Fractionation of the Combustion Products of Chlorogenic Acid: Sub-Fraction Containing Catechol Inhibits Cell Proliferation

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Running title: Toxicity of chlorogenic acid combustion products

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1 **TOC Graphic**

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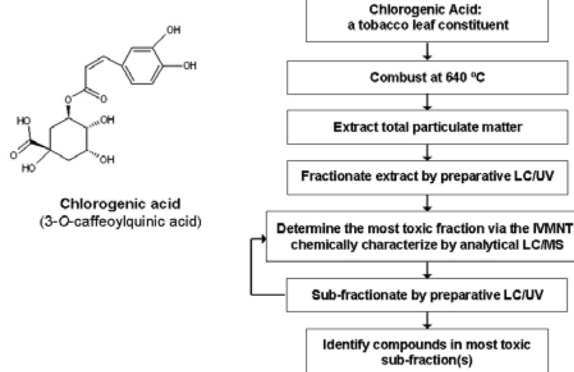

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2 **Abstract**

3

4 Chlorogenic acid is the most abundant polyphenol found in the tobacco plant, however the
5 biological effects of its combustion products remain largely unknown. In this report, various
6 fractions of chlorogenic acid combustion products were tested for the induction of
7 micronuclei in the Chinese hamster fibroblast cells (V79). The combustion products of
8 chlorogenic acid were collected onto Cambridge filters and selectively extracted with
9 dimethyl sulfoxide (DMSO), water, methanol, dichloromethane and ethyl acetate. The DMSO
10 and dichloromethane extracts induced the highest toxicity in the In Vitro Micronucleus Test.
11 However, only the extraction procedure using DMSO was highly reproducible in terms of
12 chemical composition and toxicity. Over forty compounds were identified in the DMSO
13 extract by high performance liquid chromatography coupled to electrospray time-of-flight
14 mass spectrometry (LC/TOFMS). The DMSO extract was fractionated into three major
15 fractions by preparative LC. The fraction inducing the highest degree of toxicity in the
16 micronucleus test was found to contain catechol and its derivatives. Therefore, this fraction
17 was further separated into four sub-fractions. The sub-fraction responsible for the most toxic
18 response was determined to contain catechol as its major component. The overall
19 reproducibility of the combustion, the extraction procedure and the chemical characterization
20 of the compounds responsible for the toxicity in the chlorogenic acid smoke was evaluated by
21 LC/TOFMS.

1 **Introduction**

2

3 Tobacco consists of over 2000 components and upon combustion generates more than 5000
4 compounds (*1*). Due to the highly complex nature of tobacco smoke, the exact mechanisms of
5 toxicity are still unknown. For instance, a number of lists of cigarette smoke toxicants have
6 been published in recent years, some of which have begun to estimate the relative toxicity of
7 the compounds found in tobacco smoke (*2*). However, these approaches are unable to account
8 for the complex chemical profile and potential interactions that may occur in cigarette smoke.
9 Many studies have been carried out on whole tobacco smoke in efforts to determine the
10 correlation between tobacco smoke components and their biological effects (*3-5*).

11

12 An alternative approach is to study the individual components found in leaf tobacco, which
13 upon combustion generate a variety of bioactive species. Among the major groups of
14 constituents found in tobacco, the polyphenol group accounts for about 10 % of the leaf dry
15 weight (*6, 7*). Among the polyphenols, chlorogenic acid (CA) (3-[[3-(3,4-Dihydroxyphenyl)-
16 1-oxo-2-propenyl]oxy]-1,4,5-trihydroxycyclohexanecarboxylic acid) is the most abundant
17 single constituent. It represents about 2.5 to 8 % of leaf dry weight of the tobacco plant (*8, 9*).
18 Several studies have identified components found in smoke from the combustion of CA (*10-*
19 *13*), and other studies have identified CA as well as some of its combustion products as being
20 genotoxic and carcinogenic (*14-16*). Combustion of CA principally generates pyrocatechol
21 (more commonly known as catechol), phenol, hydroquinone, quinide, benzene and benzoic
22 acid. Some of these phenolic compounds were reported to be toxic (*13, 17, 18*) and Hoffmann
23 *et al.* indicated that catechol and phenol enhanced carcinogenic processes induced by other
24 compounds such as polynuclear aromatic hydrocarbons (*19*).

25

26 A few groups have reported toxicological data on individual components found in tobacco
27 smoke (*15, 17, 20-22*). A previous study (Préfontaine et *al.* (*21*)) indicated that of twelve
28 tobacco components tested, the combustion products of the two polyphenols, CA and lignin
29 contained the most bioactive components, evaluated by the In-Vitro Micronucleus Test
30 (IVMNT). The IVMNT is an *in vitro* genotoxicity test used to identify chemicals that induce
31 the formation of small, membrane-bound deoxyribonucleic acid fragments, called
32 micronuclei, in the cytoplasm of interphase mammalian cells (*23-26*). CA is the least complex
33 and most readily available of the two above polyphenolic compounds found in tobacco,
34 therefore, it was chosen for further investigation. The objective of the current study was to

identify the toxic compounds resulting from the combustion of CA. A strategy to combust, extract, fractionate and evaluate the relative toxicity of the combustion products of CA by *in vitro* toxicological assays was designed. Our approach combines analytical chemistry and *in vitro* toxicology to expand knowledge on the toxicity of smoke constituents generated from the combustion products of one single tobacco component, CA.

Experimental Procedures

Chemicals. Chlorogenic acid (CA) (CAS 327-97-9) at ≥ 95 % purity and the following standards used for the combustion reproducibility study were supplied by Sigma-Aldrich (St. Louis, MO) and were of ≥ 99.0 % purity unless otherwise indicated: hydroquinone (123-31-9), phenol (108-95-2), *m*-cresol (108-39-4), *p*-cresol (106-44-5), *o*-cresol (95-48-7), pyrocatechol (120-80-9), resorcinol (180-46-3), 3,4-dihydroxybenzoic acid (90-50-3), caffeic acid (331-39-5), trans-cinnamic acid (140-10-3), ferulic acid (1135-24-6), 2,5-dihydroxybenzoic acid (490-79-9), *p*-hydroxybenzoic acid (99-96-7), 1,2-cyclohexanedione (765-87-7) at 97 % and *p*-coumaric acid (501-98-4) at 98 % purity. Glass wool (Pyrex© brand wool filtering fiber) was purchased from Corning (Big Flats, NY). The HPLC grade solvents used for the filter extraction and the CA combustion reproducibility study were dimethylsulfoxide (DMSO), methanol (MeOH), dichloromethane (DCM), ethyl acetate (EA), acetonitrile (ACN), acetic acid and formic acid, all purchased from Fisher Scientific (Whitby, Ontario, Canada) and used without further purification. Water used for the filter extraction was either distilled water purified using a Milli-Q® system (Millipore, Billerica, MA), which consisted of a carbon cartridge, two high-capacity mixed ion exchange cartridges and a 0.45 µm filter (Chromatographic Specialties, Brockville, Ontario, Canada) or HPLC grade water from Fisher Scientific. Formic acid for HPLC/MS studies was obtained from Fluka (Buchs, Switzerland). Benzoic acid (65-85-0) at 99.5 % purity was supplied by Laboratoire MAT (Beauport, Québec, Canada).

Appropriate ventilation measures and protection of researchers were employed for all manipulations that involved the use of organic solvents and compounds known or suspected to be toxic. The operation of all instruments used in this study was carried out according to the safety procedures recommended by the manufacturers.

1 **Sample Preparation.** Aliquots composed of 0.5 g CA dissolved in 5 mL of MeOH were
2 mixed with the aid of a vortex then deposited onto a matrix of 0.5 g of glass wool in
3 individual Petri dishes. To evaporate the MeOH, the sample was stored for at least 72 h in a
4 conditioned room at 22.5 ^{o}C with 60 % relative humidity. Following the storage period, the
5 corrected mass of CA adsorbed on the matrix was determined to ±1.0 mg by subtracting the
6 glass wool matrix and Petri dish mass (includes CA adsorbed onto the Petri dish) from the
7 total mass of the sample (mass of matrix, CA aliquot and Petri dish).
8
9 **Combustion of CA and Collection/Extraction of the Particulate Phase.** The CA sample
10 adsorbed onto the glass wool matrix was transferred from the Petri dish and packed (7.5 cm
11 bed length) into a quartz combustion tube (outer dimensions: 26.5 × 1.2 cm, wall thickness: 1
12 mm). A John Payne Tar Predictor (JPTP) (John Payne Machinery Spares Ltd., Winchester,
13 UK) apparatus was used to burn CA and collect the particulate phase of its smoke. The quartz
14 tube that contained the CA sample was automatically driven into the furnace where
15 combustion was conducted at 640 ^{o}C ±10 ^{o}C for 2 min. During combustion, atmospheric air
16 was drawn through the quartz tube at 1.8 L/min, forming smoke that passed through the
17 Cambridge filter of diameter 55 mm (Borgwaldt, Richmond, VA) which trapped the
18 particulate phase of the smoke, or total particulate matter (TPM). Silicone grease was used to
19 avoid leaking of smoke from the tubing at specific locations.
20
21 The Cambridge filter was weighed to ± 0.1 mg before and after combustion to determine the
22 mass of collected TPM. The particulate matter collected on the Cambridge filter was extracted
23 under vacuum, using a Büchner funnel, by adding drop-wise a specific volume of solvent as
24 follows. For DMSO extraction, the volume of DMSO used was that needed to obtain a final
25 concentration of 15 mg/mL of TPM, assuming 100 % extraction efficiency. For the other
26 solvents, the extraction volume was fixed at 10 mL per filter to obtain a suitable volume for
27 the subsequent biological assay. The extraction solvent was then evaporated using a rotary
28 evaporator (except when water was used) (Rotavapor-R, Büchi, Switzerland) followed by
29 lyophilization (FreeZone 4.5 L Benchtop Freeze Dry System, Labconco, Kansas, MO). The
30 dry particulate matter (DPM), which refers to the residue remaining after the evaporation of
31 the extraction solvent, was reconstituted in DMSO to give a final concentration of 15 mg/mL
32 of DPM for the water, MeOH and EA extracts and 5 mg/mL for the DCM extract. A more
33 dilute solution of the DCM extract was necessary to maintain a manageable volume since very
34 little DPM was obtained. An “extract” resulted from pooling the extraction solutions of three

