of analytes and matrix components in the injector, the detector and/or the chromatography column [9].

Analysts have long focused on modifications to sample purification procedures as a means of compensating for the matrix effect. The QuEChERS method [10] has been successfully used and adapted for the extraction of pesticide residues from various types of food sample, including fruits, such as tomato, pear, apple, orange, lemon, guava, grape, avocado. . ., vegetables, such as cabbage, carrot, lettuce, cucumber, onion. . ., rice [11], cereal grains [12], liquids and beverages, such as fruit juice, olive oil, honey [13]. . ., and processed products, such as potato chips, and crackers [14–17]. Alternative methods have been developed, based on the addition of internal labeled standards [18], calibration in the matrix [19,20], the addition of analyte protectants (*e.g.* sorbitol, γ-lactone-gulonic acid) [20], or calibration correction factors [8]. Calibration correction factors are added to both the standards and the samples. They interact strongly with the active sites of the system (silanols), thereby minimizing matrix effects [18,19]. The gas chromatograph (loading of liners and precolumns) and the mass spectrometer (source cleaning) should undergo regular maintenance, to ensure that the sensitivity and reproducibility of the GC/MS method remain high [19].

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The matrix compounds most likely to interfere with gas chromatography analysis are lipids (*e.g.* waxes, triglycerides, phospholipids), pigments (*e.g.* chlorophylls, carotenoids, melanoidins) and other molecules with a high molecular weight (*e.g.* resins) likely to dissolve in the solvents used to extract the analytes of interest [9]. A tailing off or fade-out of peaks of interest on chromatograms may be interpreted as a classic sign of a dirty detector. It must be noticed that tailing peaks can also occur when there is an interaction between the analyte and the stationary phase of the chromatographic column or because of unsuitable injection parameters (insert, injection speed, temperature, volumes. . .) [21]. Apples matrices consist of diverse components, including sugars, proteins, lipids [22–27], polyphenols [27–30] triterpenic compounds, paraffins, and alcohols [31–40], which may interfere with the analysis and contribute to matrix effects.

Positive matrix effects are stronger for pesticide molecules with particular functional groups: organophosphates (-P = O), carbamates (–O—CO—NH–), hydroxy compounds (–OH), amino compounds (–NH–), imidazoles, benzimidazoles (-N =) and urea derivatives (–NH—CO—NH–) [8,9]. Hydrophobic, non-polar compounds, such as persistent organochlorine contaminants, are less affected by positive matrix effects because they are less strongly adsorbed onto the liner surface. Organophosphates (*e.g.* chlorpyrifos, pirimiphos), organochlorides (*e.g.*, dicofol, captan), pyrethroids (*e.g.* fenvalerate, deltamethrin), azoles (*e.g.* tebuconazole, triadimefon), carbamates (*e.g.* carbaryl, pirimicarb), dinitroaniline derivatives (*e.g.* fluazinam, procymidone, trifluraline), amides (*e.g.* alachlor, butachlor), phenoxyacetic acid derivatives (*e.g.* 2,4-D-butylate, haloxyfop) and other compounds, such as piperonyl butoxide, chinomethionate, flutolanil, fluoroglycofen-ethyl, nitrofen, and hexazinone, are also typically sensitive to positive matrix effects [19]. Giacinti et al. [1] recently demonstrated negative matrix effects for flonicamid, chlorpyrifos, boscalid, fludioxonil, pirimicarb, and propargite in QuEChERS extracts of apple peel. They also demonstrated positive matrix effects for these compounds in flesh and fruit extracts. The analysis of pesticide residues by GC/MS^2 in apple peel results in higher target-analyte concentrations, at levels above the limits of detection (LOD), and a greater transfer of matrix analytes to extracts than analyses of the whole fruit.

The aim of this study was to investigate the composition of various QuEChERS extracts of peel/flesh/fruit, using an HPTLC method to determine the principal molecular markers of the apple matrix soluble in acetonitrile (sugars: fructose, glucose and sucrose, triterpenic acids, uvaol, paraffins C27-C29, phloridzin, primary fatty alcohols and polyphenols), *(i)* to identify the matrix compounds potentially responsible for the negative matrix effects in GC/MS^2, observed for flonicamid, chlorpyrifos, boscalid, fludioxonil, pirimicarb and propargite in peel extracts [1], and *(ii)* to propose a purification method for highly concentrated extracts for the limitation of these matrix effects.

2. Materials and methods

2.1. Target apple varieties

Three apple varieties (VARi) from among the most widely grown and popular in France were chosen for a previous study [1]. These varieties differ in terms of fruit color, composition, sensitivity to pests and ripening times. They were grown in various biotic and abiotic conditions and all trees were sprayed with commercial pesticide preparations according to the seasonal pest risk and the sensitivity of the variety concerned. The apples were collected from the orchard in August (VAR1), October (VAR2), or November (VAR3) and stored in a cold room at 4 °C until processing.

2.2. Selection of pesticides and matrix compounds

The matrix effects of six pesticides among the 11 selected by Giacinti et al. [1] were studied in GC/MS^2 here (Table 1). The matrix compounds likely to be present in the QuEChERS extracts of apples are also listed in Table 1.

2.3. Chemicals and materials

Chromasolv® for HPLC solvents were purchased from Sigma Aldrich (St Quentin Fallavier, France): ethyl acetate (≥97.7%), acetonitrile (≥99.9%), tetrahydrofuran THF (≥99.9%), hexane (≥97%) and isopropanol (99.9%). Chloroform HiPerSolv Chromanorm for HPLC and methanol id Reagent Ph. Eur. for HPLC-gradient grade were purchased from VWR (Strasbourg, France). Acetone Multisolvent® HPLC grade ACS ISO UV–vis Scharlau was purchased from Fischer (Illkirch, France).

Folin & Ciocalteu's phenol reagent 2N was purchased from Sigma (St Quentin Fallavier, France) and sodium carbonate Acros Organics was obtained from Fischer (Illkirch, France).

The Pestanal analytical standards and the matrix analytical standards (triterpenoids, primary fatty alcohols, paraffins, monosaccharides and polyphenols) were supplied by Sigma Aldrich (St Quentin Fallavier, France): boscalid (99.9%), captan (99.6%), chlorpyrifos (99.9%), dithianon (97.4%), flonicamid (91.9%), fludioxonil (99.9%), pirimicarb (98.5%), propargite (99.5%), pyraclostrobin (99.9%), thiacloprid (99.9%), thiamethoxam (99.7%), oleanolic acid (≥ 97%), ursolic acid (≥ 90%), uvaol (≥ 95%), 1-hexadecanol C16-OH ReagentPlus (99%), 1-octadecanol C18-OH ReagentPlus (99%), 1-eicosanol C20-OH (98%), 1-docosanol C22-OH (98%), 1-tetracosanol C24-OH (≥99%) and 1-hexacosanol C26-OH (≥97%), 1-octacosanol C28-OH (≥99%), 1-triacontanol C30-OH (≥98%), heptacosane C27 (≥98%), nonacosane C29 (≥98%), α-D-glucose (96%), D(−)-fructose (99%), sucrose (99.5%) and dihydrated phlorizin (≥98.5%).

The QuEChERS reagent (a mixture of $MgSO_4$, sodium chloride, disodium citrate and disodium hydrogen citrate; Q-Sep kit 26235), and a mixture of $MgSO_4$, primary secondary amine (PSA) and C18 (tubes 26221 + 26125), were obtained from Restek (Lisses, France).

2.4. Sample processing and preparation

The sampling procedure, extraction and purification by the QuECHERS method have been described in detail elsewhere [1]. In summary, the various samples (apple flesh, apple peel and whole apple) were ground and stored at −24 °C until extraction. Homogenized samples (10 g) were subjected to extraction in 10 mL of acetonitrile with the QuEChERS Restek Q-Sep™ salts kit. The entire supernatant (volumes ranged between 8.5-9.5 mL) was transferred to the Restek dSPE Q-Sep™ adsorbent kit (mix of one tube 26221–8 mL and two tubes 26125–1 mL each). Acetonitrile was removed by evaporation to dryness. The resulting dry extracts were then dissolved in 500 μL ethyl acetate for injection into the gas chromatograph. QuEChERS extracts were identified as follows: FRUITVAR1, 2 or 3; FLESHVAR1, 2 or 3 and PEELVAR1, 2 or 3, for the fruit, flesh and peel extracts of each apple variety, respectively.

2.5. Preparation of standards and calibration curves

2.5.1. Preparation of solvent-matched and matrix-matched pesticide standards for GC/MS^2 analysis

Pesticide standards were prepared as previously described [1]. Stocks were prepared at a concentration of about 100 ng μL^{-1} in ethyl acetate. Mixtures of standard stock solutions were diluted to give 80–8000 ng pesticide in 500 μL of ethyl acetate containing internal standards. Matrix-matched standards were obtained by spiking apple sample extracts from each variety.

Table 1
Pesticides and matrix compounds studied.

