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GC-MS fragmentation patterns of sprayed endosulfan and its sulphate metabolite in samples of *Theobroma cacao L* from a field kinetic study

Edebi N. Vaikosen [a, b, c,*], Lorraine T. Gibson [b], Christine M. Davidson [b], Bamidele I. Olu-Owolabi [a], Kayode Adebowale [a], Benjamin U. Ebeshi [c], Paul N.E Diagboya [d]

[a] Department of Chemistry, Faculty of Science, University of Ibadan, Ibadan, Nigeria

[b] Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, U. K

[c] Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, Niger Delta University, Wilberforce Island, Nigeria

[d] Department of Chemistry, Vaal University of Technology, Vanderbiljpark, South Africa

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ABSTRACT

Most environmental analytical methods for the determination of organochlorine pesticides (OCPs) are multi-residual with other organic compounds co-extracted and co-eluted. This has been observed in GC spectra using classical detectors like electron-capture detector (ECD) even after appropriate clean-up. This limitation could be resolved by using GC-MS methods which are more specific and selective. Thus, a commercial-grade endosulfan treated *Theobroma cacao* plantation was sampled. Representative samples comprising leaves, stem bark and pulp were obtained between 0.5 h and 60 d after treatment. Samples were analyzed for residual parent endosulfan (*α*- and *β*-isomers) as well as the metabolite endosulfan sulphate using an ion trap GC-MS. The retention times and chromatogram peaks obtained for various endosulfan were identified and compared with reference standards, and confirmed with National Institute of Standards and Technology (NIST) library. Results showed that the molecular ion at m/z 407 was exhibited by *α*- and *β*-endosulfan, representing the parent molecular ion $M^{+\bullet}$ ($[C_9H_6Cl_6SO_3]^{+\bullet}$). The α-isomer was more thermally stable, hence exhibited more relative abundance. Other predominant peaks were 339, 307, 277, 265, 243, 241, 207, 195, 160, 159, 99 and 75 m/z. The peak at m/z 159 was the base molecular ion. For endosulfan sulphate, the peak at m/z 422 corresponded to parent molecular ion ($M^{+\bullet}$), while m/z 424 was due to isotopic pattern characteristic of the chlorine atom. The peaks at 387, 357, 289, 272, 229, 206, 170, and 120 m/z were characteristic for the sulphate metabolite. The m/z peak at 272 was the base molecular ion, while m/z 143 may be due to metabolite diol and lactone. These results showed that the various endosulfan species can be identified and confirmed simultaneously using a GC-MS.

Keywords: Parent isomers; Lactone; Co-extraction; Co-elution; Carbene carbocation; Dichlorobenzene; Precursor molecular ions; *Theobroma cacao*

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1. INTRODUCTION

The preservation of the environment and indeed the human health from exposure to myriads of organic contaminants has become a great concern globally.[1,2] Contaminants of major concern are the persistent organic pollutants (POPs) whose environmental persistence leads to bio-accumulation and subsequently, bio-magnification in the food chain.[3,4] Simultaneously determining the presence of POPs in samples is difficult because they are most often co-extracted and co-eluted with other organic compounds; hence, the need to apply a method that would enhance easy determination.

Of the 21 compounds of concern listed in the 2009 Stockholm convention on POPs, 14 are organochlorine pesticides (OCPs) and OCPs such as dichlorodiphenyltrichloroethane (DDT), hexachloro-cyclohexane (HCH), hexachloro-benzene (HCB), chlordane and heptachlor are regarded as possible human carcinogens.[5,6] These OCPs are invaluable organic compounds because they are potent plant pesticides. However, their persistence is a major environmental side effect. Though OCPs have been banned in several countries for more than three decades, they are still found in soil, water, air, foods and in human blood serum and breast milk.[7-9]

Endosulfan is a pesticide used in growing *Theobroma cacao* L plant. The pesticide which is represented chemically as 6,7,8,9,10,10–Hexachloro–1,5,5a,6,9,9a–hexahydro–6,9–methano-2,3,4-benzo(e)dioxathiepin–3–oxide is an OCP POP as well as a prohibited pesticide.[10,11] Technical grade endosulfan is a mixture of two stereoisomers, α- and β-isomers (Fig. 1) which are metabolised into endosulfan sulphate, alcohol, ether or lactone in the environment. The sulphate is more persistent and toxic than the parent isomers.[12]

Determination of OCPs in environmental samples is often plagued with operational challenges which make accurate identification and quantification of these contaminants difficult. These challenges include co-extraction and co-elution of other organic analytes in

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environmental samples along with OCPs, and when a classical detector like the electron-capture detector (ECD) is used alongside a Gas Chromatograph, ECD suffers major limitations in that it cannot detect trace level analytes in complex environmental matrices and it also responds to other moieties other than the halogen groups within an organic compound.[13-16] Other limitations such as matrix effect and false positives have been reported.[17] These limitations may be resolved by coupling a Mass Spectrometer (MS) as detector to the GC. Such a hybrid system offers several advantages including simultaneous qualitative and quantitative determination of contaminants,[18,19] as well as high resolution and identification of unknown organic compounds by comparing with the NIST and Wiley MS databases.[20-22]

The aim of this study was to identify parent *α*- and *β*-endosulfan compounds as well as their major metabolite endosulfan sulphate simultaneously in *T. cacao* L plant tissues (leaves, stem bark and pulp) following selected time intervals (0 [0.5h], 7, 14, 21, 28, 42 and 60 d) after the application of commercial grade endosulfan in terrestrial field dissipation (TFD) or field kinetic study. The GC-MS chromatogram and mass spectra of reference endosulfan standards and the NIST library were used for the identification and confirmation of generated molecular ion fragments. Molecular formula for successive residual parent molecular ions (i.e. un-fragmented portions) were apportioned to mass spectra of m/z peaks and the eliminated fragments were elucidated in order to propose the fragmentation patterns for parent isomers and metabolite sulphate and the likely structure for each molecular ion residues.

