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The Utilization of Gas Chromatography/Mass Spectrometry in the Profiling of Several Antioxidants in Botanicals

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Introductory information regarding antioxidants in botanicals

Antioxidants are chemicals that inhibit oxidation, and certain antioxidant molecules from fruits and vegetables are thought to alleviate oxidative stress in biological systems. Oxidative stress is a process generated by excessive reactive oxygen species (ROS) in organisms, and it is considered to be involved in a number of illnesses such as cancer, arteriosclerosis, heart diseases, etc. Among these reactive oxygen species are hydroxyl radical $\text{OH}^\bullet$, superoxide radical $\text{O}_2^{\bullet -}$ and also hydrogen peroxide H_2O_2 . Reactive nitrogen species (RNS) are also present in organisms, although at lower levels. The RNS include nitric oxide $\text{NO}^\bullet$, nitrogen dioxide NO_2 , nitrosyl cation NO^+ , etc. Reducing agents are also present in aerobic organisms. Among these are ascorbic acid, glutathione, and uric acid, and these molecules maintain a limited level of ROS in the organism. The enhancement of endogenous antioxidant capability of the human body is thought to be achieved by: 1) ingesting exogenous antioxidants either as food or as dietary supplements, 2) inducing the body production of antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase, also with ingesting certain compounds, 3) inhibiting lipid peroxidation. The use of specific botanicals, either as food or as dietary supplements, has been intensively investigated for potential health benefits (see e.g. [1-5]). The reaction of antioxidants that interact with free radicals on a one-to-one basis takes place through various mechanisms. Among these are the hydrogen atom transfer (HAT), single electron transfer followed by proton transfer (SET or ET-PT), and sequential proton loss electron transfer (SPLET). For example, the HAT mechanism can be described by the following reactions (where $\text{ROO}^\bullet$ is a free radical and AH an antioxidant):

The mechanism by which antioxidant enzymes are stimulated in the human body is less well understood, but specific botanicals with “antioxidant character” are also recommended for this purpose.

Several procedures have been reported in the literature for the characterization of antioxidant properties of a material (typically food or dietary supplement). Among these are parameters such as “oxygen radical absorbance capacity” or ORAC [6-8], “ferric ion reducing antioxidant power” (FRAP) [9], “Folin-Ciocalteu reducing capacity assay” (FCR) [10], etc. The ORAC parameter can be measured by two versions of the same procedure, one indicated as hydrophilic ORAC and the other as lipophilic ORAC [6] and is expressed as μM of Trolox (TE) per g of sample. FRAP values are expressed in μM Fe^{2+} per g of sample [9]. The chemical nature of the antioxidants from different sources can vary considerably. Each compound may have different antioxidant properties, and may be considered useful for specific health benefits. Also, beneficial synergistic effects were reported for specific associations of compounds [11]. For these reasons, the analysis of individual antioxidant molecules including their identification and quantitation is important. Antioxidants from botanicals belong to different classes of molecules. Among these are the following:

1. Monoterpenoid phenols and alcohols such as: thymol, carvacol, menthol.
2. Diterpene phenols, such as: carnosic acid, carnosol, rosmanol.
3. Hydroxycinnamic type compounds such as: caffeic acid, chlorogenic acid, rosmarinic acid, p-coumaric acid, resveratrol, curcumin, eugenol, cinnamaldehyde.
4. Hydroxybenzoic acids and derivatives such as: gallic acid, protocatechuic acid, propyl gallate, tannins.
5. Benzopyrones (2- and 4-) and xanthenes such as: scopoletin, coumarin, quercetin, genistein, naringenin, diosmin, rutin, mangiferin.
6. Flavones and their derivatives such as: epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, gossypin.
7. Dihydrochalcones, such as aspalathin, notophagin.
8. Anthocyanins and anthocyanidins, such as cyanidin, pelargonidin, cyanidin glucosides.
9. Triterpene acids such as ursolic acid, oleanolic acid, betulinic acid.
10. Tocopherols, such as α , β , γ , δ -tocopherols, tocotrienols.
11. Carotenoids, β -carotene, lutein.
12. Ubiquinone, CoQ10.
13. Ascorbic acid, ascorbyl palmitate.

14. Benzodioxoles, such as myristicin, piperine, safrole.
15. Unsaturated lipids.
16. Other compounds, such as gambogic acid, gingerol, ar-turmerone, antioxidant enzymes.

Most antioxidant molecules are relatively large, and in addition, these molecules are frequently polar with groups such as OH and COOH. The high molecular weight and the high polarity of many antioxidant molecules are not conducive to the use of gas chromatography (GC) as the preferred analytical tool. If the molecule is also thermally unstable, such as lutein and carotene, the use of GC is definitely inadequate. For this reason, the analysis of many antioxidant compounds has been performed using high performance liquid chromatography (HPLC) methods [12-24]. However, GC methods can also be used for the identification of antioxidant compounds [25-27]. The use of mass spectrometric detection with its excellent capability for the determination of compound chemical formula makes GC/MS an irreplaceable tool when antioxidant analysis requires compound identification. Although significant progress has been made in using LC/MS (and LC/MS/MS) for compound identification, these techniques still remain more adequate for quantitation and not for qualitative analysis. Various procedures for the GC/MS analysis of certain antioxidants in botanicals are further described in this chapter.