Molecule	CAS No.	Classification	Chemical formula	MW ($g\,mol^{-1}$)	Log K_{ow} (1)	Boiling point (°C)
Boscalid	188425−85-6	Pesticide	$C_{18}H_{12}Cl_2N_2O$	343.21	2.96	519.6 (1)
Chlorpyrifos	39475−55-3	Pesticide	$C_9H_{11}Cl_3NO_3PS$	350.59	4.7	377.4 (1)
Flonicamid	158062−67-0	Pesticide	$C_9H_6F_3N_3O$	229.16	−0.24	381.4 ± 52.0 (1)
Fludioxonil	131341−86-1	Pesticide	$C_{12}H_6F_2N_2O_2$	248.19	4.12	382.6 (1)
Pirimicarb	23103−98-2	Pesticide	$C_{11}H_{18}N_4O_2$	238.29	1.7	326.2 (1)
Propargite	2312−35-8	Pesticide	$C_{19}H_{26}O_4S$	350.47	5.7	441.8 (1)
Oleanolic acid	508−02-1	Triterpenic acid	$C_{30}H_{48}O_3$	456.70	6.47 [41]	553.5 (exp) (1)
Ursolic acid	77−52-1	Triterpenic acid	$C_{30}H_{48}O_3$	456.70	6.43 [41]	528.0 (1)
Uvaol	545−46-0	Triterpenic diol	$C_{30}H_{50}O_2$	442.72	8.10	500.7 (1)
Chlorogenic acid	327−97-9	Polyphenol	$C_{16}H_{18}O_9$	354.31	−1.01	439.5 (2)
Phlorizin	60−81−1	Polyphenol	$C_{21}H_{24}O_{10}$	436.41	1.50	506.9 (2)
Fructose	57−48-7	Monosaccharide	$C_6H_{12}O_6$	180.16	−1.46	474.1 (2)
Glucose	50−99-7	Monosaccharide	$C_6H_{12}O_6$	180.16	−3.24 (exp)	477.4 (2)
Sucrose	57−50-1	Disaccharide	$C_{12}H_{22}O_{11}$	342.30	−3.70 (exp)	683.3 (2)
Heptacosane	593−49-7	Paraffins	$C_{27}H_{56}$	380.73	13.60	416.4 (2)
Nonacosane	630−03-5	Paraffins	$C_{29}H_{60}$	408.73	14.58	442.2 (2)
1-hexadecanol	36653−82-4	Fatty alcohol	$C_{16}H_{34}O$	242.44	6.73	322.5 (2)
1-octadecanol	112−92-5	Fatty alcohol	$C_{18}H_{38}O$	270.49	7.72	343.1 (2)
1-eicosanol	629−96-9	Fatty alcohol	$C_{20}H_{42}O$	298.55	8.70	370.4 (2)
1-docosanol	661−19-8	Fatty alcohol	$C_{22}H_{46}O$	326.60	9.68	386.7 (2)
1-tetracosanol	506−51-4	Fatty alcohol	$C_{24}H_{50}O$	354.65	10.66	412.8 (2)
1-hexacosanol	506−52-5	Fatty alcohol	$C_{26}H_{54}O$	382.71	11.65	425.7 (2)
1-octacosanol	557−61-9	Fatty alcohol	$C_{28}H_{58}O$	410.76	12.63	451.3 (2)
1-triacontanol	593−50-0	Fatty alcohol	$C_{30}H_{62}O$	438.81	13.61	461.4 (2)

(1) Chemspider.com (estimé sur KOWWIN v1.67).
(2) Calculated from smiles codes in HSPiP 4th edition v4.1.07.

Apple sample extracts were prepared as described in Section 2.4. Analyses were performed in an UltraTRACE gas chromatograph with a split/splitless injector, coupled to an ITQ900 ion trap mass spectrometer (Thermo Scientific, Courtaboeuf France). The same extracts were also analyzed with an HPTLC method.

2.5.2. Preparation of matrix compound standards for HPTLC/UV–vis characterization and terpenoid quantification

Stocks were prepared at a concentration of about $1\,g\,L^{-1}$: monosaccharides, disaccharides and phloridzin in methanol, triterpenic acids, uvaol and paraffins in THF, and fatty alcohols in chloroform. They were analyzed simultaneously with the matrix-matched pesticide standards, by HPTLC.

2.6. Extraction of total waxes from apple peels

Apples were peeled off using a mechanical apple peeler. Peels were submitted to enzymatic digestion at 25 ± 5 °C during 24 h, in a citrate buffer 20 mM (pH = 4) and containing $16000\,U\,L^{-1}$ of cellulase (from *Trichoderma longibrachiatum* ≥ $1\,U\,L^{-1}$, Sigma Aldrich, St Quentin Fallavier, France) and $23600\,U\,L^{-1}$ of pectinase (aqueous solution of *Aspergillus aculeatus* ≥ $3800\,U\,L^{-1}$, Sigma Aldrich). Citric acid anhydrous and sodium citrate were also purchased from Sigma Aldrich. Peels were then widely washed under demineralized water and freezed to −40 °C to be lyophilized in the Cryoivoire PILOT 27 (Cryonext, France). Finally, lyophilized peels were ground in an electric grinder. Total waxes were recovered by an extraction in chloroform/methanol (2:1, V/V) in a Soxhlet apparatus. After four complete cycles of solvents *i.e.* seven to eight hours, the extract was dried at 45 °C under vacuum. The total waxes were then ground into a fine powder using an electric grinder.

2.7. High-performance thin-layer chromatography coupled with UV–vis spectrophotometry

2.7.1. Chemicals and materials

Merck HPTLC silica gel $60F_{254}$ glass plates (20 × 10 cm, with a 150–200 μm-thick layer) were purchased from Chromacim (Moirans, France), HPTLC Nano-Sil NH_2/UV254 glass plates (10 × 10 cm, with a 200 μm-thick layer were obtained from Macherey Nagel (Hoerdt, France). Plates were prewashed with isopropanol, dried in an oven at 120 °C for 20 min, and stored in a desiccator until use. NH_2 plates were prederivatized just before samples application by dipping the first two centimeters of the plates in 2% formic acid (Sigma Aldrich, St Quentin Fallavier, France) in acetonitrile. The plates were allowed to dry at 50 °C during ten minutes.

The anisaldehyde-H_2SO_4 derivatization reagent was prepared by mixing 0.5 mL of anisaldehyde (98%; Sigma Aldrich, St Quentin Fallavier, France) with 50 mL of acetic acid (99%; Sigma Aldrich) and 1 mL of sulfuric acid (97%; Sigma Aldrich). The vanillin-H_2SO_4 derivatization agent was prepared by mixing 1–2 g of vanillin (99%; Alfa Aesar VWR, Strasbourg, France) with 100 mL of ethanol (99.8%; Sigma Aldrich) and 1 mL of sulfuric acid (97%; Sigma Aldrich).

2.7.2. HPTLC equipment and general procedures

Samples were applied to plates with the Autosampler ATS3 CAMAG (Muttenz, Switzerland). Ethyl acetate, methanol, chloroform or THF was used as a rinsing solvent, according to the solvent used for sample dilution. The following parameters were used: predosage volume 1000 nL, surplus volume 5000 nL, retractation volume 100 nL, delivery speed between 150 and 200 $nL\,s^{-1}$ depending on the nature of the solvent, filling speed $500\,nL\,s^{-1}$, rinsing time 10 s, compression volume 300 nL, compression time 10 s, decompression volume 240 nL. The first application position X was set at 15 mm and application position Y at 10 mm. The distance between tracks was calculated automatically from the number of deposits. The spray application mode was used, with a band velocity of $5\,mm\,s^{-1}$ and a start delay of 50 ms. Band length was fixed at 4 mm.

After migration, the plates were photographed with a digital camera (8 megapixels, with 1.5 μm pixels, f/2.2) under a UV lamp functioning at a power of 4W, at 254 nm (BioBlock Scientific, France), and in daylight after derivatization.

For derivatization, the plates were dipped in a solution of anisaldehyde-H_2SO_4 or vanillin-H_2SO_4 with a TLC Immersion Device III CAMAG (Muttenz, Switzerland), at an immersion speed of $2\,cm\,s^{-1}$, with an immersion time of 3 s. They were then dried in an oven at 100 °C for 8 min or 10 min respectively.

The plates were also scanned at 200 nm before chemical derivatization and at 500 nm after chemical derivatization, in the TLC Scanner 3 SC3 CAMAG, with deuterium and tungsten lamps (Muttenz, Switzerland). The slit dimension was set to 4 × 0.1 mm micro, the scanning speed to 5 mm s^{-1} and data resolution was set to 50 μm $step^{-1}$. Remission and absorption were selected for the measurement type and mode, respectively. A second-order optical filter was used, and the detector mode and sensitivity were automatic.

The retardation factor Rf values were evaluated as the substance position with respect to the position of the solvent front measured from the sample application position.

The HPTLC autosampler and scanner, data acquisition and processing were controlled with WinCats 1.4.6.2002 Planar Chromatography Manager from CAMAG.

2.8. Analysis and quantification of matrix analytes in apple extracts

Volumes of 0–10 μL of QuEChERS extracts and standards were applied to HPTLC silica gel glass plates with ATS3 as described in Section 2.5.2. (Table S1 in the Supplementary material section). The spots were allowed to dry at room temperature for 30 min.

Migration was then carried out in a flat-bottomed glass chamber (24 × 24 × 8 cm) saturated with 45 mL of a mixture of hexane, chloroform and methanol (3:6:1, v/v/v). The solvent front was 60 mm from the edge of the plates. The plates were allowed to dry at room temperature for 30 min, derivatized by a solution of anisaldehyde-H_2SO_4 and then photographed and scanned as described in Section 2.5.2.

Triterpenoids (oleanolic and ursolic acids and uvaol) were quantified in the QuEChERS extracts by HPTLC-UV–vis at 500 nm, after chemical derivatization with anisaldehyde-H_2SO_4 reagent. Triterpenoid contents (in μg 100 g^{-1} of extract) were evaluated by comparing their peak areas with those for calibration curves plotted using Microsoft Excel:Mac (2011). The levels of all the other components were determined by studying the areas of their chromatographic peaks on different densitograms.

