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ORIGINAL PAPER

Comprehensive gas chromatography coupled to mass spectrometry for the separation of pesticides in a very complex matrix

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Abstract The present research is focused on the development of a comprehensive two-dimensional gas chromatography–rapid scanning quadrupole mass spectrometric (GC x GC-qMS) methodology for the analysis of trace-amount pesticides contained in a complex real-world sample. Reliable peak assignment was carried out by using a recently developed, dedicated pesticide MS library (for comprehensive GC analysis), characterized by a twin-filter search procedure, the first based on a minimum degree of spectral similarity and the second on the interactive use of linear retention indices (LRI). The library was constructed by subjecting mixtures of commonly used pesticides to GC x GC-qMS analysis and then deriving their pure mass spectra and LRI values. In order to verify the effectiveness of the approach, a pesticide-contaminated red grapefruit extract was analysed. The certainty of peak assignment was attained by exploiting both the enhanced separation power of dual-oven GC x GC and the highly effective search procedure.

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Introduction

An effective analytical method, to be defined as such, must not only allow the qualitative/quantitative elucidation of major food components but also be sufficiently sensitive to enable the determination of trace-amount organic contaminants. The analysis of these components in food products may be directed to the assessment of food authenticity and quality, the control of an industrial process, and the detection of molecules with a possible beneficial or, more importantly, an adverse effect on humans. It is clear, therefore, that a primary aim for food chemists is the improvement and development of analytical methods [1].

The preliminary analysis of pesticides is normally carried out by means of single GC column in combination with a group-selective or element-selective detector such as an electron capture or nitrogen phosphorous system. In order to achieve positive peak assignment, mass spectrometric structural elucidation is required [2]. Ideally, full mass spectra relative to totally separated micro-contaminants (from the rest of the matrix compounds) and high sensitivity should be attained; in practice, this is not often the case.

Monodimensional chromatographic processes are still the most widely used for the separation of target analytes in real-world samples. The complexity of the latter often greatly exceeds the peak capacity of any single capillary

column. Hence, many detected peaks will be the summation result of two or more co-eluting analytes. The routes preferred by many analysts to elude this type of disadvantage are either directing the column effluent to an MS ion source, or increasing the chromatographic resolving power. In an MS system, as is well known, multiple-component effluent bands are transformed into a bunch of ions which are separated and then detected on a mass basis. The confirmation of analyte identity may be achieved by selecting specific ions (SIM mode), but then precious spectral information is lost; this may be avoided by using deconvolution procedures [3, 4]. Although the potential of MS systems, in terms of detection, is unrivalled, the importance of a satisfactory chromatographic process must be equally considered. In fact, spectral interpretation is always more easy and reliable when pure mass spectra relative to entirely resolved compounds are acquired. In this respect, both analytical dimensions present the same mutual importance and should be fully exploited.

Comprehensive two-dimensional gas chromatography (GC x GC), introduced in 1991 [5], is generally achieved on two columns connected in series, with a "modulator" device located between them, which enables the entrapment, re-concentration and then injection of continuous fractions of the primary column effluent onto the secondary capillary. During the second dimension run time (equal to the modulating period), the interface is engaged in the following entrapment process. The benefits in terms of separation power are enormous: ideally, the peak capacity becomes the product of the peak capacity in each dimension. The addition of a third MS dimension to a GC x GC setup, leads to the most powerful tool today available for volatile and semi-volatile analysis. The GC x GC methodology, which has been thoroughly reviewed [6–8], has been applied to the analysis of pesticides [9–11]: the experiments reported have demonstrated the main advantages of comprehensive GC-MS over conventional GC-MS in this field of research, namely the avoidance of highly selective sample preparation procedures, the attainment of purer MS spectra and the great increase in sensitivity.

The first aim of the present work was to exploit the enhanced resolving power of dual-oven comprehensive GC for the separation of pesticides contained in a highly complex natural sample (red grapefruit). The second objective and main novelty of the research was positive structural elucidation by using a recent laboratory-constructed pesticide comprehensive GC-qMS library with a twin-stage filter process. A similar approach has been recently used for the determination of coffee analytes in a solid-phase microextraction GC-quadrupole (q) MS experiment [12] and of allergens in a commercial perfume GC x GC-qMS experiment [13]. In both cases a laboratory-constructed "flavour and fragrance" MS library was employed.