2. Experimental procedures for extending the GC analysis to larger molecules

The GC/MS analysis has considerable advantages compared to other analytical techniques. Besides the simplicity of the procedure, the technique can be used for definite identification of each compound based on its MS spectrum. Also, GC/MS provides separation with excellent resolution of the compounds, and is suitable for quantitation when standards are available. The area counts in the chromatograms can be measured and expressed as normalized area counts reported to the peak area of an internal standard. This type of presentation of results, does not provide quantitative levels for compounds, but allows for the determination of which sample has a higher or a lower level of a given compound. The disadvantage of the technique is caused by the need for volatility and certain thermal stability for the compounds to be analyzed. These restrictions limit the use of GC to larger and non-volatile molecules. However, several procedures are used for extending the capability of gas chromatography for the analysis of these types of molecules. Among these procedures are specific techniques for sample preparation, in particular the derivatization of the analytes. Other procedures include certain GC instrument settings such as the use of hydrogen as carrier gas, selection of appropriate chromatographic column, selection of the type of injection port, and a GC oven gradient with high final temperatures, etc. Derivatization of analytes can be beneficial in a variety of circumstances in GC, such as when the polarity of the analyte is too high and does not elute from the column, when a desired separation is not achievable, when the peak shape of a com-

pound is not good, or when the analyte is not stable in the injection port of the GC. Many antioxidants fit this scenario, and for this reason derivatization is frequently used in GC/MS analysis of antioxidants from botanicals. A variety of chemical reactions are utilized for analytes derivatization. These reactions can be alkylations, arylations, silylation, acylation, additions to carbon-heteroatom multiple bonds, etc. Hydrolysis and formation of smaller molecules (e.g. from lipids) is another type of chemical reaction used as sample preparation step for GC analysis. Of particular interest for the derivatization of many antioxidant molecules is silylation. Many antioxidant molecules contain OH and COOH groups, and these can be easily derivatized using silylation. For this reason, silylation is a preferred technique used for extending the range of analysis by GC/MS of antioxidants. However, in spite of the utility of GC/MS for antioxidant analysis it must be emphasized that it offers only a limited window in the whole range of antioxidant compounds present in botanicals, and heavier molecules may still need to be analyzed using HPLC methods.

Larger molecules, even after derivatization typically require specific conditions for the GC separation, such as temperature gradient up to a relatively high temperature. Modern GC ovens are designed to be able to reach temperatures as high as 400 °C, but the limiting factor regarding the oven temperature is typically the stability of the stationary phase of the column. Depending on the nature of the stationary phase, the columns may be stable up to 360 °C, and only special ones may stand higher temperatures. Such temperatures are necessary in certain instances for the elution of heavier compounds from the chromatographic column. The typical split/splitless injection port, with relatively high temperatures (e.g. around 300 °C) is frequently adequate for the analysis of larger molecules. However, some compounds decompose in the standard split/splitless injection port and “cold on-column” injection is necessary for obtaining acceptable results [28,29].

The identification of the compounds in the chromatogram is typically performed using the library search capability of the GC/MS instrument and a mass spectral library (e.g. NIST8, NIST11, Wiley_9THL, Wiley Registry 10th Ed., etc.). However, the mass spectra of most antioxidant molecules, in particular in silylated form, are not available in standard mass spectral libraries. For this reason, the identification of unknown antioxidant molecules in a natural product material may be difficult. Valuable information can be obtained from separate analysis of standard compounds (if available), or from the comparison of spectra of unknowns with those of expected similar molecules that are available as standards and can be directly analyzed. Special procedures can be used that help with identification of silylated compounds, by using derivatization with deuterated silylating reagents. As an example, the use of d₉-BSTFA [d₉-bis(trimethylsilyl)-trifluoroacetamide] that generates deuterated TMS (trimethylsilyl) derivatives allows the detection of the number of silyl groups in a compound (and implicitly of the number of OH or COOH groups). This can be done by comparing the masses of the same compound when silylated with the deuterated reagent and when silylated with non-deuterated reagent. For each TMS group a difference of 9 a.m.u. is noticed between the two types of silylated compounds. One additional procedure that helps with the identification of an unknown compound is based

on high resolution mass spectra. The spectrum with high resolution can be obtained using MS instruments that were initially recommended for spectra in unit resolution, by using specific post acquisition programs and internal calibration (e.g. MassWorks, Cerno Bioscience, Danbury, CT 06810 USA). Such programs allow the determination of the probable empirical molecular formula of unknown compounds.

Common procedures for the quantitation of specific compounds, such as calibration curves using standards can be applied for the quantitation in case the compound derivatization is not strongly influenced by the sample matrix. In some cases, the standard addition technique for quantitation (see e.g. [30]) gives better results as compared to external calibration. In both cases, the unavailability of standards may limit the possibilities for quantitation.

