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Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric Determination of Quassin and Neoquassin in Fruits and Vegetables

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Quassia amara wood chips are used by organic farmers as a valid alternative to synthetic insecticides. The powder of *Q. amara* contains high levels of quassin, neoquassin and picrasinoside B. In this study we developed a liquid chromatography mass spectrometry method for the rapid and accurate quantification of the insecticide quassinoids on fruits and vegetables. Quassinoids were extracted from fruits and vegetables with acetonitrile and separated on a Zorbax Column Eclipse XDB C8 by isocratic elution with a mobile phase consisting of water and methanol with 0.1% of formic acid. Using a high-performance liquid chromatograph coupled with an electrospray ionization tandem mass spectrometer (HPLC/ESI-MS/MS), quassinoids were selectively and simultaneously detected monitoring the multiple reaction (MRM) transitions of proton adduct precursor ions: *m*/*z* 389.5 → 222.9 for quassin, 391.5 → 372.9 for neoquassin and 576.1 → 394.5 for picrasinoside B. For all quassinoids calibration was linear over a working range of 1 and 100 μg/kg with $r > 0.991$. Limit of determination (LOD) and limit of quantification (LOQ) for both quassinoids were 0.5 and 1 μg/kg respectively while for picrasinoside B they were 5 and 10 μg/kg. Quassinoid recoveries ranged from 85.3% to 105.3% with coefficients of variation between 2.5% and 12.8% for fruit and vegetables. The presence of interfering compounds in the fruit and vegetable extract was evaluated and found to be minimal. Due to the linear behavior it was concluded that the multiple reaction transitions of precursor ions can be used for analytical purposes, i.e. for identification and quantification of quassin, neoquassin, and picrasinoside B in fruit and vegetable extracts at trace levels.

KEYWORDS: HPLC/ESI-MS/MS; LC-Chip/QTOF; quassin; neoquassin; picrasinoside B; bitterwood; tomato; pepper; eggplant; apple; pear; grape

INTRODUCTION

Extracts from the wood of *Quassia amara* L. or *Picrasma excelsa* L. (Simaroubaceae), native to Central America, Brazil, and Surinam, are often referred to as "quassin" or "bitterwood" (*1*, *2*). Commercial extracts of *Quassia* are known to contain a mixture of the bitter principles named quassinoids. Among these, the most abundant are quassin and neoquassin (*3*) belonging to the terpenes class; moreover quassinoid glycosides are also present (*4*) (**Figure 1**).

Several studies reported that quassinoids have an insecticidal activity acting as antifeedant and insect-growth regulator (IGR) (*5*−*8*) as well as nematocidal (*9*). Evans et al. (*10*, *11*) suggested that inhibition of the enzyme tyrosinase, that plays an important role in the cuticle sclerotization of insects, explains the insecticidal activity of quassinoids. Cuendet and Liao (*12*, *13*) identified the inhibition of protein synthesis as a probable mechanism of action. In plant protection practice an aqueous extract is prepared from *Quassia* chips at the site of application and applied at the rate of 18 g/ha. These extracts roughly contain 0.2% of quassin and neoquassin, substances toxic to sucking insects (*14*). Quassia is not inserted in the Annex I of the European Directive 91/414/EEC, but it is provided with a default maximum residue level (MRL) of 0.01 mg/kg.

To date, very little is known about residue level of organic insecticides in foods because of the lack of easy, rapid, inexpensive and rugged analytical methods. Taylor et al. reported an ultra-performance liquid chromatography time-of-flight mass spectrometry (UPLC−TOFMS) multiresidue determination of the sum of quassin and neoquassin in strawberry (*15*). Modern residue monitoring programs, however, are expected to be responsive in the latest development in agriculture and new legislation. For this reason we have developed a fast LC−MS/MS analytical procedure for the identification and unambiguous quantification of quassin, neoquassin, and picrasinoside B in fruits and vegetables.