Experimental

Samples and sample preparation

The pure standard pesticides (the names are listed in Table 1) were attained from Sigma–Aldrich (Milan, Italy). The pesticide solutions were prepared in a 1:1 mixture of cyclohexane and ethyl acetate (1–2 ppm for GC-qMS and 0.1–0.2 ppm for GC x GC-qMS). A mixture of C_7 to C_{30} hydrocarbons was obtained from Sigma–Aldrich.

A stock standard solution, containing 150 ppm of imizalil and demeton-S-methyl, was prepared. Calibration curves, limits of detection (LOD) and limits of quantitation (LOQ) were derived by using serial dilutions of the stock solution.

The red grapefruit extract was prepared by following the ISTISAN 23/97 guidelines, in the part regarding the isolation of pesticides from vegetable products.

GC-qMS parameters

The GC-qMS system consisted of a Shimadzu GCMS-QP2010 system (Shimadzu, Milan, Italy), equipped with a split-splitless injector maintained at 300 °C and an AOC-20i autoinjector (Shimadzu).

An SLB-5MS (silphenylene polymer) 30 m×0.25-mm I. D., 0.25-μm film thickness column (Supelco, Milan, Italy) was used and was temperature programmed from 70 to 300 °C at 2 °C min^{-1}. Injection volume was 1 μL, performed in the splitless mode. Helium was used as carrier gas at a constant head pressure of 48.0 kPa.

The following MS parameters were used: interface temperature, 250 °C; MS ionization mode, electron ionization; detector voltage, 0.9 kV; acquisition mass range, 50–495 u; scan speed, 5,000 u s^{-1}; acquisition mode, full scan; scan interval, 0.15 s; solvent delay, 10 min. Data were collected by the GCMS Solution software (Shimadzu).

GC x GC-qMS parameters

The comprehensive GC-qMS system consisted of a Shimadzu GC x GCMS-QP2010 system. The primary and secondary GC ovens were connected by a heated transfer line (300 °C). The primary gas chromatograph was equipped with a split-splitless injector maintained at 300 °C and an AOC-20i autoinjector. A KT-2006 loop modulation system (under Zoex Corporation license) was retrofitted onto the secondary oven of the Shimadzu GC x GCMS-QP2010 system. The loop modulator is a thermal modulation device employing a continuous nitrogen cold jet and a pulsed nitrogen hot jet (375 ms). A modulation period of 6 s was applied.

Table 1 Standard pesticides, their activities, and their relative LRI values determined in both GC-qMS and GC x GC-qMS applications

	Pesticide	GC-MS	GC x GC-MS	Activity
		LRI	LRI	
1	Methamidophos	1228	1241	Acaricide
2	Dichlorvos	1244	1249	Acaricide
3	Propamocarb	1393	1399	Fungicide
4	Chlormefos	1433	1437	Insecticide
5	Etridiazole	1445	1449	Fungicide
6	Propham	1459	1462	Herbicide, plant growth regulator
7	Chloroneb	1505	1508	Fungicide
8	Carbaryl	1510	1517	Acaricide, insecticide
9	Molinat	1533	1538	Herbicide
10	Heptenophos	1567	1572	Acaricide, insecticide
11	Propoxur	1606	1609	Acaricide, insecticide
12	Demethon-S-methyl	1614	1620	Acaricide, insecticide
13	Diphenilamine	1621	1625	Fungicide
14	Ethoprophos	1630	1635	Insecticide
15	Chlorpropham	1657	1660	Herbicide, plant growth regulator
16	Trifluralin	1671	1674	Herbicide
17	Sulfotep	1671	1675	Acaricide, insecticide
18	Benfluralin	1676	1679	Fungicide
19	Phorate	1686	1691	Acaricide, insecticide
20	Promecarb	1689	1695	Insecticide
21	Dichloran	1717	1723	Fungicide
22	Dimethoat	1718	1724	Acaricide, insecticide, nematicide
23	Carbofuran	1735	1740	Acaricide, insecticide, nematicide
24	Simazine	1748	1743	Algicide, herbicide
25	Atrazine	1748	1752	Herbicide
26	Propazine	1756	1761	Herbicide
27	Dioxathion	1758	1763	Acaricide, insecticide
28	Fonofos	1773	1779	Insecticide
29	Terbuthylazine	1773	1779	Herbicide
30	Propetamphos	1774	1779	Acaricide
31	Propyzamide	1789	1778	Herbicide
32	Pyrimethanil	1789	1796	Fungicide
33	Diazinon	1790	1794	Fungicide
34	Tefluthrin	1821	1826	Insecticide
35	Pirimicarb	1836	1840	Insecticide
36	Phosphamidon	1860	1866	Insecticide, nematicide
37	Chloropyriphos-methyl	1873	1879	Insecticide
38	Metribuzin	1873	1882	Herbicide
39	Prothoate	1873	1881	Acaricide, insecticide
40	Vinclozolin	1883	1889	Fungicide
41	Tolclofos-methyl	1884	1892	Fungicide
42	Alachlor	1889	1896	Herbicide
43	Metalaxyl	1905	1910	Fungicide
44	Ametryn	1907	1912	Herbicide
45	Fenchlorphos	1908	1912	Insecticide
46	Fenpropidin	1928	1936	Fungicide
47	Pirimiphos-methyl	1938	1942	Insecticide
48	Terbutryn	1939	1944	Herbicide
49	Dichlofluanid	1947	1965	Acaricide
50	Malathion	1959	1965	Acaricide, insecticide
51	Metolachlor	1963	1968	Herbicide
52	Chlopyriphos	1971	1976	Acaricide, insecticide, nematicide
53	Fenthion	1976	1983	Insecticide
54	Chlorthal-dimethyl	1977	1985	Herbicide