3. Examples of GC/MS analysis of botanicals containing antioxidant molecules

A large number of botanicals contain antioxidant compounds, and their properties are extensively reported in the literature (see e.g. [31,32]). Also numerous studies were dedicated to individual botanical composition and content of antioxidants. Two examples of botanicals studied using GC/MS of directly silylated natural material (e.g. leaves) are further described. The silylation technique starts with 50 mg solid sample which is weighed (with 0.1 mg precision) in GC vials (2 mL screw top vials with screw caps with septa, Agilent, Wilmington, Delaware 19808). The silylation is done to all the compounds containing active hydrogens, such as acids, alcohols, or amines. The result is the formation of various trimethylsilyl (TMS) derivatives. A reagent and a solvent are used for the silylation process, and the procedure does not require a separate extraction step. From various available reagents, it was determined that bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchloro-silane (TMCS) gives the best results. The preferred solvent was found to be N,N-dimethylformamide (DMF). The solvent used in this study contained as internal standard *tert*-butylhydroquinone. The DMF solution with internal standards was prepared using 100 mL DMF and 40 mg *tert*-butylhydroquinone (all compounds from Aldrich/Sigma Saint Louis, MO 63178-9916). The final DMF solution contained 0.4 mg/mL *tert*-butylhydroquinone. For the analysis, to each vial were added 0.4 mL DMF with internal standards and 0.8 mL BSTFA with 1% TMCS (Aldrich/Sigma Saint Louis, MO 63178-9916). The vials were kept at 78° C (in a heating block) for 30 min., and were allowed to cool at room temperature for another 30 min. After cooling the solution, each vial was filtered through a 0.45 µm PTFE filter (VWR Suwanee, GA 30024) into screw top vials with screw caps and septa, which were used for the GC/MS analysis. This procedure can be scaled down when either the amount of sample is small, or when using the derivatization reagent *d*₉-BSTFA (available from CDN Isotopes, Pointe Claire, Canada H9R 1H1). The analysis was done using a GC/MS instrument (such as Agilent 6890/5973 system from Agilent, Wilmington, Delaware 19808). The GC/MS conditions are given in Table 1.

Parameter	Description	Parameter	Description
GC column	DB-5MS*	Carrier gas	Hydrogen
Column dimensions	30 m long, 0.25 mm id.	Flow mode	Constant flow
Film thickness	0.25 μm	Flow rate	1.0 mL/min
Initial oven temp.	50°C	Nominal initial pressure	7.57 psi
Initial time	0.5 min	Split ratio	30 : 1
Oven ramp rate	3°C/min	Split flow	29.8 mL/min
Oven final first ramp	200°C	GC outlet	MSD
Final time first ramp	0 min	Outlet pressure	Vacuum
Oven ramp rate	4°C/min	Transfer line heater	300°C
Oven final temp.	320°C	Ion source temp.	230°C
Final time	10 min	Quadrupole temp.	150°C
Total run time	90.5 min	MSD EM offset	100 V
Inlet temp.	300°C	MSD solvent delay	7.0 min
Inlet mode	Split	MSD acquisition mode	scan
Injection volume	1.0 μL	Mass range	33 to 1050 a.m.u.

*Note: equivalent columns can be used, such as ZB-5 HT Inferno, etc.

Table 1. GC/MS operating parameters for silylated compounds analysis.

As shown in Table 1, the separation used hydrogen as a carrier gas, and a relatively high final oven temperature.

3.1. Example of green tea analysis

Green tea (leaves of *Camellia sinensis*) is a well known botanical with antioxidant properties [33-35]. For the green tea evaluated in this study (commercially available from Shanghai Tiantan Intern. Trading Co., Ltd.) the ORAC values (both lipophilic and hydrophilic) were $1150 \pm 20 \mu\text{M TE/g}$, and FRAP was $2200 \pm 15 \mu\text{M Fe}^{2+}/\text{g}$. The chemical composition of green tea leaf can be studied using the GC/MS technique after direct silylation of the dry leaf. The chromatogram of silylated green tea is shown in Figure 1.

The identification of the main peaks from Figure 1 can be viewed in Table 2 where the retention times for individual compounds are listed.

Some of the spectra of the silylated compounds are not available in common mass spectral libraries. The spectra of silylated epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate(EGCG), and chlorogenic acid, as obtained using standards, are shown in Figures 2 to 5.

Figure 1. Chromatogram of silylated green tea dry leaf. The identification of main peaks is given in Table 2. (I.S. elutes at 29.65 min).

Antioxidant compounds	Ret. Time	Other main compounds	Ret. Time
Caffeine	37.79	Phosphate	16.63
Gallic acid	42.23	Malic acid	25.76
Epicatechin	63.83	5-Oxoproline	26.42
Catechin	64.32	Fructose	37.20
Epigallocatechin (EGC)	65.18	Quinic acid	39.03
α -Tocoferol (trace)	68.03	Glucose	43.23
Chlorogenic acid (trace)	68.11	Myoinositol	45.93
Epicatechin gallate	78.14	Sucrose	59.80
Epigallocatechin gallate (EGCG)	78.83	Disaccharide	61.77
Gallocatechin gallate	79.41	Disaccharide ?	72.18

Table 2. Compound identification for green tea chromatogram shown in Figure 1

Fragmentation indicated in Figures 2 to 5 can be verified using silylation with d_9 -BSTFA. Figure 6 shows the spectrum of d_9 -silylated epigallocatechin gallate.