1 Cambridge filters unless otherwise stated. Extracts were then aliquoted into 1.5 mL vials and
2 stored in the dark at -80 ^{o}C. All toxicity and chromatography experiments using the extracts
3 were performed in duplicate, unless stated otherwise.
4
5 **Mammalian Cell Cultures.** The cellular lineage used for the IVMNT assay was an
6 internationally registered V79 Chinese hamster cell line (lung fibroblast) obtained from the
7 European collection of Cell Cultures (V79 86041102 lot 04/C/016). Cells were cultured in
8 complete culture medium (Dulbecco Modified Eagle Medium, DMEM; Gibco, Grand Island,
9 NY) supplemented with 10 % (v/v) heat-inactivated Fetal Bovine Serum (FBS) and 0.5 %
10 (v/v) penicillin/streptomycin (50 units/mL, 50 μg/mL), both from Gibco. Cells were
11 resuspended by trypsinization (0.1 % Trypsin, 1.06 mM EDTA; Gibco) at 37 ^{o}C.
12 Subcultivation of cells was performed two times per week (1.0 to 2.0×10^5 cells) into a
13 75 cm^3 Corning flask.
14
15 ***In Vitro* Micronucleus Test (IVMNT).** The IVMNT was performed with V79 Chinese
16 hamster fibroblast cells without metabolic activation (S9 fraction). Cells were grown in
17 25 cm^3 flasks at a concentration of 5.0×10^5 cells/mL in 10 mL of DMEM for 24 h. The
18 culture medium was then replaced by the DMSO-dissolved extracts added to DMEM at the
19 following concentrations to which the cells were exposed for three hours: 5, 10, 15 and 20 μg
20 of DPM (or TPM) per mL of DMEM. The positive control was mitomycin C (MMC, 0.8
21 μg/mL; Sigma-Aldrich) and the negative control was DMSO (1 % (v/v) in DMEM). After the
22 3 h exposure, cells were rinsed twice with Hanks' Balanced Salt Solution (HBSS, Gibco) and
23 re-incubated for 17 h in DMEM containing 3 μg/mL cytochalasin B (which blocks cellular
24 division, but does not block nuclear division). Cells were harvested by trypsinization, re-
25 suspended in culture medium at 1.0×10^5 cells/mL and centrifuged onto microscopic slides at
26 1200 rpm for 8 min using a Cytospin 3 (Shandon, London, UK). Slides were then air dried,
27 fixed in 90 % methanol (9 min at -20 ^{o}C) and stained with Acridine Orange solution for 30 s
28 (12.5 mg/100mL of 1X-PBS; Sigma-Aldrich). Finally, slides were scored at 400×
29 magnification according to Fenech's criteria (*27*). The percentage of micronuclei, which is a
30 measure of genotoxicity, was determined by first selecting 1000 binucleated cells and then
31 counting the number of these having at least one micronucleus detected, as follows:
32

1 *Micronuclei (%)*

2 $$\% \, Micronuclei = \left(\frac{No. of \ binucleated \ cells \ with \ one \ or \ more \ micronuclei}{Total \ No. of \ binucleated \ cells} \right) \times 100$$

3

4 where a micronucleus is defined as a particle surrounded by distinct borders, having a
5 maximum of one third the size of the main nucleus and lying inside the cytoplasm (*28*). The
6 percentage of inhibition of cell proliferation was calculated by first determining the
7 Cytokinesis-Block Proliferation Index (CBPI) (*27*) as follows:

8

9 *Inhibition of cell proliferation (%)*

10 $$CBPI = \left(\frac{No. binucleated \ cells + 2[No. of \ tri\text{-}, tetra\text{-} \ and \ multi\text{-}nucleated \ cells]}{Total \ No. cells \ - \ mitotic \ cells} \right)$$

11 $$\% \, Inhibition \ of \ cell \ proliferation = 100 - \left(\left[\frac{mean \ CBPI \ sample \ dose}{mean \ CBPI \ solvent \ control} \right] \times 100 \right)$$

12
13

14 The average and relative standard deviation (RSD) for the percentages of micronuclei and
15 inhibition of cell proliferation were calculated from duplicate experiments.

16

17 **Reproducibility Study of the CA Combustion.** The precision of the combustion of CA was
18 evaluated by HPLC using a Waters 2695 Separation Module with a Waters 715 Ultra Wisp
19 automatic injector (Milford, MA). Detection was achieved with a Waters 2475 Multi
20 wavelength fluorescence detector (FD). The instrument was controlled by ChemStation Plus
21 Family software version A.08.03 (Agilent Technologies). Separation was achieved on a
22 Spherisorb, ODS2 analytical column (5 µm particles, 4.6 × 150 mm) from Waters.

23

24 Reproducibility of the combustion method was determined by comparing the quantity of
25 selected phenolic compounds obtained from four different combustions (*24, 29*), but using
26 only 25% of the TPM from each. A quarter of each Cambridge filter (one per combustion)
27 was extracted with 10 mL of 1% (v/v) aqueous acetic acid for 30 min on an orbital shaker.
28 The four extracts from the four combustions were each filtered through a 0.45 µm filter, of
29 which, 2 mL was collected for analysis by HPLC/FD. The volume of each extract injected
30 was 10 µL. Separation was achieved by gradient elution (0 to 100 % ACN in 1 % (v/v)

aqueous acetic acid over 46 min) at a mobile phase flow rate of 1.2 mL/min. The total run time was 66 min. Quantification was achieved by external calibration as follows. A stock solution of 1.00 mg/mL of each standard compound was prepared in 1 % (v/v) aqueous acetic acid. From the stock solutions, six working solutions, ranging from 0 to 50 µg/mL, were prepared in 1 % (v/v) aqueous acetic acid, filtered through a 0.45 µm filter and transferred into 2 mL amber vials. A 20 µL volume of each working solution was injected in duplicate and a standard calibration curve was made by plotting the concentration of the working solutions versus their respective peak areas.

Reproducibility Study of the Extraction with DMSO and DCM. HPLC/MS was used to assess the precision of the DMSO and DCM extraction procedure. The instrument consisted of an 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) directly interfaced with an Agilent electrospray ionization single quadrupole mass spectrometer (LC/MSD). Injections of 5 µL (75 µg of product per injection) of DMSO or DCM extracts (the latter having been re-suspended in DMSO) were made onto an Eclipse XDB-C18 analytical column (5 µm particles, 4.6 × 150 mm) from Agilent Technologies. Separation was achieved using a gradient elution of 0 to 80% MeOH in 0.1 % (v/v) aqueous formic acid over 24 min at a flow rate of 0.5 mL/min. The total run time was 30 min. For mass spectrometric detection, ions were generated in negative electrospray mode with 4000 V applied on the capillary. The fragmentor was set at 70 V and the drying gas (N2) was heated at 300°C and run at 10 L/min. Spectra were acquired from m/z 75 to m/z 575 at a rate of 0.94 s/cycle. The reproducibility of the method of extraction by DMSO and DCM was determined by comparing the peak areas (for duplicate injections) of the following thirteen phenolic reference compounds consistently found in the four different DMSO extracts: catechol, hydroxyquinone, 4-methylcatechol, 4-vinylcatechol, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, 4-ethylcatechol, 1-(3,4-dihydroxyphenyl) ethanone, p-coumaric acid, coumaric acid, hydrocaffeic acid, quinic acid and caffeic acid methylester.

Analytical Separation of the DMSO Extract. Accurate mass-based identification of several products found in the DMSO extract was achieved using an Agilent 1100 HPLC system directly interfaced with an electrospray ionization Time-of-Flight mass spectrometer (LC/TOFMS) 6120 series from Agilent Technologies. The LC/TOFMS instrument was controlled by Agilent Mass Hunter software, and the data was processed by Analyst QS software (Agilent Technologies/Sciex). Samples were diluted 1:100 in HPLC grade water and

1 2 μL aliquots (0.3 μg of product per injection) were injected onto the Eclipse XDB-C18
2 analytical column. The chromatographic separation was performed in gradient mode (0 to 80
3 % MeOH in 0.1 % (v/v) aqueous formic acid over 45 min) at a flow rate of 0.5 mL/min. The
4 total run time was 60 min. For MS detection, ions were generated in negative electrospray
5 mode with 4000 V applied on the capillary. The fragmentor was set at 200 V and the heated
6 drying gas (N_2 at 350°C) was run at 12 L/min. Spectra were acquired from m/z 50 to m/z 300
7 at a rate of 0.94 s/cycle.
8
9 **Preparative Fractionation of the DMSO Extract.** The LC system used for preparative
10 fractionation of the DMSO extract consisted of a Gilson 215 LC Handler with 156 UV-VIS
11 absorbance detector (Middletown, WI) directly interfaced with an LCQ single quadrupole
12 mass spectrometer from Thermo Fisher Scientific (Waltham, MA). The instrument was
13 controlled by XCalibur software, version 1.3 (Thermo Fisher) and Gilson Unipoint software.
14 DMSO extracts (15 mg/mL) were injected (1.8 mL) and separations were performed on a
15 Prevail C18 preparative column (5 μm particles, 22 × 250 mm) from Alltech (Lexington, KY)
16 by gradient elution (0 to 80 % MeOH in 0.1 % (v/v) aqueous formic acid over 20 min) at a
17 flow rate of 15 mL/min. The total run time was 30 min and the UV signal was recorded at 254
18 nm concomitant to monitoring the MS signal. Fractions of 8 mL each were collected every
19 39.1 s into borosilicate disposable culture tubes (10 × 100 mm; Fisher Scientific) and then
20 pooled to give three major fractions spanning the following time intervals: 0-14.2 min, 14.2-
21 23.5 min and 23.5-30 min. A second injection of 1.8 mL (27 mg) was treated identically and
22 pooled with the corresponding major fractions from the first injection in round bottom flasks.
23 The three (pooled) fractions were reduced in volume using a rotary evaporator for
24 approximately 10 min at 30 ^{o}C under a moderate rotation speed. The flasks were then
25 immersed and rotated in acetone/dry-ice to induce uniform sample freezing. Finally, the
26 remaining liquids were lyophilized overnight and re-suspended in 50% MeOH (aq),
27 transferred into pre-weighed vials which were again rotavapped, lyophilized and weighed to
28 obtain the correct mass for each fraction. The quantities of the products obtained were, 39.5
29 mg, 22.5 mg and 7.9 mg respectively, for the first through third pooled fractions. The
30 fractions were stored at -80 ^{o}C in clear glass vials. Approximately 29 % more material was
31 collected than was injected (69.9 mg collected versus 54 mg injected, by calculation). This
32 discrepancy is probably due to residual DMSO in the first fraction that can not be entirely
33 evaporated by lyophilization.
34