2. EXPERIMENTAL

2.1. Materials, sampling and sample pre-treatment

The OCP mixed reference standard of 20 components (mix AB#1) containing 200 µg/mL each (Aldrin; α-HCH; β-HCH; δ-HCH; γ-HCH; cis-Chlordane; trans-Chlordane; 4,4'-DDD;

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4,4'-DDE; 4,4'-DDT; Dieldrin; α-Endosulfan; β-Endosulfan; Endosulfan sulphate; Endrin; Endrin aldehyde; Endrin ketone; Heptachlor; Heptachlor epoxide; Methoxychlor - in hexane:toluene (1:1) solution) was commercially obtained from Restek Corporation, USA. Analytical grade reagents (dichloromethane (DCM), n-hexane, acetone, petroleum ether and acetonitrile) were used throughout the study. Stock standard solutions were prepared in hexane, with a working concentration range of 200 - 1000 µg/L of mixed OCPs reference standard containing *α*- and *β*-endosulfan and endosulfan sulphate was prepared in hexane from the stock solution (20,000 µg/L). Sodium sulphate (anhydrous) and silica gel 60 extra-pure (60–120 mesh) for column chromatography were from BDH limited (Poole, England).

Representative samples of *T. cacao* comprising of leaves, stem bark and pulp were collected after the application of 1.4 kg ai/ha (0.5% ai) of commercial grade endosulfan to *T. cacao* farm at intervals of 0.5 h (day 0), 7, 14, 21, 28, 42 and 60 d. The samples were wrapped in aluminium foils and stored in an ice chest cooler for immediate transportation to the laboratory for analysis. Then, the fresh leaves, bark and epicarp of the pod were blended separately before extraction using a Kenwood BL237 table top blender. After each blending, the cup of the blender was thoroughly cleaned and rinsed with acetone to prevent cross contamination.

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2.2 Sample Extraction and Clean Up

Modified USEPA method 3570 (2001)[23] and that of Yeboah *et al*. (2003)[24] were employed for the extraction of OCPs from the samples. To 10 g of blended plant tissues, 2 g of anhydrous sodium sulphate was added in an amber extraction flask. This was shaken vigorously with 60 mL mixture of petroleum ether:ethyl acetate (2:3) for 30 minutes (using a Thermo Scientific reciprocating/orbital shakers, model MaxQ) at 80-100 rpm and thereafter allowed to stand for 5 min in a fumehood for solvent to separate from solid matrix. The supernatant was carefully filtered through a Whatman filter paper containing 2 g of

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anhydrous sodium sulphate into a graduated 50 mL glass measuring cylinder to obtain a 30 mL filtrate (equivalent to 5 g of sample). The filtrate was evaporated in a round-bottom flask using a rotary evaporator to 5 mL at 80°C. This was then transferred into a 20 mL beaker. The flask was rinsed thrice with 2 mL of hexane into the same 20 mL beaker and further evaporated using nitrogen gas to 2 mL residue. The resultant residue was cleaned using silica gel. Cleaned extract was reduced to 2 mL and transferred into an amber glass vial for GC-MS analysis.

2.3 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The hexane reconstituted cleaned extracts from *T. cacao* were analysed with a Thermo-Finnigan Trace GC Ultra (Waltham, MA, USA) equipped with an AS 2000 Tray Auto-sampler (Thermoquest), splitless injector, coupled to an ion trap mass spectrometer (MS) (Polaris Q) with *X*calibur data software processor. Chromatographic separation was achieved with a HP-5MS capillary column of 30m length × 0.25mm i.d. × 0.25µm film thickness (Agilent J&W Scientific Co., Folsom, CA, USA). The oven temperature was programmed, which was initially held at 80 ºC for 5 min, and was increased to 473 K at a rate of 293 K/min, held for 5 min and then raised to 553 K at a rate of 283 K/min and held for 2 min. The flow rate of the carrier gas (helium, 99.99% purity) was kept constant at 1.18 mL/min. Splitless injection mode at an injection temperature of 250 ºC was carried out at a pressure of 79.5 kPa. The linear velocity and total flow of the carrier gas were 10.0 cm/sec and 32.7 mL/min, respectively. The GC-MS interface line and ion source temperatures were 533 K and 523 K respectively. The GC-MS retention times obtained for *α*-endosulfan, *β*-endosulfan and endosulfan sulphate in *T. cacao* samples were identified from retention times recorded for each corresponding reference standards. These identified chromatogram peaks were further confirmed using the mass spectra peaks of the reference standard and National

