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Determination of diquat and paraquat in olive oil by ion-pair liquid chromatography–electrospray ionization mass spectrometry (MRM)

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Abstract

For the first time a method for determination of herbicides diquat (DQ) and paraquat (PQ) in olive oil was developed utilising liquid chromatography–electrospray ionization mass spectrometry (MRM). *n*-Hexane/10 mM HFBA aqueous solution partitioning was used as the extraction method. Separation was carried out in an Xterra C8 column (100 × 21 mm, 3 μm), using the gradient mode. Solvent A was a HFBA aqueous solution (5 mM, pH 2) and solvent B acetonitrile/methanol 75/25 (v/v). Peaks used for quantification were m/z = 157 (diquat) and m/z = 158 (paraquat). Detection limit found for both diquat and paraquat was 4 μg kg^{-1}. The method can also be applied for determination of chlormequat (CQ, quantification peak m/z = 58), the detection limit being 0.3 μg kg^{-1}. Such limits are clearly lower than the MCLs commonly applied to olive oil as reference criteria (5 times MCLs in olives). Good reproducibilities (day to day and run to run) were obtained.

The method allowed us to check that even though DQ and PQ residues had been detected in soils from olive grove plantations, they did not pass to olive oil.

Keywords: LC-mass spectrometry; Olive oil; Pesticides; Quaternary ammonium compounds; 1,1′-ethylene-2,2′-bipyridinium ion (diquat); 1,1′-dimethyl-4,4′-bipiridynium ion (paraquat); 2-chloroethylmethylammonium ion (chlormequat)

1. Introduction

Herbicides and crop protection products in general are essential in modern agriculture. However, their use means a potential risk to humans, animals and the environment. Therefore, there has been a great interest in the presence of these compounds in food, drinking water and soils. One particularly difficult type of herbicide is the group of quaternary ammonium salts, also known as quats.

The quaternary ammonium compounds diquat (1,1′-ethylene-2,2′-bipyridinium ion, DQ), paraquat (1,1′-dimethyl-4,4′-bipyridinium ion, PQ) and chlormequat (2-chloroethyltrimethylammonium ion, CQ) are commonly used herbicides. Although available as bromide, chloride and methylsulphonate salts, commercial herbicide formulations contain almost exclusively diquat dibromide (CAS Registry number 85-00-7), paraquat dichloride (CAS Registry number 1910-42-5) and chlormequat chloride (CAS Registry number 999-81-5) as the active ingredients (Meister & Sine, 1996).

Spain produces around 34% of olive oil in the world, its olive groves being mainly concentrated in Andalusia, a region highly dependant on agriculture. Therefore, the

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council of Andalusia is making a continuous effort to preserve high quality characteristics of olive oil.

DQ and PQ have been extensively used as herbicides in olive crops. They are toxic to algae, fish, and other aquatic organisms such as crayfish and insects (USEPA, 1989). Acute oral LD_{50} (rats) for diquat (400 mg kg^{-1}) and paraquat (155 mg kg^{-1}) are relatively low (Verschueren, 1983). The adverse health effects of acute and chronic exposure to humans are well documented (Murphy, 1986). DQ produces lung damage although it is not concentrated selectively as PQ. However, diquat has severe toxic effects on the central nervous system that are not typical of paraquat poisoning.

The United States Environmental Protection Agency (EPA) maximum contaminant limits (MCL) for diquat and paraquat in drinking water are 20 and 3 μg L^{-1}, respectively. The European Union has not regulated the levels of these compounds in drinking water and continue to apply the values of 0.1 μg L^{-1} for individual pesticides and 0.5 μg L^{-1} for total pesticides (Nuñez, Moyano, & Galcerán, 2004)

There is currently no legislation on the MCL values in oils. However, values of 5 times the admitted limits in olives are commonly accepted. Using such a thumb-rule, maximum contamination levels of 0.25 mg kg^{-1} (DQ and PQ) and 0.5 mg kg^{-1} (CQ) would be applied.