The masses of different ions are explained in Figure 6 in comparison with those shown in Figure 5. For example, the mass 693 a.m.u. is obtained from the ion with mass 648 a.m.u. by adding 5×9 a.m.u. resulting from d_9 groups, which indicates 5 TMS groups on this fragment. This spectrum is in agreement with the suggested fragmentation from Figure 5. A similar result

as shown for the spectrum of epigallocatechin gallate, can be obtained for any other silylated compound.

Besides the similarity in the spectrum profile, the d_9 -silylated compounds have a similar retention time as those silylated with non-isotopically labeled BSTFA, and the chromatogram also has a similar profile, as shown in Figure 7, that displays two time windows between 58 min and 80 min from a green tea water extract derivatized with BSTFA and for the same extract derivatized with d_9 -BSTFA.

Figure 2. Spectrum of silylated epigallocatechin (EGC) (from standard compound), ret. time 65.18 min, MW = 738.31 (Note: The molecular weight does not consider the natural isotope distribution of elements and it is based only on the nuclidic masses of a single isotope).

Figure 3. Spectrum of silylated chlorogenic acid (from standard compound), ret. time 68.11 min, MW = 786.33.

Figure 4. Spectrum of silylated epicatechin gallate (ECG) (from standard compound), ret. time 78.14 min, MW = 946.37.

Figure 5. Spectrum of silylated epigallocatechin gallate (EGCG) (from standard compound), ret. time 78.83 min, MW = 1034.40. At higher instrument sensitivity, small peaks at 1034 a.m.u. and 1019 a.m.u. (loss of 15 a.m.u.) can be seen.

Figure 6. Spectrum of d_9 -silylated epigallocatechin gallate. Masses of fragments are shown as related to those in the spectrum of non-deuterated compound displayed in Figure 5.

Figure 7. Two time windows between 58 min and 80 min from a green tea water extract derivatized with BSTFA (A) and for the same extract derivatized with d_9 -BSTFA (B).

The quantitation of epigallocatechin and epigallocatechin gallate in the green tea was also evaluated in this study. For this purpose, the initial peak area was measured in the chromatogram of the silylated green tea sample. This was followed by the addition of 500 µg and 1000 µg of the two compounds (as solution in DMF) to 50 mg green tea sample with silylation. In order to avoid peak overloading, the silylated solution that was filtered through a 0.45 µm PTFE filter. The samples were analyzed by GC/MS and the peak areas were measured. The results are illustrated in Figure 8 that represents the peak area measurement normalized by the internal standard area (0.04 mg/mL *tert*-butylhydroquinone after 1/10 dilution) versus the compound addition.

Figure 8. Peak area for epigallocatechin (EGC) and epigallocatechin gallate (EGCG) for a green tea sample, and for the same type of sample with 500 µg and 1000 µg of the two compounds added.

From the trendline equations, it can be calculated that the green tea contained about 2.307 mg EGCG/50 mg sample, and 1.720 mg EGC/50 mg sample. This is equivalent to 46.14 mg/g EGCG and 34.41 mg/g EGC. These levels are in the range reported in other studies for green tea [34]. Green tea from different sources may have different levels of antioxidants, and silylation followed by GC/MS analysis is an excellent tool for comparing these levels.

3.2. Example of rosemary analysis

Rosemary (dry leaf) (*Rosmarinus officinalis*) is another botanical with antioxidant properties [22,23]. For rosemary (dry leaf) evaluated in this study (commercially available from American Spice Trading Co.) the ORAC values (both lipophilic and hydrophilic) were 620 ± 20 µM TE/g, and FRAP was 800 ± 15 µM Fe²⁺/g. The chemical composition of rosemary leaf can be studied using the GC/MS technique after direct silylation of the dry leaf similarly to green tea.

The chromatogram of silylated rosemary is shown in Figure 9, and the identification of the main peaks can be viewed in Table 3 where the retention times for individual compounds are listed.

Figure 9. Chromatogram of silylated rosemary dry leaf. The identification of main peaks is given in Table 3. (I.S. elutes at 29.67 min).

Antioxidant compounds	Ret. Time	Other main compounds	Ret. Time
Catechol lactate	45.33	Camphor	11.09
Caffeic acid	47.60	Borneol	14.21
Rosmaricin	55.68	Malic acid	25.77
Carnosic acid	57.54	Pentose (ribose ?)	34.71
Carnosol	58.90	Fructose	37.24
Rosmanol	60.19	Quinic acid	39.01
Rosmarinic acid	72.69	Glucose	40.05, 43.24
Oleanolic acid	74.02	Myoinositol	45.91
Betulinic acid	74.35	Sucrose	59.79
Ursolic acid	74.77	Disaccharide	69.90
Betulonic acid	75.23	Disaccharide ?	83.78

Table 3. Compound identification for rosemary chromatogram shown in Figure 9

Some of the spectra of the silylated compounds are not available in common mass spectral libraries. The spectra of silylated rosmarinic acid, carnosic acid, carnosol, rosmanol, rosmarinic acid, oleanolic acid, betulinic acid, ursolic acid, and betulonic acid are shown in Figures 10 to 18.