Chemical Characterization and Separation of Fraction 2. The LC/TOFMS system described above was used for the chemical characterization of the most bioactive fraction of the DMSO extract. This was achieved by first using the lower resolution LC/MSD system (see section on Reproducibility studies) to optimize the separation of a test mixture representative of fraction 2, comprised of the following 7 standards: caffeic acid, benzoic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, trans-cinnamic acid, 4-hydroxy-3-methoxycinnamic acid and 2,5-dihydroxybenzoic acid. This test mixture was injected onto four different stationary phases: Synergi Polar-RP (4 µm, 4.6 × 150 mm), Synergi Hydro-RP (4 µm, 4.6 × 150 mm), Gemini C18 (5 µm, 4.6 × 150 mm) and Gemini C6-Phenyl (5 µm, 4.6 × 150 mm), all from Phenomenex (Torrance, CA). Separations were carried out under nine different gradient elution conditions by varying the initial MeOH concentration as follows: 10, 15, 20, 25, 30, 35, 40, 45 and 50 %, in 0.1 % (v/v) aqueous formic acid, with the gradient applied up to 75 %, over the first 24 min in each case. The best gradient conditions were transferred to the higher resolution LC/TOFMS instrument and applied to the separation of fraction 2 components. Samples were first diluted 100 fold in 50 % MeOH (aq) to make them compatible with the dynamic range of the LC/TOFMS, then injections of 2 µL (corresponding to 0.3 µg of product) were made on the four columns listed above. The total run time was 30 min at a flow rate of 0.5 mL/min.

Preparative Sub-fractionation of Fraction 2. To sub-fractionate "fraction 2" of the DMSO extract by preparative LC, an injection of 2.0 mL was made on the instrument described for preparative fractionation of the DMSO extract. Samples (4.24 mg/mL in 75% (v/v) MeOH (aq)) were injected in duplicate and separations were performed on an AXIA packed Synergi Polar-RP preparative column (4 µm particles, 21.2 × 100 mm) from Phenomenex. A Polar-RP security guard prep cartridge (15 × 21.2 mm) from Phenomenex was installed upstream of the preparative column. The chromatographic separation was performed in gradient mode (15 to 75 % MeOH in 0.1 % (v/v) aqueous formic acid over 20 min) at a flow rate of 6 mL/min. The total run time was 30 min and the UV signal was monitored at 254 nm concomitant with the MS signal. Fractions of 4 mL each were collected every 19.8 s into borosilicate disposable culture tubes (10 × 100 mm; Fisher Scientific) and then pooled to give four large sub-fractions spanning the following time intervals: 12.0-15.2 min, 15.2-16.4 min, 16.4-21.2 min and 21.2-30 min. Each pooled sub-fraction was placed in a round-bottom flask and was treated as described above during the first fractionation step. The amounts of product obtained for the first through fourth pooled sub-fractions were 2.16 mg, 1.49 mg, 5.87 mg and 9.07 mg

respectively. A 1.8 mg portion of (major) fraction 2 of the DMSO extract was used for control studies. Samples were kept at -80°C in clear glass vials until utilization.

Chemical Characterization of Sub-fraction 1. The LC/TOFMS system described above was used for the accurate mass identification of products present in the sub-fraction displaying the highest toxicity. Samples were diluted 1:100 in 50 % (v/v) MeOH (aq) and injections of 2 µL aliquots were performed on the Polar-RP column (4 µm, 4.6 × 150 mm) followed by separation by gradient elution (0 to 80 % MeOH in 0.1 % (v/v) aqueous formic acid over 24 min) at a flow rate of 0.5 mL/min. The total run time was 15 min.

Statistical Analysis. The results for the combustion reproducibility study were tested for comparison of linearity between different groups of either extracts or fractions using the Analysis of Covariance (ANCOVA) method. For comparison between the DMSO extracts, the percentages of micronuclei (genotoxicity) and inhibition of cell proliferation were taken as the direct quantitative variable, the dose of exposure as the quantitative dependent covariable and the extraction solvent was taken as the qualitative covariable for two replicates. ANCOVA compares the dose-response linearity between each extract. Significant differences between extracts were determined by the Duncan's multiple comparison test and were considered significant when $p < 0.05$. Toxicological data obtained from the IVMNT for the different solvent extracts and fractionation studies were analyzed using XLSTAT software, version 7.5 (Addinsoft© Brooklyn, NY).

ANOVA was used to evaluate the toxicity results where the dose, the CA extracts, the fractions and the sub-fractions were all considered as factors. The dose by extract/fraction interaction was also included in the model. In order to assess differences between the CA extracts/fractions for the different doses, the dose by extract interaction was investigated using multiple comparisons. More specifically, the extracts/fractions were analyzed by the Fisher least significant difference multiple comparison test with a Bonferroni correction to type 1 error to ensure that the overall risk was kept under $\alpha = 5\%$. In all cases, the background level of genotoxicity generated by the control solvent (1% DMSO) was subtracted from the micronuclei percentage values for all samples. As a result of the statistical analysis, the data were grouped as follows: A, B or C. Samples sharing the same letter lie within the same group and are not statistically different.

1 **Results and Discussion**

2

3 The various toxicological studies carried out on tobacco smoke have been generally related to
4 the combustion products of whole tobacco (*30-32*). The aim of our study was to characterize
5 the toxicity of the combustion products of one individual tobacco component, CA, which is
6 the major polyphenolic component of tobacco. A few toxicological studies have reported on
7 the genotoxicity of CA (*14*) and its combustion products (*13*), (*19*), (*21*). In addition, some
8 chemical studies have been published on the identification of CA combustion products (*10*)
9 (*12*). However, no previous study on relating genotoxicity to the chemical composition of the
10 combustion products of CA has been made. The combustion conditions used in this study
11 were chosen based on the range of temperatures found during the combustion of cigarettes,
12 which occur between 300 °C and 900 °C and higher (*33*). The precision of our chemical
13 analyses required a robust and reproducible means of simulating the combustion of CA, which
14 is why the JPTP apparatus was employed. Furthermore, this study was carried out at a single
15 combustion temperature of 640 °C for simplicity.

16

17 **Reproducibility Study of the CA Combustion.** In order to understand and quantify any
18 variability in the toxicological and/or chemical analyses, it was deemed important to evaluate
19 the precision of the CA combustion method. This was assessed by comparing: a) the phenolic
20 content in four different extracts by HPLC/FD and b) the genotoxicity and the degree of
21 inhibition of cell proliferation between three of the four extracts using the IVMNT. The
22 Cambridge filters were extracted with 1% (v/v) aqueous acetic acid for this study because this
23 solution is known to extract phenolic compounds well (*29*).

24

25 HPLC/FD showed that the concentration of hydroquinone, resorcinol, catechol and phenol
26 (reported as a function of the quantity of TPM extracted per quarter filter) varied with an
27 average RSD of 15.5% (and median RSD of 12.6%) (Table 1). *Para*-cresol was often below
28 the limit of quantification. The high polarity of the solvent may have impeded the extraction
29 of *p*-cresol, thus explaining why the latter was barely detected. To evaluate the relative
30 proportion of each compound, their concentrations were normalized relative to hydroquinone
31 (Table 2) for each experiment to eliminate the sampling error associated with extracting only
32 ¼ of the filter pad. The relative (i.e. normalized) concentrations of the phenolic compounds
33 resorcinol, catechol and phenol determined by HPLC/FD, showed an average of 11.1 % RSD
34 (Table 2). As seen in Table 1, the concentration of phenol varied the most among the 4

combustions; its RSD was over twice that of the other phenolic compounds. Although phenol is the most volatile of the five species, ineffective trapping was ruled out as a source of its high variability because the temperature did not exceed 45 °C at the Cambridge filter pad position.

The IVMNT method was chosen to measure the extracted TPM bioactivity because it is one of the *in vitro* toxicity tests recommended for tobacco smoke studies by the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) and Health Canada (*23, 25, 26*). Overall, although a variation of 11.1 % in normalized phenolic content was present between the extracts, this did not translate into a similar variation in bioactivity. The IVMNT data (Figure 1a and 1b) showed that the percentages of micronuclei and inhibition of cell proliferation among different extracts were not significantly different as per the Duncan's multiple comparison test. Therefore, we decided to continue with this method of combustion using the JPTP. However, to reduce the impact of the high variability between combustions, we pooled the extracts from three independent combustions to obtain one final pooled extract, which was then divided into equal aliquots and stored at -80°C for subsequent toxicological and chemical assays.