2.9. Analysis of matrix analytes by the HTpSPE clean-up procedure of Oellig & Schwack [42]

Pesticide and triterpenic acid standards were applied onto HPTLC Nano-Sil NH_2 and silica gel glass plates with ATS3 as described in Section 2.5.2. The spots were allowed to dry at room temperature for 30 min.

Two-dimensional chromatography was performed in a flat-bottomed glass chamber (18 × 15 × 3 cm). Acetonitrile (12 mL) was used as the first mobile phase to a migration distance of 75 mm. After drying, acetone (12 mL) was used as the second mobile phase, in the backwards direction to a migration distance of 46 mm. The plates were allowed to dry at room temperature for 30 min. They were then dipped in a solution of the derivatization agent, with the TLC Immersion Device III and dried in oven (8 min for anisaldehyde or 10 min for vanillin), photographed and scanned as described in Section 2.5.2.

2.10. Determination of the total phenolic content of QuEChERS extracts by the method of Folin & Ciocalteu [43]

The total phenolic content of the QuEChERS extracts in acetonitrile was determined before concentration to dryness. These extracts were diluted in water ($n = 4$), with adjustment of the dilution for the measurement of absorbances in the middle of the calibration curve. The calibration curve as obtained with six standards of chlorogenic acid, with concentrations ranging from 0 to 100 mg L^{-1}. Standards and samples (20 μL in each case) were dispensed into the wells of a 96-well plate, together with 10 μL of Folin & Ciocalteu reagent. The plate was shaken for 10 s and 170 μL of 2.36% sodium carbonate in water was added. The plate was shaken and then incubated at 45 °C for 10 s every 15 min. After 45 min of incubation and shaking cycles, absorbance at 760 nm was read on a UV–vis SpectroStarNano spectrophotometer (Labtech).

The results are expressed in micrograms of chlorogenic acid equivalents per 100 g of fresh matrix.

2.11. Relationship between the matrix effect in GC/MS^2 and the amount of matrix compounds in the extracts

The matrix effect (%ME) was calculated from the slopes of the calibration curves obtained in solvent (S_s) and in matrix (S_m) (Table S2 in the Supplementary material section):

$$\%ME = (S_m - S_s)/S_s \times 100$$

The matrix effect values were then plotted against the peak areas on HPTLC densitograms. Finally, matrix effect values were plotted against total phenolic and triterpenoid contents, which were quantified for all QuEChERS extracts.

2.12. Statistical analysis

The software XLSTAT V 2015.2.01. (Addinsoft, Paris, France) was used to calculate the Pearson's correlation coefficients (r).

3. Results and discussion

3.1. Study of the target matrix analytes in QuEChERS extracts of apples

Analytical conditions were established for separation of the targeted matrix analytes by functional group. The HPTLC plate was first scanned at different UV wavelengths. Five of the eight standards were detected at 200 nm (see Fig. 1B). Sugars, paraffins and fatty alcohols do not absorb at 200 nm because they lack chromophores. Triterpenoids have few chromophores and they absorb only at 200 nm in the UV domain. Phlorizin moved to an Rf of 0.05. All the pesticides were retarted at the same Rf value, 0.52. The oleanolic and ursolic acids were overlapped at an Rf of 0.58, whereas uvaol was retarded at an Rf of 0.62.

The compounds on the HPTLC plate were then derivatized with anisaldehyde. Anisaldehyde was chosen for this step because it enhances the detection of triterpenoids, sugars and steroids. Triterpenoids appear as violet zones on a pink background (see Fig. 1A). Sugars give dark green zones, whereas phlorizin gives an orange zone. The track of paraffins shows light green zones at Rf = 0.42 and fatty alcohols give a white halo enclosed by a pink line at Rf = 0.75 in this system. Paraffins and fatty alcohols were not visible after derivatization with anisaldehyde as well as the pesticide mixture. The plate was scanned at 500 nm. Five of the eight standards were detected in these conditions: the sugar mixture, phlorizin, and triterpenoids (see Fig. 1C). The sugar mixture remained very close to the deposit line. Anisaldehyde derivatization did not enhance the detection of paraffins, alcohols or pesticides. Paraffins and fatty primary alcohols were detected after derivatization with primuline at Rf = 0.99 and Rf = 0.76 respectively (data not shown). Derivatization with anisaldehyde facilitated the qualitative and quantitative HPTLC analysis of the target matrix compounds in apple matrices spiked with pesticides, without interaction between these compounds and the pesticides themselves.

The separate study of flesh, fruit and peel QuEChERS extracts by HPTLC-UV–vis revealed the presence of triterpenic acids and uvaol, and many other matrix compounds (see Fig. S1 in the Supplemen-

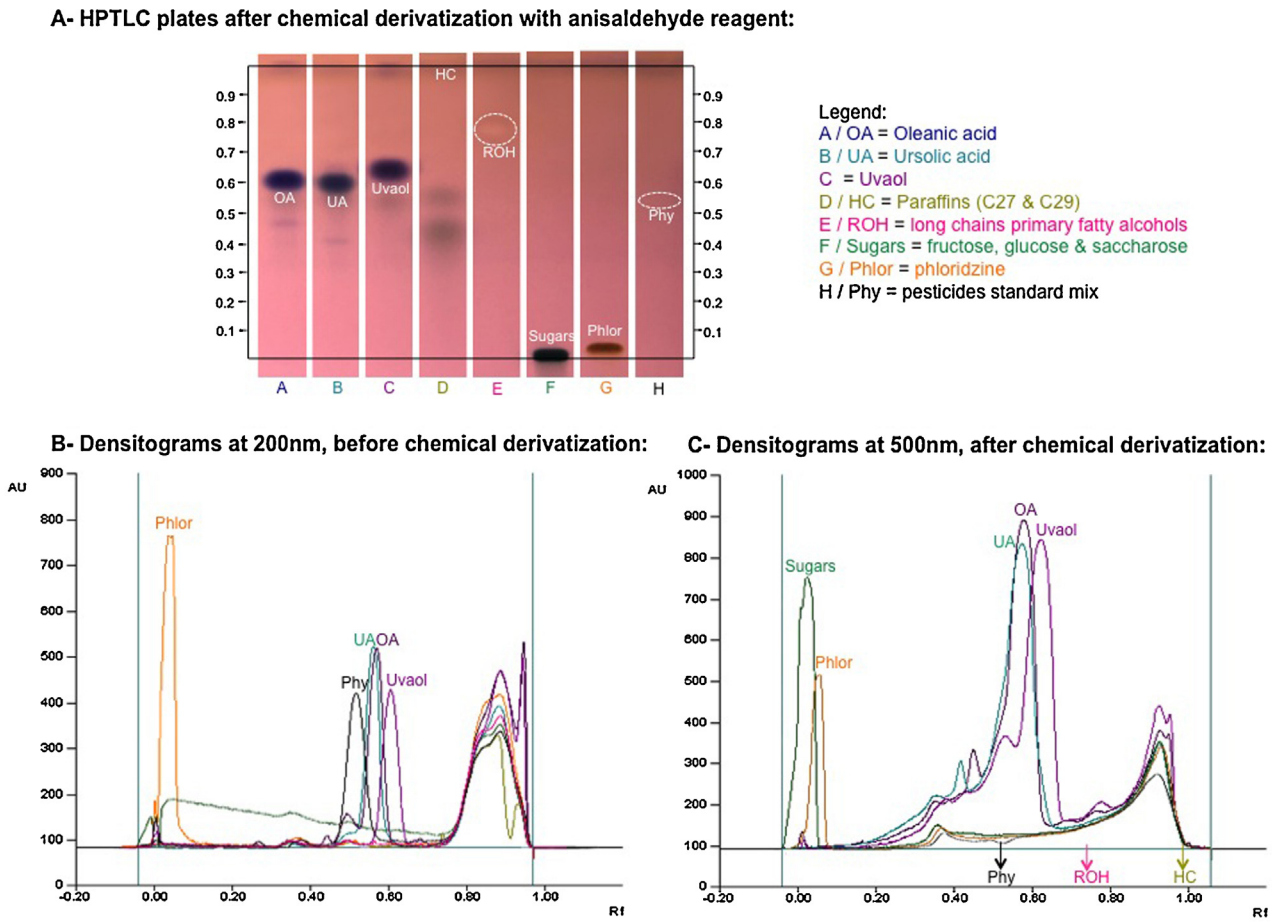

Fig. 1. HPTLC (Silica Gel plates) profiles and densitograms for matrix analyte standards before (A) and after (B and C) derivatization with anisaldehyde reagent.

tary material section). Thirteen zones of matrix compounds (MCi) were detected on the densitograms for FLESHVAR1 and FLESHVAR2, and 10 were detected on that for FLESHVAR3. Fourteen zones of MCi were detected for FRUITVAR1 and 15 each for FRUITVAR2 and FRUITVAR3. Sixteen zones of matrix compounds were detected for PEELVAR1, and 17 each for PEELVAR2 and PEELVAR3.

The densitograms differed in terms of the nature (some compounds detected and others not detected) and/or concentrations of the compounds identified (see Fig. 2A and B). On the basis of the Rf values of the matrix analytes standards, glucose and fructose were detected in all samples. The compounds at Rf = 1 may be wax esters. Phlorizin and paraffins may have been present in the extracts. However, the principal difference between matrices observed was the presence of triterpenic acids in fruit and peel QuEChERS extracts and the absence of these compounds from flesh extracts ($< 0.05\ mg\ kg^{-1}$, see Fig. 2B and Fig. S2 in the Supplementary material section). Triterpenoid concentration and total phenolic contents are shown in the histograms in Fig. 3.