Table 1 (continued)

	Pesticide	GC-MS	GC x GC-MS	Activity
		LRI	LRI	
55	Fenpropimorph	1984	1990	Fungicide
56	Triadimefon	1990	1997	Fungicide
57	Nitrothal-isopropyl	2007	2013	Fungicide
58	Isopropalin	2027	2034	Herbicide
59	Metazachlor	2039	2046	Fungicide
60	Cyprodinil	2037	2043	Fungicide
61	Penconazole	2048	2054	Fungicide
62	Fipronil	2052	2059	Acaricide, insecticide
63	Chlorfenvinphos	2059	2064	Acaricide, insecticide
64	Quinalphos	2067	2075	Acaricide
65	Procymidone	2076	2084	Fungicide
66	Triadimenol	2076	2084	Fungicide
67	Methidiathion	2099	2103	Insecticide
68	Bromophos-ethyl	2097	2105	Acaricide
69	Tetrachlorvinphos	2112	2121	Acaricide, insecticide
70	Ditalimfos	2128	2137	Fungicide
71	Mepanipyrim	2134	2141	Fungicide
72	Chlorfenson	2148	2157	Acaricide
73	Hexaconazole	2150	2161	Fungicide
74	Imazalil	2156	2165	Fungicide
75	Profenofos	2165	2174	Insecticide
76	Oxadiazon	2182	2189	Herbicide
77	Diclobutrazol	2191	2201	Fungicide
78	Buprofezin	2191	2201	Insecticide
79	Kresoxim-methyl	2200	2208	Fungicide
80	Fluazifop-butyl	2234	2242	Herbicide
81	Oxadixyl	2260	2272	Fungicide
82	Triazophos	2299	2309	Acaricide, insecticide, nematicide
83	Propiconazole I	2333	2342	Fungicide
84	Trifloxystrobin	2339	2344	Fungicide
85	Propiconazole II	2345	2357	Fungicide
86	Nuarimol	2370	2382	Fungicide
87	Bromopropylate	2461	2470	Acaricide
88	Piperophos	2464	2471	Acaricide, insecticide
89	Bifenthrin	2463	2472	Fungicide
90	Tebufenpyrad	2497	2505	Acaricide
91	Fenazaquin	2499	2511	Insecticide
92	Tetradifon	2522	2533	Acaricide, insecticide

The column set consisted of two capillaries, which were serially connected by a SGE mini-union (Austin Texas, USA). The conventional apolar first dimension was an SLB-5MS 30 m×0.25-mm I.D., 0.25-μm film thickness column and the secondary polar fast column was an Omegawax [100% poly(ethylene glycole)], 1 m× 0.10-mm I.D., 0.10-μm film thickness (Supelco). The primary column temperature was programmed from 70 to 300 °C at 2 °C min^{-1}, while the second column was maintained at a 30 °C constant higher temperature. Injection volume was 1 μL, performed in the splitless mode. Helium was used as carrier gas at a constant head pressure of 242.7 kPa.