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3 Institute of Standards and Technology (NIST) search library software (version 2012) installed
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5 on the instrument.
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9 ## 3. RESULTS AND DISCUSSION
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11 ### *3.1 Identification and confirmation*
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13 Table 1 shows seventeen and fifteen characteristic molecular ions from the GC-MS analysis
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15 for α-endosulfan and β-endosulfan and endosulfan sulphate respectively, that were identified
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17 and confirmed for *T. cacao* samples using their reference standards and NIST library. Figure
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19 2, represents the total-ion-count (TIC) chromatogram for mixed OCP reference standards,
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21 with peaks at 18.52, 20.15 and 21.11 mins being retention times for α-endosulfan, β-
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23 endosulfan and endosulfan sulphate respectively. These peaks were used to identify the GC-
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25 MS spectra obtained for *α*-endosulfan, *β*-endosulfan and endosulfan sulphate in *T. cacao*
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27 samples collected from day 0 to day 60 (Figure 3). The mass spectra showed the presence of
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29 only parent isomers at day 0 (*α*-endosulfan -18.71 min; β-endosulfan -20.29 min) (Figure 3a),
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31 while endosulfan sulphate was significantly identified from day 14 (Figure 3b and 3c). This
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33 implied that the endosulfan sulphate is a metabolite of the parent isomers.
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39 ### *3.2 Molecular ions generated from α- and β-endosulfan fragmentation*
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41 The molecular ion (m/z) peaks obtained from the fragmentation of both endosulfan isomers
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43 were generally the same. The electron ionization (EI) mass spectra at m/z 407 (Figure 4a)
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45 exhibited by both isomers is indicative of the presence of the parent molecular ion $M^{+\cdot}$ -
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47 $[C_9H_6Cl_6SO_3]^{+\cdot}$. The base peak (most abundant molecular ion) for both isomers in the *T.*
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49 *cacao* samples was observed at m/z 159 or 160. This may have resulted from consecutive
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51 losses of various components from the parent molecular ion at m/z 407. Predominant m/z
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53 ratio peaks observed in the EI spectra from the fragmentation of residual endosulfan isomers
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55 were as follows; – 407, 339, 307, 277, 265, 243, 241, 207, 195, 160, 159, 99 and 75. These
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57 selected molecular ions are comparable with m/z peaks obtained for endosulfan reference
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standard, NIST library spectra (Figure 4, Table 1) and some other biological studies.[25-27]. The first predominant fragment formed at m/z 339 was due to the loss of the component $HClO_2^{\bullet}$ from the parent molecular ion at m/z 407. This loss represented a mass unit of 68 resulting in the molecular ion structure of $C_9H_6Cl_5SO^+$ (Scheme 1). Sinha et al., (2004) [27] and Sarafin and Winterscheidt (1985) [28] have reported mass unit at m/z 339. However, Sinha *et al.*, (2004) reported a loss of H_4SO_2 corresponding to a molecular ion structure of $C_9H_2Cl_6O^+$. To obtain the most abundant specie at m/z 159 (or 160) and other lower molecular ions several losses of mass units were observed via the molecular ions at m/z 307 and 295 (Scheme 1). The formation of molecular ion m/z at peaks 307 and 295 were due to losses of SO_2Cl (μ mass 100) and $CHSO_2Cl$ (μ mass 112) respectively from the parent molecular ion $M^{\bullet+}$. All elements have at least one stable isotope, however, chlorine ($^{35, 37}Cl$), sulphur ($^{32, 33, 34}S$) and oxygen ($^{16, 17, 18}O$) are known to exist in multiple stable isotopes. The peaks at m/z 277 (represented as molecular ion 5b) and 241 may be due the sequential removal of the OH_2 and $Cl^{\bullet}$ groups respectively from m/z 295 as precursor (Scheme 1), while m/z 277 (molecular ion 5a) may have resulted from the elimination of CH_2O (30 μ mass) from the m/z peak at 307. Also, the loss of H_2O could have resulted to the peak at 277 (5a) from m/z 295. The peaks at 295 and 277 have been reported as significant fragments in the mass spectra of endosulfan.[28] The molecular ion m/z 207 may be represented as $[C_8Cl_5H_3]^+$ - 2 [^{35}Cl], due to the loss of the most dominant isotopic chlorine. This peak was the base ion for α-endosulfan reference standard, while the β-isomer (reference standard) had a relative abundance of 72%. Also, it has been reported to have high intensity, with relative abundance ≥70%.[28,29] There are two routes or pathways for the fragmentation of the m/z at 277 to form m/z 207 (8a). This is either by the loss of a chlorine molecule (Cl_2) or by two consecutive losses of chlorine free radicals. In addition, the loss of C_4HCl_2 (mass unit 122) from m/z 207 (8a) led to the formation of the peak m/z 85, which was observed in the samples, reference standards and

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NIST library. Its relative abundance and intensity ranged from 32 -56 % for *T. cacao* samples, while 32 – 48% and 28% for reference standard and the NIST library respectively for both isomers (Table 1). The peak at m/z 195 is the incident molecular ion from m/z 265 due to the elimination of 2Cl. It was very significant with strong intensity and relative abundance ranging from 84 - 93 % and 75 -96% for α- and β-isomers, respectively, for *Theobroma cacao* samples. It was the most abundant peak besides the base molecular ion at m/z 159 for both isomers. The same trend was observed for the endosulfan reference standard, however in the NIST library it was recorded as the base molecular ion for β-isomer (Figure 4). The loss of 36 mass unit (-HCl), followed by the cleavage of the dichloro methylene bridge led to the formation of $C_7H_6Cl_2$ at m/z 159 - which also is the most abundant molecular ion for *T. cacao* samples and the reference standard.

The peaks at m/z 133 and 99 were observed in all *T. cacao* samples, standard endosulfan and NIST library; these may have been due to the successive exit of C_3H_2Cl or C_3H_3Cl species (74 mass units) and Cl free radical (36 mass units) respectively from m/z 207. Isotopic ^{13}C may have been eliminated in C_3H_2Cl.

3.3 Fragmentation scheme for endosulfan sulphate molecular ions

Characteristic and notable mass spectra peaks obtained for endosulfan sulphate as metabolite in *T. cacao* plant tissue were comparable to those of reference standard and NIST search library (Figure 5). Also, corresponding m/z peaks have been presented in Table 1 alongside the reference standard and NIST search library.

The peak at m/z 422 corresponded to endosulfan sulphate parent molecular ion (M^+), while m/z 424 represented as molecular ion $(M+2)^{+\bullet}$ was attributed to isotopic pattern characteristic of the chlorine atom which also exist as ^{37}Cl isotope. The m/z peaks at 387, 357, 289, 272, 229, 206, 170, 143 and 120 are characteristic for endosulfan sulphate and have been reported

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in biological matrices.[28-30] The fragmentation scheme for the metabolite formed between days 7 and 60, showed the loss of 35/36 mass units ($M^{+\bullet}$ – Cl/HCl), 75 mass units ($M^{+\bullet}$ - $CH_2ClO^{\bullet}$) and 150 mass units ($M^{+\cdot}$ – $SCl_2O_3^{\bullet}$) simultaneously to obtain m/z peaks at 387, 357 and 272, respectively (Scheme 2). The relative abundance of the fragment at peak m/z 272 ranged between 62% and 100% in *T. cacao*. The peak at m/z 272 was obtained as the base peak for reference standard endosulfan sulphate and has been reported as base peak in some biological studies.[26,29] This is contrary to the 62 – 100% range obtained in *T. cacao* samples, while the NIST library base peak was at m/z 387, with the peak at m/z 272 being next most abundant having a relative abundance of about 90%. Further loss of a chlorine atom from the base peak at m/z 272 had resulted to the formation of the m/z peak at 237 in *T. cacao* (54 – 80%), reference standard (90%) and NIST library (51%), with percent relative abundance in parenthesis. The m/z peak at 239 was distinct for plant tissues, with percent relative abundance ranging from 76 - 100%. This peak also showed more relative abundance over m/z 272 in *T. cacao* especially at day 60 in a ratio of 3:2 (Figure 6). The abundance of this peak may have been enhanced by environmental factors, since mass spectra of reference standard and NIST library for endosulfan sulphate showed m/z 272 as the base peak (Figure 5). The m/z peak at 239 has been reported as the base molecular ion for endosulfan lactone.[27,29] Endosulfan lactone is also a metabolite of endosulfan and it is formed from the microbial action on parent endosulfan in the environment.[30,31] The strong presence of this peak in plant tissues portrayed that endosulfan lactone and other metabolites such as endosulfan diol and endosulfan ether may have been formed[32], however these metabolites were not monitored in this study. The intense peak at m/z 289 (62% RA) was due to the loss of $^{\bullet}SO_2$ and Cl_2 from the parent molecular ion ($M^{+\cdot}$ –$Cl_2SO_2^{\bullet}$ or successive losses of $^{\bullet}SO_2$ and $2Cl^{\bullet}$ from precursor molecular ion at m/z peak 387. The loss of $SCl_2O_3^{\bullet}$ from parent molecular ion yielded m/z 272 (i.e, $M^{+\bullet}$ - $SCl_2O_3^{\bullet}$), with a further consecutive loss of C_2H_3O