Figure 10. Spectrum of silylated rosmarinic acid, ret. time 55.68 min, MW = 489.27 (Note: the presence of two silyl groups on this molecule has been verified using silylation with d_9 -BSTFA).

Figure 11. Spectrum of silylated carnosic acid, ret. time 57.54 min, MW = 548.32.

Figure 12. Spectrum of silylated carnosol, ret. time 58.90 min, MW = 474.26.

Figure 13. Spectrum of silylated rosmannol, ret. time 60.19 min, MW = 562.30.

Figure 14. Spectrum of silylated rosmarinic acid, ret. time 72.69 min, MW = 720.28.

Figure 15. Spectrum of silylated oleanolic acid or 3 β -hydroxyolean-12-en-28-oic acid (from standard), ret. time 74.02 min, MW = 600.44.

Figure 16. Spectrum of silylated betulinic acid, or (3 β)-3-Hydroxy-lup-20(29)-en-28-oic acid (from standard), ret. time 74.35 min, MW = 600.44.

Figure 17. Spectrum of silylated ursolic acid or 3- β -hydroxy-urs-12-en-28-oic acid (from standard), ret. time 74.77 min, MW = 600.44.

Figure 18. Spectrum (tentative) of silylated betulonic acid or 3-oxo-lup-20(29)-en-28-oic acid, ret. time 75.23 min, MW = 598.42.

The spectrum of silylated betulonic acid has a similar pattern to that of betulonic acid, except for several fragments being lower by two a.m.u. It was assumed that during silylation, the carbonyl group in position 3 is enolised and silylated.

The GC/MS analysis with direct derivatization of dry leaf of a botanical has various specific advantages compared to other analysis techniques. Besides its simplicity, the technique allows a detailed identification of the compounds seen in the chromatogram, allows a comparison of peak intensity between different types of botanicals, and quantitation when standards are available. An example of the application of this technique is the study of stability upon heating of rosemary regarding its antioxidant level. Starting from room temperature the heating was performed at three intervals up to 120 °C, for two hours. The variation in normalized area counts in the chromatograms of silylated leaf heated at different temperatures is shown in Figure 19. The results show that carnosic acid and rosmarinic acid have the tendency to decrease as the leaves are heated, while other antioxidant compounds are not affected by the heating in the indicated range.

3.3. Other applications of direct silylation and GC/MS analysis

A variety of other botanicals (leaves, rhizomes, or other plant parts) containing antioxidant molecules form silyl derivatives can be analyzed by GC/MS. Among the compounds that can be identified by silylation and GC/MS are: vitexin, isoorientin, mangiferin, gossypin, delphinidin and other cyanidins, quercetin, tocoferol, coumaroyl quinic acid, ar-turmerone, curcumin, leucocyanidin gallate, etc. Some of the mass spectra of these molecules are easily identifiable, but in other cases, the identification is less obvious. In cases of glucosides (and C-glucosides), for example, the set of ions 147, 204, 217, 305 that are characteristic for the carbohydrate (glucose) moiety may lead to the conclusion that the chromatographic peak

Figure 19. Variation with temperature in the normalized area counts of several antioxidant compounds (rosmarinic level is shown x 10 for being in scale) in rosemary heated between 20 °C and 120 °C, for two hours.

belongs to a carbohydrate, since carbohydrates are frequently present in plant extracts. As an example, the spectrum of silylated isoorientin (luteolin-6-C-glucoside) is given in Figure 20. In this spectrum, the presence of MW - 15 ion caused by the loss of a CH_3 from the silyl group, which is typical for silyl derivatives is a good indication of the parent molecule isoorientin which has MW = 1024.41.

Figure 20. Spectrum of silylated isoorientin, (luteolin-6-C-glucoside) ret. time 82.68 min, MW = 1024.41. The peak with 1009 a.m.u. resulting from the loss of CH_3 is seen in the spectrum.

4. GC/MS analysis of triglycerides with antioxidant character

Some triglycerides present in botanicals, usually from the fruits or from seeds, are known to have antioxidant character. This character is caused by the presence of polyunsaturation in the long chain hydrocarbon moiety of the fatty acids (PUFAs) that are typically part of the triglyceride molecules. PUFAs (free or as triglyceride) have a scavenging potential toward reactive oxygen/nitrogen (ROS/RNS) species [36]. Several nomenclature systems are used for the fatty acids, a common one being omega-x (ω - x, or n - x). The value of x indicates the position of the double bond which is the closest to the terminal methyl of the hydrocarbon chain of the acid, with counting from the terminal methyl. For example, linoleic acid is a n - 6 or an omega-6 acid. Triglycerides formed from omega-3 acids, besides the antioxidant character, are considered essential fatty acids, since they cannot be synthesized by the human body and are related to additional health benefits. Common analysis of triglycerides is done either for the intact compound, or after hydrolysis and derivatization of the acid with methyl groups [37], or with silyl groups [29,38].