The cationic character of quats makes their determination difficult. Capillary electrophoresis (CE) (Carneiro, Puignou, & Galcerán, 2000; Vinner, Stievenart, Humbert, Mathieu, & Lhermitte, 2001) and ion-pair high–performance liquid chromatography (HPLC) using UV detection (Carneiro, Puignou, & Galcerán, 1994) are the methods of choice for ionic species, although the use of selective electrodes (Ruhling, Schafer, & Ternes, 1999; Saad, Ariffin, & Saleh, 1998) have also been reported. An inherent disadvantage of these methods is the lack of analytical specificity, which may result in identification and quantification difficulties, especially in complex matrices. The US Environmental Protection Agency (EPA) recommends methods where identification is confirmed by mass spectrometry (MS).

EPA method 549.2 employs reversed phase/ion-pair extraction utilizing C8 SPE cartridges or disks to isolate diquat and paraquat from drinking waters followed by ion-pair LC with ultraviolet (UV) or photodiode array (PDA) detection (Hodgenson, Bashe, Eichelberger, Munch, & Jashe, 1997). Marr and King (1997) have developed an LC/(IonSpray) MS/MS method for diquat and paraquat in water that requires no sample concentration. With no concentration factor detection limits were 1 and 5 μg L^{-1} for diquat and paraquat, respectively.

Different techniques of mass spectrometry coupled to either capillary electrophoresis (Lazar & Lee, 1999; Nuñez, Moyano, & Galcerán, 2002) or HPLC (Castro, Moyano, & Galcerán, 1999; Taguchi, Jenkins, Crozier, & Wang, 1998) have been described for determination of DQ and PQ in waters, the former being the most appropriate one. The US Food and Drug Administration recommend the use of mass spectrometry utilizing three ions to confirm the presence of the compound (Makovi & Mahon, 1999). Multiple reaction monitoring (MRM) performed on triple quadrupole instruments (Castro, Moyano, & Galcerán, 2001a; Evans, Startin, Goodall, & Keely, 2001) has become the preferred quantitative tool. Moreover, the ion or ions to be used for quantification may be selected either before or after acquisition since a full product spectrum is obtained and they can be changed without the need of further analysis. Furthermore, the full-scan product spectra obtained can be used to rule out false positives.

In developing a LC-(ESI)MS method for diquat and paraquat, chromatographic conditions from existing LC-UV methods cannot be directly adapted as they typically employ non-volatile buffers or non-volatile ion-pair reagents. In the case of LC-(ESI)MS methods, reagents that form volatile ionic pairs (such as the heptafluorobutyric (HFBA), pentafluoropropionic (PFPA) or trifluoroacetic (TFA) acids) are required (Carneiro et al., 1994; Castro et al., 1999).

In the present paper, a fast, sensitive and selective method for determination of DQ and PQ in oils (especially olive oil) is described. Chlormequat is not used in olive trees as an herbicide. However, it has been included in the present study due to the possibility of contamination by nearby plantations. In principle, considering the extreme lipophobicity of the bipyridyl herbicides, it is quite unlikely to find such residues in olive oil. Nevertheless, they could appear at ppb levels. This is particularly critical in the case of organic oils which are, by definition, supposed not to contain pesticides. Therefore, we must ensure that such oils fulfil the legislation. This work must be seen in the context of a project on improvement of the quality of olive oil carried out in our department and supported by Junta de Andalucia.

2. Materials and methods

2.1. Chemicals

Diquat dibromide, paraquat dichloride and chlormequat chloride were provided by Riedel–de Haën (Seelze, Germany). Acetonitrile and methanol (LC-MS purity) together with heptafluorobutyric acid (HFBA) and *n*-hexane were purchased from Sigma–Aldrich (Steinheim, Germany). Formic acid was provided by Merck (Darmstadt, Germany), trifluoroacetic acid (TFAA) and pentafluoropropionic acid (PFPA) from Sigma–Aldrich (Milwaukee, USA) and ammonium hydroxide solution together with heptafluorobutyric

acid (HFBA) from Sigma–Aldrich (Steinheim, Germany). Milli-Q purified water was used throughout this study.

2.2. Sample extraction

Due to the high polarity of the studied herbicides, an *n*-hexane/10 mM HFBA water solution partition was selected for extraction. Moreover, polystyrene test tubes were used throughout the present study in order to avoid binding of the quats to the surfaces of glasswares.