MATERIALS AND METHODS

Chemicals. Acetonitrile and methanol were of HPLC grade (Baker, Milan, Italy), and formic acid 99% and chloroform were purchased from

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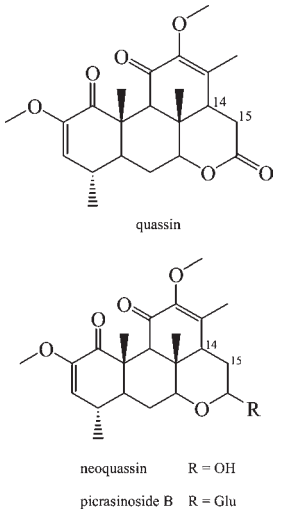

Figure 1. Chemical structures of quassin, neoquassin, and picrasinoside B extracted from powder wood of *Quassia amara*.

Sigma Aldrich (Milan, Italy). Water was distilled and filtered through a Milli-Q apparatus (Millipore, Milan, Italy) before use. The bark powder of *Q. amara* was kindly provided by Cerrus (Varese, Italy). Standards of quassin, neoquassin, and picrasinoside B were isolated from the methanolic extract of the powder of *Q. amara* with a purity greater than 95% using a semipreparative thin layer chromatography method. Thin-layer chromatography (TLC) aluminum sheets, 20 × 20 cm silica gel 60 F254, were purchased from Merck (Milan, Italy). TLC plates were developed with a mixture of chloroform−methanol (9:1 v/v); for neoquassin, quassin and picrasinoside B the retention factors (Rf) at 254 nm were 0.10, 0.46, 0.81 respectively.

Apparatus and Chromatography. *HPLC−MS/MS Analysis.* A Varian tandem mass spectrometer (Palo Alto, CA) consisting of a ProStar 410 autosampler, two ProStar 210 pumps, and a 1200 L triple quadrupole mass spectrometer equipped with an electrospray ionization source was used. Varian MS workstation version 6.9 software was used for data acquisition and processing. The column used was a 150 mm, 4.6 mm i.d., 5 μm, Zorbax Eclipse C8 from Agilent (Milford, MA). The mobile phase consisted of (A) methanol and (B) bidistilled water both containing 0.1% formic acid. The elution was with methanol−water (50:50, v/v) for 20 min. The mobile phase, previously degassed with high-purity helium, was pumped at a flow rate of 0.4 mL/min and the injection volume was 10 μL. The electrospray ionization source mass spectrometer was operated in the positive ion mode. The electrospray capillary potential was set to 64 V while the shield was at 525 V. Nitrogen at 50 mTorr was used as a drying gas for solvent evaporation. The atmospheric pressure ionization (API) housing and drying gas temperatures were kept at 54 and 375 °C respectively. The scan time was 1 s, and the detector multiplier voltage was set to 1450 V, with an isolation width of *m/z* 1.2 for the quadrupole 1 and *m/z* 2.0 for the quadrupole 3. Proton adducts of the parent compounds quassin, neoquassin and picrasinoside B were subjected to collision-induced dissociation using argon at 3.80 mTorr in multiple reaction-monitoring (MRM) mode. The atmospheric pressure chemical ionization (APCI) was operated in the positive mode. The capillary potential was set to 65 V, the APCI torch at 450 °C, and the shield at 750 V. Nitrogen at 48 mTorr was set at 400 °C. Full scan spectra were obtained in the ranges of 200−800 amu for quassin, neoquassin, and picrasinoside B, scan time of 0.75 amu, scan width of 0.70 amu, and detector at 1450 V. For APCI the atmospheric pressure ionization (API) housing was kept at 50 °C.

HPLC−MS Q-TOF Analysis. Quassinoids were confirmed by reverse-phase HPLC on an Agilent 1200 series HPLC system fitted with microchip technology using a Zorbax 300 SB-C18 (5 μm, 43 mm × 75 μm) (Agilent, Santa Clara, CA). The HPLC conditions were as follows: flow rate, 0.4 μL/min; solvent A, 0.1% formic acid in water; solvent B, acetonitrile (A:B; 40:60 v/v). Samples (1 μL) were then analyzed by ESI in positive mode using an Agilent 6520 time of flight (TOF) MS. Mass spectrometric data were acquired in the range, *m/z* 100−1000 with an acquisition rate of 1.35 spectra/s, averaging 10,000 transients. The source parameters were adjusted as follows: drying gas temperature 250 °C, drying gas flow rate 5 L/min, nebulizer pressure 45 psi, and fragmentor voltage 150 V. Data acquisition and processing were done using Agilent MassHunter Workstation Acquisition software v. B.02.00.