The following MS parameters were used: interface temperature, 250 °C; MS ionization mode, electron ionization; detector voltage, 0.9 kV; acquisition mass range, 50–495 u; scan speed, 10,000 u s^{-1}; acquisition mode, full scan; scan interval, 0.05 s (20 Hz); solvent delay, 10 min. Data were collected by the GCMS Solution software and by using its export function; the ASCII data were converted into a matrix with rows corresponding to a 6-s duration, and data columns covering all successive second dimension 6-s chromatograms using the Comprehensive Chromatography Converter 1.0 software (Shimadzu). Contour representation of the 2D chromatograms was achieved through the same software.

Results and discussion

GC x GC-qMS library construction

Pesticide mixtures, each containing 90–100 compounds, were subjected to GC x GC-qMS analysis. Contour plot maps were constructed for every mixture, one of which is shown in Fig. 1 (the 92 components are listed in Table 1). GC x GC method optimization is rather a delicate matter and, therefore, a great deal of time was spent in properly tuning the experimental parameters (column set, head pressure and the two temperature programs) in order to achieve the best possible separation. As can be observed, the totally separated 2D peaks are nicely distributed across the double axis TIC chromatogram, demonstrating the orthogonality of the apolar–polar column set. Other options were tested (mediumly polar–polar, polar–mediumly polar, polar–apolar) but did not provide the same degree of analyte separation (data not shown). The helium linear velocities during the entire run time were also determined, corresponding approximately to initial values of 25.2 and 261.7 cm s^{-1} in the first and second dimension, respectively; these values decreased to 18.8 and 207.4 cm s^{-1} at the end of the application. These values were calculated as