The extraction procedure was as follows: 2 g of oil are weighed in a 15 mL polystyrene test tube and dissolved in 2 mL of *n*-hexane. Then, 2 mL (1 + 1) of a 10 mM aqueous solution of HFBA are added. The system is shaken for 30 min, centrifuged and put into the fridge at 4 °C for, at least, 2 h. Finally, the lower aqueous phase is taken for direct analysis.

For analyses of residues in soils, 100 mL of a MeOH:-H_2O (80:20 v/v) mixture were added to 25 g of soil sample. The mixture was shaken for 30 min and centrifuged, sampling from the liquid phase for direct analysis by HPLC-MS/MS.

2.3. Standards

Stock standard solutions of quats, 200 mg L^{-1} were prepared in water. Working solutions were prepared ($n = 3$) by diluting the stock solutions in matrix extract at 5 different concentrations in the range 0.1–5 mg kg^{-1} for HPLC-DAD and 8 concentrations in the range 0.1–700 μg kg^{-1} for HPLC-MS/MS experiments. All solutions were stored in polystyrene vials in order to prevent adsorption.

Standards in the matrix extract are stable at least for 10 days or 1 month depending on them being kept at room temperature or 4 °C, respectively.

2.4. Fortifications

In recovery studies, 2 g pesticide-free olive oil sample was fortified at 5 times the LOQ value for each analyte. The mixture was shaken for 30 min and then procedure described in sample extraction section was applied.

2.5. HPLC-DAD conditions

Optimization of chromatographic separation was performed on a Waters 2695 liquid chromatograph (Milford, MA, USA) equipped with a quaternary solvent delivery system, an autosampler and a Waters 2996 DAD detector ($\lambda = 310$ and 258 nm for DQ and PQ, respectively). Five different chromatographic columns were tested: Restek Allure Acidix (100 × 2.1 mm, 3 μm), Tracer Kromasil C8 (100 × 2.1 mm, 5 m), Tracer Excel C8 (100 × 2.1 mm, 5 μm), Tracer Kromasil C18 (150 × 2.1 mm, 5 μm) (Teknokroma, Sant Cugat del Vallés, España) and Waters Xterra C8 (150 × 2.1 mm, 3μm) (Milford, MA, USA). Moreover, three different ion-pair reagents (HFBA, PFPA and TFAA) were tested. Gradient elution was used for optimal separation of quats. Solvent A was a HFBA aqueous solution (5 mM, pH 2) and solvent B acetonitrile/methanol 75/25 (v/v). Pure solvent A was used for 3 min; then, its concentration was changed from 100% to 88% in 9 min and then to 40% in 2 min, keeping these conditions 10 min. Finally, the program is switched to 100% A in 5 min and kept for at least 10 min before starting a new analysis.

Total flow: 0.20 mL min^{-1}; column temperature: 40 °C; injection volume: 10 μL.

2.6. HPLC-MS/MS conditions

HPLC conditions were the same as described above but injecting only 5 μL. MS analyses were performed on a triple-quadrupole LC-MS equipment (Varian LC-MS 1200L, Torrance, CA, USA).

Optimization of MS/MS conditions was performed for each analyte, infusing solutions of ca. 5 mg/L of such an analyte in MeOH/H_2O 50/50 (v/v) into the electrospray source. Therefore, for each analyte, the values of the voltages applied to the sampling cone, focusing lenses, collision cell and quadrupoles were optimized in the MRM (multiple reaction monitoring) mode by continuous infusion in order to achieve the highest sensitivity as possible.

General MS/MS conditions were as follows: the nebulizing and auxiliary gas pressures (N_2) were set at 50 psi and the curtain gas pressure (N_2) at 25 psi. Source and desolvation temperature were 45 and 275 °C, respectively. Capillary voltage (positive ionization mode) was 5.0 kV and shield voltage 0.4 kV. Electron multiplier was set at 1.8 kV. Scan time: 1.8 scan/s. Dwell time: 0.01 s. Argon was used as collision gas at 3.9×10^{-5} psi.