4.1. Triglyceride hydrolysis and fatty acids methylation

The formation of methyl esters from triglycerides is typically done in one operation that produces both the hydrolysis of the triglyceride and the methylation of the free acids formed in the hydrolysis. Methyl esters of the fatty acids (FAME) can be obtained using various reagents [30,39]. Common procedures use methanol and H_2SO_4 , methanol and BF_3 [40-42] or methanol and HCl . One standard procedure [43] starts with the addition to 100 mg lipid in a 50 mL round bottom flask with condenser. To the flask is added 4 mL of a 0.5 M methanolic solution of NaOH . The solution is boiled until fat globules disappear. Then, 5 mL solution of BF_3 in methanol (125 g BF_3/L) is added and the boiling is continued for 2-3 min. Then about 5 mL of heptane is added and boiled for another minute. The mixture is allowed to cool and 15 mL saturated solution of NaCl is added. About 1 mL heptane is collected from the upper layer and is dried over anhydrous Na_2SO_4 . The solution is diluted with heptane if necessary for the GC analysis. Detection for the GC can be either flame ionization (FID) or mass spectrometry (MS). A number of variants of this methylation procedure are reported in the literature (e.g. [44]). For example, one variant starts with 200–500 mg lipid which is boiled with 5 mL 0.5 N NaOH or KOH in methanol for 3–5 min. To this mixture is added 15 mL of an esterification solution, and the mixture is refluxed for 3 min. The esterification solution is prepared by adding 2 g NH_4Cl to 60 mL methanol and 3 mL conc. H_2SO_4 which are then refluxed together for 15 min. The esterified acids are transferred into a separation funnel containing 25 mL petroleum ether and 50 mL water. The water is discarded and the organic phase is washed twice with 25 mL water. The resulting organic phase can be concentrated, dried with Na_2SO_4 , and analyzed by GC. The reactions taking place are described as follows:

The analysis of FAME can be performed following various procedures. One such procedure uses a SP2560 100 m x 0.25 mm column with 0.2 µm film for separation. This is a highly polar biscyanopropyl column specifically designed to separate geometric position isomers of fatty acid methyl esters. The recommended GC conditions are given in Table 4.

Parameter	Description	Parameter	Description
Initial oven temp.	100°C	Injection volume	1.0 µL
Initial time	4.0 min	Carrier gas	Helium
Oven ramp rate	3°C/min	Flow mode	Constant flow
Oven final temp.	240°C	Flow rate	0.75 mL/min
Final time	15 min	Linear flow rate	18 cm/s
Total run time	65.6 min	Split ratio	200 : 1
Inlet temp.	225°C	GC outlet	FID
Inlet mode	Split	Detector temperature	300°C

Table 4. GC operating parameters for methyl ester analysis.

The procedure allows the separation of over 60 FAME. Other columns and shorter run times can be utilized if a less detailed separation is desired. For example, a SP2380 30 m x 0.25 mm column with 0.2 µm film can be used, with oven starting at 150 °C and gradient to 250 °C at 4 °C/min, helium carrier gas at 20 cm/s (at 150 °C), and FID detector at 260 °C. In these conditions, a typical GC/MS chromatogram obtained for linseed oil is shown in Figure 21.

Figure 21. GC/MS chromatogram for methyl esters in linseed oil.

Other procedures to generate methyl esters are also reported in the literature [38].

4.2. Triglyceride hydrolysis and fatty acids silylation

Hydrolysis and formation of silyl derivatives of fatty acids is another procedure used for lipid analysis. The analysis starts with the hydrolysis of the triglycerides. For this purpose, 0.3 to 0.5 mg lipid (precisely weighed) was treated with 50 μ L solution of 2M KOH in ethanol. The mixture was heated in a 2 mL capped vial for 30 min at 78 $^{\circ}$ C in a heating block, to generate potassium salts of the fatty acids. After that, the cap of the vial was removed, and the ethanol evaporated. Complete evaporation of ethanol, which takes 3-5 min, is necessary to avoid the formation of small proportions of ethyl esters when HCl is further added. To the vial, 25 μ L solution of 6M HCl was added to neutralize the base and change the organic acid potassium salts into free acids. Then, 750 μ L of *n*-nonane was added to extract the free acids. The nonane solution was treated in the vial with about 0.2 g of anhydrous Na₂SO₄ for drying. From the dry nonane solution, 500 μ L were transferred into a separate 2 mL vial, treated with 25 μ L of pyridine, 100 μ L of dimethylformamide (DMF) that contains 400 μ g/mL of tert-butylhydroquinone (TBHQ), and with 300 μ L bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS). The TBHQ is used as a chromatographic standard. The vials with the samples were heated at 78 $^{\circ}$ C for 30 min, followed by GC/MS analysis. The analysis of the samples was performed using a GC/MS instrument (Agilent 7890/5975 system, Wilmington, DE, USA), equipped with a Zebron ZB-50 column (Phenomenex, Torrence, CA 90501-1430, USA) that was 60 m long, 0.25 mm i.d., and 0.50 μ m film thickness. The recommended parameters for the GC/MS analysis are given in Table 5