Triterpenic acid concentration was higher in peel than in fruit extracts. Peel extracts from VAR2 and VAR3 had triterpenic acid concentrations twice that for VAR1. Uvaol concentrations in VAR2 extracts were higher than those in extracts from the other two varieties. Indeed, this compound was detected in flesh and its concentration in peel extracts from VAR2 were 10 times those in peel extracts from VAR1 and VAR3. Ursolic acid is one of the major cyclic components of the cuticular waxes of apples [38–40,44].

Total phenolic contents followed a pattern very different from that of triterpenoid content. The values are generally normalized by expression as gallic acid equivalents. However, the results in Fig. 3 are expressed as chlorogenic acid equivalents, because this compound is more prevalent in apples.

In VAR1, total phenolic content was similar for flesh, fruit and peel extracts, at about 5 mg chlorogenic acid equivalent per kg of matrix. In VAR2, the total phenolic content of the fruit extract was about twice that of the flesh and peel extracts. VAR3 had the highest total phenolic content of all the varieties tested, about four to 10 times higher than those for the other varieties. In VAR3, total phenolic content was higher for the flesh extract than for the fruit and peel extracts. The total phenolic content of the peel extract was about half that of the flesh and fruit extracts.

As it is stated in the literature, the different extracts studied here may not have contained the same polyphenols, with the polyphenol composition of the extract depending on the origin of the matrix. Indeed, the distribution of polyphenols in apple depends on the functional groups of these molecules. For example, flavonols and flavanols are found in the peel, whereas the phenolic acids are mostly located in the flesh. The core and seeds are richer in dihydrochalcones than the peel [45]. Expressing the results as chlorogenic acid equivalents may have resulted in the concentrations of some polyphenols being overestimated and those of others underestimated, depending on the absorbance of the complex they form with Folin & Ciocalteu reagent. However, the differences observed may reflect differences in anthocyanin content. Anthocyanins are characteristic polyphenols responsible for the red color of some apple varieties, such as VAR1 and VAR3. Indeed, the peel of VAR1 and VAR3 yielded red and pink extracts, respectively. Apples from VAR2 markedly less red in color and their extracts were yellow-orange in color if oxidization occurred during

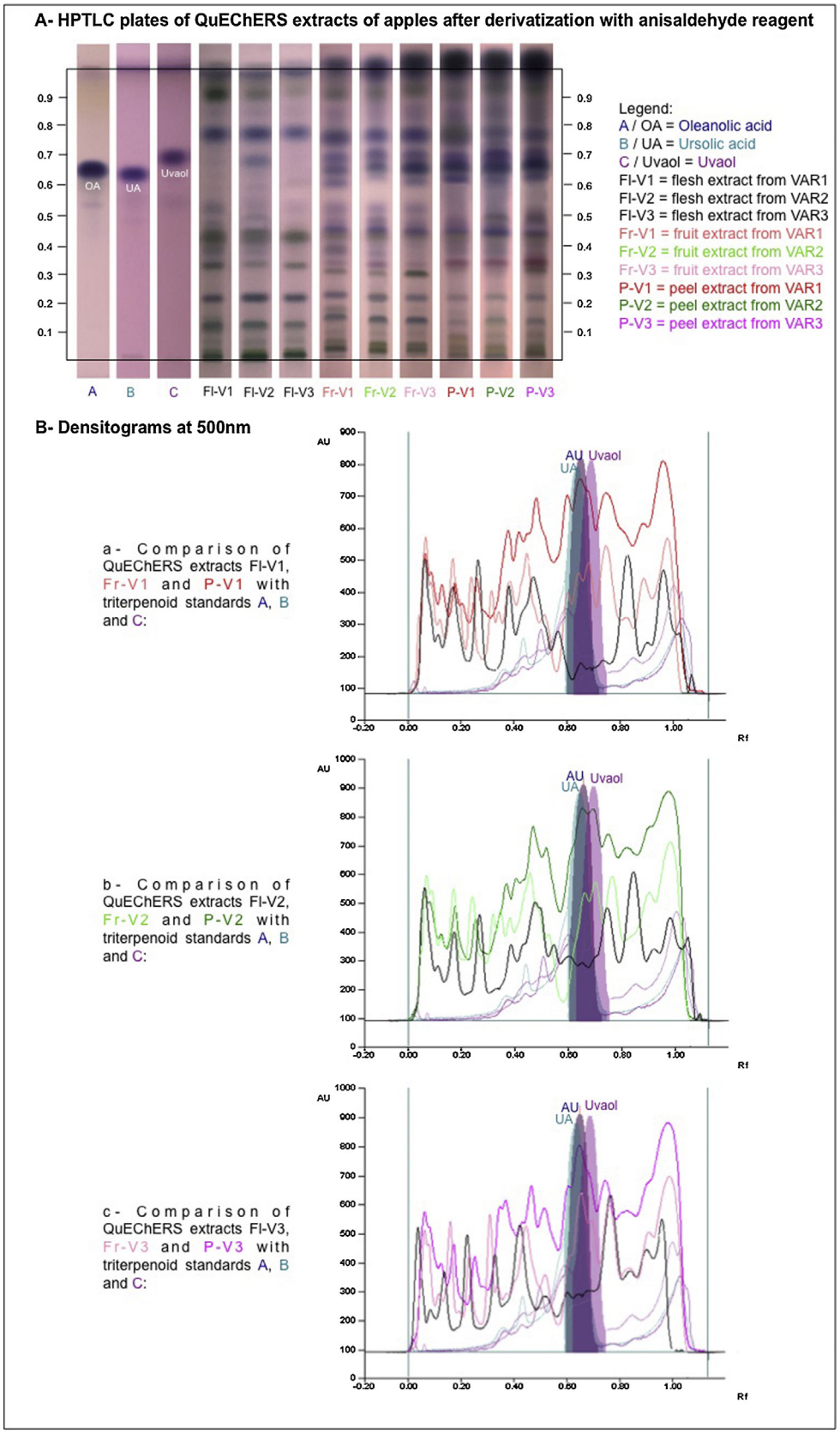

Fig. 2. HPTLC (Silica Gel plates) profiles (A) and densitograms at 500 nm (B) after the derivatization with anisaldehyde of QuEChERS extracts of apples from VAR1 (a), VAR2 (b) and VAR3 (c).

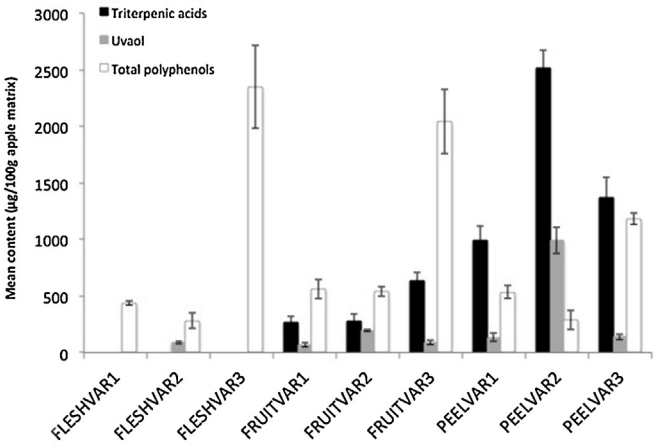

Fig. 3. Mean terpenoid and polyphenol concentrations ($n = 5$) in QuEChERS extracts from different apple matrices.

grinding, and greenish yellow otherwise. For all varieties, oxidization of the sample during grinding resulted the extract being more yellow in color. However, anthocyanins are particularly sensitive to oxidation. Nevertheless, after the SPE dispersive step of the QuEChERS extraction procedure, all the extracts were similarly yellow (see Fig. S3 in the supplementary material section).

It could be argued that the purification step, during which matrix analytes are likely to be adsorbed onto the stationary phases (PSA, C18, GCB), and the sensitivity of some molecules to oxidation may account for the pattern of total phenolic content observed.

3.2. Relationship between negative matrix effects in GC/MS^2 ion trap and co-injected matrix analytes

An analysis of the pesticide content of these extracts without normalization of the peak responses in GC/MS^2 ion trap experiments showed that the extent of the matrix effect depended on the pesticide molecules (Table S2 in the Supplementary material section). Negative results were obtained for peel extracts, whereas positive results were obtained for flesh extracts. Statistical analysis revealed an impact of the nature of the apple matrix but no significant effect of apple variety. Fludioxonil and boscalid were the pesticides most sensitive to matrix effects [1].

The occurrence of a matrix effect reflects the nature and quantities of analytes from the matrix co-extracted during the QuEChERS procedure. As the chemical compositions of the flesh and the peel of apples were different (See Section 3.1.), it was therefore hypothesized that the negative matrix effects observed in peel extracts might reflect the presence, in sufficiently large quantities, of one or several molecules able to interfere with pesticides during hot splitless injection.

An HPTLC/UV–vis analysis of all the extracts led to the identification of a large number of analytes (Section 3.1. and see Fig. S1 in the Supplementary material section) likely to be involved in the observed matrix effects.

To examine the relationship between the MCi matrix data containing peak area responses for the nine apple matrices and the matrix effect data for each pesticide, Pearson correlation coefficients were calculated. A strong significant correlation was observed (p-values < 0.05) between all the pesticides and the content of oleanolic and ursolic acids ($-0.833 < r < -0.954$; see Table S3 in the Supplementary material section).