Standard and Working Solutions. Stock standard solutions of quassin, neoquassin, and picrasinoside (1000 mg/L) were prepared in water by weighing 0.01 g of the pure analyte into a 10 mL volumetric flask and diluting to volume. An intermediary mixed standard solution was prepared daily by diluting with water. Standard working solutions of quassinoids were prepared by diluting the mixed standard solution with the extract obtained from the untreated matrix of fruits and vegetables. Levels of calibration for all compounds, including a blank, were 1, 5, 10, 20, 50, and 100 μg/kg respectively. All standard solutions were stored in the dark at −20 °C until usage.

Efficiency. *A. Standard Curves and Linearity.* A six-point standard curve for each quassinoid was prepared. Standard solutions were prepared in triplicate containing all three quassinoids at 1, 5, 10, 20, 50, and 100 μg/kg. Calibration curves were created by plotting the concentration of each compound against the standard peak area of the monitored transition. Simple linear regression analysis was performed to calculate the slope and intercept. The correlation coefficient (*r*) for each quassinoid was also determined.

B. Repeatability. To evaluate precision, repeatability of both the instrument and the analytical procedure proposed was determined. Intermediate precision was calculated performing six injections of the same standard at 10, 20, 50, and 100 μg/kg for six consecutive days.

Extraction of Quassin and Neoquassin from Fruits and Vegetables. Fresh and uncontaminated fruits and vegetables were purchased at local markets in Cagliari, Italy. Samples were analyzed, unwashed, and in a raw state. Samples of fruits or vegetables were placed in a blender/cutter (Malavasi, Bologna, Italy) and chopped for 30 s.

A portion (5 g) of well-homogenized chopped tomatoes, peppers, eggplants, peaches, apples, and grape was weighed in a 40 mL screw-capped glass tube, and 2 g of sodium chloride and 20 mL of acetonitrile were added. The tube was agitated for 15 min in a rotary shaker at 9 rpm (FALC Instrumentals, Bergamo, Italy) at room temperature, and 1 mL of the mixture was evaporate to dryness under a gentle nitrogen stream. The residue was dissolved with 200 μL of the mobile phase previously reported and submitted to the chromatographic analysis in the MRM mode.

Recovery Assays. A 50 μL aliquot of pesticide solution at the desired standard concentration was added to each 5 g sample of untreated tomatoes, peppers, eggplants, peaches, apples, and grape. The fortification levels were 1, 5, 10, 50, and 100 μg/kg. The samples were allowed to settle for 30 min prior to the extraction. They were later processed according to the above extraction procedure. Four replicates for each level were analyzed by HPLC/ESI-MS/MS analysis.

RESULTS AND DISCUSSION

Multiple Reaction Transitions. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were tested for the determination of quassinoid levels both in positive and in negative mode respectively. ESI in the negative mode and APCI in the negative and positive mode did not give any signal when infusion took place at the rate of 0.6 mL/h of standard solutions of quassin, neoquassin, and picrasinoside B at 1000 μg/L. Thus, ESI in the positive mode was chosen for the identification, quantification, and confirmation of quassinoids in fruit and vegetable extracts for its most intense response.

A full scan spectrum of quassinoids was acquired with a scan range of 150−700 amu, scan time 0.75 amu, scan width 0.70 amu, detector at 1200 V (**Figure 2**). The electrospray capillary potential as well as shield and needle voltage was optimized for each compound. Quassin and neoquassin showed similar ESI-MS behavior and fragmentation patterns with proton and sodium adducts (**Table 1**).The full scan spectrum for quassin shows the most intense ions at *m/z* 389.5 corresponding to the molecular ion $[M + H]^+$ and the ion at *m/z* 411.5 corresponding to the sodium adduct $[M + Na]^+$. Neoquassin shows an intense ion at *m/z* 391.5 corresponding to the molecular ion and two signals at *m/z* 413.5