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yielding of a distinctive m/z peak at 229 (>90% RA). The m/z peak at 206 was as a result of the elimination of CH_2, Cl_4 and SO_2 groups (mass units) from the parent molecular ion or protonated parent molecular ion. The structural representation for the peak at m/z 206 is a carbene carbocation, with the carbene carbon at the methylene bridge of the parent molecular ion for endosulfan (See Scheme 2). Sinha et al., (2004) has reported the formation of carbene in the fragmentation of endosulfan.[26]. The successive losses of 36 (-2OH) and 26 ($-C_2H_2$) mass units from molecular ion at m/z peak 206 resulted in the formation of m/z peaks at 170 and 143, respectively – these peaks are characteristic for endosulfan sulphate in the NIST library. Similarly, the peak at m/z 272 further fragmented by consecutive losses of CH_3Cl_2O and C_2H_2 to yield m/z 170 and 143 respectively, with the two chlorine atoms in 1, 2-positions of the latter molecular ion (dichlorobenzene molecular ion) compared to 1,4-position via the m/z 206 route.

The peak at 143 (dichlorobenzene molecular ion) was observed for the parent isomers and endosulfan sulphate in *T. cacao* samples, reference standards and NIST library (Table 1), while it is reported to have shown prominence in the diol and lactone metabolites.[27,29] This implies that the peak at m/z 143 is characteristic of all endosulfans.

4. CONCLUSION

Commercial grade endosulfan (*α*- and *β*-isomers) and its' major metabolite endosulfan sulphate (a substructure of parent compound) were simultaneously identified successfully after application to *T. cacao* using their reference standards by GC-MS. In addition, the mass spectra for the reference standards and the NIST library were used to confirm the presence of parent isomers, endosulfan sulphate and other metabolites such as the diol and lactone that were formed from environmental activities, although they were not monitored using their reference standards and NIST library spectra. The m/z fragments obtained for residual parent endosulfan and endosulfan sulphate in field kinetic studies were comparable to those of the

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NIST library. The molecular fragment at 143 m/z is a significant peak for the identification and confirmation of endosulfans (α- and β-isomers and metabolites such as sulphate, diol and lactone).

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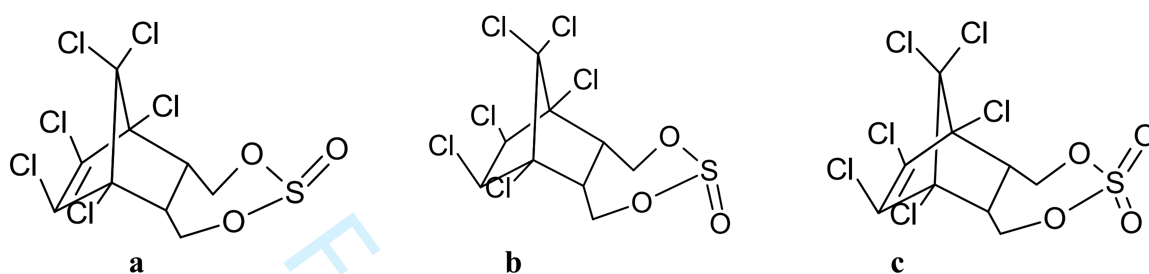

Figure 1. Chemical structures of (**a**) α-endosulfan, (**b**) β-endosulfan and (**c**) endosulfan sulphate

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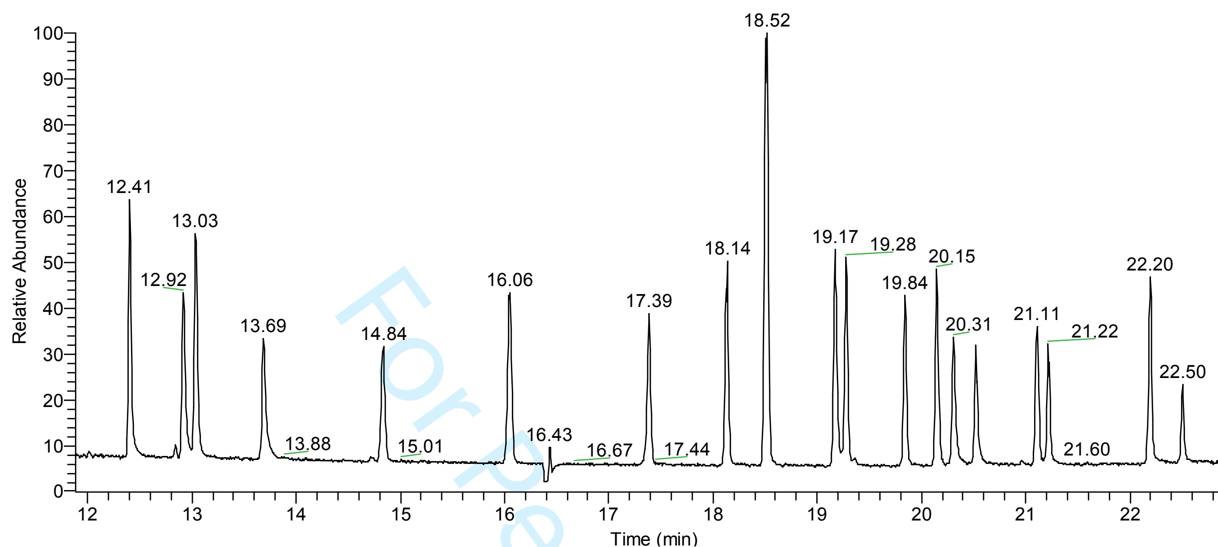