3.3. Relationship between the negative matrix effect observed in GC/MS^2 and triterpenic acid contents

Matrix effect values were plotted against triterpenic acids content (See Fig. 4) A linear relationship was observed between matrix effect and triterpenic acids content for each pesticide ($0.72 < R^2 < 0.88$).

Matrix effects are known to be controled by a number of factors simultaneously. In hot splitless injection, for example, many compounds of different volatilities may accumulate, leading to interference resulting in a globally positive or negative matrix effect. In flesh extracts, in which no triterpenic acids were detected, matrix effects were generally positive and very strong for fludioxonil and boscalid (between 200 and 400%). These effects were much weaker (between 50 and 100%) for flonicamid, pirimicarb and chlorpyrifos. Propargite is a particular case, for which the matrix effect is always negative, regardless of the nature of the matrix. In VAR2 and VAR3, the values are really closed to zero, meaning that the matrix effect for this compound was almost non-existent in these flesh extracts.

Thus, flesh extracts probably contain compounds that enhance the chromatographic signal in GC/MS^2 in the absence of triterpenic acids. It has been stated in the literature that compounds like sorbitol can act as analytes' protectants during hot splitless injection [20]. Sugars are the main components of flesh. It is likely that analytes from flesh matrices could enhance the pesticides' chromatographic responses and cause a positive matrix effect.

In 1993, Erney et al. [3,46] were the first to identify "matrix-induced chromatographic response enhancement" in analyses of organophosphates in milk and butter extracts. They claimed that, when standards are injected in solvents, analytes could be adsorbed onto the active sites of the injector and column, or degraded by catalytic thermodecomposition at these sites (presence of metals and free silanols on the glass liners). When standards are injected in matrix, the matrix compounds block these sites, globally enhancing the standard signals. The matrix effect thus results from the analytes co-extracted from the matrix, generally in larger amounts than the analytes of interest. The smallest amounts (ultratraces) of co-extracted analytes are associated with the most positive matrix effects. Quantitative data may be unreliable if calibration curves are not prepared in matrix extracts.

At the same time, the number of new active sites increases, because of the progressive accumulation of non-volatile compounds in the inlet liner and in front of the column. This could lead to a decrease in the chromatographic signal, referred to by Hajslova & Cajka as "matrix-induced diminishment" [6]. These two phenomena occur almost simultaneously, and it is therefore impossible to exert any real control over the formation of new active sites due to the accumulation of non-volatile analytes. Kowalski et al. [7] recently showed that pesticide molecules could react with matrix compounds and degrade during hot splitless injection, and even during extraction steps in some cases.

Apple peel contains large amounts of cuticular lipids. The soluble waxes protecting the cuticle consist of long-chain (n = 20-40 carbon atoms) molecules and terpenoids, of various degrees of volatility (Table 1). These molecules can accumulate in the inlet liner and create active sites capable of reacting with pesticides, decreasing the amount of pesticide reaching the mass detector. The propensity of each pesticide to interact with the active molecules may result in matrix effects of various strengths.

3.4. So what happens during hot splitless injection to suppress the pesticide signal during co-injection with triterpenic acids?

In gas phase, a proton transfer reaction can occur between a compound HA which can be deprotonated, and a compound B,

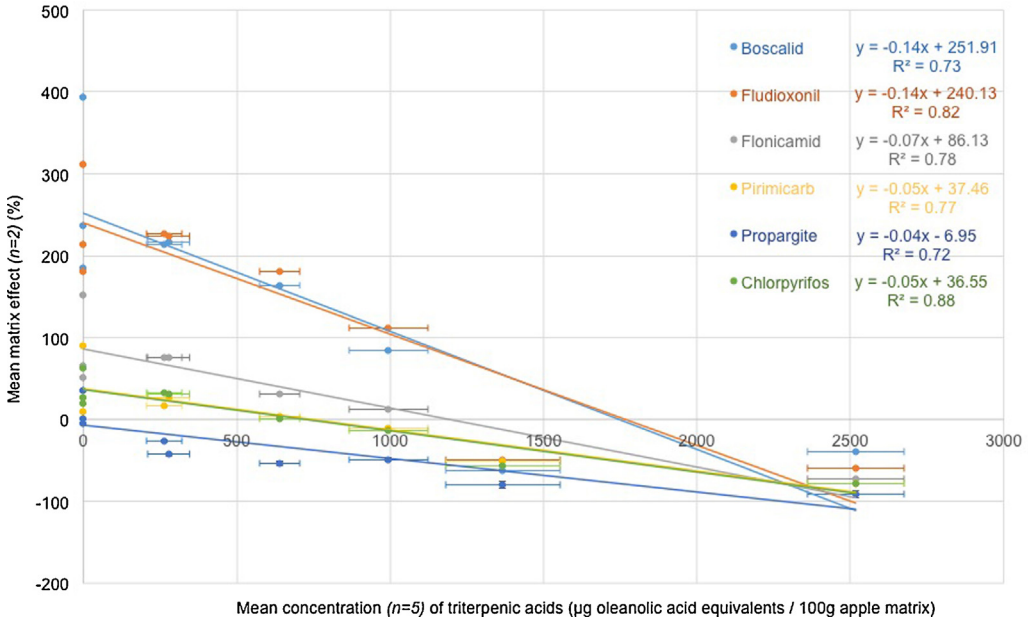

Fig. 4. Linear regression between the matrix effect and triterpenic acid content in the different apple matrices.

which can be protonated. The gas phase acidity (GA) of HA is the free energy change for the reaction $HA \rightarrow A^- + H^+$. The gas phase basicity (GB) is the free energy of the reaction $BH^+ \rightarrow B + H^+$ [47–49].

Triterpenic acids are reactive species because of their carboxylic acid group COOH, whose proton can undergo a proton transfer reaction. Boscalid (I), chlorpyrifos (II), flonicamid (III), fludioxonil (IV) and pirimicarb (V) contain nitrogen involved in different amino groups that could behave as bases. Pesticides (I) to (V) contain heterocyclic amines: (I) to (III) have a pyridine ring, (IV) a pyrrole ring and (V) a pyrimidine ring. (I), (III) and (V) have an amide group. (III) and (IV) have a nitrile group. Propargite has no basic amino groups (See Fig. S4. in the Supplementary material section).

The relationship between the matrix effect on boscalid and fludioxonil and triterpenic acid content is the strongest of all the six pesticides (similar slopes a = −0.14, see Fig. 4). The relationship between the matrix effect on pirimicarb and triterpenic acid content is similar to that for chlorpyrifos (a = −0.05) and less pronounced than for flonicamid (a = −0.07). Finally, propargite is less sensitive to matrix effects: the slope of the linear regression for the relationship between the matrix effect on propargite and triterpenic acid content was lowest for this compound (a = −0.04).

Most GB values for nitrogen compounds are available in the literature [50,51] but not for the targeted pesticides. It was observed that gas phase basicity increases from nitriles < amines < amides < pyroles < pyrimidines < pyridines. The nature, position and number of substituants on the nitrogen function greatly modify the gas basicity values. Substituants act on protonation by the electrostatic effects and the resonance of π electrons [49]. There are globally two kinds of substituants in the gas phase: the ones that participate to charge stabilization (alkyl groups, aromatic cycles) and those that destabilize the charges in the protonated forms (halogens) [50]. It has been observed that para-substituants are more influent than meta and ortho-substituants.

Propargite, that does not contain nitrogen, is the less basic of all the six pesticides and does not highly interact with triterpenic acids. Pesticides containing pyridine cycles (boscalid, chlorpyrifos and flonicamid) are expected to have the most important GB values. The GB value of pyridine is 898.1 kJ mol^{-1}. It decreases to 862 kJ mol^{-1} in *p*-trifluoromethylpyridine. Boscalid is then expected to have the most important GB value whereas chlorpyrifos the least. In fludioxonil, the preferred site of protonation could be the cyano nitrogen atom. The protonated form is highly stabilized by the pyrrole and the aromatic rings. This could explain the high reactivity of the fludioxonil with the triterpenic acids.

Following the injection of highly concentrated extracts, the chromatographic system becomes dirty, suppressing the ion signal. Efficient cleaning is thus required, with replacement of the inlet liner and the pre-column (and/or the removal of several centimeters at the start of the column, particularly if it is not preceded by a pre-column) and cleaning of the ion volume and the detector source. Merely cleaning the detector does not restore sensitivity: signal suppression seems to occur mostly upstream, in the chromatograph. Marked fouling of the inlet liner has been observed, with successive injections of highly concentrated extracts leading to a greenish-yellowish deposit within the glass liner. Thus, if injections of highly concentrated extracts are required to highlight the amounts of pesticides in more representative samples, as demonstrated in a previous study [1], then effective clean-up techniques for extracts are essential.

3.5. Purification of apple peel QuEChERS extracts by HPTLC, to minimize negative matrix effects in subsequent GC/MS^2 ion trap analysis

A recently developed clean-up concept in the multi-residue analysis of pesticides based on planar solid phase extraction followed by chromatographic analysis [42] could be used to purify extracts. Oellig & Schwack used thin-layer chromatography (TLC) to separate pesticides from matrix compounds and to focus them into a sharp zone, followed by extraction of the target zone through TLC–MS Interface before chromatography. Their method was used to analyze a QuEChERS extract of peel from organic VAR1 apples with a standard mixture of target pesticides, both on HPTLC NH_2 plates and on TLC Silica foils (Data not shown). Overlapping of pesticides with some matrix compounds at Rf = 0.68 was observed, regardless of the nature of the stationary phase. Further investigations were then undergone.