3. Results and discussion

3.1. Optimisation of HPLC separation in HPLC-DAD equipment

Selected wavelengths for detection of DQ and PQ were 310 and 258 nm, respectively, corresponding to the maxima in the UV spectra. Different HPLC-DAD tests were performed in order to establish the optimum quats separation conditions. As described above, gradient elution between solvent A (5 mM HFBA aqueous solution, pH 2) and solvent B (acetonitrile/methanol 75/25 (v/v)) was used. Special emphasis was laid on the separation of analytes and other products present in

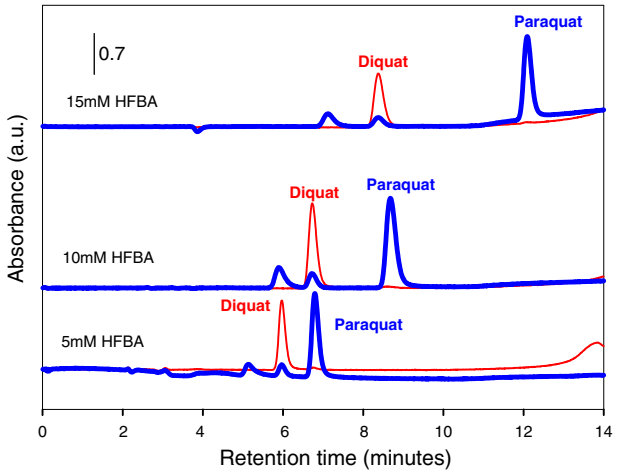

Fig. 1. HPLC-DAD chromatography. Influence of HFBA concentration on chromatographic resolution of diquat and paraquat (5 mg kg^{-1} each one).

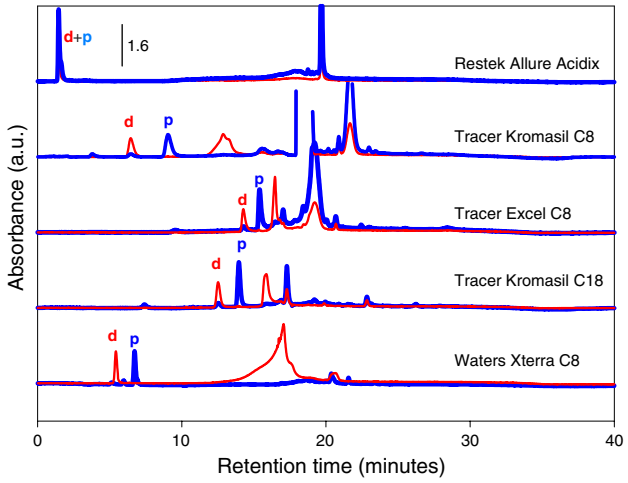

Fig. 2. HPLC-DAD experiments: Influence of column type on chomatographic resolution of diquat (d) and paraquat (p) (5 mg kg^{-1} each one).

the matrix, since the existence of interferences in electrospray ionization due to matrix effect is described in the literature (Ardrey, 2003). Initially, a Waters Xterra C8 (150 × 2.1 mm, 3 μm) column, was used. Solvent B composition (acetonitrile: methanol 75:25 v/v) was found to be the one providing an optimum sensitivity in positive electrospray for the selected analytes. Addition of methanol reduces the formation of adducts. Concentration of ion-pair reagents was chosen as low as possible, in order to avoid the contamination of the mass spectrometer probe and source. In this sense, Fig. 1 allows us to show, taking the example of HFBA as the ion-pair reagent, that 5 mM HFBA is good enough for separation of diquat and paraquat. Under the same working conditions, separation of both quats was not possible with TFAA or PFPA. Therefore, 5 mM HFBA was chosen. However, a more concentrated (10 mM) HFBA aqueous solution was used in sample extraction procedure, since this way recoveries obtained were significantly higher (in all cases over 92%, the standard deviation being below 7% for $n = 6$). An additional point to take into account is that the volume of HFBA solution cannot be too high in order not to co-extract other chemicals. Therefore, paraquat recovery passes from 96 ± 3 to 112 ± 7 when 5 mL of 15 mM HFBA (instead of 2) are used.

As for the injection volume used in HPLC-MS/MS experiments (5 μL), it was low so that there were no matrix effects (Ardrey, 2003). Once gradient elution program had been optimized, some other chromatographic columns were tested. Results are shown in Fig. 2. As depicted in such a figure, the Waters Xterra C8 column was the one leading to a better separation (both between paraquat and diquat and from the other possible interferences), analytes appearing in less than 10 min.