Parameter	Description	Parameter	Description
Initial oven temp.	50 $^{\circ}$ C	Carrier gas	Hydrogen
Initial time	0.5 min	Flow mode	Constant flow
Oven first ramp rate	10 $^{\circ}$ C/min	Flow rate	0.71 mL/min
Final oven temp. first ramp	200 $^{\circ}$ C	Nominal initial pressure	12.05 psi
Final time first ramp	0 min	Split ratio	20 : 1
Oven second ramp rate	3 $^{\circ}$ C/min	Split flow	14.20 mL/min
Final oven temp. second ramp	250 $^{\circ}$ C	GC outlet	MSD
Final time second ramp	0 min	Outlet pressure	Vacuum
Oven third ramp rate	20 $^{\circ}$ C/min	Transfer line heater	300 $^{\circ}$ C
Final oven temp. third ramp	300 $^{\circ}$ C	Ion source temp.	230 $^{\circ}$ C
Final time third ramp	2 min	Quadrupole temp.	150 $^{\circ}$ C
Total run time	36.66 min	MSD EM gain	2.0
Inlet temp.	300 $^{\circ}$ C	MSD solvent delay	8.0 min
Inlet mode	Split	MSD acquisition mode	scan
Injection volume	0.5 μ L	Mass range	33 to 550 a.m.u.

Table 5. GC/MS operating parameters for silylated acids analysis.

The peak identification was performed using both standards (when available) and mass spectra library searches (on NIST 08 library). The chromatography allows excellent separation of acids in the range C6 to C27, and differentiate isomers such as oleic and elaidic acid. Quantitation of fatty acids was obtained using calibration curves. A typical total ion chromatogram (TIC) for the fatty acids as TMS derivatives from a commercial vegetable cooking oil hydrolysate sample is shown in Figure 22.

Figure 22. GC/MS chromatogram of TMS derivatives of fatty acids from a commercial cooking oil hydrolysate sample. Peak identification can be obtained using the data from Table 6.

No.	Compound	Ret. time	MW	Identifying ions	Formula	Symbol	Area %
1	Glycerin 3TMS (not shown)	10.74	308.64	205, 218	C ₁₂ H ₃₂ O ₃ Si ₃		0.15
2	Unknown	14.61	?	192, 163	?		0.24
3	Internal standard (I.S.)	16.41					
4	Column bleed	21.03					
5	Palmitic acid TMS	23.13	328.613	313, 328	C ₁₉ H ₄₀ O ₂ Si	C16:0	9.52
6	Palmitoleic acid TMS	23.30	326.597	311, 326	C ₁₉ H ₃₈ O ₂ Si	C16:1 Z-9	0.07
7	Stearic acid TMS	27.27	356.667	341, 356	C ₂₁ H ₄₄ O ₂ Si	C18:0	2.56
8	Oleic acid TMS	27.35	354.651	339, 354	C ₂₁ H ₄₂ O ₂ Si	C18:1 Z-9	20.33
9	Elaidic acid TMS (trans-9-C18:1)	27.49	354.651	339, 354	C ₂₁ H ₄₂ O ₂ Si	C18:1 E-9	2.09
10	Linoleic acid TMS	27.82	352.635	337, 352	C ₂₁ H ₄₀ O ₂ Si	C18:2 Z,Z-9,12	59.72
11	Linolenic acid TMS	28.50	350.62	335, 350	C ₂₁ H ₃₈ O ₂ Si	C18:3 Z,Z,Z-6,9,12	4.99
12	Arachidic acid TMS	31.70	384.721	369, 384	C ₂₃ H ₄₈ O ₂ Si	C20:0	0.13
13	11-Eicosenoic acid TMS	31.80	382.705	367, 382	C ₂₃ H ₄₆ O ₂ Si	C20:1 Z-11	0.06
14	Docosanoic acid TMS (behenic)	34.63	412.78	397, 412	C ₂₅ H ₅₂ O ₂ Si	C22:0	0.14

Table 6. Peak identification and relative peak area for the chromatogram of TMS derivatives of fatty acids from a commercial vegetable cooking oil hydrolysate sample.

4.3. Analysis of intact triglycerides

For the analysis of triglycerides as whole molecules, a solution containing about 0.5 mg/mL lipid in n-nonane (b.p. 151 °C) was made from each sample. This solution was analyzed directly by GC, in conditions described in Table 7. The GC was equipped with a Rtx®-65TG column, 30 m x 0.25 mm, with 0.1 µm film thickness (Restek, Bellefonte, PA 16823, USA). Similar separation was obtained using a CP-Tap column, 25 m x 0.25 mm, 0.1 µm film (Varian, Walnut Creek, CA 94598, USA) in the same conditions as in Table 7. The GC can be used either with FID detection or MS detection. The conditions for the MS and FID detectors are shown in Table 8.

Parameter	Description	Parameter	Description
Initial oven temperature	130 °C	Inlet mode	Ramped
Initial time	1.0 min	Inlet initial temperature	130 °C
Oven temp. rate first ramp	30 °C/min	Initial time	0.1 min
Final temperature first ramp	300 °C	Inlet temperature rate	150 °C/min
Final time	0.0 min	Final temperature	300 °C
Oven temp. rate second ramp	4.0 °C/min	Injection volume	0.2 µL
Final temperature second ramp	365 °C	Carrier gas	H ₂
Final time	7.0 min	Flow mode	Constant flow
Total run time	29.92 min	Flow rate	0.8 mL/min
Inlet	Cold on column		

Table 7. GC operating parameters.