Effect of Extraction Solvent. Selective solvent extraction was used to initiate the chemical characterization study of CA combustion products. The five solvents, used in parallel, were DMSO (polarity index (P)=7.2, dipole moment (DM)=3.96), water (P=9, DM=1.85), MeOH (P=5.1, DM=1.70), DCM (P=3.1, DM=1.60) and EA (P=4.4, DM=1.78), thus yielding five different extracts. These solvents were chosen due to their different polarity index values and because of the limited selectivity and high variability observed with 1% (v/v) aqueous acetic acid as an extraction solvent. Schlotzhauer and Chortyk showed that tobacco directly extracted with solvents of various polarity or "extraction strength" yielded extracts of different chemical composition (*34*). According to their miscibility and polarity index, the most hydrophilic products are preferentially extracted by DMSO, water and MeOH, whereas less hydrophilic products are found in the DCM and EA extracts. Generally, the phenolic compounds have amphiphilic properties, and thus should be found in every extract. In a previous work (*35*), GC/MS analysis of the five extracts of CA combustion products showed the presence of phenolic compounds such as catechol, phenol, hydroquinone, ethyl catechol, benzoic acid and quinic acid in most of the extracts.

Based on their chemical composition and the relative amounts of each combustion product, certain extracts among the five tested were expected to induce a higher degree of genotoxicity and/or inhibit cell proliferation than others by the IVMNT. The percentage of micronuclei, or genotoxicity, is shown in Figure 2a for the five extracts. At a dose of 20 µg/mL, the genotoxicity induced by the DCM extract was significantly different from the water and MeOH extracts, but not from the DMSO and EA extracts. The inhibition of cell proliferation is shown in Figure 2b for the five extracts. At doses of 5 and 10 µg/mL there was no statistically significant difference in the genotoxicity induced, as evaluated by ANOVA. However, at doses of 15 and 20 µg/mL, the inhibition of cell proliferation induced by the DCM extract was significantly different from all other extracts except DMSO at 15 µg/mL, and except water and DMSO at 20 µg/mL. The negative values observed for the inhibition of cell proliferation reflect cell growth. Overall, the DMSO and DCM extracts induced higher biotoxicity compared to extracts obtained using water, EA and MeOH.

DCM is the least polar of the solvents tested and thus would be expected to extract phenolic compounds, which are known to be bioactive. DMSO on the other hand possesses excellent solvating powers; it dissolves both polar and non-polar compounds. Furthermore, a low concentration of DMSO (1 % (v/v) in DMEM) has low toxicity (*36*), which was why the other extraction solvents were reconstituted in DMSO for the IVMNT assays.

Reproducibility Study of the Extraction with DMSO and DCM. Based on the results comparing extraction by five different solvents, the precision of the DMSO and DCM extraction procedures was evaluated by LC/MS to ensure a robust and reliable method. Four independent combustion experiments were carried out for both DMSO and DCM. Each extract obtained was injected in duplicate. The abundance (peak areas) of thirteen reference compounds found in the extracts were monitored (Table 3). Retention times were highly reproducible (≤ 0.1% RSD) across the four extracts tested for both extraction solvents. The peak area precision of the DMSO extraction (< 10 % RSD) was nine times better than that of DCM (data not shown). This may have been due to the volatile nature of DCM; evaporation may have occurred during the extraction procedure leading to less reproducible results. Therefore, DCM extraction was not further investigated. In addition, DMSO was observed to extract a larger number of compounds, which is in keeping with its good solvating strength. With respect to biological activity, the DMSO extracts were not further tested by the IVMNT since the results above showed that variation in genotoxicity and inhibition of cell

proliferation was minimal even though phenolic content varied greatly (15.5 % average RSD, Table 1).

Analytical Separation of the DMSO Extract. Accurate mass determination by LC/TOFMS was used to identify the main components, and class of components, in the whole DMSO extract (Figure 3). Over forty compounds were identified by negative ionization mode, which was used because the majority of the combustion products possessed alcohol and/or acidic functional groups. These results guided the choice of which fractions to pool for preparative LC.

Several phenolic compounds were present in the DMSO extract, which is consistent with previous studies of CA (*10, 12, 13*). Based on the complexity of the combustion products of a single tobacco component like CA, we can only begin to imagine the complexity of whole tobacco smoke. Although the DMSO extract was bioactive according to the IVMNT, it was difficult to identify the specific compounds responsible for bioactivity. Therefore, it was necessary to further simplify the extract. Some potential techniques to achieve this include: filtration, centrifugation, liquid-liquid extraction, solid-phase extraction and sample fractionation, among others. Fractionation by preparative scale LC was chosen based on its ability to divide the sample into precise portions having sufficient quantity for further analysis by the IVMNT.

Preparative Fractionation of the DMSO Extract. The DMSO extract was fractionated by preparative LC/UV (detection at 254 nm) into three major fractions, as indicated by the dotted lines in Figure 3. This allowed for determination of the difference in toxicity between fractions and presumably a convergence on the compounds responsible for the observed toxicity. The first fraction, which was selected to include quinic acid-related compounds and other non UV-absorbing species, contained 39.5 mg of product. The second fraction (22.5 mg) included catechol and its derivatives while the third (7.9 mg) comprised more hydrophobic compounds. Biotoxicity was assessed by the IVMNT. As illustrated (Figures 4a and 4b), among the three major fractions tested, fraction 2 induced the highest percentages of micronuclei and inhibition of cell proliferation compared to fractions 1 and 3. The increased level of toxicity generated by fraction 2 was likely due to the presence of phenolic compounds found in that fraction. Figure 4a shows that the whole DMSO extract as well as the second fraction induced the highest percentage of micronuclei. However, only the (whole) DMSO

extract induced a significantly higher percentage of micronuclei at a dose of 35 μg/mL. Figure 4b shows that fraction 2 and the (whole) DMSO extract induced a significantly higher inhibition of cell proliferation compared to fractions 1 and 3 at doses of 15 to 35 μg/mL.

Chemical Characterization and Separation of Fraction 2. Due to its overall higher bioactivity, the second fraction was re-analyzed by LC/TOFMS with accurate mass measurement to assess its chemical composition. Fraction 2 was found to contain catechol and its derivatives (methyl-catechol, ethyl-catechol and vinyl-catechol), phenol, hydrocaffeic acid, 1-(3,4-dihydroxyphenol)ethanone, 3,4-dihydroxybenzoic acid, *p*-hydroxycinnamic acid, *p*-coumaric acid, caffeic acid methylester, caffeic acid and hydroxybenzoic acid (Figure 3). Among these, the last 8 compounds (hydrocaffeic acid to hydroxybenzoic acid) have not been previously reported as carcinogens, mutagens or teratogens as opposed to catechol, phenol and caffeic acid (*37*). As previously discussed, catechol and its derivatives are known to be responsible for induction of micronuclei and toxicity in the micronuclei assay (*18*) and thus could be responsible for the increased level of bioactivity of fraction 2.

The analytical separation of fraction 2 was optimized with respect to peak resolution with the objective of sub-fractionating it for further analysis to identify the compounds responsible for its bioactivity. Based on the compounds identified in fraction 2, a test mixture of seven standard compounds was prepared and a series of different stationary phases and eluant compositions were evaluated on the LC/MSD instrument as described in Materials and Methods. The best resolution for the test mixture was obtained with a 15 (or 20) to 75 % MeOH in 0.1 % (v/v) aqueous formic acid gradient over 23 min using the Polar-RP column (data not shown). This column, which is composed of an ether-linked phenyl stationary phase with polar end-capping, most likely enabled a more selective interaction with the aromatic compounds improving their resolution. Subsequently, fraction 2 was analyzed under the optimized conditions by LC/TOFMS. This enabled separation of the quite abundant and bioactive catechol from three isomers of hydroxybenzoic acid (data not shown).

Preparative Sub-fractionation of Fraction 2 and Chemical Characterization of Sub-fraction 1. The optimized analytical separation conditions used for fraction 2 of the DMSO extract were transferred to a Polar-RP preparative column for sub-fractionation. Figure 5 shows how we generated the four major sub-fractions of fraction 2 by preparative LC. The genotoxicity induced by these four sub-fractions, as well as by major fraction 2 and by the

1 whole DMSO extract is shown in Figure 6a. No statistically significant difference (α=0.05)
2 was measured by ANOVA between the six samples compared at the lower dose range (5 and
3 10 µg/mL). Whereas, at the dose ranges corresponding to 15 and 20 µg/mL there were
4 statistically significant differences in terms of generation of micronuclei between sub-fraction
5 4, the DMSO extract and sub-fraction 2. The inhibition of cell proliferation induced by the
6 four sub-fractions, by fraction 2 and by the whole DMSO extract is compared in Figure 6b.
7 Sub-fraction 1 and the DMSO extract induced a higher percentage of inhibition of cell
8 proliferation but were only significantly higher compared to sub-fractions 2 and 3 at doses of
9 15 and 20 µg/mL. Overall, the IVMNT showed that sub-fraction 1 induced the highest degree
10 of genotoxicity and inhibition of cell proliferation compared to the other sub-fractions.
11
12 Subsequently, sub-fraction 1 was analyzed by LC/TOFMS (Figure 7) and found to contain
13 catechol as the major component, 3,4-dihydroxybenzoic acid and a third, less abundant
14 compound with the empirical formula $C_6H_8O_2$. Based on this formula, some logical structures
15 were deduced. One possible compound is 1,2-cyclohexanedione, for which no toxicology
16 information was found in the literature A set of standards of 1,2-cyclohexanedione were
17 prepared, but they were inactive in terms of toxicological response in the dose range of 5-20
18 µg/mL. A second possibility may be one of the isomers of dihydroxycyclohexadiene.
19 Unfortunately, no standards were commercially available to test biotoxicity by the IVMNT.
20 To the best of our knowledge, toxicological data is also not available for any of these isomers.
21 Further structural analysis of the $C_6H_8O_2$ compound was beyond the scope of this study.
22
23 The second compound identified, 3,4-dihydroxybenzoic acid, is not known to be either
24 genotoxic or an inhibitor of cell proliferation (*37*). Catechol, on the other hand, which was *ca.*
25 10-fold more abundant than 3,4-dihydroxybenzoic acid (Figure 7), was confirmed to be
26 genotoxic and inhibit cell proliferation as seen in Figures 8a and 8b for catechol standards (5-
27 20 µg/mL dose range) assessed by the IVMNT. These results support previous findings in
28 terms of the toxicological response (*38*) and in terms of catechol being a product of the
29 combustion of CA (*11, 13, 16, 18, 19*).
30
31 **Conclusion**
32
33 A multidisciplinary study comprising solvent extraction, fractionation, bioassay and state-of-
34 the-art LC/MS allowed us to systematically narrow in on the biotoxic components in the