3.2. HPLC-DAD results

HPLC-DAD chromatograms of 5 olive-oil extractions known not to contain DQ or PQ are depicted in Fig. 3 together with the blank (10 mM HFBA aqueous solution). As can be seen, spectra of all matrices are quite similar for all five oils. Furthermore, some co-extracted compounds interfere in PQ detection (Fig. 3, $\lambda = 258$ nm) but not in DQ detection (Fig. 3, $\lambda = 310$ nm). This is clearly seen in Fig. 4 where the HPLC-DAD chromatogram of a sample before and after fortification (5 mg kg^{-1} of both, DQ and PQ), is shown.

3.3. HPLC-DAD calibration

The calibration linear curves were obtained in the concentration range 0.25–5 mg kg^{-1} and the correlation coefficients, r, were 0.999 for both DQ and PQ. The relative standard deviation for the repeatability was below 4% and 3% for DQ and PQ, respectively ($n = 5$). Standard deviation value for the reproducibility was below 5% and 6% for DQ and PQ, respectively ($n = 5$). Limits of detection found with this technique (0.13 mg kg^{-1} for both DQ and PQ) are higher than the current MCLs admitted for such products in olives (0.05 mg kg^{-1}). Therefore, a more sensitive technique (MS) seems to be necessary, even though there is currently no legislation on MCLs of such residues in olive oil, as commented in Section 1.

3.4. Mass spectrometry experiments

Total ion chromatogram (TIC) and extracted ion chromatograms of CQ ($m/z = 122$), DQ ($m/z = 183$) and PQ ($m/z = 185$) for a standard solution containing

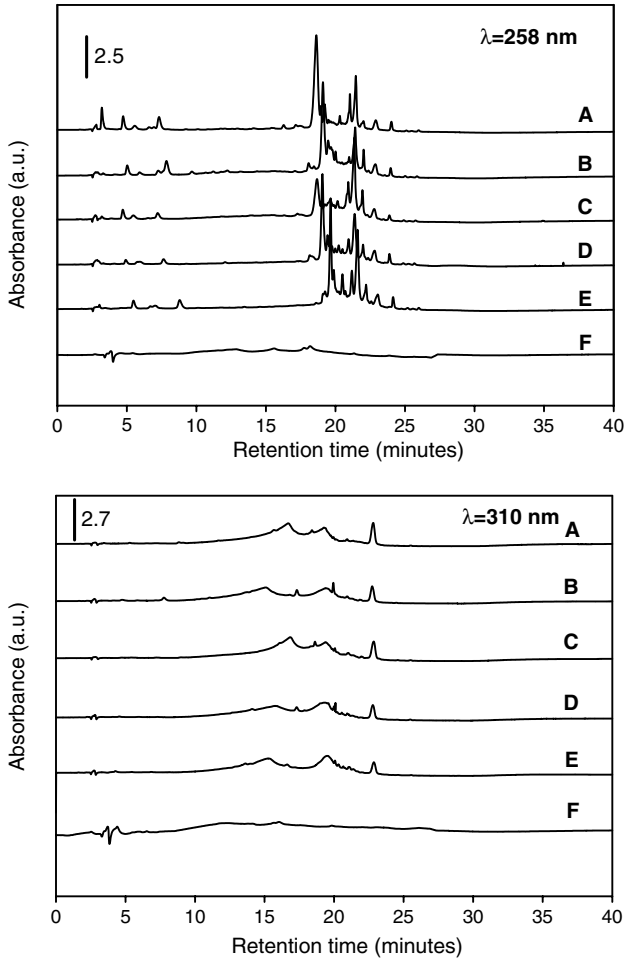

Fig. 3. HPLC-DAD chromatograms corresponding to matrices from extraction of 5 different olive oil samples (A–E) together with the blank (F, 10 mM HFA aqueous solution) at $\lambda = 258$ nm (upper graph) and $\lambda = 310$ nm (lower graph).