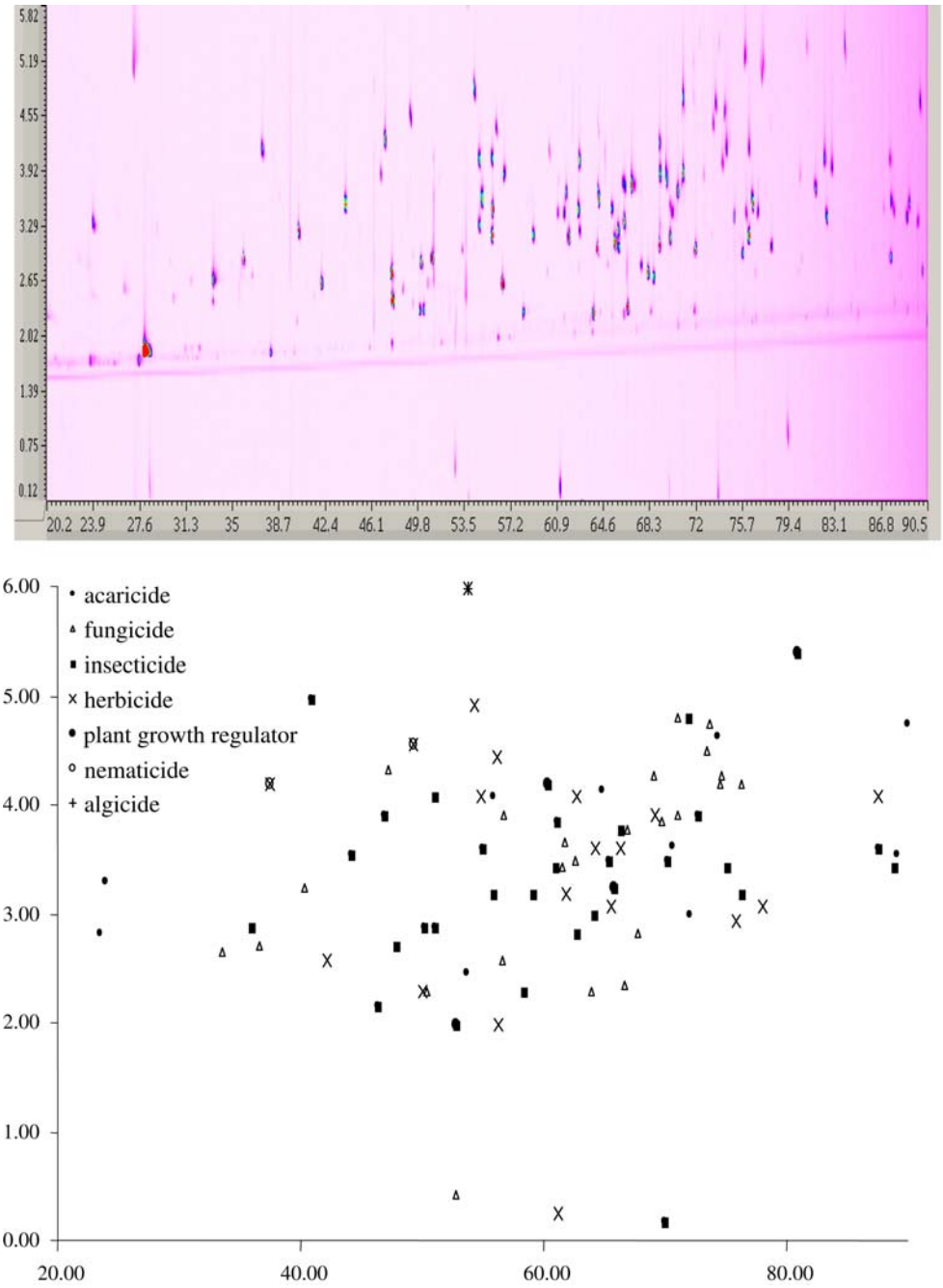

Fig. 1 TIC 2D chromatogram expansion relative to the GC x GC-qMS analysis of a mixture of 92 standard pesticides (*top*), dot plot relative to the TIC 2D chromatogram (*bottom*)

described in the literature [14]. In this specific application, the constant pressure mode, rather than a pressure gradient, enabled a better separation of analytes eluting later in the chromatogram (from 60 min onwards). In general, the far-above-optimum gas linear velocities in the second dimension are necessary for the rapid elution of the re-injected components, while the slightly-lower-than-ideal gas velocities in the first dimension are required to attain sufficient samplings per peak (ideally at least three).

A dot plot, constructed using the identified peak apex retention time coordinates and enabling the easier visualization of the 92 separated contaminants, is also illustrated in Fig. 1. Pure mass spectra and GC x GC LRI values were derived for each compound and then included in the MS library. Although outside the scope of the paper, it must be noted that a GC-qMS library (a 30 m×0.25-mm I.D.× 0.25-μm film thickness SLB-5MS column was used) was also constructed through analyses carried out on less complex mixtures, by using the same procedure (LRI values for the 92 pesticides are also listed in Table 1). The LRI values were calculated from the unconverted GC x GC chromatogram as follows: the alkane and the pesticide mixture were analysed. In both applications, the retention time of the central peak was considered in the case of an odd number of modulated analyte peaks, while in the case of an even number of modulated peaks the central retention time between the two most internal peaks was considered. It is evident, from Table 1, that the LRI differences between the monodimensional and bidimensional applications are limited, and much lower than those previously observed [13], which were related to the excessive retention of polar compounds (i.e. alcohols) on the secondary polar column. In the present research, the use of independent temperature programming (not used in the previous research) in the second dimension (a constant 30 °C higher temperature was applied), eliminated analyte wrap-around, apart from three pesticides located along the x-axis. These components presented retention times of less than 7 s on the secondary column. Higher temperature differences were tested and, although analyte wrap-around was completely avoided, a series of compounds were not sufficiently separated (data not shown). It must be emphasized that the LRIs attained cannot be considered as bidimensional, as the separation in the second dimension is neglected; hence, the secondary column retention information is lost and the calculated values are essentially monodimensional. Although a standard method for the calculation of 2D space LRIs has not yet been adopted, a series of approaches have been recently introduced [15, 16].

In any GC x GC experiment, detector capabilities must always be considered: the rise time should be short, the acquisition rate high and the contribution towards band enlargement negligible (low internal volumes). The GC x GC-qMS expansion shown in Fig. 1 is essentially the summation of 700 rapid 6-s chromatograms (= 70 min). The pure analyte bands coming off the micro-bore column presented peak base widths within the 200- to 700-ms range. The rapid quadrupole mass spectrometer used in this research generated a sufficient scan speed (20 Hz) for the acquisition of from three to ten pure spectra for all peaks spreading across the entire chromatogram. The data acquisition rate was sufficient for quantitative purposes in many but not in all cases: peaks with less than six data points were not quantified.

