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 \( \text{OH}^* \), superoxide radical \( \text{O}_2^* \) and also hydrogen peroxide \( \text{H}_2\text{O}_2 \). Reactive nitrogen species (RNS) are also present in organisms, although at lower levels. The RNS include nitric oxide \( \text{NO}^* \), nitrogen dioxide \( \text{NO}_2 \), nitrosyl cation \( \text{NO}^+ \), 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 ROO\(^*\) is a free radical and AH an antioxidant):
ROO• + AH → ROOH + A•

ROO• + A• → ROOA

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 Fe²⁺ 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 xanthises 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. Triterpenic 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 antioxidants 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 botanics. 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 easy 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 botanics, 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)-trifluoracetamide] 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 \( \text{tert}-\text{butylhydroquinone} \). The DMF solution with internal standards was prepared using 100 mL DMF and 40 mg \( \text{tert}-\text{butylhydroquinone} \) (all compounds from Aldrich/Sigma Saint Louis, MO 63178-9916). The final DMF solution contained 0.4 mg/mL \( \text{tert}-\text{butylhydroquinone} \). 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 \( \mu \)m 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 \( \text{d}_9\)-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.
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 ± 20 μM TE/g, and FRAP was 2200 ± 15 μM Fe²⁺/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.

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

| 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.*
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 d9-BSTFA. Figure 6 shows the spectrum of d9-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 x 9 a.m.u. resulting from d9 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 d₉-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 d₉-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$_7$-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$_7$-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 DMP) 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](image-url)  
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 ECG. 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.

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

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

| 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     |
Some of the spectra of the silylated compounds are not available in common mass spectral libraries. The spectra of silylated rosmarinic, 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, 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<sub>9</sub>-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 rosmanol, 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 3β-hydroxyolean-12-en-28-oic acid (from standard), ret. time 74.02 min, MW = 600.44.
Figure 16. Spectrum of silylated betulinic acid, or (3β)-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 betulinic 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 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 sylilation 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
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](image-url)

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 H2SO4, methanol and BF3 [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 BF3 in methanol (125 g BF3/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 Na2SO4. 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 NH4Cl to 60 mL methanol and 3 mL conc. H2SO4 which are than 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 Na2SO4 and analyzed by GC. The reactions taking place are described as follows:

\[
\begin{align*}
\text{RCOO-CH}_3
+\text{H}_2\text{SO}_4 / \text{NH}_4\text{Cl} & \rightarrow \text{RCOONa} \\
\text{CH}_3\text{OH} & \rightarrow \text{RCOO-CH}_3
\end{align*}
\]
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.

![GC/MS chromatogram for methyl esters in linseed oil](image-url)

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 μL solution of 2M KOH in ethanol. The mixture was heated in a 2 mL capped vial for 30 min at 78 °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 μL solution of 6M HCl was added to neutralize the base and change the organic acid potassium salts into free acids. The nonane solution was treated in the vial with about 0.2 g of anhydrous Na\textsubscript{2}SO\textsubscript{4} for drying. From the dry nonane solution, 500 μL were transferred into a separate 2 mL vial, treated with 25 μL of pyridine, 100 μL of dimethylformamide (DMF) that contains 400 μg/mL of tert-butylhydroquinone (TBHQ), and with 300 μL 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 °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 μm film thickness. The recommended parameters for the GC/MS analysis are given in Table 5.