Figure 2. TIC chromatogram of OCPs mixed standard._12.41 –α-HCH; 12.92 – γ-HCH; 13.03 – β-HCH; 13.69-δ-HCH; 14.84-Heptachlor; 16.06-Aldrin; 17.37-heptachlor epoxide; 18.14-trans-chlordane; **18.52**-α-endosulfan; 19.17-ppDDE; 19.28-Dieldrin; 19.84-Endrin; **20.15**-β-endosulfan; 20.31-opDDD; 20.53-Endrin aldehyde; **21.11**-Endosulfan sulphate; 21.22-ppDDT; 22.20-Endrin ketone; 22.50-Methoxychlor

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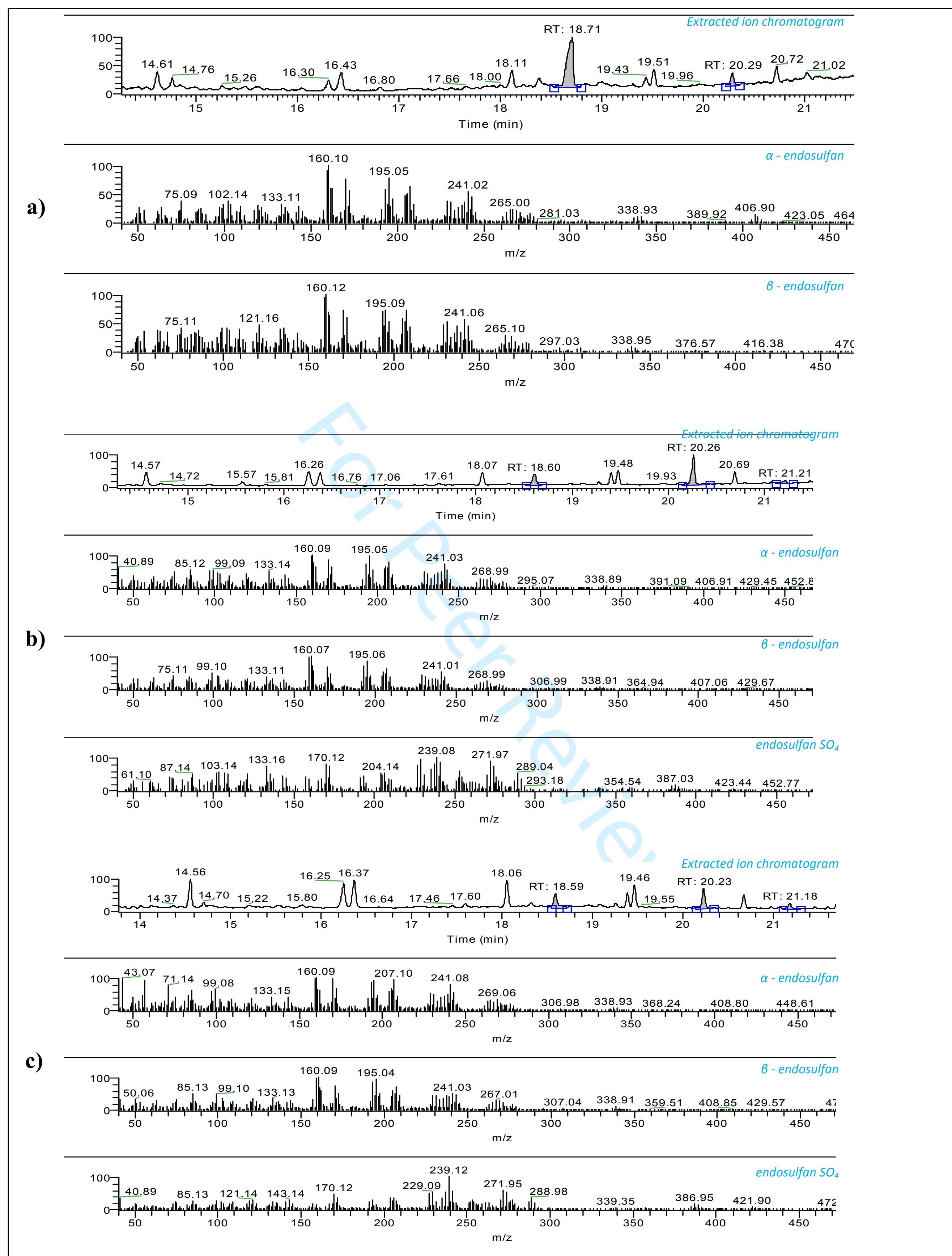

Figure 3: Extracted ion count chromatogram and mass spectra of α-endosulfan, β-endosulfan and endosulfan sulphate **(a)** day 0 in leaves; **(b)** day 14 in cocoa pods; and **(c)** day 60 in stem bark