particulate matter produced from the combustion of CA. Extraction with DMSO followed by successive chromatographic fractionation combined with accurate mass identification and use of the IVMNT for bioactivity identified catechol, 3,4-dihydroxybenzoic acid and a minor, unidentified constituent ($C_6H_8O_2$) as being components of the most bioactive sub-fraction of CA combustion products. 3,4-Dihydroxybenzoic acid has not been reported to be genotoxic or an inhibitor of cell proliferation. Catechol, on the other hand, was the major component present in the most toxic sub-fraction and is known to be toxic. By testing catechol standards alone, we were able to confirm that catechol is indeed genotoxic and blocks cell proliferation in the dose working range. We suspect that catechol is therefore the major component responsible for the bioactivity resulting from the whole DMSO extract. Furthermore, a positive correlation was established between CA (compared to other polyphenolic compounds) found in tobacco and catechol and ethyl-catechol found in smoke (*11, 13*). This demonstrates that in terms of chemistry, our approach of studying a single component is not only valid but is also relevant. The relationship between CA and catechol would support the reduction of CA in tobacco in order to reduce catechol.

Our research carried out on the combustion products of CA may not be directly correlated to cigarette smoke due to the fact that the combustion of a single tobacco component does not take into account possible interactions between multiple components during combustion. Also, the conditions of tobacco combustion, such as heating rate and atmospheric gas concentration are known to influence the relative proportions of the products (*33*). However, our methodology allows for the analysis of a simpler product mixture. Also, we cannot directly relate the toxicological results from the *in vitro* assays to *in vivo* toxicity since there are detoxification pathways involved in the latter. Finally, only the compounds detected by LC/MS in negative mode were accounted for. Nonetheless, our approach combining toxicology with chemical identification has contributed to a better understanding of the toxicity of a single tobacco component, CA.

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Tables

Table 1: Phenolic compounds identified by HPLC/FD in extracts generated from CA combustion. The TPM was extracted from ¼ of each of four Cambridge filters with 1% (v/v) aqueous acetic acid.

CA combustion replicates	TPM qty on ¼ Cambridge filter (mg)	Phenolic content (µg/mg TPM)					Avg
		Hydroquinone	Resorcinol	Catechol	Phenol	p-Cresol	
λexcitation (nm)	-	285	270	270	270	270	
λemission (nm)	-	325	310	310	298	305	
Combustion 1	27.8	16.1	0.8	32.9	10.2	0.1	
Combustion 2	33.2	19.5	1.0	40.4	16.1	0.1	
Combustion 3	32.9	19.2	1.0	34.1	10.4	< LOD	
Combustion 4	38.2	15.1	0.9	31.2	9.1	0.1	
Avg	33.0	17.5	0.9	34.7	11.5	0.1	
RSD (%)	12.9	12.6	10.4	11.6	27.5	[a]N/C	15.5

[a]N/C: not calculated.

Table 2: Normalized content relative to hydroquinone (from Table 1).

CA combustion replicates	Normalized quantity relative to hydroquinone					Avg
	Hydroquinone	Resorcinol	Catechol	Phenol	p-Cresol	
Combustion 1	1.00	0.052	2.042	0.633	0.006	
Combustion 2	1.00	0.053	2.076	0.826	0.007	
Combustion 3	1.00	0.053	1.775	0.539	0.001	
Combustion 4	1.00	0.060	2.067	0.604	0.005	
Avg	-	0.054	1.990	0.651	0.005	
SD	-	0.004	0.144	0.124	0.003	
RSD (%)	-	7.1	7.2	19.0	N/C	11.1

Table 3: Reproducibility of the extraction by DMSO (n = 4), with respect to peak area for thirteen phenolic compounds identified by LC/MSD (negative mode).

Reference Product Name	Average Peak Area (10^3)	RSD (%)
catechol	19 ± 1	5.3
hydroxyquinone	400 ± 30	7.5
4-methylcatechol	29 ± 2	6.9
4-vinylcatechol	710 ± 40	5.6
2-hydroxybenzoic acid	160 ± 10	6.3
4-hydroxybenzoic acid	310 ± 20	6.5
4-ethylcatechol	210 ± 20	9.5
1-(3,4-dihydroxyphenyl)ethanone	25 ± 2	8.0
p-coumaric acid (isomer 1)	170 ± 10	5.9
coumaric acid (isomer 2)	34 ± 3	8.8
hydrocaffeic acid	120 ± 6	5.0
quinic acid	54 ± 3	5.6
caffeic acid methylester	30 ± 3	10.0

List of Figures

Figure 1: Graphs showing the genotoxic activity (% Micronuclei), a), and inhibition of cell proliferation b), induced by DMSO extracts from three independent combustions of 0.5 g of CA, on V79 cells exposed for 3 h without metabolic activation. Dose refers to the quantity of TPM or DPM (μg) per mL of medium. The three extracts were tested in the Duncan's test ($\alpha = 0.05$) and no statistically significant difference was found. Combustion 1: ····◆···· . Combustion 2: – ■ –. Combustion 3: —▲—. Error bars indicate standard deviation.

Figure 2: Graphs showing the genotoxic activity (% Micronuclei), a), and inhibition of cell proliferation, b), induced by DCM, DMSO, water, EA and MeOH extracts generated from combustions of 0.5 g of CA, on V79 cells exposed for 3 h without metabolic activation. Dose refers to the quantity of TPM (μg) per mL of medium. n = 2 for all the condensates except DCM and DMSO, where n = 4. The dose/extraction solvent interactions were analyzed by the Fisher least significant difference multiple comparison test with a Bonferroni correction to type 1 error to ensure that the overall risk was kept under $\alpha = 0.05$. Letters A, B and AB designate different statistical groups. Error bars have been removed for clarity.

Figure 3: Total ion chromatogram of the DMSO extract showing the CA combustion products, which were analyzed by LC/TOFMS in negative mode. The dashed lines have been overlaid to represent the compounds isolated in the three main fractions collected.

Figure 4: Graphs showing the genotoxic activity (% Micronuclei), a), and inhibition of cell proliferation b), induced by (whole) DMSO extract, Fraction 1, Fraction 2 and Fraction 3. All other conditions as in Fig. 2. Error bars have been removed for clarity.

Figure 5: Preparative LC chromatogram (254 nm UV trace) of Fraction 2 of the DMSO extract showing the sub-fractions collected. The dotted lines have been overlaid to represent the four main sub-fractions collected.

Figure 6: Graphs showing the genotoxic activity (% Micronuclei), a), and inhibition of cell proliferation b), induced by DMSO extract, Fraction 2, Sub-fraction 1, Sub-fraction 2, Sub-

1 fraction 3 and Sub-fraction 4. All other conditions as in Fig. 2. Error bars have been removed
2 for clarity.
3
4 **Figure 7:** Base peak LC/TOFMS chromatogram (upper most trace) and extracted ion
5 chromatograms (lower traces) of sub-fraction 1 of fraction 2 of the DMSO extract of CA
6 combustion products. Separation conditions are given in the Experimental section.
7
8 **Figure 8:** Graphs showing the genotoxic activity (% Micronuclei), a), and inhibition of cell
9 proliferation b), induced by DMSO extract and catechol standards. All other conditions as in
10 Fig. 1. DMSO: —■—. Catechol standard: - ▲ - -. Error bars indicate standard deviation.