and m/z 373.6 corresponding to the $[M + Na]^+$ adduct and to the ion $[M - H_2O]^+$ respectively. On the other hand, picrasinoside B showed the sodium adduct ion at m/z 575.6, and a fragment ion at m/z 391.5 $[M - 162]^+$ corresponding to the loss of a glucose moiety. The presence of the fragment ion at m/z 391.5 suggested that picrasinoside B originated from neoquassin. This was confirmed by cochromatography of the standard of neoquassin with the products deriving from the glycosidic cleavage of a solution picrasinoside B treated with HCl (0.1 M) at 60 °C for 30 min. Quassinoids were also identified and confirmed by a high resolution mass spectrometer LC-Chip/QTOF. High resolution spectra showed the fragments expected for all the quassinoids of interest with an error below 4.1 ppm (**Table 2**).

The formation of a protonated adducts in the source was utilized to provide increased selectivity for all quassinoids except for picrasinoside B where a sodium adduct was observed. Scanning for the protonated adducts ion as the precursor ion, mass fragments were produced by collision-induced dissociation (CID) using argon at 3.80 mTorr. The collision energy was optimized to achieve the highest sensitivity. The CID resulted in the formation of ions unique to each compound. As seen in **Table 3**, a product ion scan of the individual compounds produces unique mass fragments for each quassinoid showing a common feature, losing one or two water molecules. The most intense transition was chosen to provide selective detection of quassinoids in the fruit and vegetable extracts without the need of a further purification step. Selected reaction monitoring of the precursor−product ion transitions were the following: m/z 389.5 → m/z 222.9 for quassin, m/z 391.5 → m/z 372.9 for neoquassin, m/z 576.1 → m/z 394.5 for picrasinoside B. For the HPLC/ESI-MS/MS quantitation of quassinoids an external standard method was used. Peak areas obtained from the MRM of quassinoids standards were used for the quantitative determination.

Method Development. The chromatographic separation of quassinoids was achieved with an isocratic elution in the reverse phase mode, and the retention times of quassinoids were 6.98, 7.43, 10.35 min for picrasinoside B, quassin, and neoquassin, respectively. Using a fruit and vegetable extract, which is usually a complex mixture, the selectivity of the MRM transitions for all quassinoids was determined. The result of the chromatographic separation indicates the presence of interfering extract components; however the relative abundance of these compounds is minimal by comparison of the total peak area (**Figure 3**).