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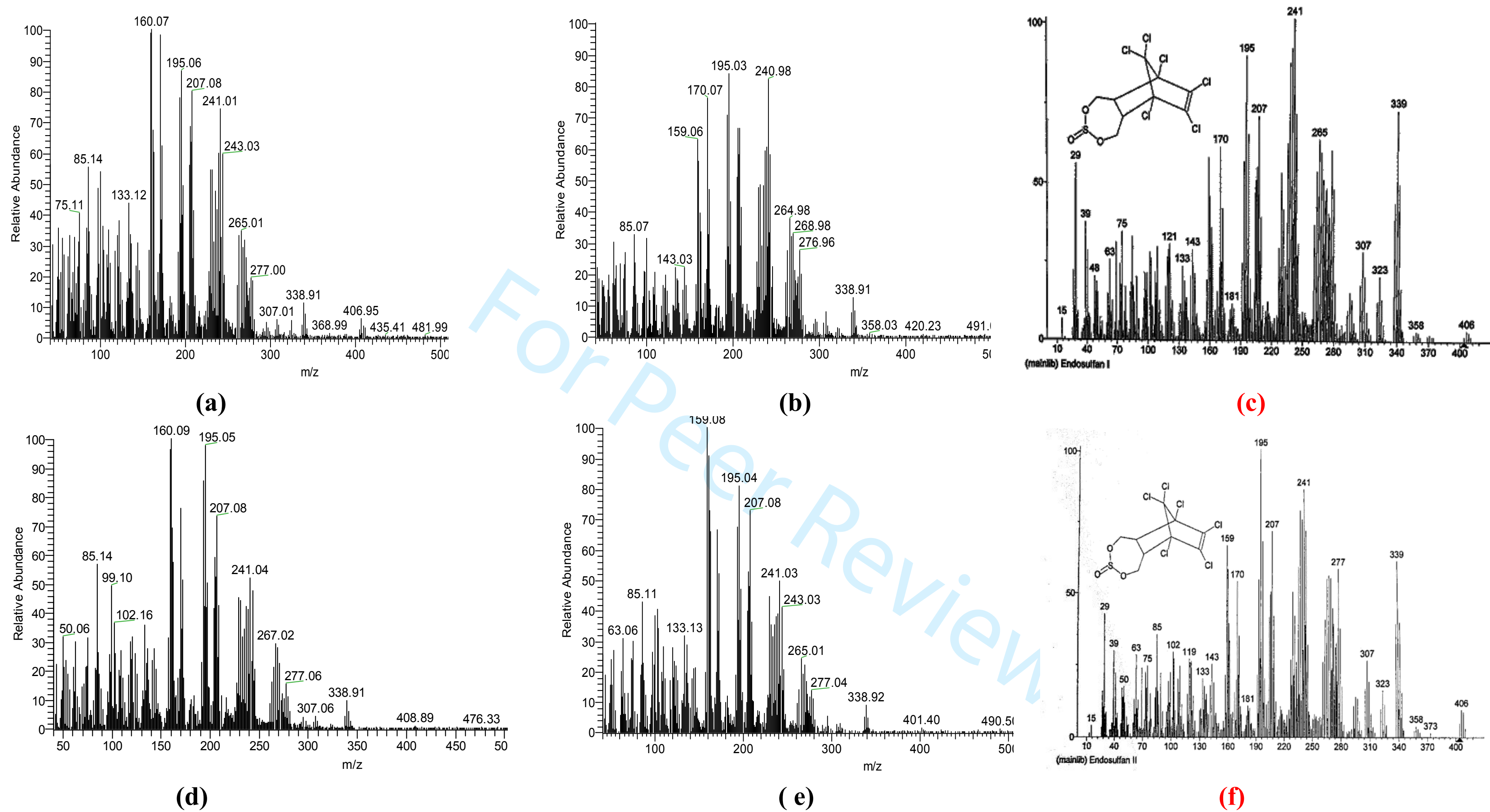

Figure 4. Extracted mass spectra for α-endosulfan in (a) *Theobroma cacao* (b) reference standard (c) NIST library; β-endosulfan in (d) *Theobroma cacao* (e) reference standard (f) NIST library

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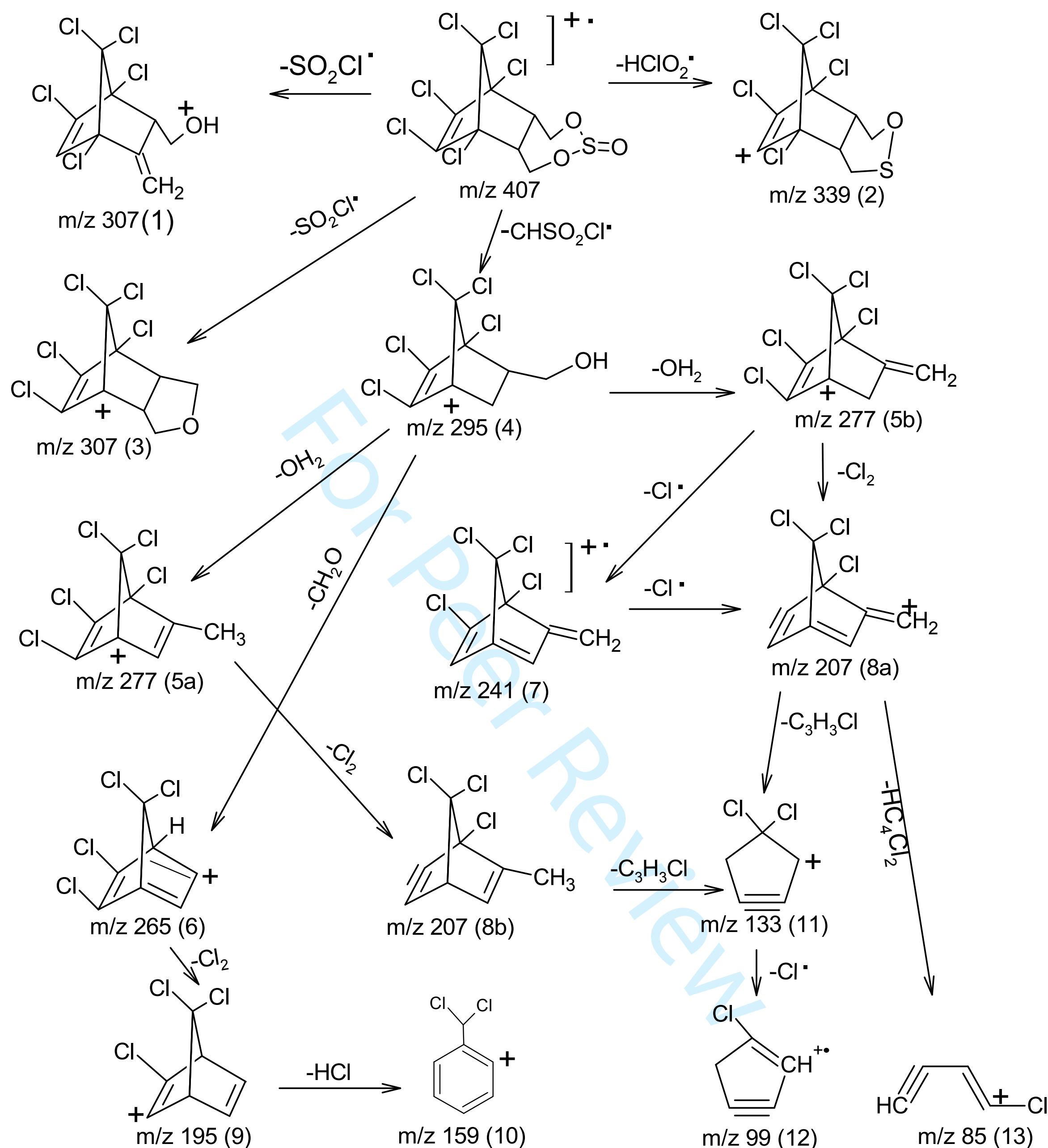