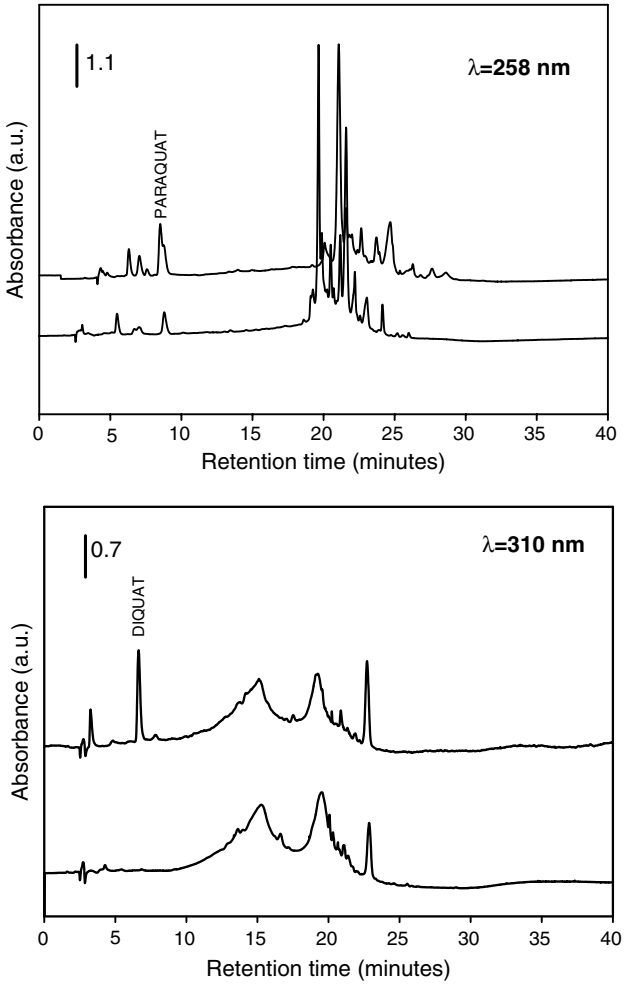

Fig. 4. Fortified sample containing paraquat (5 mg kg^{-1}, upper graph) and diquat (5 mg kg^{-1}, lower graph). For comparative reasons, the corresponding matrix before fortification is also included.

5 mg kg^{-1} of each herbicide in matrix extract are depicted in Fig. 5.

MRM conditions for the selected analytes are shown in Table 1. As can be seen, three peaks were selected for each analyte (quantification one, marked with an asterisk, and two other ones for confirmation). Collision energies corresponded to the maximum response for each ion, as shown in Fig. 6. DQ spectrum shows a precursor peak at $m/z = 183$ ascribed to $[cat^{2+}\text{-}H^+]^+$ and two other minor peaks at $m/z = 168$ and 157 corresponding to the loss of CH_3 and C_2H_2, respectively, from the precursor peak. Similar results were found by Taguchi et al. (1998) and Grey, Nguyen, and Yang (2002).

As far as PQ spectrum is concerned, it shows a precursor peak at $m/z = 185$ assigned to $[cat^{2+}\text{-}H^+]^+$ and two minor peaks at $m/z = 170$ and 158, resulting from the loss of CH_3 and HCN, respectively (Taguchi et al., 1998). Other authors (Castro, Moyano, & Galcerán, 2001b; Nuñez et al., 2002) suppose that a unielectronic reduction of dications with the formation of a radical cation ($cat^{2+} + 1e^- \rightarrow cat^{+\cdot}$) takes place in the ES probe. Subsequent loss of an H species could lead to the formation of the base peaks of DQ and PQ, the above mentioned daughter ions originating on their fragmentation.

Regarding chlormequat, the most intense peak appearing at $m/z = 58$ is ascribed to $[cat^+\text{-}ClC_2H_4]^+$ whereas minor peaks at $m/z = 122$ and 63 correspond to $[ClCH_2CH_2\text{-}N^+\text{-}(CH_3)_3]^+$, that is $(cat)^+$, and $[cat^+\text{-}N(CH_3)_3]^+$, respectively.

Note that the three studied compounds are quite stable, thus not allowing much fragmentation.

Precursor and quantification ions, limits of detection (signal/noise = 5), and correlation coefficients (r) obtained for calibration curves of PQ, DQ and CQ together with the linearity intervals are depicted in Table 2.