On HPTLC NH_2 plates prederivatized with 2% formic acid in acetonitrile, triterpenic acids were retarded at Rf = 0, far away

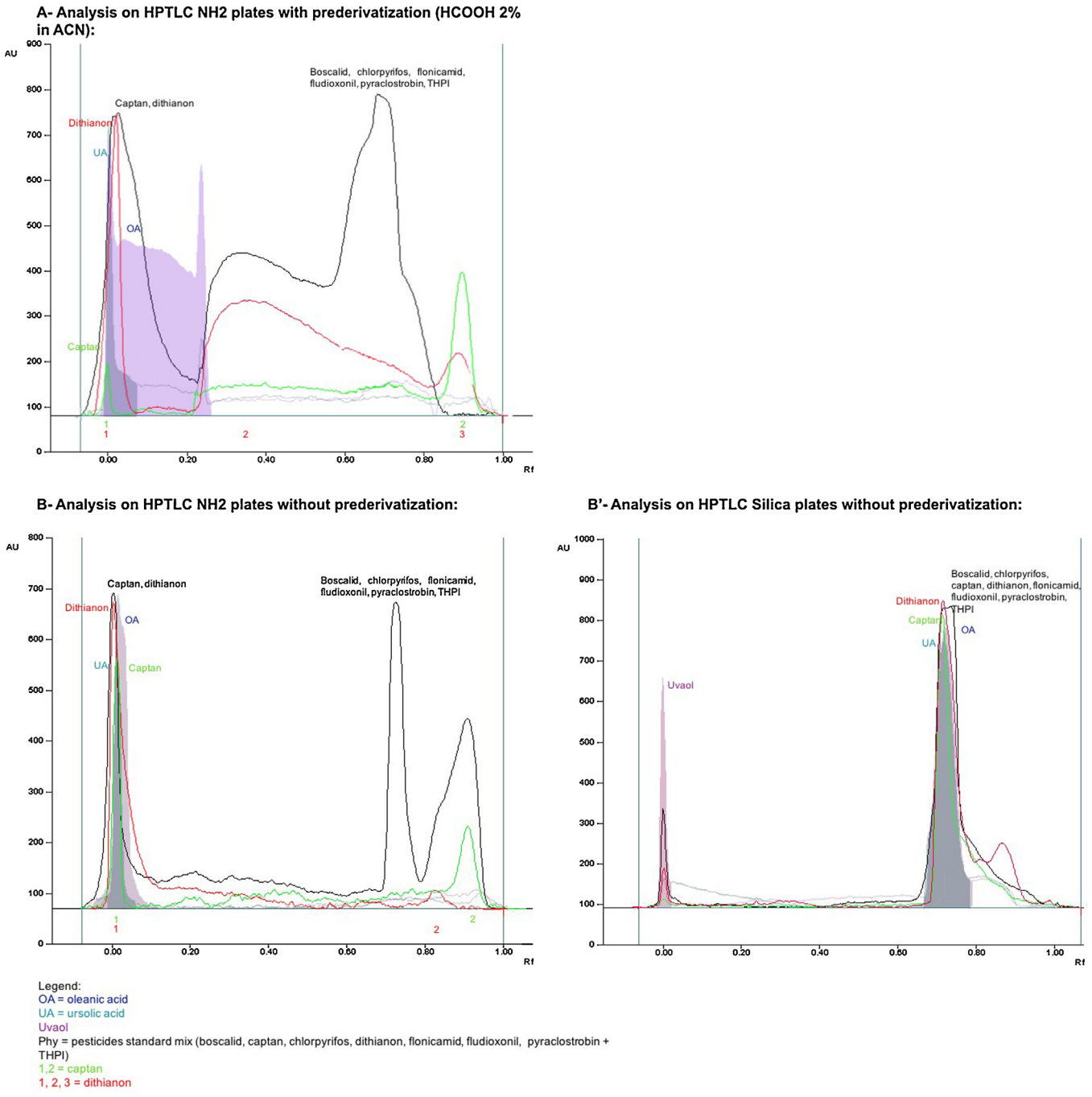

Fig. 5. Densitograms at 200 nm of pesticide and triterpenic acid standards on (A) HPTLC NH_2 plates after prederivarization with formic acid (2% in acetonitrile), (B) HPTLC NH_2 plates without prederivatization and (B') HPTLC Silica Gel plates without prederivarization, based on the HTpSPE clean-up method developed by Oellig & Schwack [42].

from boscalid, chlorpyrifos, flonicamid, fludioxonil and boscalid, all retarded at Rf = 0.68 as well as tetrahydrophtalimid, the metabolite of captan (see Fig. 5A). It is worth noticing that captan and dithianon were not retarded at the same Rf that of the other pesticides. In this analysis, the signal of captan showed two peaks at Rf = 0 and Rf = 0.9 and dithianon three peaks at Rf = 0, Rf = 0.35 (tailing peak) and Rf = 0.9. On HPTLC NH_2 plates without prederivatization, triterpenic acids, captan and dithianon were all retarded at Rf = 0 whereas the other pesticides were retarded at Rf = 0.68 (see Fig. 5B). Prederivatization did not change the densitrographic profile, anyway the oleanolic acid turned into a well-defined Gaussian-like peak. On HPTLC Silica plates, all standards were retarded at Rf = 0.68 (See Fig. 5B').

The method developed by Oellig and Schwack well separates triterpenic acids from boscalid, chlorpyrifos, flonicamid, fludioxonil and pyraclostrobine. As the negative matrix effects observed in GC/MS^2 seem to be due to the presence of large amounts of triterpenic acids, it is an effective method to purify highly concentrated peel extracts. The main drawback is the lack of recovery of captan and dithianon, two pesticides out of the 11 mostly used to treat apple orchards in South West of France [1]. Using Silica plates did not solve the problem by overlapping all the compounds.

The HPTLC method optimized in this study for the analysis of matrix compounds and the quantitation of the triterpenoids (see Fig. 6A), separated pesticides (Rf = 0.58) and triterpenic acids (Rf = 0.62). All the eleven previous studied targeted pesticides

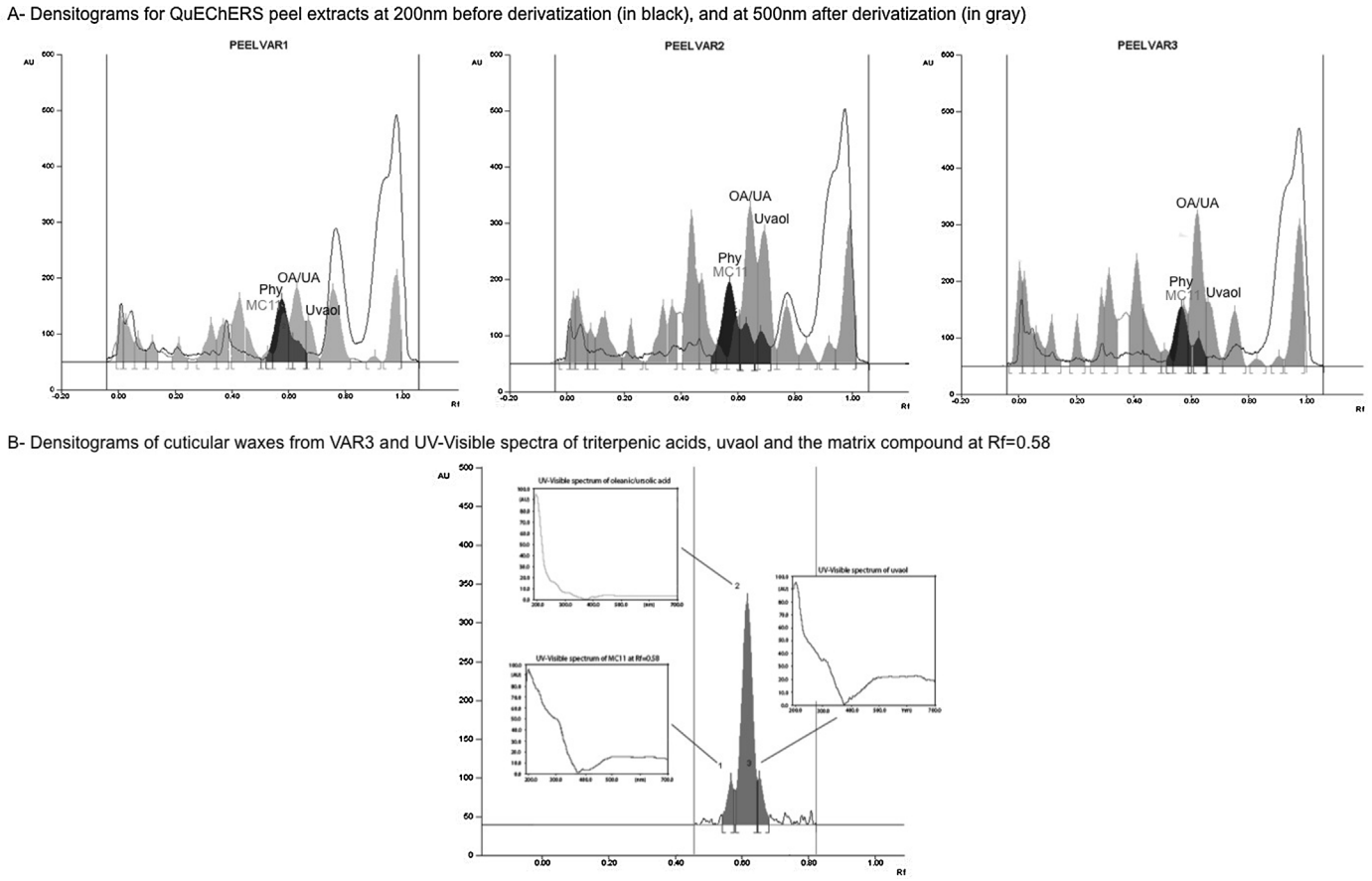

Fig. 6. Densitograms of QuEChERS extracts of peel spiked with pesticide standards before and after chemical derivatization, at 200 nm (in gray) and 500 nm (in black) (A) and HPTLC/UV–vis characterization of terpenoids in apple cuticular waxes (B).