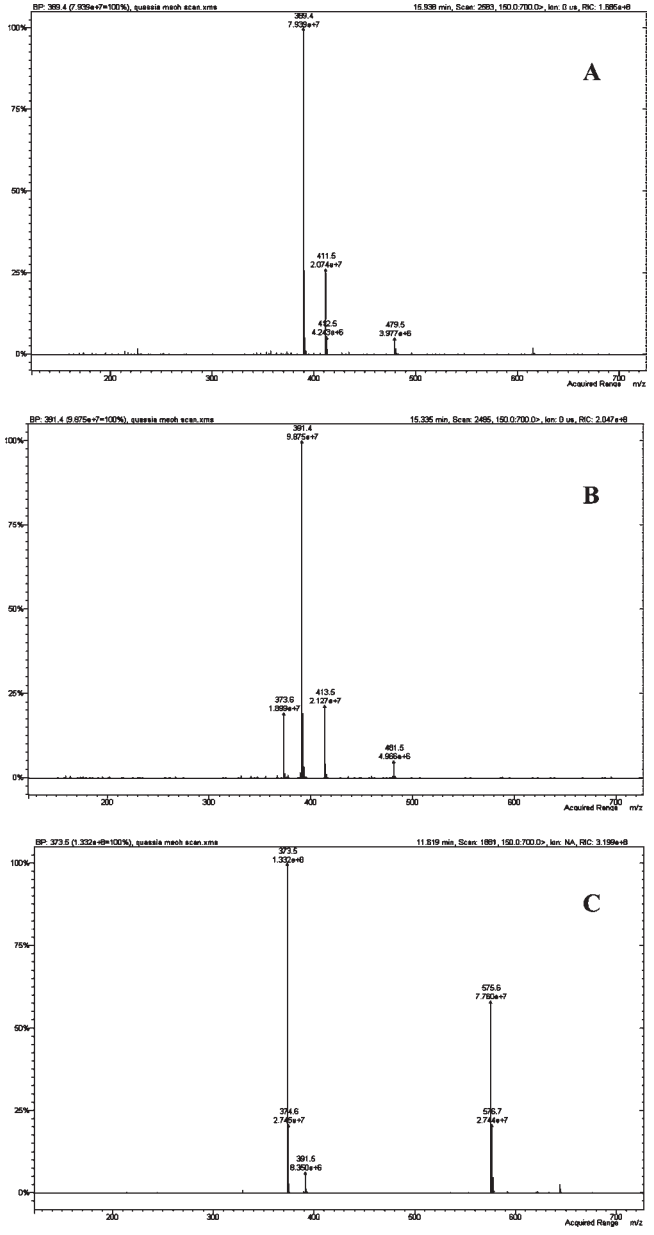

Figure 2. Positive ion electrospray full scan mass spectra of quassin (**A**), neoquassin (**B**), and picrasinoside B (**C**).

Table 2. LC-Chip/QTOF Characteristics of Quassinoids Proton Adducts of Interest

compound	formula	theor mass	measd mass	error (ppm)
quassin	$C_{22}H_{29}O_6$	389.1959	389.1966	1.8
neoquassin	$C_{22}H_{31}O_6$	391.2115	391.2101	3.6
picrasinoside B	$C_{28}H_{41}O_{11}$	553.2722	553.2699	4.1

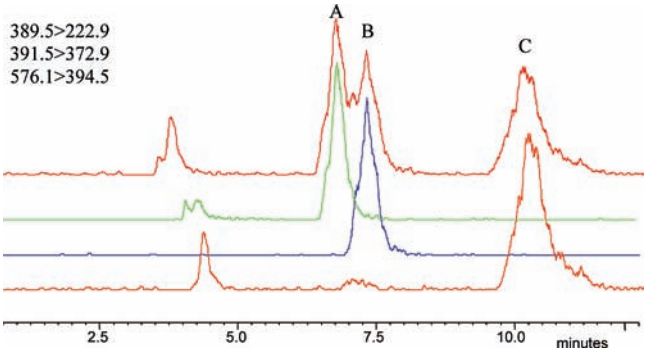

Figure 3. Ion chromatograms of picrasinoside B (**A**), quassin (**B**), and neoquassin (**C**) in a pear extract fortified at 1 μg/kg for quassin and neoquassin and 10 μg/kg for picrasinoside B.

Table 1. HPLC/ESI-MS Characteristics of Quassinoids of Interest

compound	molecular wt	LC/MS (ESI) m/z (% relative abundance)
quassin	388	389.5 $[M + H]^+$ (100); 411.5 $[M + Na]^+$ (25); 479.5 unknown (10)
neoquassin	390	391.5 $[M + H]^+$ (100); 413.5 $[M + Na]^+$ (24); 373.6 $[M - H_2O]^+$ (20); 481.5 unknown (6)
picrasinoside B	552	373.5 $[M - \text{glucose} - H_2O]^+$ (100); 575.6 $[M + Na]^+$ (70); 391.5 $[M - \text{glucose}]^+$ (6)

Table 3. Analyte HPLC/ESI-MS/MS Characteristics, Transitions, and Instrument Conditions for the Analysis of Quassinoids in Food

compound	$t_{R(LCMS)}$ (min)	log *P*	transition (*m*/*z*) parent	transition (*m*/*z*) product	CE (V)
quassin	7.43	2.4	389.5 $[M + H]^+$	222.9 $[M - C_{10}H_{14}O_2]^+$	−20
neoquassin	10.35	2.2	391.5 $[M + H]^+$	372.9 $[M - H_2O]^+$	−15
picrasinoside B	6.98	0.7	576.1 $[M + Na]^+$	394.5 $[M + Na - Glu]^+$	−38

Table 4. Matrix Effect Calculated as Slope Ratios of the Calibration Curves for Eggplant and Apple

compound	$slope_{solv}/slope_{egg}$	$slope_{solv}/slope_{app}$
quassin	1.10	1.14
neoquassin	1.09	1.02
picrasinoside B	1.36	1.38