MS Parameter	Description	FID Parameter	Description
MSD transfer line	300 °C	Detector temperature	300 °C
Ion source temperature	230 °C	H ₂ flow	30 mL/min
MSD EM gain	2.0	Air flow	400 mL/min
MSD solvent delay	3.0 min	Make up flow N ₂	25 mL
MS operating mode	Scan EI+		
Mass range a.m.u.	50 – 800 a.m.u.		

Table 8. ID and MS operating parameters.

Using the conditions previously described, the chromatogram of a commercial vegetable cooking oil with FID detection is shown in Figure 23, and with MS detection is shown in Figure 24.

Figure 23. Chromatogram of a commercial vegetable cooking oil generated using FID detection.

Figure 24. Chromatogram of a commercial vegetable cooking oil generated using MS detection (total ion chromatogram or TIC). Peak identification following retention times as given in Table 9.

	Compound	Formula	Ret. time in MS	MW	Identifying ions	Area % from MS	Triglyc. % from FID
1	Dipalmitin olein	C53H100O6	16.34	833.380	551, 577, 339	0.51	0.27
2	Dipalmitin linolein	C53H98O6	16.60	831.364	551, 575, 335	1.42	0.85
3	Palmitin stearin olein	C55H104O6	17.55	861.434	579, 605, 341	0.34	0.19
4	Palmitin diolein	C55H102O6	17.73	859.418	577, 603, 339	4.14	2.65
5	Palmitin stearin linolein	C55H102O6	17.81	859.418	579, 603, 341	2.14	1.37
6	Palmitin olein linolein	C55H100O6	18.00	857.402	577, 601, 339	12.51	9.19
7	Palmitin dilinolein	C55H98O6	18.28	855.386	575, 599, 337	15.93	13.44
8	Palmitin linolein linolenin	C55H96O6	18.61	853.370	573, 575, 335,	1.40	1.36
9	Linolein distearin	C57H106O6	18.93	887.472	605, 341, 264	1.03	0.70
10	Triolein	C57H104O6	19.13	885.456	603, 339, 264	5.34	4.56
11	Distearin olein	C57H108O6	19.23	889.488	605, 341, 262	5.31	3.17
12	Diolein linolein	C57H102O6	19.44	883.440	603, 339, 262	8.36	8.63
13	Stearin olein linolein	C57H104O6	19.53	885.456	603, 341, 262	8.68	6.87
14	Dilinolein olein	C57H100O6	19.77	881.424	601, 339, 262	16.37	20.21
15	Trilinolein	C57H98O6	20.11	879.408	599, 337, 262	12.90	19.39
16	Dilinolein linolenin	C57H96O6	20.53	877.392	597, 599, 337	3.62	6.59

Table 9. Peak identification, relative peak area for the MS chromatogram and % triglyceride from FID measurement for a commercial vegetable cooking oil.

The peak identifications can be done based on the mass spectra of each compound. For example, the mass spectrum of palmito-linoleo-olein is given in Figure 25.

Figure 25. Mass spectrum of palmito-linoleo-olein (the correct position of substituents is unknown).

The structures of several diagnostic ions in the spectrum of a triglyceride species that contains palmityl, linoleyl, and oleyl fatty acids in the molecule is given in Figure 26.

Figure 26. The structures of several diagnostic ions in the spectrum of palmito-linoleo-olein.

Heavier triglycerides are less amenable for direct GC analysis. For example, direct measurement of triglycerides esterified with more than two linolenic acids is not possible in the chromatographic conditions previously described. As an example, the TIC trace for a sample of linseed oil generated in the same conditions as the chromatogram from Figure 25 is given in Figure 27 [29].

Figure 27. Chromatogram of linseed oil generated using MS detection. Peak identification following retention times as given in Table 9 and in Table 10.

Many peaks from this chromatogram are identical to those described in Table 9. However, a few additional triglycerides were identified (some tentatively) in linseed oil and they are given in Table 10.

	Compound	Formula	Ret. time in MS	MW	Identifying ions
1	Palmito oleino linolenin	C55H98O6	18.33	855.386	577, 599, 573
2	Dioleino linolenin?	C57H100O6	19.82	881.424	604, 599, 339
3	Stearo linoleo linolenin	C57H100O6	20.28	881.424	603, 601, 597
4	Oleino linoleo linolenin	C57H98O6	21.45	879.408	597, 599, 601
5	Oleino dilinolenin	C57H96O6	21.95	877.392	595, 599, 335

Note: Ions with m/z value in bold result from the loss of a fatty acid residue from the triglyceride molecule.

Table 10. Peak Identification, for Extra Peaks in the MS Chromatogram for Linseed Oil.

5. Conclusions

GC/MS is a very useful technique for the analysis of antioxidants in botanicals, although many antioxidant molecules are large and/or contain numerous polar groups. GC methods have limitations regarding their capability to be used for the analysis of heavier and less volatile molecules. However, the use of derivatization of the analytes, and special selection of the GC settings allow the extension of the applicability for this technique. The unique capability to identify molecular species based on EI+ mass spectra makes GC/MS an invaluable tool in the analysis of antioxidants in botanicals.

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