overlapped at Rf = 0.58 and were detectable only before chemical derivatization at 200 nm, whereas the triterpenic acids were also detectable at 500 nm, after chemical derivatization with the anisaldehyde-H_2SO_4 reagent. Derivatization highlighted another zone of matrix compounds MC11 at the same Rf as pesticides (see Fig. S1 in the Supplementary material section) on the densitograms of peel extracts (see Fig. 6A). This zone was also detected in the cuticular waxes (see Fig. 6B). UV spectra were registered for the three zones of matrix compounds in cuticular waxes from apples of VAR3: MC11 at Rf = 0.58, triterpenic acids at Rf = 0.62 and uvaol at Rf = 0.66 (see Fig. 6B). After derivatization, MC11 was detected as a purple spot on a pink background with a Rf very close to those of triterpenoids, particularly triterpenic acid. Moreover, a study of its UV spectrum, with only one λ_{max} at 200 nm, as for the terpenoids studied, suggested that his molecule might also be a terpenoid. The correlation between MC11 and the calculated matrix effects in GC/MS^2 was not significant ($p > 0.05$, see Table S3 in the Supplementary material section).

This method does not provide the same separation efficiency between pesticides and triterpenic acids as the one of Oellig and Schwack, anyway it separates pesticides and triterpenic acids allowing at the same time the recovery of captan and dithianon, two pesticides widely used in French apple orchards. Used as a purification step before hyphenated HPTLC/GC/MS^2 ion trap techniques for example, it would help to minimize matrix effects occurring during pesticides analysis in highly concentrated QuEChERS extracts from apple samples.

4. Conclusion

The previously published GC–MS^2 method [1] was successfully extended to the identification of matrix analytes in QuEChERS extracts of fruit, flesh and peel, through HPTLC analysis of the major molecular markers representative of each type of matrix. Peel extracts are characterized by particularly high levels of oleanolic and ursolic acids (10–25 mg kg^{-1}). There is a relevant relationship between negative matrix effects and the concentration of these acids. According to gas phase acid-base chemistry, the pesticides can react as proton acceptor and the triterpenic acids as proton donor. Boscalid and fludioxonil were the pesticides found to interact most with triterpenic acids. They are more basic than flonicamid, pirimicarb, chlorpyrifos and propargite, due to the nature of their nitrogen protonable site and the substituants involved in the stabilization of the charges of the protonated species. The higher concentrations of triterpenic acids in peel extracts suppress the signal, this effect being less marked for flonicamid, pirimicarb, chlorpyrifos and propargite.

The proposed HPTLC method involves the overlapping of pesticides combined with their separation from most of the matrix compounds, including oleanolic and ursolic acids in particular. Thus, the combination of this method with a TLC/MS Interface could result in a more efficient clean-up of QuEChERS extracts before GC analysis. This method could be transposed to purification on columns or by flash chromatography.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.12.056.

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Table S1

Analytical parameters for the characterization of matrix compounds and the quantification of terpenoids in QuEChERS extracts by HPTLC/UV-Visible

Sample	Volume (µL) used for characterization	Volume (µL) used to investigate the ME*/matrix compounds relationship	Volume (µL) used for terpenoid quantification
QuEChERS extracts FRUITVAR1, 2 & 3 (*n*=5)	10	1	4
QuEChERS extracts FLESHVAR1, 2 & 3 (*n*=5)	10	1	10
QuEChERS extracts PEELVAR1, QuEChERS extracts PEELVAR2 & 3 (*n*=5)	10	1	2 1
Pesticide mixture (0.2 mg L^{-1})	10	-	-
Oleanolic acid (1 g L^{-1})	5	1	0 – 1.5
Uvaol (1 g L^{-1})	5	1	0 – 1.5
Ursolic acid (1 g L^{-1})	5	-	-
Paraffins C27 and C29 (0.5 g L^{-1} each)	5	-	-
Primary fatty alcohols (0.125 g L^{-1} each)	5	-	-
Sugar mixture (1 g L^{-1} each)	5	-	-
Phlorizin (1 g L^{-1})	5	-	-

**ME = matrix effect*

Table S2

Matrix effect values evaluated without normalization of chromatographic peak area (from a previous study by Giacinti *et al*., 2016, [1])

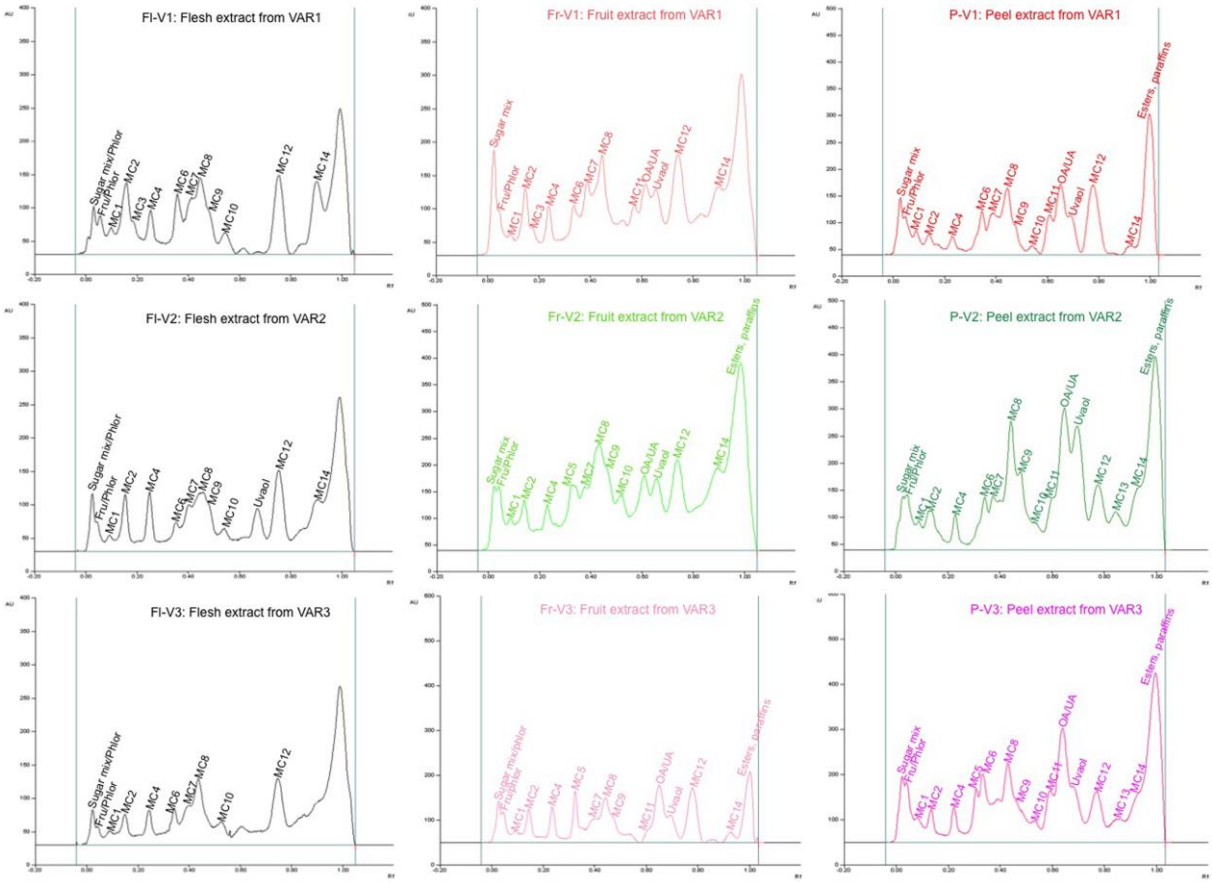
Active agent	FLESH VAR1	FLESH VAR2	FLESH VAR3	FRUIT VAR1	FRUIT VAR2	FRUIT VAR3	PEEL VAR1	PEEL VAR2	PEEL VAR3
Flonicamid	168	68	48	72	69	23	27	-73	-50
	136	64	54	79	81	38	-3		-53
Pirimicarb	98	25	5	20	17	-2	-6	-78	-49
	81	28	14	13	37	9	-16		-53
Propargite	35	7	-6	-25	-42	-57	-35	-91	-80
	36	-5	-3	-27	-43	-50	-63		-80
Chlorpyrifos	59	29	16	30	24	-8	-3	-78	-57
	66	24	23	34	37	9	-24		-56
Fludioxonil	315	214	176	208	222	177	135	-60	-52
	308	212	184	244	226	183	89		-47
Boscalid	418	246	183	202	219	146	96	-40	-71
	370	228	188	225	213	182	73		-53

Table S3

Pearson correlation coefficients between matrix effects and matrix compounds MCi levels (areas of peaks) measured in the nine studied apple extracts (The coefficients in bold are significant).