Scheme 1: Proposed fragmentation scheme for α and β-endosulfan and structures of selected molecular ions

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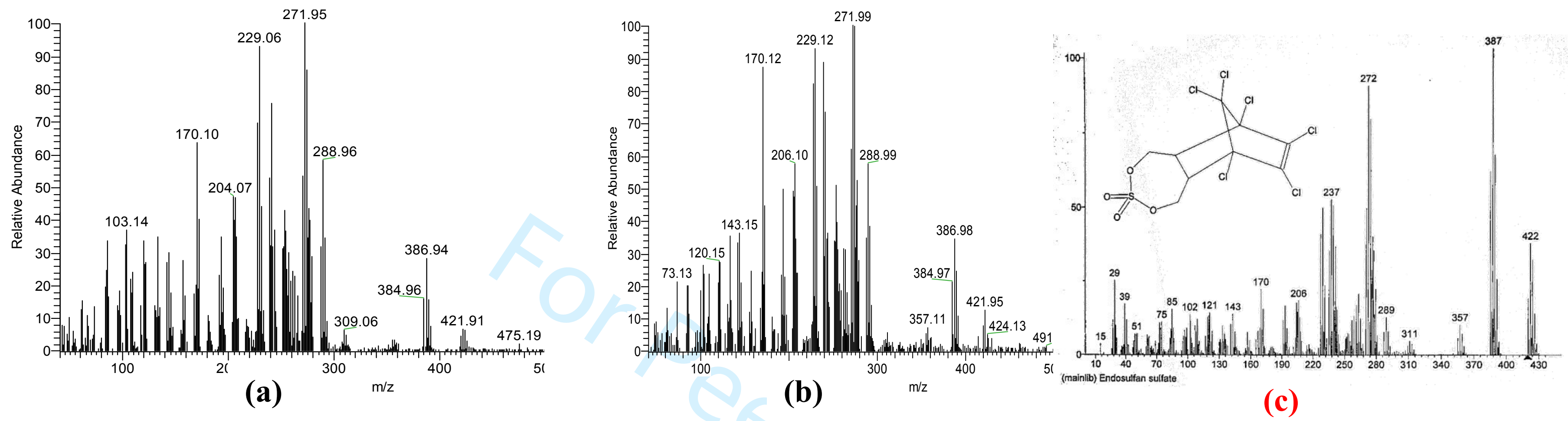

Figure 5: Extracted mass spectra for endosulfan sulphate in (a) *Theobroma cacao* (b) reference standard (c) NIST library (ver. 12)

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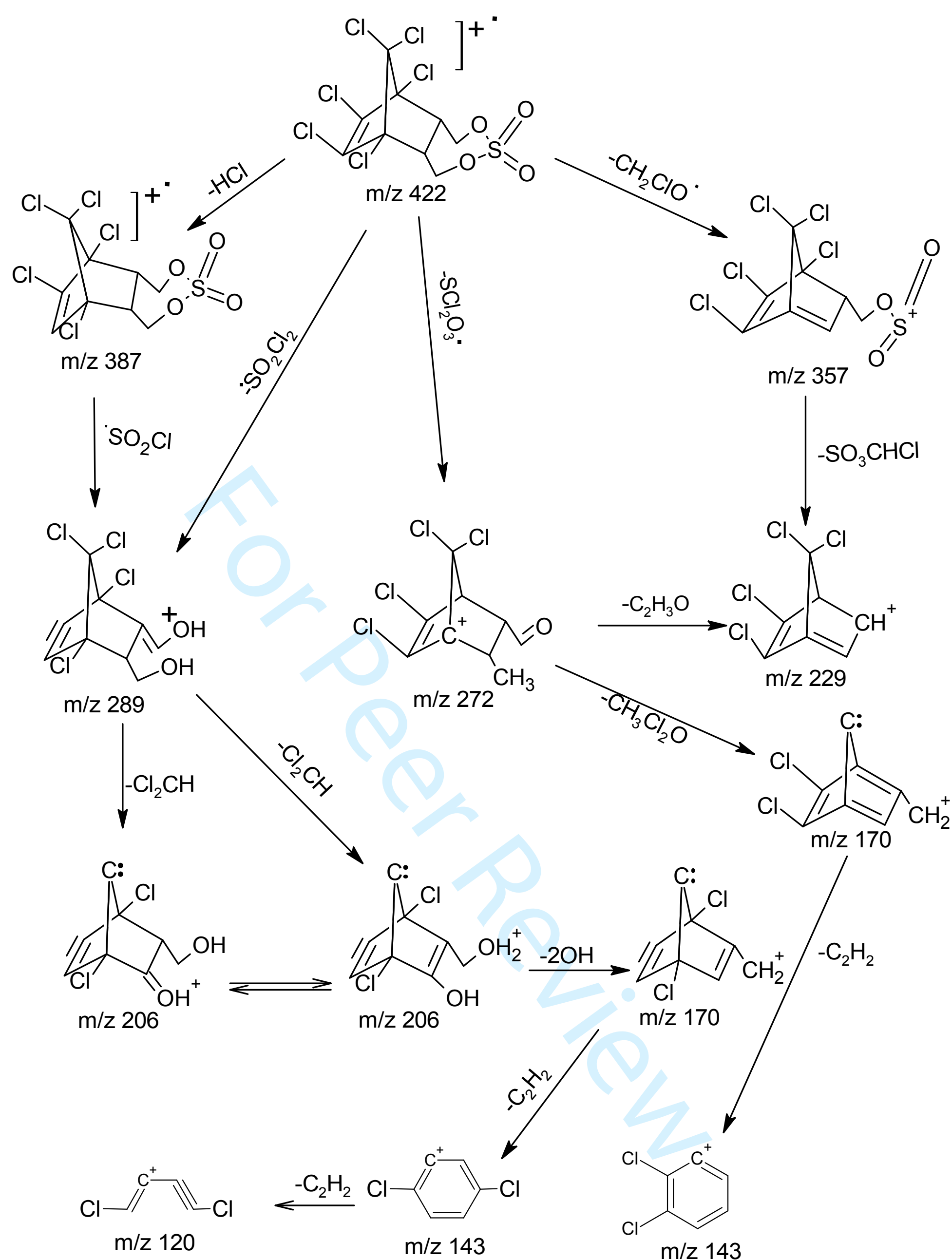