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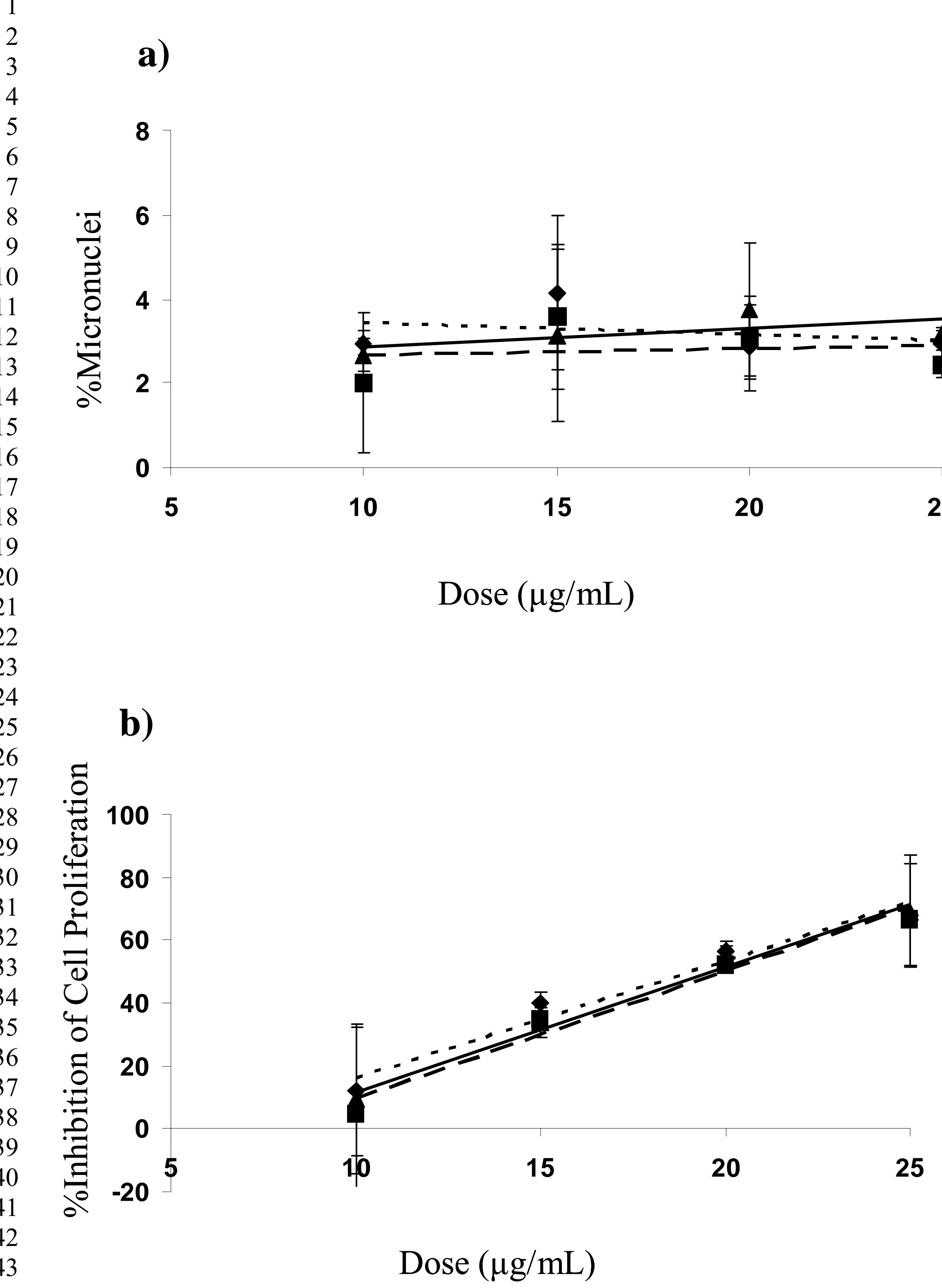

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48 TPM or DPM (µg) per mL of medium. The three extracts were tested in the Duncan's test
49 ($\alpha = 0.05$) and no statistically significant difference was found. Combustion 1: ····◆···· .
50 Combustion 2: – ■ – –. Combustion 3: —▲—. Error bars indicate standard deviation.
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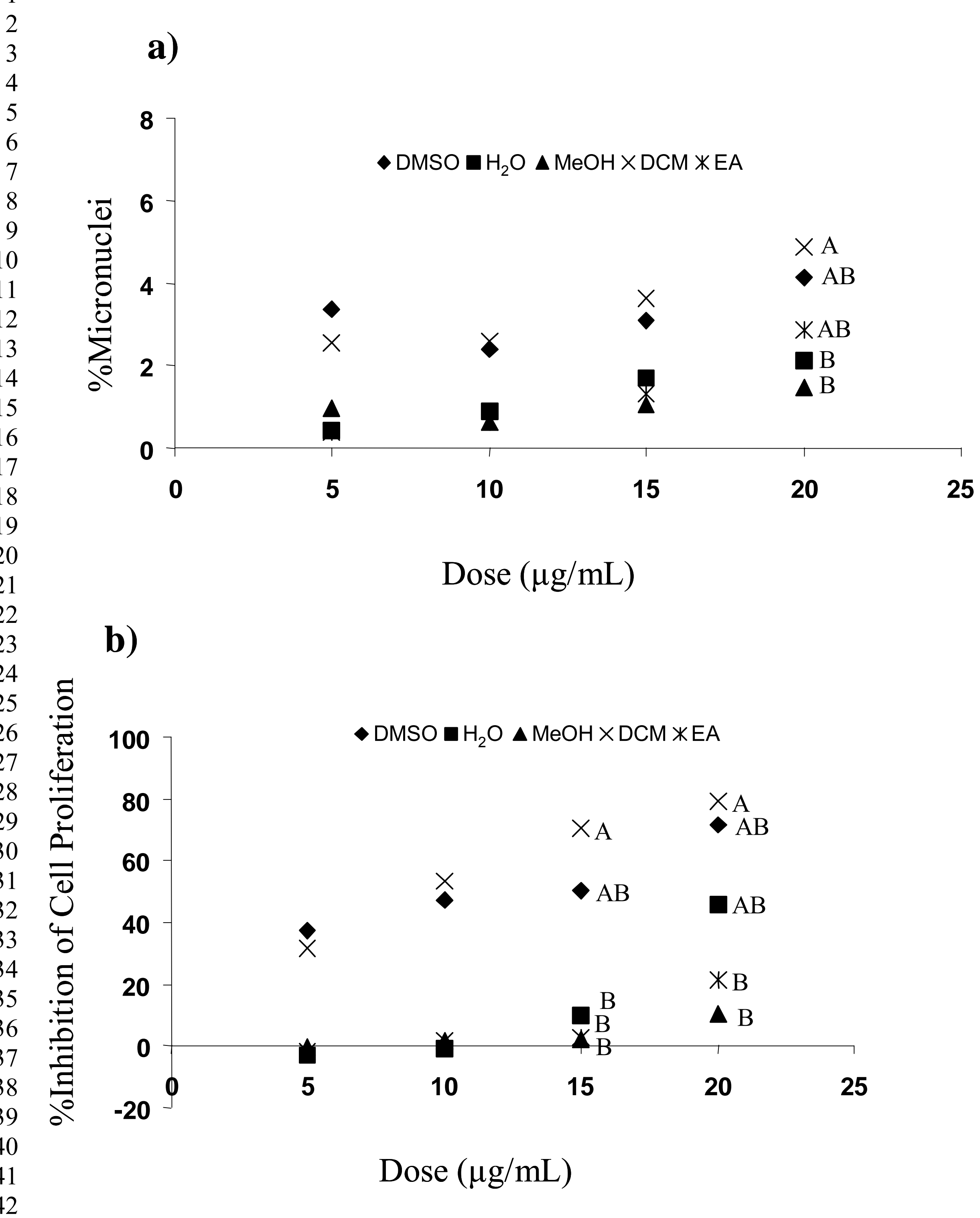

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50 type 1 error to ensure that the overall risk was kept under $\alpha = 0.05$. Letters A, B and AB
51 designate different statistical groups. Error bars have been removed for clarity.

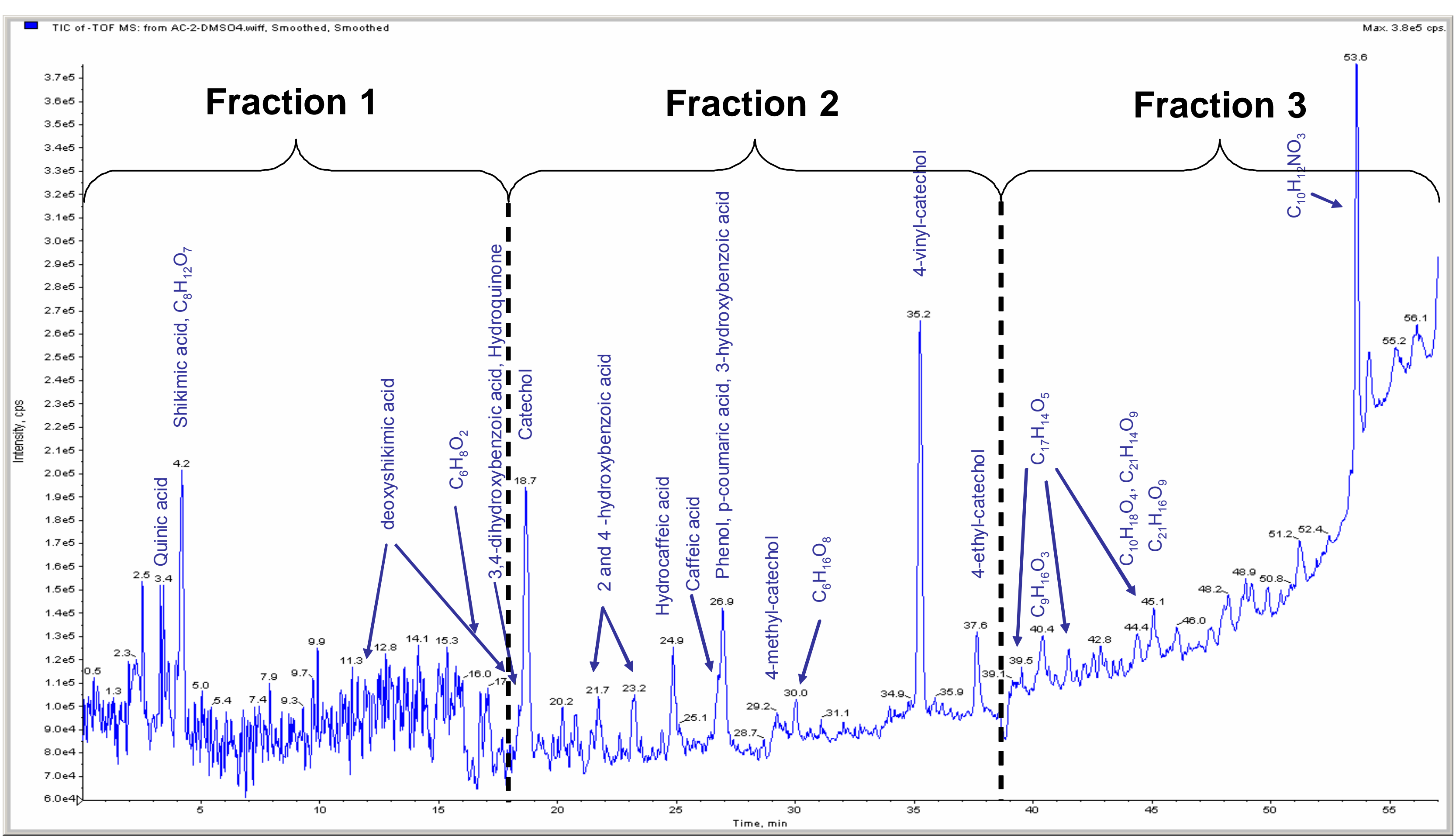

Figure 3: Total ion chromatogram of the DMSO extract showing the CA combustion products, which were analyzed by LC/TOFMS in negative mode. The dashed lines have been overlaid to represent the compounds isolated in the three main fractions collected.