It is commonly accepted that working conditions (such as mass spectrometer used or mobile phase composition)

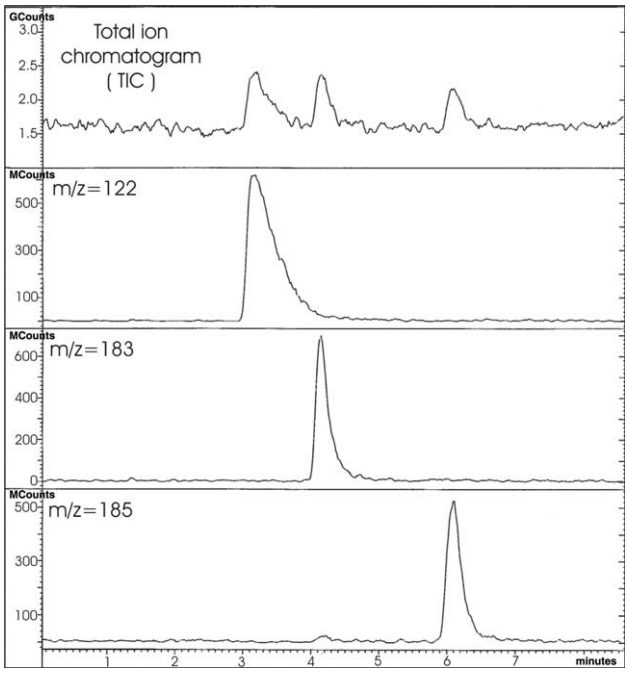

Fig. 5. Total ion chromatogram (TIC) and extracted ion chromatograms of CQ (m/z = 122), DQ (m/z = 183) and PQ (m/z = 185) for a standard solution containing 5 mg kg^{-1} of each herbicide in matrix extract.

Table 1
MRM conditions for the different quats

Quat	Collisions monitored by HPLC-MS/MS		
	Precursor ion	Monitored ion	Collision energy (V)
Diquat	183	157[a]	20
		168	23
		183	8
Paraquat	185	158[a]	21
		170	22
		185	8
Chlormequat	122	58[a]	22
		63	20
		122	11

[a] Quantification ion.

can influence on the resulting peaks in mass spectrometry (Castro et al., 2001b; Taguchi et al., 1998).

In order to study the effect of matrix on the results, MS/MS spectra of standard solutions containing 0.5 mg kg^{-1} of each herbicide in both 5 mM HFBA aqueous solution and matrix extract were recorded. Experimental conditions were the ones described in Table 1. For both solutions the relative intensity of the peaks obtained for the analytes was almost the same. Moreover, quantification peak areas both in the mobile phase and matrix extract were quite similar the difference included in the standard deviation of the repetitivity. In conclusion, under our experimental conditions no matrix effect was observed.