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Scheme 2. Proposed fragmentation scheme for endosulfan sulphate and structures of selected molecular ions

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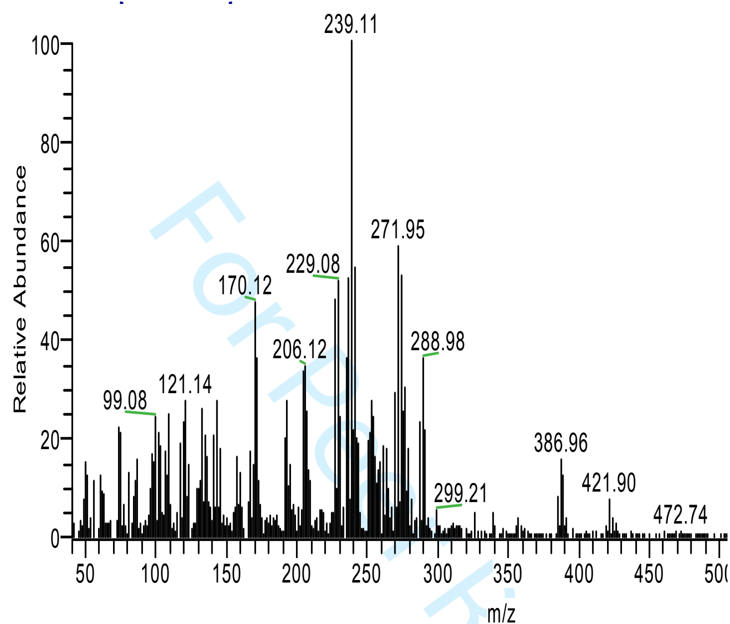

Figure 6. Extracted mass spectrum with base peak at m/z 239 obtained for *Theobroma cacao* at day 60 (characteristic for lactone metabolite)

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Table 1: Selected GC-MS molecular ions for α-, β-endosulfan and endosulfan sulphate in *Theobroma cacao* vegetation, standards and NIST library spectra using EI mode

Parent compound								Metabolite			
α-endosulfan (m/z peaks)				β-endosulfan (m/z peaks)				Endosulfan sulphate (m/z peaks)			
Sample	Standard	NIST	Reference	Sample	Standard	NIST	Reference	Sample	Standard	NIST	Reference
75 (32-40)	75 (26)	75 (32)	-	75 (32-42)	75 (32)	75 (24)	-	75 (43)	73 (20)	75 (10),	75 (30)***
85 (32-56)	85 (32)	85 (28)	-	85 (42 -44)	85 (42)	85 (28)	-	102 (60)		102 (12),	102 (30)***
99	99	99		99	99	99		120 (48)	120 (24)	121 (13)	
133 (32-36)	133 (23)	133 (24)	-	133 (28-45)	133 (32)	133 (22)	-	143 (46)	143 (34)	143 (11)	
143(27)	14324	143(25)	-	143 (25 -38)	143 (21)	143(26)	-	170 (77)	170 (84)	170 (23)	170 (28)***
159 (94-100)	159 (62)	159 (60)	159 (77)* (66) **	159 (98-100)	159 (100)	159 (60)	-	206 (62)	206 (54)	206 (21)	206 (20)***
160(100)	160(58)	160 (48)	-	160 (72-100)	160 (90)	160 (58)	-				
170 (97)	170 (75)	170 (60)		170 (75)	170 (62)	170 (56)	-	229 (92)	229 (91)	229(49)	229(60)***
195 (84 – 93)	195 (84)	195 (89)	195 (100)*, **	195 (75 – 96)	195 (82)	195 (100)	-	237 (54-80)	237 (90)	237(51)	237(40) ***
207 (75-86)	207 (100)	207 (70)	-	207 (57 -78)	207 (74)	207 (72)	-	239 (76-100)			
241(62 - 72)	241 (82)	241 (100)	-	241(40-68)	241 (52)	241 (89)	-	272 (60-100)	272 (100)	272 (89)	272(89)** (100) ***
243 (48 – 58)	243 (54)	243 (70)	-	243 (34 -40)	243 (40)	243 (70)	-	289 (60)	289 (55)	289 (13)	289(10)***
265 (20 -34)	265 (34)	265 (63)	265 (51)* (41)**	265 (18-20)	265 (24)	265 (53)	265 (51)* (41)**	311 (5)	311 (PBND)	311 (5)	357(5)***
277 (15 - 20)	277 (20)	277(60)	-	277 (10 -20 %)	277 (12)	27754	-	357 (4)(PBND)	357 (8)	357 (10)	357 (10)***
307 (7 - 8)	307 (7)	307 (26)	-	307 (8 - 10)	307 (1)	307 (26)	-	387 (28)	387 (32)	387 (100)	387 (100)** (20)***
339 (4- 22)	339 (15)	339 (71)	339 (17)* (61) (41) **	339 (10 - 12)	339 (10)	339 (62)	339 (17)* (61) (41) **	422 (10)	422 (14)	422 (28)	422 (3)** (10)***
407 (7 -30)		406 (5)	407 ****	407 (5 -7)	406 (1)	406 (10)	-		424 (8)		

Note: PBND- Present but not distinct; %- Percentage relative abundance in parenthesis; Reference: *Pfleger, Maurer and Weber (1992). **Sinha *et al.,* (2004). ***Bajaja *et al.,* (2010). **** Sarafin and Winterscheidt (1985a)

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