| Parameter                        | Description                  | Parameter                  | Description    |
|----------------------------------|------------------------------|----------------------------|----------------|
| Initial oven temp.               | 50°C                         | Carrier gas                | Hydrogen       |
| Initial time                     | 0.5 min                      | Flow mode                  | Constant flow  |
| Oven first ramp rate             | 10°C/min                     | Flow rate                  | 0.71 mL/min    |
| Final oven temp. first ramp      | 200°C                        | Nominal initial pressure   | 12.05 psi      |
| Final time first ramp            | 0 min                        | Split ratio                | 20 : 1         |
| Oven second ramp rate            | 3°C/min                      | Split flow                 | 14.20 mL/min   |
| Final oven temp. second ramp     | 250°C                        | GC outlet                  | MSD            |
| Final time second ramp           | 0 min                        | Outlet pressure            | Vacuum         |
| Oven third ramp rate             | 20°C/min                     | Transfer line heater       | 300°C          |
| Final oven temp. third ramp      | 300°C                        | Ion source temp            | 230°C          |
| Final time third ramp            | 2 min                        | Quadrupole temp            | 150°C          |
| Total run time                   | 36.66 min                    | MSD EM gain                | 2.0            |
| Inlet temp.                      | 300°C                        | MSD solvent delay          | 8.0 min        |
| Inlet mode                       | Split                        | MSD acquisition mode       | scan           |
| Injection volume                 | 0.5 μL                       | 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         | C12H32O3Si3         | 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       | C19H40O2Si         | C16:0        | 9.52   |
| 6   | Palmitoleic acid TMS            | 23.30     | 326.597 | 311, 326       | C19H38O2Si         | C16:1 Z-9    | 0.07   |
| 7   | Stearic acid TMS                | 27.27     | 356.667 | 341, 356       | C21H44O2Si         | C18:0        | 2.56   |
| 8   | Oleic acid TMS                  | 27.35     | 354.651 | 339, 354       | C21H42O2Si         | C18:1 Z-9    | 20.33  |
| 9   | Elaidic acid TMS (trans-9-C18:1) | 27.49   | 354.651 | 339, 354       | C21H42O2Si         | C18:1 E-9    | 2.09   |
| 10  | Linoleic acid TMS               | 27.82     | 352.635 | 337, 352       | C21H40O2Si         | C18:2 Z,Z-9,12 | 59.72  |
| 11  | Linolenic acid TMS              | 28.50     | 350.62  | 335, 350       | C21H38O2Si         | C18:3 Z,Z,Z-6,9,12 | 4.99   |
| 12  | Arachidic acid TMS              | 31.70     | 384.721 | 369, 384       | C23H46O2Si         | C20:0        | 0.13   |
| 13  | 11-Eicosenoic acid TMS          | 31.80     | 382.705 | 367, 382       | C23H46O2Si         | C20:1 Z-11   | 0.06   |
| 14  | Docosanoic acid TMS (behenic)   | 34.63     | 412.78  | 397, 412       | C25H52O2Si         | 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                        | H2                |
| 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        | H2 flow                            | 30 mL/min         |
| MSD EM gain                       | 2.0           | Air flow                           | 400 mL/min        |
| MSD solvent delay                 | 3.0 min       | Make up flow N2                    | 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 Linolein distearin      | C57H106O6   | 18.93           | 887.472     | 605, 341, 264    | 1.03           | 0.70                |
| 9 Triolein                | C57H104O6   | 19.13           | 885.456     | 603, 339, 264    | 5.34           | 4.56                |
| 10 Distearin olein        | C57H108O6   | 19.23           | 889.488     | 605, 341, 262    | 5.31           | 3.17                |
| 11 Diolein linolein       | C57H102O6   | 19.44           | 883.440     | 603, 339, 262    | 8.36           | 6.63                |
| 12 Stearin olein linolein | C57H104O6   | 19.53           | 885.456     | 603, 341, 262    | 8.68           | 6.87                |
| 13 Linolein olein linolein| C57H100O6   | 19.77           | 881.424     | 601, 339, 262    | 16.37          | 20.21               |
| 14 Trilinolein            | C57H98O6    | 20.11           | 879.408     | 599, 337, 262    | 12.90          | 19.39               |
| 15 Distearin linolein     | 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).](image-url)
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.
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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References

[1] D. Huang, B. Ou, R. L. Prior, The chemistry behind antioxidant capacity assays, J. Agric. Food Chem., 53 (2005) 1841-1856.

[2] H. Wang, G. Cao, R. L. Prior, Total antioxidant capacity of fruits, J. Agric. Food Chem., 44 (1996) 701-705.
[3] S. M. Henning, C. Fajardo-Lira, H. W. Lee, A. A. Youssefian, V. L. W. Go, D. Heber, Catechin content of 18 teas and a green tea extract supplement correlates with the antioxidant capacity, *Nutrition and Cancer*, 45 (2003) 226–235.

[4] European Food Safety Authority, Use of rosemary extracts as a food additive, *The EFSA Journal*, 721 (2008) 1-29.

[5] D. Del Rio, A. Rodriguez-Mateos, J. P. E. Spencer, M. Tognolini, G. Borges, A. Crozier, Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases, *Antioxidants & Redox Signaling*, 18 (2013) 1818-1892.