Variables	Flonicamid	Pirimicarb	Propargite	Chlorpyrifos-ethyl	Fludioxonil	Boscalid	Sugar mix/Phlor	Fru/Phlor	MC1	MC2	MC3	MC4	MC5	MC6	MC7	MC8	MC9	MC10	MC11	OA/UA	Uvaol	MC12	MC14	Etsers/paraffins
Flonicamid	**1**																							
Pirimicarb	**0.990******	**1**																						
Propargite	**0.913*****	**0.917*****	**1**																					
Chlorpyrifos-ethyl	**0.987******	**0.970******	**0.897*****	**1**																				
Fludioxonil	**0.977******	**0.960******	**0.866****	**0.988******	**1**																			
Boscalid	**0.987******	**0.978******	**0.921*****	**0.969******	**0.976******	**1**																		
Sugar mix/Phlor	-0.572	-0.591	**-0.714***	-0.579	-0.603	-0.647	**1**																	
Fru/Phlor	-0.549	-0.536	**-0.736***	-0.583	-0.597	-0.614	**0.772***	**1**																
MC1	-0.243	-0.224	-0.329	-0.297	-0.345	-0.334	**0.694***	**0.789***	**1**															
MC2	0.232	0.196	0.041	0.160	0.180	0.261	0.179	0.357	0.287	**1**														
MC3	0.651	0.613	0.582	0.573	0.551	0.619	-0.062	-0.299	0.022	0.301	**1**													
MC4	0.470	0.413	0.219	0.520	0.485	0.421	0.079	0.130	0.051	0.590	0.169	**1**												
MC5	0.127	0.138	-0.190	0.163	0.240	0.141	-0.073	0.216	-0.224	0.265	-0.285	0.478	**1**											
MC6	-0.025	-0.066	0.059	-0.104	-0.165	-0.081	0.321	0.232	0.611	0.180	0.503	-0.187	**-0.739***	**1**										
MC7	-0.307	-0.329	-0.383	-0.297	-0.375	-0.431	**0.857****	0.520	**0.667***	-0.085	0.187	0.076	-0.336	0.498	**1**									
MC8	-0.182	-0.291	-0.367	-0.175	-0.190	-0.219	0.507	0.514	0.367	0.590	0.108	0.508	0.053	0.418	0.368	**1**								
MC9	-0.489	-0.451	-0.512	-0.559	-0.536	-0.451	0.401	**0.718***	0.580	0.551	-0.401	-0.042	0.089	0.139	0.003	0.278	**1**							
MC10	0.337	0.355	0.402	0.325	0.264	0.336	-0.447	0.030	0.107	0.359	-0.140	0.391	0.048	0.042	-0.406	0.075	0.288	**1**						
MC11	-0.543	-0.562	-0.576	-0.544	-0.540	-0.610	**0.724***	0.457	0.566	-0.312	-0.020	-0.423	-0.350	0.495	**0.746***	0.235	0.078	-0.647	**1**					
OA/UA	**-0.834****	**-0.834****	**-0.954******	**-0.856****	**-0.833****	**-0.861****	**0.815****	**0.845****	0.526	0.090	-0.369	-0.217	0.111	0.158	0.510	0.431	0.577	-0.403	0.666	**1**				
Uvaol	-0.635	**-0.691***	**-0.720***	-0.647	-0.613	-0.605	**0.695***	0.616	0.387	0.503	-0.247	0.097	0.009	0.212	0.314	**0.708***	0.663	-0.212	0.386	**0.732***	**1**			
MC12	0.383	0.306	0.150	0.454	0.462	0.304	0.131	0.032	0.212	0.040	0.217	0.475	0.186	0.152	0.295	0.411	-0.354	-0.083	0.295	-0.118	-0.035	**1**		
MC14	0.657	0.610	0.436	0.630	0.605	0.622	0.039	0.100	0.244	**0.798****	0.537	**0.842****	0.227	0.183	0.062	0.479	0.079	0.419	-0.364	-0.299	0.079	0.429	**1**	
Esters/paraffins	**-0.790***	**-0.757***	**-0.854****	**-0.820****	**-0.828****	**-0.829****	**0.692***	**0.922*****	**0.669***	0.097	-0.519	-0.195	0.081	0.173	0.420	0.320	**0.738***	-0.036	0.508	**0.904*****	0.592	-0.213	-0.268	**1**

Legend: degrees of "statistical significance": *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001


Fl-V1: Flesh extract from VAR1
Sugar mix/Phlor
Fru/Phlor
MC1
MC2
MC3
MC4
MC6
MC7
MC8
MC9
MC10
MC12
MC14
Fr-V1: Fruit extract from VAR1
Sugar mix
Fru/Phlor
MC1
MC2
MC3
MC4
MC5
MC7
MC8
MC11
OA/UA
Uvaol
MC12
MC14
P-V1: Peel extract from VAR1
Esters, paraffins
Sugar mix
Fru/Phlor
MC1
MC2
MC4
MC6
MC7
MC8
MC9
MC10
MC11
OA/UA
Uvaol
MC12
MC14
Fl-V2: Flesh extract from VAR2
Sugar mix/Phlor
Fru/Phlor
MC1
MC2
MC4
MC6
MC7
MC8
MC9
MC10
Uvaol
MC12
MC14
Fr-V2: Fruit extract from VAR2
Esters, paraffins
Sugar mix
Fru/Phlor
MC1
MC2
MC4
MC5
MC7
MC8
MC9
MC10
OA/UA
Uvaol
MC12
MC14
P-V2: Peel extract from VAR2
Esters, paraffins
Sugar mix
Fru/Phlor
MC1
MC2
MC4
MC6
MC7
MC8
MC9
MC10
MC11
OA/UA
Uvaol
MC12
MC13
MC14
Fl-V3: Flesh extract from VAR3
Sugar mix/Phlor
Fru/Phlor
MC1
MC2
MC4
MC6
MC7
MC8
MC10
MC12
Fr-V3: Fruit extract from VAR3
Esters, paraffins
Sugar mix/phlor
Fru/Phlor
MC1
MC2
MC4
MC5
MC7
MC8
MC9
MC11
OA/UA
Uvaol
MC12
MC14
P-V3: Peel extract from VAR3
Esters, paraffins
Sugar mix
Fru/Phlor
MC1
MC2
MC4
MC6
MC8
MC9
MC10
MC11
OA/UA
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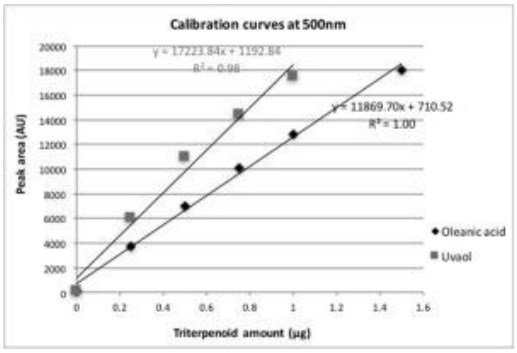
MC12
MC13
MC14



Matrix compound	Oleanic acid	Uvaol
Linear domain (µg)	0.0 – 1.5	0.0 – 1.0
LOD (mg kg^{-1} flesh matrix)	0.05	0.025
LOD (mg kg^{-1} fruit matrix)	0.13	0.06
LOD (mg kg^{-1} peel matrix)	0.25 – 0.50	0.13 – 0.25
LOQ (mg kg^{-1} flesh matrix)	0.10	0.05
LOQ (mg kg^{-1} fruit matrix)	0.26	0.13
LOQ (mg kg^{-1} peel matrix)	0.50 – 1.00	0.26 – 0.50

A- Peel extracts in acetonitrile (a, b, c) and purified extract (d):


a- VAR1

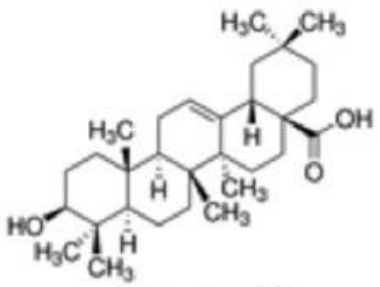

b- VAR2


c- VAR3

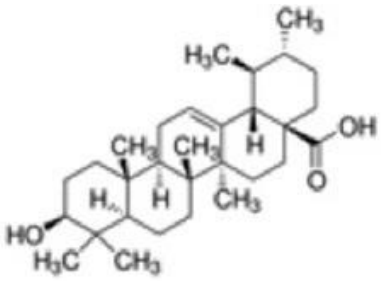

d- dSPE

B- Peel extracts from VAR1 in acetonitrile: non oxidized (1) and oxidized (2)


(1) (2)

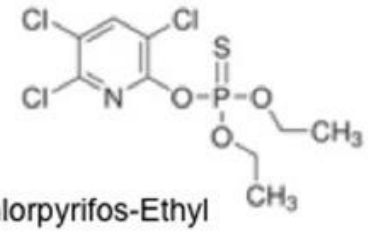


Oleanic acid

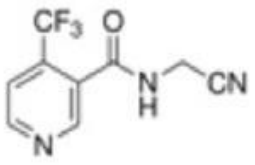


Ursolic acid

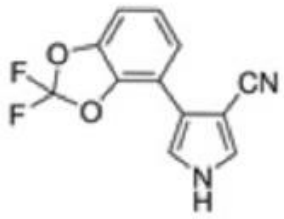
Boscalid



Chlorpyrifos-Ethyl

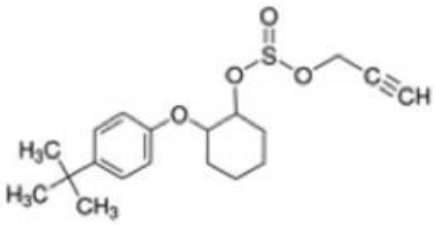


Flonicamid



Fludioxonil

Pirimicarb



Propargite