Table 5. Recoveries of Quassinoids on Fruits and Vegetables ($n = 4$)

	fortification levels (μg/kg)	quassin (% ± RSD)	neoquassin (% ± RSD)
grape	1	89.6 ± 4.3	103.6 ± 8.9
	5	92.7 ± 3.5	94.8 ± 3.7
	10	85.3 ± 2.9	90.2 ± 5.8
	50	90.4 ± 5.7	107.8 ± 9.6
	100	93.2 ± 8.3	88.7 ± 4.5
apple	1	86.6 ± 3.3	92.6 ± 5.9
	5	98.6 ± 10.9	97.1 ± 4.8
	10	105.2 ± 12.4	90.5 ± 6.2
	50	87.9 ± 5.3	100.8 ± 7.7
	100	96.1 ± 3.8	98.4 ± 9.1
peach	1	93.3 ± 7.9	96.0 ± 5.8
	5	90.8 ± 11.6	90.8 ± 7.5
	10	94.2 ± 8.1	104.2 ± 9.6
	50	99.3 ± 7.6	102.7 ± 6.9
	100	87.5 ± 4.7	93.6 ± 8.1
pepper	1	91.4 ± 6.3	90.9 ± 7.3
	5	104.7 ± 11.4	97.3 ± 4.6
	10	95.8 ± 3.6	88.3 ± 3.1
	50	89.9 ± 4.1	100.5 ± 11.4
	100	90.8 ± 5.9	98.7 ± 6.8
tomato	1	91.3 ± 4.9	99.6 ± 8.3
	5	97.5 ± 4.2	92.0 ± 4.4
	10	92.4 ± 9.3	105.3 ± 7.9
	50	88.7 ± 5.1	98.4 ± 6.2
	100	101.7 ± 9.8	95.0 ± 3.8
eggplant	1	87.8 ± 4.3	91.7 ± 6.1
	5	97.0 ± 6.4	97.5 ± 7.3
	10	91.8 ± 7.1	91.4 ± 8.2
	50	95.9 ± 7.8	102.6 ± 12.8
	100	89.7 ± 4.3	95.9 ± 2.5

The calibration of quassinoids was linear over a working range of 1 and 100 μg/kg with $r > 0.991$. For efficiency experiment standard solutions of quassinoids with concentration ranging from 1 to 100 μg/kg were injected for the analysis in HPLC/ESI-MS/MS in a MRM experiment. For the precision experiment under conditions of repeatability the highest and the lowest variation coefficient of variation were 9.84 and 3.20% for fruits and vegetables, while for the intraday experiment the highest and the lowest variation coefficient of variation were 11.30 and 4.69% for fruits and vegetables.

For the establishment of limit of quantification and detection (LOQ, LOD), 1000 μg/kg standard solutions of quassinoids was gradually diluted with the mobile phase. Each individual standard was injected three times. LOD, calculated at a signal/noise ratio of 3, for both quassin and neoquassin, was 0.5 μg/kg, and 5 μg/kg for picrasinoside B, while LOQ at a signal/noise ratio of 10 was 1 μg/kg for quassin and neoquassin, and 10 μg/kg for picrasinoside B, respectively.

Recovery Studies. Although the fruit and vegetable extracts were complex mixtures, no interfering components were detected in the chromatographic separation. The matrix effect (**Table 4**) for the two extracts was calculated as slope ratios of the calibration curve prepared in solvent with the slope of the calibration curve prepared with the corresponding extract. We observed the highest matrix positive effect for picrasinoside, while for quassin and neoquassin the matrix effect was minimal. Quassinoid recoveries ranged from 85.3% to 105.3% with coefficients of variation between 2.5% and 12.8% for fruits and vegetables (**Table 5**).

We report for the first time a liquid chromatography−tandem mass spectrometry method with electrospray ionization in the positive mode for the simultaneous identification and quantitation of residues of quassin and neoquassin insecticides in fruit and vegetables. The possibility of using the protonated adduct of quassinoids for the quantification at residue levels has been investigated. The adduct fragmentation was stable and intense. The proposed LC−MS/MS analytical method for the determination of quassinoids in fruit and vegetables has been demonstrated to be adequate, fast, precise, accurate and robust and can be easily used by regulatory laboratory for the determination of residue levels quassinoids in fruit and vegetables.

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