A fundamental issue in quadrupole mass scanning is related to the inconsistency of mass spectra across a peak, defined as “peak skewing”. The direct negative consequence of this effect is that a single acquired spectrum would present different ion abundances with respect to the averaged mass spectrum, present in the library. This issue was investigated for several contaminants and was determined to generate only limited spectral differences: a reconstructed pesticide peak (fonofos) along with three successive acquired spectra are illustrated in Fig. 2.

GC x GC-qMS analysis of a contaminated red grapefruit extract

A contaminated red grapefruit extract was subjected to GC x GC-qMS analysis (Fig. 3a). As can be seen, the matrix is extremely complex with thousands of compounds visible on the 2D space plane. Obviously, the same experimental parameters as in the previous application were applied; however, although a substantial part of the contour plot is occupied, extensive wrap-around is evident. This did not represent a problem, as the analysis was aimed towards the identification of specific target compounds.

A 2D chromatogram 10-min expansion, with an indicated pesticide (imizalil), is shown in Fig. 3b. The software mass spectra matching procedure worked as follows: library hits with a lower than 90% probability (filter 1) and with an LRI, with respect to the calculated unknown peak value, outside an acceptable retention index window (filter 2) are automatically deleted. The index window range was ±10 units for the setup used in the GC x GC application. The excellent spectral search result for imizalil is shown in Fig. 4a; in this case, the presence of filter 2 would not have been necessary as only a single component with the minimum degree of required spectral similarity was present in the library. A further 2D chromatogram 10-min expansion, with another totally resolved contaminant (demeton-S-methyl), is shown in Fig. 3c. The extreme complexity of this part of the chromatogram is evident. In this case the search procedure proved to be of great help: a retention index of 1615 was automatically calculated by the software for the indicated peak. The unfiltered library search provided a series of

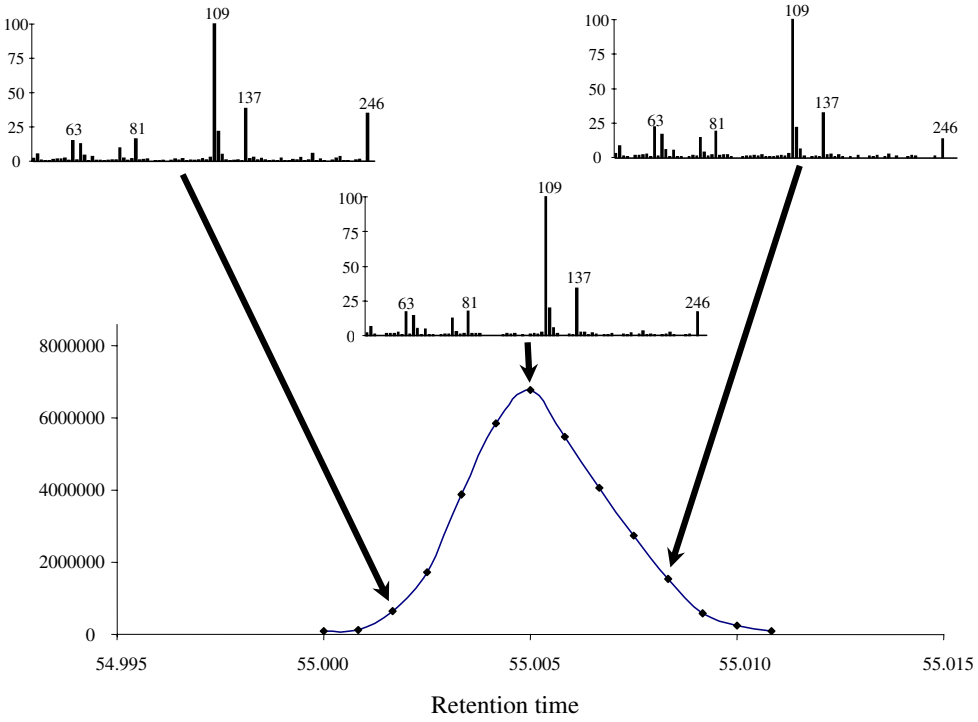

Fig. 2 Re-constructed unconverted 2D peak relative to fonofos with three successive spectra acquired across the peak