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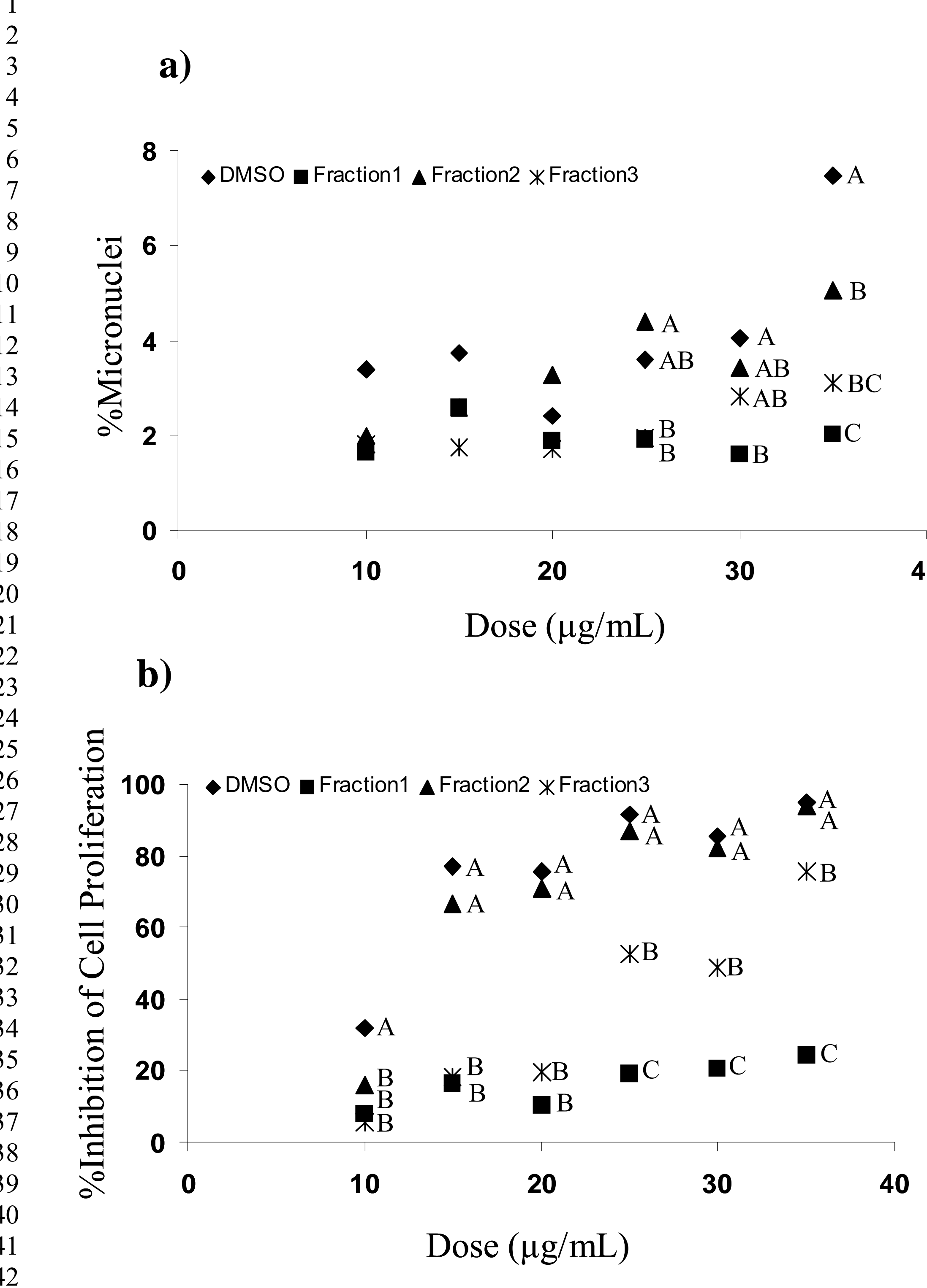

47 **Figure 4:** Graphs showing the genotoxic activity (% Micronuclei), a), and inhibition of cell
48 proliferation b), induced by (whole) DMSO extract, Fraction 1, Fraction 2 and Fraction 3. All
49 other conditions as in Fig. 2. Error bars have been removed for clarity.
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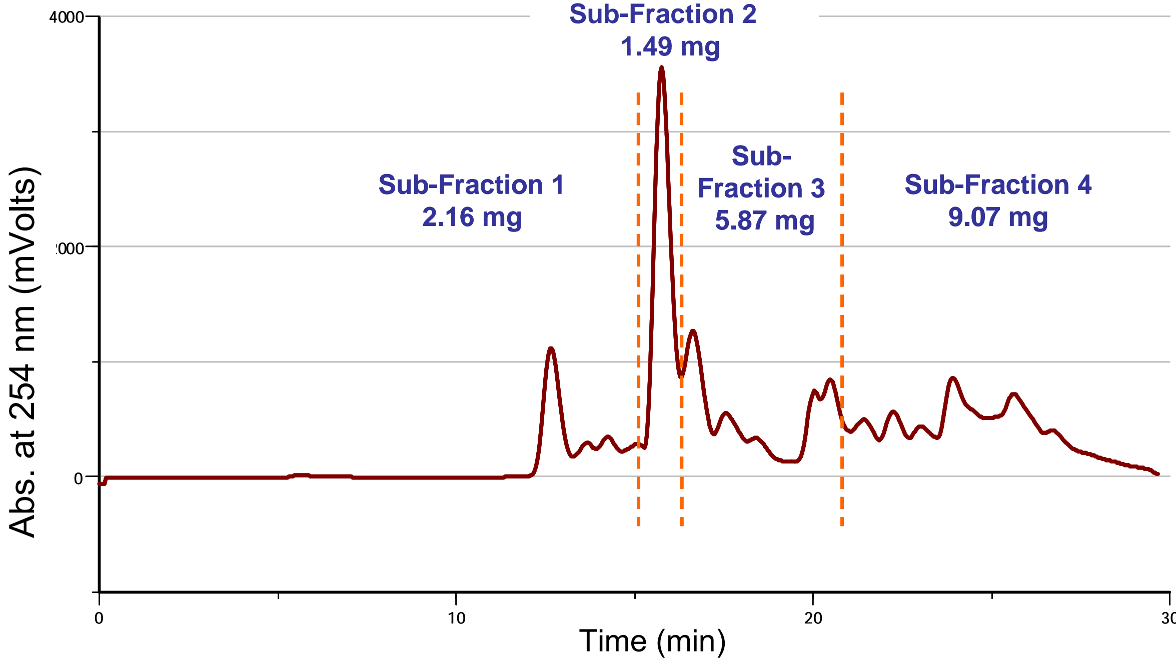

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2 **Figure 5:** Preparative LC chromatogram (254nm UV trace) of Fraction 2 of the DMSO extract showing the sub-fractions collected. The dotted lines
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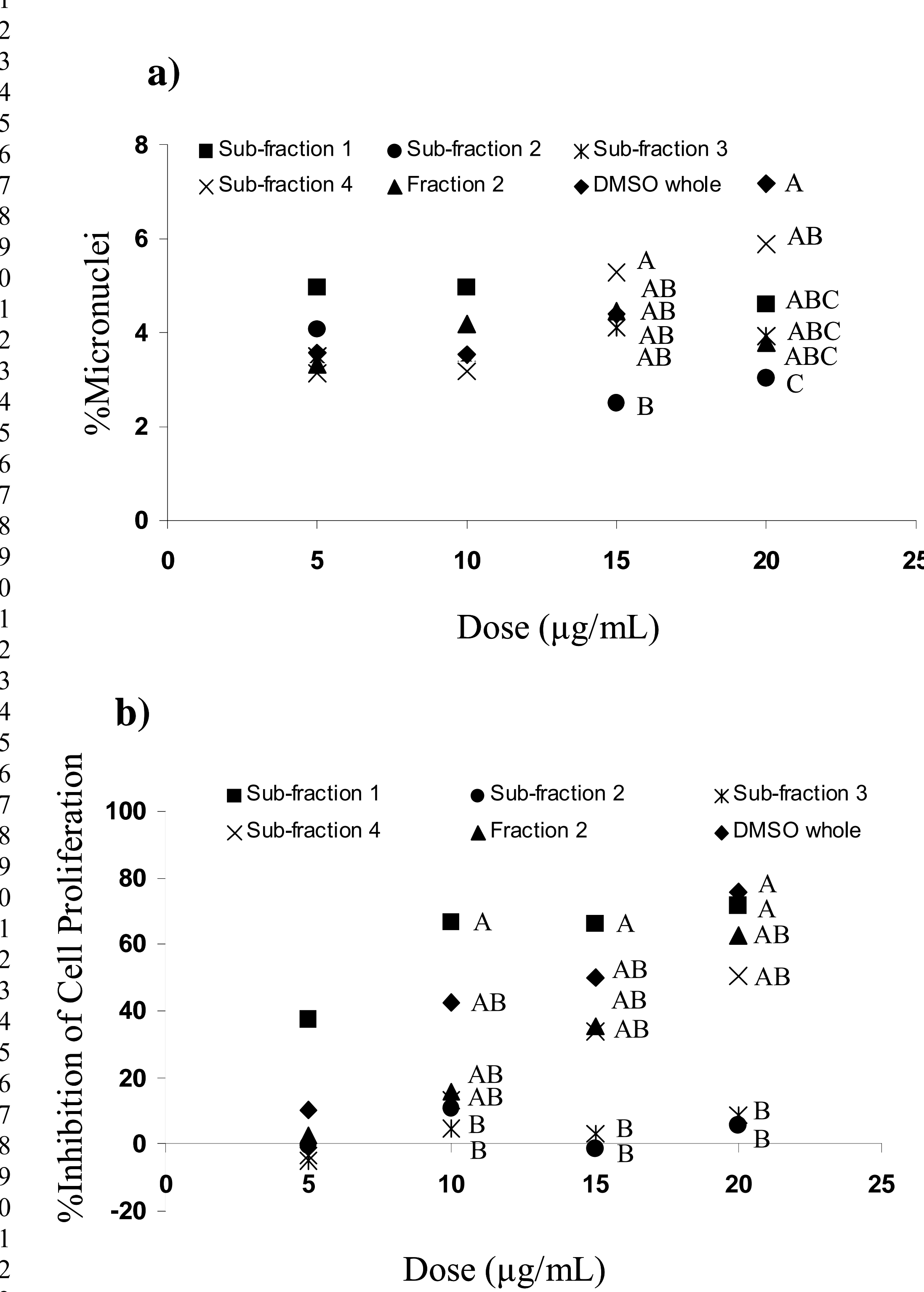