[6] G. Cao, H. M. Alessio, R. G. Cutler, Oxygen-radical absorbance capacity assay for antioxidants, *Free Radical Biology & Medicine*, 14 (1993) 303-311.

[7] G. Cao, A. H. Wu, H. Wang, R. L. Prior, Automated assay of oxygen radical absorbance capacity with the Cobas Fara II, *Clin. Chem.* 41 (1995) 1738–1744.

[8] B. Ou, M. Hampsch-Woodill, R. L. Prior, Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe., *J. Agric. Food Chem.*, 49 (2001) 4619–4626.

[9] I. F. F. Benzie, J. J. Strain, The ferric reduction ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay, *Anal. Biochem.*, 239 (1996) 70-76.

[10] O. Folin, V. Ciocalteu, On tyrosine and tryptophane determinations in proteins, *J. Biol. Chem.*, 73 (1927) 627–650.

[11] A. Khaffif, S. P. Schantz, T.-C. Chou, D. Edelstein, P. G. Sacks, Quantitation of chemopreventive synergism between (−)-epigallocatechin-3-gallate and curcumin in normal, premalignant and malignant human oral epithelial cells, *Carcinogenesis*, 19 (1998) 419–424.

[12] D. Del Rio, A. J. Stewart, W. Mullen, J. Burns, M. E. Lean, F. Brighenti, A. Crozier, HPLC-MS analysis of phenolic compounds and purine alkaloids in green and black tea. *J. Agric. Food Chem.*, 52 (2004) 2807–2815.

[13] M. N. Irakli, V. F. Samanidou, I. N. Papadoyannis, Development and validation of an HPLC method for the simultaneous determination of tocopherols, tocotrienols and carotenoids in cereals after solid-phase extraction, *J. Sep. Sci.* 34 (2011)1375–1382.

[14] S. Y. Li, Y. Yu, S. P. Li, Identification of antioxidants in essential oil of radix Angelicae sinensis using HPLC coupled with DAD-MS and ABTS-based assay, *J. Agric. Food Chem.*, 55 (2007) 3358–3362.

[15] D. Han, K. H. Row, Determination of luteolin and apigenin in celery using ultrasonic-assisted extraction based on aqueous solution of ionic liquid coupled with HPLC quantification, *J. Sci. Food Agric.*, 91 (2011) 2888–2892.
[16] M. Parvu, A. Toiu, L. Vlase, P. E. Alina, Determination of some polyphenolic compounds from Allium species by HPLC-UV-MS, *Nat. Prod. Res.* 24 (2010) 1318–2134.

[17] T. M. Rababah, K. I. Ereifej, R. B. Esoh, M. H. Al-u’datt, M. A. Alrababah, W. Yang, Antioxidant activities, total phenolics and HPLC analyses of the phenolic compounds of extracts from common Mediterranean plants, *Nat. Prod. Res.* 25 (2011) 596–605.

[18] C. Proestos, N. Chorianopoulos, G. J. Nychas, M. Komaitis, RP-HPLC analysis of the phenolic compounds of plant extracts: investigation of their antioxidant capacity and antimicrobial activity. *J. Agric. Food Chem.*, 53 (2005) 1190–1195.

[19] M. M. Naidu, B. N. Shyamala, J. R. Manjunatha, G. Sulochanamma, P. Srinivas, Simple HPLC method for resolution of curcuminoids with antioxidant potential, *J. Food Sci.*, 74 (2009) C312–C318.

[20] M. Reto, M. E. Figueira, H. M. Filipe, C. M. M. Almeida, Chemical composition of green tea (Camellia sinensis) infusions commercialized in Portugal, *Plant Foods Hum. Nutr.*, 62 (2007) 139–144.

[21] S. M. Henning, C. Fajardo-Lira, H. W. Lee, A. A. Youssefian, V. L. W. Go, D. Heber, Catechin content of 18 teas and a green tea extract supplement correlates with the antioxidant capacity, *Nutrition and Cancer*, 45 (2003) 226–235.

[22] F.J. Señoráns, E. Ibañez, S. Cavero, J. Tabera, G. Reglero, Liquid chromatographic–mass spectrometric analysis of supercritical-fluid extracts of rosemary plants, *J. Chromatog. A*, 870 (2000) 491–499.

[23] L. Almela, B. Sánchez-Muñoz, J. A. Fernández-López, M. J. Roca, V. Rabe, Liquid chromatographic–mass spectrometric analysis of phenolics and free radical scavenging activity of rosemary extract from different raw material, *J. Chromatog. A*, 1120 (2006) 221-229.