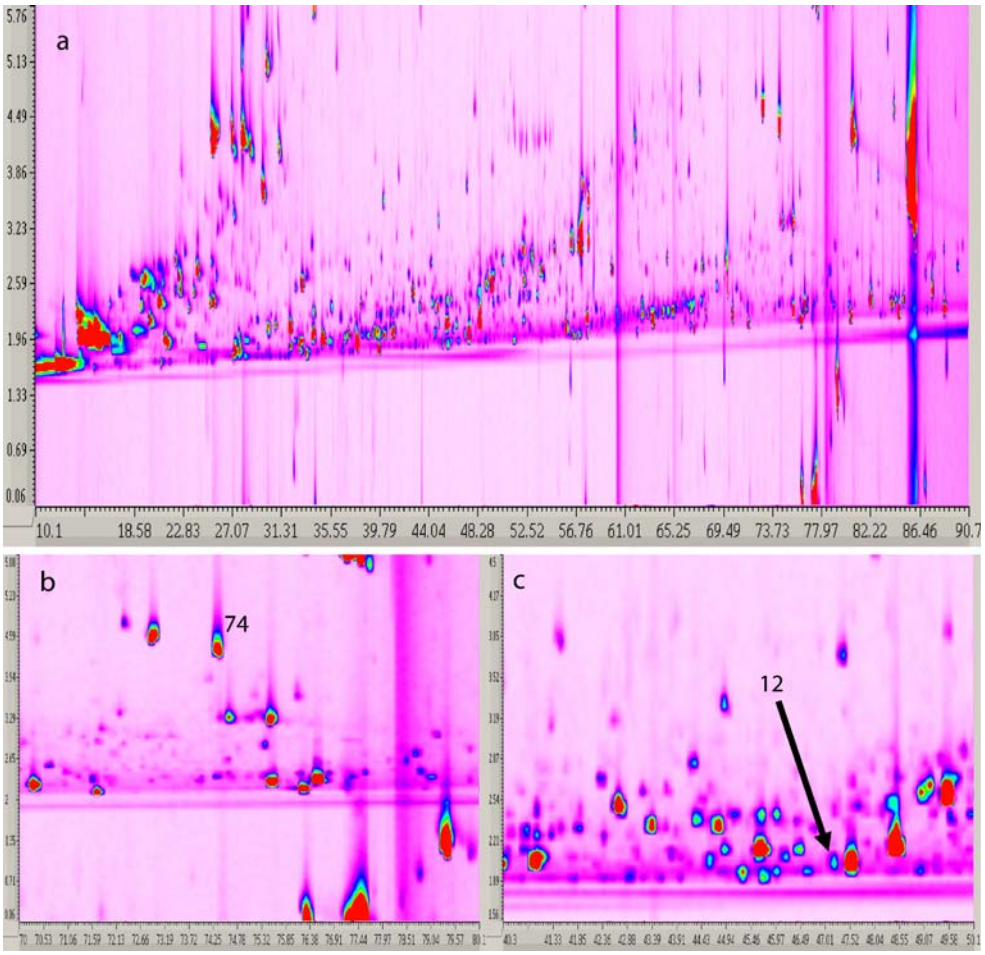

Fig. 3 **a** TIC 2D chromatogram expansion relative to the GC x GC-qMS analysis of a contaminated red grapefruit extract, **b** TIC 2D chromatogram location of imizalil, **c** TIC 2D chromatogram location of demeton-S-methyl