44 **Figure 6:** Graphs showing the genotoxic activity (% Micronuclei), a), and inhibition of cell
45 proliferation b), induced by DMSO extract, Fraction 2, Sub-fraction 1, Sub-fraction 2, Sub-
46 fraction 3 and Sub-fraction 4. All other conditions as in Fig. 2. Error bars have been removed
47 for clarity.

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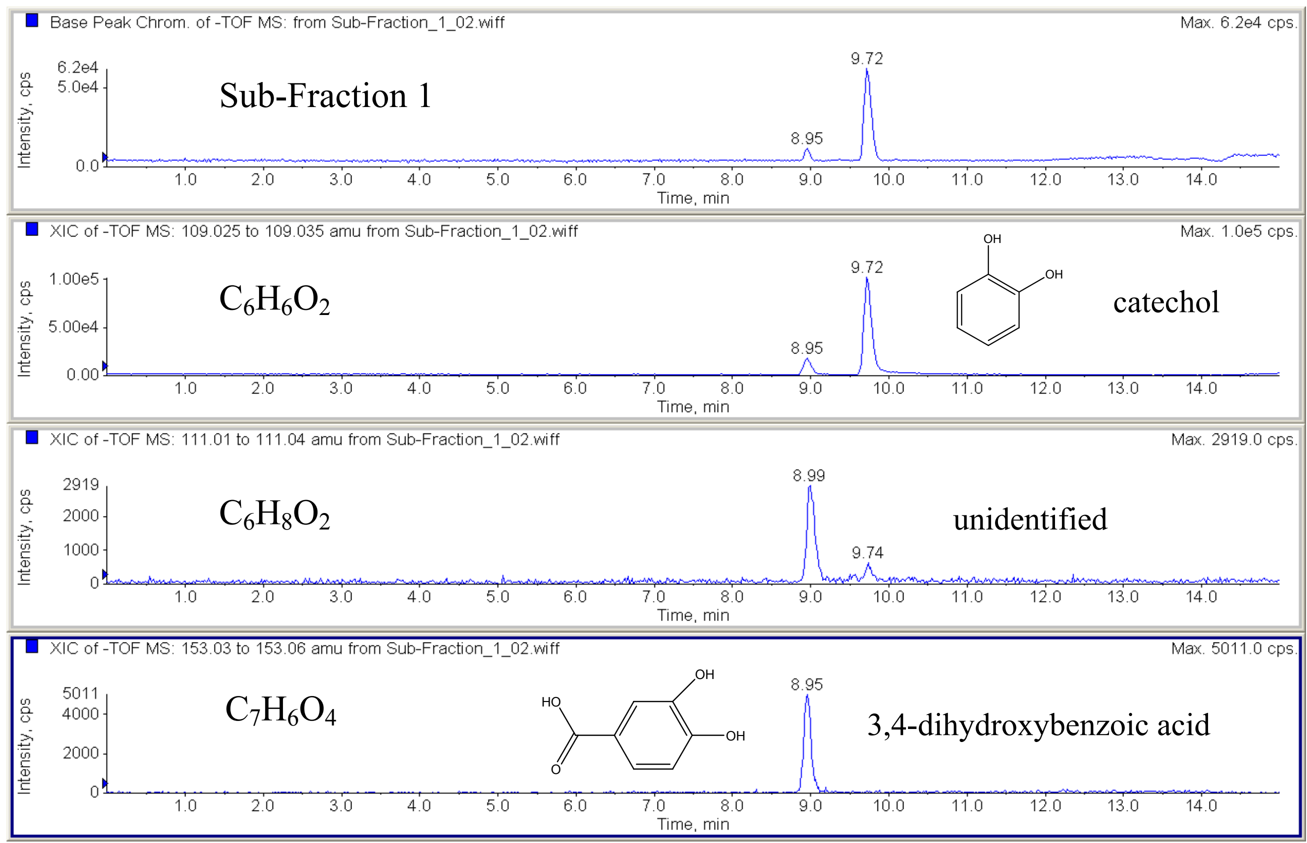

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3 **Figure 7:** Base peak LC/TOFMS chromatogram (upper most trace) and extracted ion chromatograms (lower traces) of sub-fraction 1 of fraction 2

4 of the DMSO extract of CA combustion products. Separation conditions are given in the Experimental section.

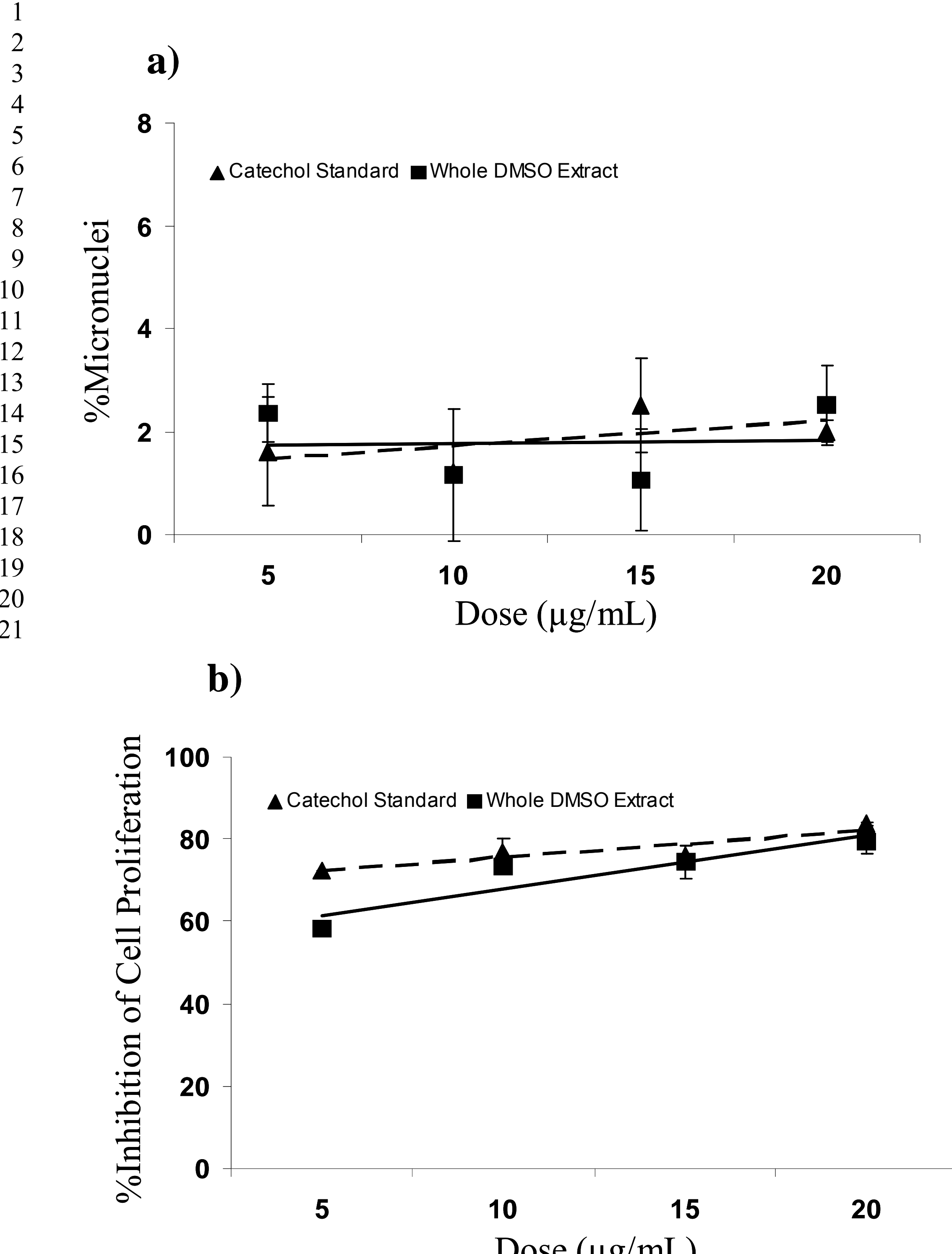

Figure 8: Graphs showing the genotoxic activity (% Micronuclei), a), and inhibition of cell proliferation b), induced by DMSO extract and catechol standards. All other conditions as in Fig. 1. DMSO: —■—. Catechol standard: - -▲- -. Error bars indicate standard deviation.