[24] M. Herrero, M. Plaza, A. Cifuentes, E. Ibáñez, Green processes for extraction of bioactives from rosemary. Chemical and functional characterization via UPLC-MS/MS and in-vitro assays, *J. Chromatog. A*, 1217 (2009) 2512-2520.

[25] C. D. Stalikas, Extraction, separation, and detection methods for phenolic acids and flavonoids, (Review), *J. Sep. Sci.*, 30 (2007) 3268-3295.

[26] R. J. Robbins, phenolic acids in foods: an overview of analytical methodology, *J. Agric. Food Chem.*, 51 (2003) 2866-2887.

[27] I. Arslan, A. Celik, Chemical composition and antistaphylococcal activity of an endemic Salvia chrysophylla stapf. naturally distributed in Denizli province (Turkey) and its vicinity, *Pak. J. Bot.*, 40 (2008) 1799-1804.

[28] Z. Penton, Determination of neutral lipids by high temperature GC, Part I. Triglycerides and cholesteryl esters in foods, *Varian Application Note*, No. 22b.
[29] S. C. Moldoveanu, Y. Chang, Dual analysis of triglycerides from certain common lipids and seed extracts, J. Agric. Food Chem., 59 (2011) 2137-2147.

[30] S. C. Moldoveanu, V. David, Sample Preparation in Chromatography, Elsevier, Amsterdam, 2002.

[31] F. Shahidi, C. -T. Ho (eds.), Antioxidant Measurement and Applications, ACS Symposium Ser. 956, ACS, Washington, 2007.

[32] D. J. Charles, Antioxidant Properties of Spices, Herbs and Other Sources, Springer, New York, 2013.

[33] S. M. Henning, C. Fajardo-Lira, H. W. Lee, A. A. Youssefian, V. L. W. Go, D. Heber, Catechin content of 18 teas and a green tea extract supplement correlates with the antioxidant capacity, Nutrition and Cancer, 45 (2003) 226–235.

[34] M. Reto, M. E. Figueira, H. Mota Filipe, C. M. M. Almeida, Chemical composition of green tea (Camellia sinensis) infusions commercialized in Portugal, Plant Foods Hum. Nutr. 62 (2007) 139–144.

[35] H. N. Graham, Green tea composition, consumption, and polyphenol chemistry, Preventive Med. 21 (1992) 334-350.

[36] D. Richard, K. Kefi, U. Barbe, P. Bausero, F. Visioli, Polyunsaturated fatty acids as antioxidants, Pharmacol. Res., 57 (2008) 451-455.

[37] W. W. Christie, http://lipidlibrary.aocs.org/topics/ester_93/refs.htm

[38] S.C. Moldoveanu, Profiling of lipids from fruit and seed extracts, pp. 73-123, in Su Chen (ed.) Lipidomics: Sea Food, Marine Based Dietary Supplement, Fruit and Seed, Transworld Res. Network, Kerala, India, 2012.

[39] D. R. Knapp, Handbook of Analytical Derivatization Reactions, J. Wiley, New York, 1979.

[40] D. E. Albertyn, C. D. Bannon, J. D. Craske, N. T. Hai, N. L. Harper, K. L. O’Rourke, C. Szonyi, Analysis of fatty acids methyl esters with high accuracy and reliability I Optimization of flame-ionization detectors with respect of linearity, J. Chromatogr., 247 (1982) 47-61.

[41] C. D. Bannon, J. D. Craske, N. T. Hai, N. L. Harper, K. L. O’Rourke, Analysis of fatty acids methyl esters with high accuracy and reliability II Methylation of fats and oils with boron trifluoride methanol, J. Chromatogr., 247 (1982) 63-69.

[42] C. D. Bannon, G. J. Breen, J. D. Craske, N. T. Hai, N. L. Harper, K. L. O’Rourke, Analysis of fatty acids methyl esters with high accuracy and reliability III Literature review of and investigation into the development of rapid procedures for the methoxide-catalysed methanalysis of fats and oils, J. Chromatogr., 247 (1982) 71-89.

[43] AOAC official method 969.22. Fatty acids in oils and fats. Preparation of methyl esters boron trifluoride method first action 1969
[44] AOAC Official method 996.06 Fat (Total, Saturated, and Unsaturated) in Foods.