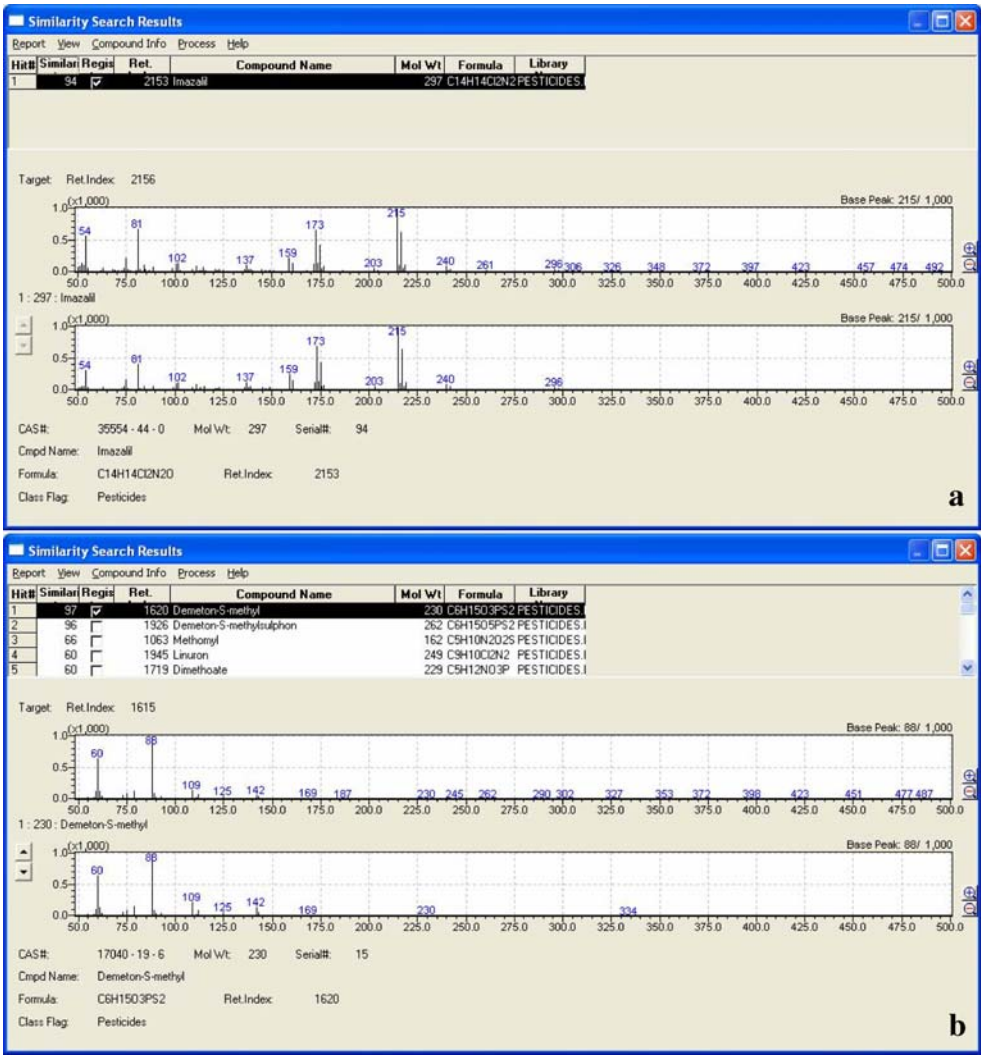

Fig. 4 **a** Filtered search for imizalil, **b** unfiltered search for demeton-S-methyl

possible matches, five of which are shown in Fig. 4b. The application of filter 1 (minimum 90% similarity) led to the exclusion of all but two components, while the additional employment of filter 2 (1605–1625 LRI window) eliminated the demeton-S-methylsulphon match (LRI=1926). In both cases the quality of the experimental spectra were excellent.

Four-point calibration curves (in the 15.0- to 1.0-ppm range) were derived for imizalil and demeton-S-methyl, with regression coefficients of 0.9997 and 0.9994, respectively. Grapefruit sample concentrations of 13.2 ppm for imizalil (maximum residue level, 5 ppm) and 4.3 ppm for demeton-S-methyl (maximum residue level, 0.4 ppm) were determined. LOQs, which were determined by analysing serial dilutions of the standard pesticide solution and by measuring the mean noise value (sample blanks were injected) plus ten standard deviations of the blank mean, equalled 0.98 ppm for imizalil and 0.28 ppm for demeton-S-methyl. LODs, which were determined by considering three standard deviations of the blank mean, equalled 0.31 ppm for imizalil and 0.11 ppm for demeton-S-methyl.

Conclusions

The GC-qMS elucidation of pesticide profiles within complex food samples is an arduous challenge, even when the MS system is pushed to its full potential. Whenever the main objective is to separate and identify target contaminants from hundreds or even thousands of interfering analytes, the best solution is certainly comprehensive two-dimensional gas chromatography in combination with mass spectrometry. As observed in the present research, even the delivery of pure effluent bands to an MS system is sometimes not enough for unambiguous peak assignment, which is due to the fact that many pesticides are characterized by structural similarity and, hence, near-to-identical spectra profiles. We

have demonstrated that, in such cases, the use of a dual-filtered library search procedure enables a more reliable identification of experimental MS spectra.

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