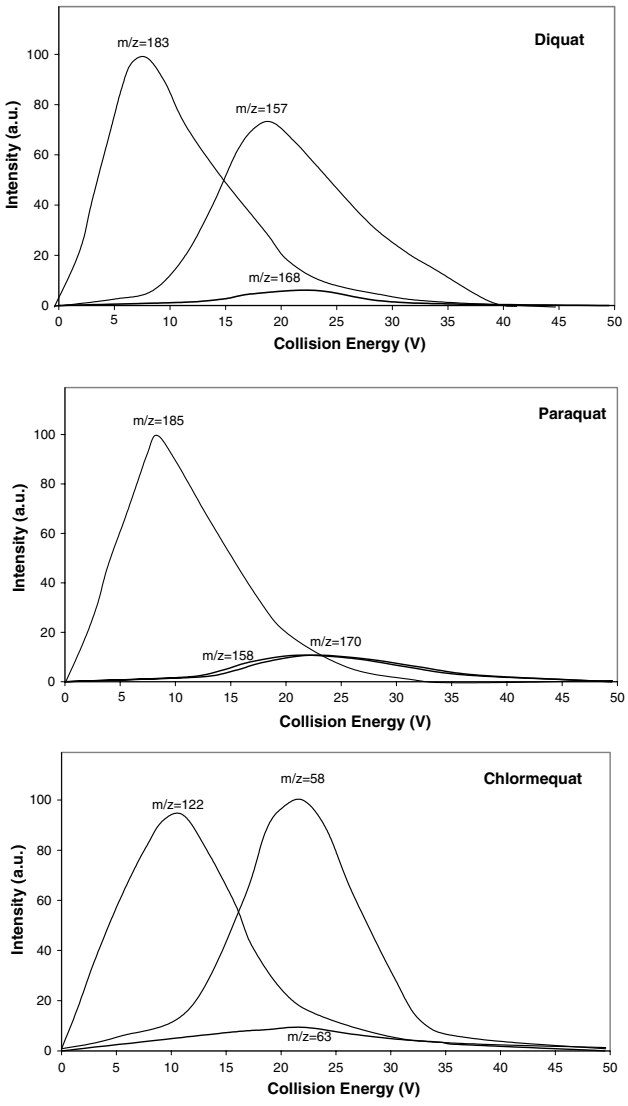

Fig. 6. Intensity of the different m/z ratios as a function of collision energy (V) for diquat, paraquat and chlormequat.

Castro et al. (1999) performed experiments using a single quadrupole mass spectrometer finding that coelution of DQ and PQ led to a decrease in the PQ peak. However, in our case, such an effect was not observed due to the use of a triple quadrupole system. In order to rule out the possibility of the so-called coelution effect, two different analysis of a same sample containing 5 mg kg^{-1} of PQ and DQ were performed. Chromatographic conditions were selected so that with a similar mobile phase composition PQ and DQ were coeluted in one case whereas both analytes were clearly resolved in the other. In both cases DQ and PQ peak areas obtained in MS/MS technique were similar, thus concluding that there is no effect due to the coelution. This is hardly surprising, since the first quadrupole let us selectively choose a peak corresponding to one of the analytes discarding the other, in contrast to single-quadrupole equipments.

Table 2
Calibration data for determination of CQ, DQ and PQ in olive oil samples by HPLC-MS/MS

Analyte	Precursor ion	Ion used for quantification	LOD (μg kg^{-1})	R	$y = ax + b$
Diquat	183	157	4	0.998	$y = 0.84x - 11.6$
Paraquat	185	158	4	0.998	$y = 0.54x - 4.5$
Chlormequat	122	58	0.3	0.996	$y = 0.65x + 14.9$

Linearity intervals: 8–670 μg kg^{-1} (DQ); 8–500 μg kg^{-1} (PQ) and 0.6–700 μg kg^{-1} (CQ).

3.5. Quality parameters

The relative standard deviation for the repeatability was <5% ($n = 5$) and for reproducibility was <9% ($n = 5$). LODs found for DQ (4 μg kg^{-1}), PQ (4 μg kg^{-1}) and CQ (0.3 μg kg^{-1}) do by far fulfil the current requirements of legislation.

3.6. Applications

In order to test the applicability of the method, different quat-free olive oils were spiked at the EU maximum residue level for olives. In all the cases standard deviations ($n = 5$) were lower than 11%. A previous determination of the herbicides in soils from olive groves plantations taken at the olive harvesting time, showed values in the intervals 0–250 μg kg^{-1} (DQ) and 0–60 μg kg^{-1} (PQ). The method was applied to 100 olive oil samples corresponding to the 2001–2004 period, not finding residues of DQ, PQ or CQ. Therefore, such residues do not seem to constitute a current problem for olive oil. In contrast, low and medium polarity herbicides have been analyzed during the above mentioned period by GC-MS/MS. We found in many samples residues of such herbicides (simazine and diuron mainly) at levels over five times the value legislated for olives. Interestingly, after the use of simazine was forbidden, terbutylazine appeared. A manuscript on that is currently in preparation.

4. Conclusions

A method for the analysis of diquat and paraquat in vegetable oils was developed using liquid–liquid partition, followed by ion-pair liquid chromatography and mass spectrometry. The sample preparation is compatible with the chromatographic system, which, in turn, is compatible with the electrospray ionization process. Detection limits are low (4 μg kg^{-1} for diquat and paraquat) and could be improved using a solid-phase extraction preconcentration step. The method was also applied to chlormequat, its detection limit being 0.3 μg kg^{-1}. The use of MRM technique allows a high sensitivity. Moreover, the full-scan product spectra obtained can be used to rule out false positives.

Olive oil samples analyzed corresponded to the 2001–2004 interval, not finding residues of the above-mentioned herbicides, even though they have been detected in some soil samples.

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